

Charles University  
Faculty of Pharmacy in Hradec Králové

Department of Analytical Chemistry



**Stability Studies of Oral Liquid Preparations Using HPLC**

Stabilitní studie tekutých perorálních přípravků s využitím HPLC

DISSERTATION

(article-based)

*Supervisor: Prof. RNDr. Petr Solich, CSc.*

*Consultant: Assoc. Prof. PharmDr. Ludmila Matysová, Ph.D.*

Hradec Králové, 2017

Mgr. Lukáš Zahálka

*„Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně (pod vedením svého školitele prof. RNDr. Petra Solicha, CSc. a konzultantky doc. PharmDr. Ludmily Matysové, Ph.D.). Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.“*

V Hradci Králové dne 11. 7. 2017

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Mgr. Lukáš Zahálka

## Poděkování

Tímto bych rád vyjádřil poděkování mému školiteli prof. RNDr. Petru Solichovi, CSc. a konzultantce doc. PharmDr. Ludmile Matysové, Ph.D. za jejich odborné vedení během mého doktorského studia a za koordinaci plodné spolupráce mezi Katedrou analytické chemie, Katedrou farmaceutické technologie (doc. PharmDr. Zdeňka Šklubalová, Ph.D.) a Fakultní nemocnicí v Motole (PharmDr. Sylva Klovrzová, Ph.D.).

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# Abstrakt

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

Katedra analytické chemie

**Kandidát:** Mgr. Lukáš Zahálka

**Školitel:** prof. RNDr. Petr Solich, CSc.

**Název disertační práce:** Stabilitní studie tekutých perorálních přípravků s využitím HPLC

Na českém trhu je dle databáze Státního ústavu pro kontrolu léčiv dostupných 59 tis. registrovaných léků, přičemž 8 tisíc z nich bylo v červenci 2017 obchodovaných; přesto existují terapeutické požadavky, které nemohou být těmito komerčně dostupnými léky splněny z důvodu nevhodnosti dostupných lékových forem pro použití např. pro pediatrické pacienty.

Proto byla vytvořena spolupráce mezi Farmaceutickou fakultou v Hradci Králové (Katedra analytické chemie a Katedra farmaceutické technologie) a Fakultní nemocnicí v Motole k řešení zmiňovaných terapeutických potřeb vývojem magistraliter formulací perorálních tekutých přípravků vybraných účinných látek.

Každý projekt byl složen ze tří hlavních částí. První částí byl vývoj několika variant lékových formulací s ohledem na různé požadavky (např. přípravky bez cukru či bez konzervantů). Tato část byla vypracována na Katedře farmaceutické technologie. Druhá (vývoj HPLC metody) a třetí (provedení vlastní stabilitní studie) část byly následně provedeny na Katedře analytické chemie.

V rámci této práce byly vyvinuty magistraliter perorální tekuté přípravky tří účinných látek a byla hodnocena jejich stabilita při různých skladovacích podmínkách:

- 1) Perorální roztoky s obsahem propranololu – neselektivní  $\beta$ -blokátor; léčba infantilních hemangiomů.
- 2) Perorální roztoky s obsahem sotalolu –  $\beta$ -blokátor s antiarytmickými vlastnostmi; léčba ventrikulární a supraventrikulární tachykardie u dětí.
- 3) Perorální roztoky s obsahem furosemidu – diuretikum; léčba hypertenze a edému spojeného se srdečním selháním včetně plicního edému u dětí.

# Abstract

Charles University, Faculty of Pharmacy in Hradec Králové

Department of Analytical Chemistry

**Candidate:** Mgr. Lukáš Zahálka

**Supervisor:** Prof. RNDr. Petr Solich, CSc.

**Title of Doctoral Thesis:** Stability Studies of Oral Liquid Preparations Using HPLC

According to the database of the Czech State Institute for Drug Control there are 59 thousand of registered drugs available in the Czech Republic, out of which 8 thousand drugs were marketed in July 2017; however, there are still some therapeutic needs that cannot be met by using of these commercially available drugs because of unsuitability of available dosage forms for being used, *e.g.*, in pediatric patients.

Thus, cooperation was established between the Faculty of Pharmacy in Hradec Králové (Department of Analytical Chemistry and Department of Pharmaceutical Technology) and the University Hospital in Motol (Prague) to address such therapeutic needs by developing of extemporaneous formulations of oral liquid preparations containing selected pharmaceutical active ingredients.

Each project was composed of three major parts. First part was to develop several versions of drug formulations according to various requirements (*e.g.*, sugar-free or preservative-free). This part was accomplished by the Department of Pharmaceutical Technology. The second (development of HPLC method) and the third (stability study conducting) part was then carried out at the Department of Analytical Chemistry.

Finally, extemporaneous oral liquid preparations containing three active substances were developed and their stability under various storage conditions was evaluated:

- 1) Propranolol oral liquid solutions – nonselective  $\beta$ -blocker; treatment of infantile hemangioma.
- 2) Sotalol oral liquid solutions –  $\beta$ -blocker with anti-arrhythmic properties; treatment of ventricular and supraventricular tachycardia in children.
- 3) Furosemide oral liquid solutions – diuretic; treatment of hypertension and edema associated with heart failure including pulmonary edema in children.

# Content

<b>1</b>	<b>INTRODUCTION.....</b>	<b>9</b>
1.1	BASIC DEFINITIONS.....	10
1.2	BRIEF HISTORY OF ANALYTICAL SCALE LIQUID CHROMATOGRAPHY .....	11
1.2.1	“Birth” of Liquid Chromatography .....	11
1.2.2	HPLC.....	11
1.2.3	UHPLC / UPLC® and Future .....	13
1.2.4	Nobel Prizes Related Directly/Indirectly to Chromatography .....	14
1.3	LC SYSTEM.....	16
1.4	GENERAL PRINCIPLES OF LIQUID CHROMATOGRAPHY .....	19
1.4.1	Retention .....	22
1.4.2	Selectivity.....	24
1.4.3	Peak Symmetry .....	25
1.4.4	Resolution.....	26
1.4.5	The van Deemter Equation .....	26
1.4.6	Pressure Drop.....	29
1.4.7	Column Efficiency.....	30
1.5	CHROMATOGRAPHIC SEPARATION PRINCIPLES.....	34
1.5.1	Normal Phase Chromatography.....	34
1.5.2	Reversed Phase Chromatography .....	36
1.5.3	Hydrophilic Interaction Chromatography .....	44
1.5.4	Chiral Separations .....	45
1.5.5	Size Exclusion Chromatography .....	46
1.5.6	Ion Exchange Chromatography.....	46
<b>2</b>	<b>HPLC/UHPLC INSTRUMENTATION .....</b>	<b>48</b>
2.1	COLUMNS .....	49
2.1.1	Type of Packing Material (Porous, Nonporous, Monolithic) .....	50
2.1.2	Base Material (Silica, Zirconia, Alumina, Polymers) .....	51
2.1.3	New Generation 1.3 µm Core-Shell Particles Column.....	53
2.1.4	Monolithic Columns.....	54
2.1.5	Column Backpressure .....	55
2.2	PUMPS.....	56
2.3	INJECTORS.....	58
2.4	DETECTORS .....	59
2.4.1	UV Detectors.....	59

2.4.2	<i>Combination of Detectors</i> .....	62
2.5	HPLC AND HEALTH & SAFETY.....	64
2.5.1	<i>Human Hazard Potential of Selected Mobile Phase Components</i> .....	64
<b>3</b>	<b>VALIDATION</b> .....	<b>69</b>
3.1	ANALYTICAL PROCEDURE.....	70
3.2	ACCURACY.....	71
3.3	PRECISION.....	72
3.4	SPECIFICITY.....	74
3.5	DETECTION LIMIT.....	75
3.6	QUANTIFICATION LIMIT.....	76
3.7	LINEARITY AND RANGE.....	78
3.8	ROBUSTNESS.....	80
3.8.1	<i>System Suitability</i> .....	80
<b>4</b>	<b>STABILITY TESTING</b> .....	<b>82</b>
4.1	STABILITY TESTING OF DRUG SUBSTANCE.....	83
4.1.1	<i>Stress Testing</i> .....	83
4.1.2	<i>Selection of Batches</i> .....	83
4.1.3	<i>Container Closure System</i> .....	84
4.1.4	<i>Specification</i> .....	84
4.1.5	<i>Testing Frequency</i> .....	84
4.1.6	<i>Storage Conditions</i> .....	85
4.1.7	<i>Evaluation</i> .....	87
4.1.8	<i>Statements/Labeling</i> .....	88
4.2	STABILITY TESTING OF DRUG PRODUCT.....	89
4.2.1	<i>Photostability Testing</i> .....	89
4.2.2	<i>Selection of Batches</i> .....	89
4.2.3	<i>Container Closure System</i> .....	89
4.2.4	<i>Specification</i> .....	90
4.2.5	<i>Testing Frequency</i> .....	90
4.2.6	<i>Storage Conditions</i> .....	91
4.2.7	<i>Evaluation</i> .....	95
4.2.8	<i>Statements/Labeling</i> .....	95
<b>5</b>	<b>REFERENCES</b> .....	<b>96</b>
<b>6</b>	<b>ABBREVIATIONS</b> .....	<b>100</b>
<b>7</b>	<b>AIMS</b> .....	<b>102</b>
<b>8</b>	<b>LIST OF PUBLICATIONS &amp; AUTHOR'S CONTRIBUTION</b> .....	<b>103</b>

<b>9</b>	<b>ARTICLES RELATED TO THE DISSERTATION – COMMENTARY.....</b>	<b>105</b>
9.1	PUBLICATION NO. I .....	106
9.1.1	<i>Commentary ad Publication No. I .....</i>	<i>106</i>
9.2	PUBLICATION NO. II .....	107
9.2.1	<i>Commentary ad Publication No. II .....</i>	<i>107</i>
9.3	PUBLICATION NO. III .....	108
9.3.1	<i>Commentary ad Publication No. III .....</i>	<i>108</i>
9.4	PUBLICATION NO. IV .....	109
9.4.1	<i>Commentary ad Publication No. IV .....</i>	<i>109</i>
9.5	PUBLICATION NO. V .....	110
9.5.1	<i>Commentary ad Publication No. V .....</i>	<i>110</i>
<b>10</b>	<b>CONCLUSION.....</b>	<b>112</b>
<b>11</b>	<b>FULL-TEXTS OF RESEARCH ARTICLES RELATED TO THE DISSERTATION.....</b>	<b>114</b>



# 1 Introduction

High performance liquid chromatography (HPLC, or for short LC) is the most commonly used chromatographic technique to determine drugs in pharmaceutical preparations and in biological material. In LC the mobile phase is a liquid, forced through a column packed with a material that retards the analytes introduced into the system. The analytes are injected into the flow of mobile phase just in front of the separation column. The outlet of the column is connected to a detector where the eluted substances are detected.

This work used HPLC instrumentation for conducting stability assays of newly developed formulations of extemporaneous oral liquid preparations that are designated particularly for administration in pediatric patients.[1]

## 1.1 Basic Definitions

International Union of Pure and Applied Chemistry (IUPAC) has defined chromatography and liquid chromatography (LC) as follows:

### **Chromatography:**

*“A physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction.”*

### **Liquid chromatography:**

*“A separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or on a plane.*

*Note: Present-day liquid chromatography generally utilizing very small particles and a relatively high inlet pressure is often characterized by the term high-performance (or high-pressure) liquid chromatography, and the acronym **HPLC**.”[2]*

## 1.2 Brief History of Analytical Scale Liquid Chromatography

### 1.2.1 “Birth” of Liquid Chromatography

Chromatography is based on a single principle and is the invention of one individual: the principle is the passing of a solution through an adsorbing column and the inventor is **Michael Tswett**. Tswett possibly was influenced by the work of Goppelsroeder, whose experiments on capillary analysis he cites in one of his first papers. Goppelsroeder first described this subject as early as 1861 and, much later, in his well-known monograph (1901). The basic observations were, however, made by Goppelsroeder’s teacher, Schoenbein (1861, 1864) who observed selective adsorption of the components of a mixture by the different heights to which they rose when a strip of filter paper was dipped into the solution. Schoenbein made his first observations during his classic studies of ozone, when he impregnated paper with potassium iodide-starch or other ozone reagents. At the age of 34, Tswett, who was the son of a Russian father and an Italian mother, described the fundamental principle and technique of chromatography in an eight-page article (1906) which reached the Editors of the *Berichte der Deutschen Botanischen Gesellschaft* on **June 21, 1906**, a date which we may accept as the official birthday of chromatography.

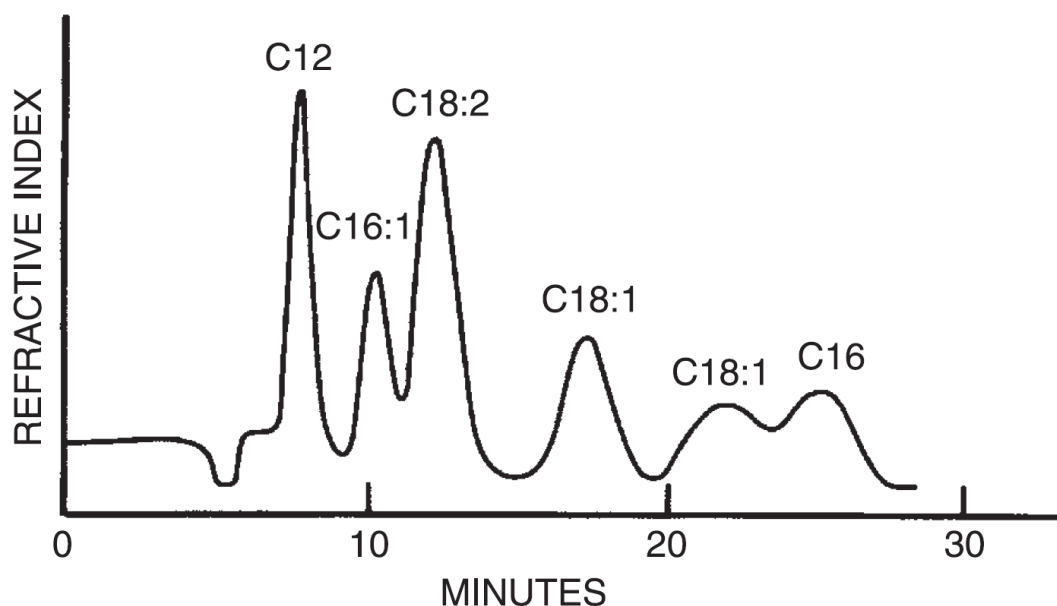
Tswett coined the name chromatography (from the Greek words *chroma*, meaning color, and *graph*, meaning writing – literally “color writing”) to describe his colorful experiment (separation of leaf pigments). Curiously, the Russian name Tswett means color.[3-4]

### 1.2.2 HPLC

Chromatography was discovered by Tswett in the form of liquid-solid chromatography (LSC), but its development continued for over 50 years primarily in the form of gas chromatography (GC) and partially as thin-layer and liquid-liquid chromatography. Rebirth of liquid chromatography in its modern form and its enormously fast growth had driven this to be the dominant analytical technique in the twenty-first century which can be attributed in the most part to the pioneering work of Prof. **Csaba Horvath** at Yale

University. In the mid-1960s Prof. Horvath, who previously worked on the development of a porous layer open-tubular columns for GC, had decided to use for LC small glass beads with porous layer on their surface to facilitate the mass transfer between the liquid phase and the surface. Columns packed with those beads developed a significant resistance to the liquid flow, and Prof. Horvath was forced to build an instrument that allowed development of a continuous flow of the liquid through the column. This was the origin of high-performance liquid chromatography (HPLC), and the actual name for this separation method was introduced by Prof. Horvath in **1970** at the Twenty-first Pittsburgh Conference in Cleveland, where he gave this title to his invited talk. The acronym HPLC originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 3.5 MPa (500 psi; 35 bar). This was called **high pressure liquid chromatography**, or HPLC. The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 40 MPa (6,000 psi; 400 bar) of pressure, and incorporated improved injectors, detectors, and columns. HPLC really began to take hold in the mid-to late-1970s.

With continued advances in performance during this time (smaller particles, even higher pressure), the acronym HPLC remained the same, but the name was changed to **high performance liquid chromatography**. Later in 2001, Horvath further defined the meaning of the word “performance” as “an aggregate of the efficiency parameters” such as efficiency, selectivity, reproducibility, speed, sensitivity, automation, data handling, versatility and complete control over operational variables. The first separation on a chemically modified surface with an aqueous eluent, which later got the name “**reversed-phase**,” was also invented by Horvath, he demonstrated the first reversed-phase separation of fatty acids on pellicular glass beads covered with graphitized carbon black (Figure I). High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals.[3, 5]



**Figure I.** Separation of fatty acids on pellicular graphitized carbon black from the mixture of ethanol and  $10^{-4}$  M aqueous NaOH. Refractive index detection [5]

### 1.2.3 UHPLC / UPLC® and Future

In 2004, further advances in instrumentation and column technology were made to achieve very significant increases in resolution, speed, and sensitivity in LC. Columns with sub 2-micron porous particles (1.7 micron) and instrumentation with specialized capabilities designed to deliver mobile phase at 100 MPa (15,000 psi; 1,000 bar) were needed to achieve a new level of performance. A new system had to be holistically created to perform ultra-performance liquid chromatography, now known as UPLC® technology (UltraPerformance LC® and UPLC® are registered trademarks of Waters Corporation). As vendors entered the market with similar instruments UHPLC (Ultra-high performance liquid chromatography) was coined as a way to refer to instruments similar to UPLC®. Thus UHPLC and UPLC® in fact are the same techniques and these two terms are understood to be synonymous.

In 2005, just year after launching the UPLC® technique by Waters, Novakova *et al.* published paper called “*Advantages of application of UPLC in pharmaceutical analysis*” in which they concluded UPLC® advantages compared to HPLC: The separation mechanisms are still the same, chromatographic principles are maintained while speed, sensitivity and resolution is improved. This all supports easier method transfer from HPLC to UPLC and its revalidation. The main advantage was particularly a significant reduction

of analysis time, which meant also reduction in solvent consumption. Experiments showed 4.7–6.9 times analysis shortening, while solvent consumption decreased 5.6–8.5 times. From this point of view, UPLC® is more convenient for complex analytical determination of pharmaceutical preparations. Analysis duration, solvent consumption and consequently analysis cost is a very important aspect in many analytical laboratories. Moreover, the time spent with new method optimization is saved. The time needed for method development experiments, for column equilibration or re-equilibration while using gradient elution and for method validation is much shorter.

Today the basic research is being conducted by scientists working with columns containing even smaller (1-micron-diameter) particles and instrumentation capable of performing at 690 MPa (100,000 psi; 6,900 bar). This provides a glimpse of what we may expect in the future. [3, 6-8]

#### **1.2.4 Nobel Prizes Related Directly/Indirectly to Chromatography**

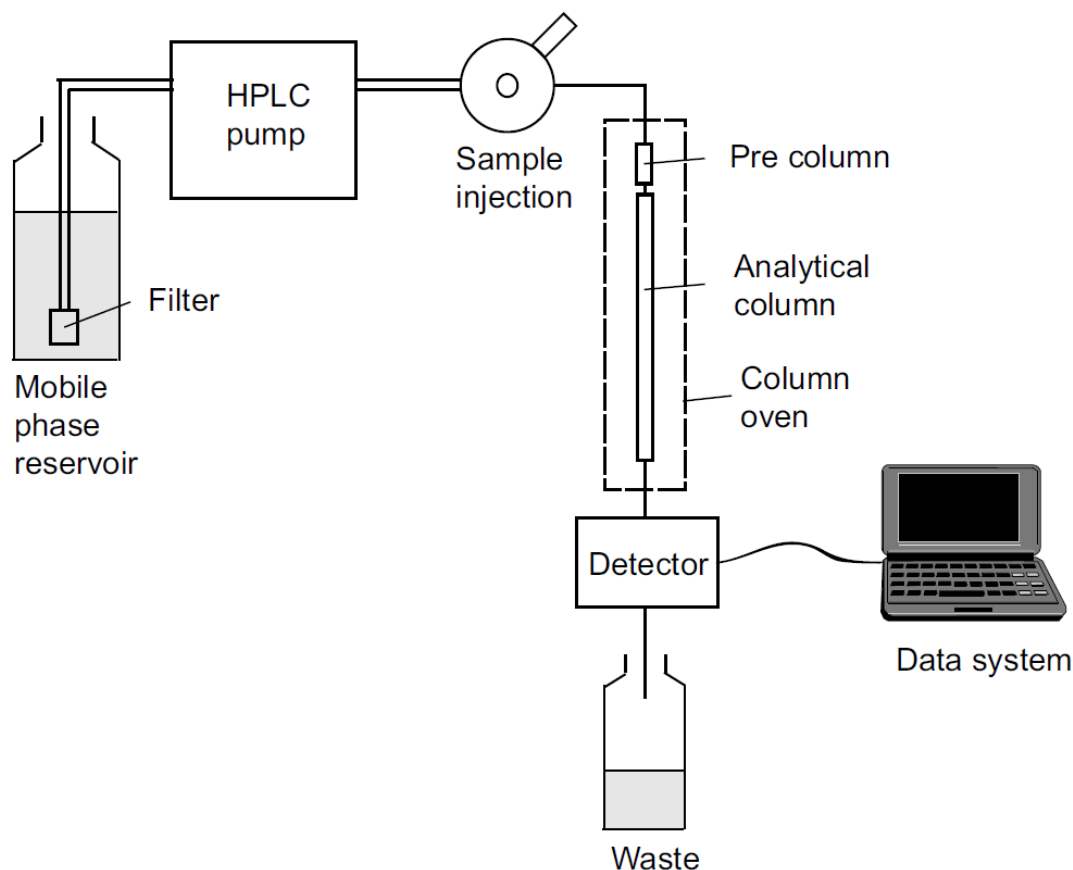
- The Nobel Prize in Chemistry **1948** was awarded to **Arne Tiselius** "for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins". (Note: According to the IUPAC definition of chromatography, separation techniques making use of electric current, *i.e.*, the electrophoretic techniques, in a formal way also count as chromatography.)
- The Nobel Prize in Chemistry **1952** was awarded jointly to **Archer John Porter Martin** (1/2) and **Richard Laurence Millington Syngé** (1/2) "for their invention of **partition chromatography**": When a drop of a liquid containing a mixture of various substances is placed on paper, the liquid begins to spread out on the paper. The various substances in the mixture spread at different speeds, however, which gives rise to marks on the paper with different colors. In the 1940s Archer Martin and Richard Syngé used this and similar phenomena in gas mixtures, for example, to develop **different types of chromatography - methods for separating substances in mixtures and for determining the composition of mixtures**.
- The Nobel Prize in Chemistry **1958** was awarded to **Frederick Sanger** "for his work on the **structure of proteins, especially that of insulin**": Proteins, which are molecules made up of chains of amino acids, play a pivotal role in life processes in our cells. One important protein is insulin, a hormone that regulates sugar content

in blood. Beginning in the 1940s, Frederick Sanger studied the composition of the insulin molecule. **He used acids to break the molecule into smaller parts, which were separated from one another with the help of electrophoresis and chromatography.** Further analyses determined the amino acid sequences in the molecule's two chains, and in 1955 Frederick Sanger identified how the chains are linked together.

- The Nobel Prize in Chemistry **1972** was divided, one half awarded to Christian B. Anfinsen "for his work on ribonuclease, especially concerning the connection between the amino acid sequence and the biologically active conformation", the other half jointly to **Stanford Moore and William H. Stein** "for their contribution to the understanding of the connection between chemical structure and catalytic activity of the active centre of the ribonuclease molecule".
- The Nobel Prize in Chemistry **2002** was awarded "for the development of methods for identification and structure analyses of biological macromolecules" with one half jointly to **John B. Fenn** and Koichi Tanaka "for their development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules" and the other half to Kurt Wüthrich "for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution". John B. Fenn's work: When electrically charged molecules - ions - are accelerated by an electrical field, their speed depends on the ion's charge and weight. By measuring the time it takes for the ions to pass a certain distance, the incidence of different molecules in a test can be determined. It was impossible, however, to use this technique on large molecules, such as proteins, before large ions could be produced in gaseous form. In 1988 John Fenn showed that when a test sample is sprayed with an electrical field, small charged drops are formed, and when the water evaporates, ions in gaseous form remain. This is in fact the basic principle of widely used **liquid chromatography – mass spectrometry ionization technique, electrospray**.<sup>[9-19]</sup>

## 1.3 LC System

The main components of an LC system are shown in Figure II.



**Figure II.** Main structure of an LC system [1]

The separation columns are typically made of steel tubes 5–25 cm long and packed with the stationary phase. It is in the column that substances are separated and thus the column is “the heart” of the chromatograph. The other parts of the chromatograph are optimized individually and carefully put together to optimize the separation efficiency of the total system.

The three main parts of the LC system: the solvent delivery, the separation column and the detector are all vital and indispensable units. The separation occurs when the mobile phase is pumped at a constant flow rate through the column bringing the separated analytes to the detector.



In a standard analytical HPLC system the typical flow rate of the mobile phase through the column is 0.5–2.0 ml/min but can be in the range from 0.01 to 10 ml/min. The small particle size of the column packing materials result in a back pressure of 3–30 MPa (440–4400 psi; 30–300 bar) and in certain cases (UHPLC) up to 120 MPa (17,400 psi; 1200 bar), when the mobile phase is pumped through. The pumps used must be able to pump the mobile phase at a constant flow rate against a high pressure. Any particle in the samples injected collects at the top of the column and gradually blocks the column, with an increase in back pressure as the result. This results in a decrease in flow rate that compromises the analytical result. Therefore HPLC pumps are equipped with a regulation mechanism that keeps the flow rate constant, and a gradual blockage thus results in an increase in back pressure while the flow is kept constant.

Degassing may be required for efficient pump and detector operation, particularly for aqueous organic mobile phase and for gradient forming devices using low pressure mixing. Degassing is used to prevent gas bubble formation when different solvents are mixed or the mobile phase is depressurized. Dissolved oxygen in the mobile phase is a source of reduced sensitivity and poor baseline stability for ultraviolet, fluorescence and electrochemical detectors. Degassing can be carried out by applying a vacuum above the solvent, heating with vigorous stirring, ultrasonic treatment, sparging with helium or by using a gas permeable membrane. The latter principle is widely used in on-line solvent degassers – the solvent flows through a narrow-bore polymer tube housed in a chamber connected to vacuum.

The substances to be separated must be dissolved in a liquid that is miscible with and not stronger eluting than the mobile phase. Typical injection volumes are 5–100  $\mu\text{L}$ . The injection systems are optimized to inject the solution under high pressure directly into the flowing mobile phase just before the column inlet.

The detectors provide an electronic response to the analytes. The response is processed by a computer system that displays the results as chromatograms. The whole analysis process can be automated and controlled by the computer system. When an autosampler is used as the injector the LC system can work 24 h daily. Analytical chemical data, including quantitative calculations can be reported continuously by the computer system.

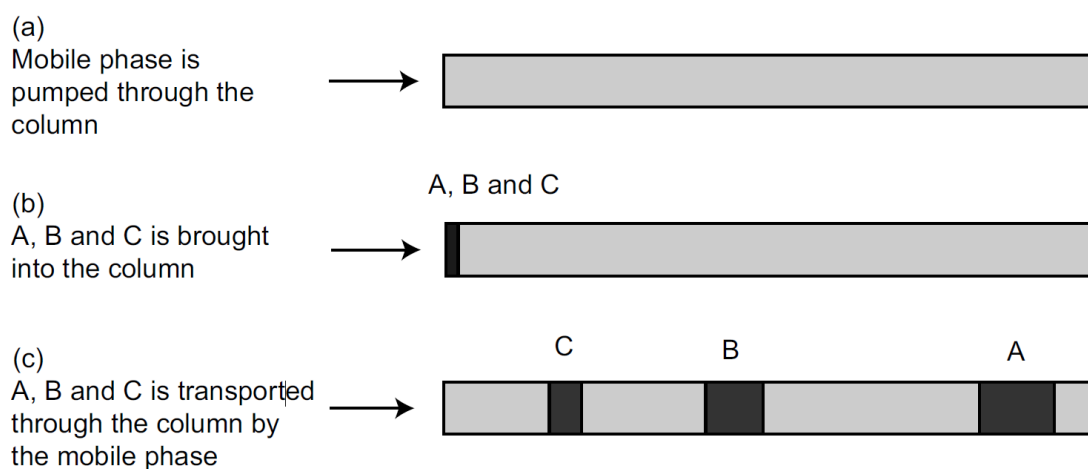
The broad applicability and the high degree of automation of HPLC are among the reasons why this technique has gained such a dominant position in analysis of food,

pharmaceuticals and biofluids. HPLC is important in drug research and development because:

- HPLC with UV detection provides accurate, precise and robust methods for the quantitative determination of drugs in pharmaceutical preparations.
- HPLC is very suitable for monitoring the stability of drug substances and drugs in drug preparations and for quantifying decomposition.
- HPLC is very well suited for the determination of drugs and their metabolites in biological material.[1, 20]

## 1.4 General Principles of Liquid Chromatography

There are two phases in chromatography: a phase that moves (**mobile phase; MP**) and a phase that is stationary (**stationary phase; SP**). In LC the SP is solid particles that are packed into a tube, so that they remain stationary inside the tube. The tube with the SP is called a **column**. The MP is pumped through the column at a certain speed. If the SP retards the transport of the components through the column, they will migrate through the column at a lower speed than the MP itself. If the SP retards the components of the sample differently, the components will migrate with different speeds through the column. This is illustrated in Figure III. In this example component A is not retained by the SP and consequently A migrates through the column with the same speed as the MP. Even if the analyte molecules do not interact with the column packing material, the analyte needs some time to pass through the column. This time is usually called **hold-up time, dead time, or void time**. The corresponding volume is either **the void volume, the volume of the liquid phase in the column, or the dead volume**. Analyte retention volume that exceeds the column void volume is essentially the volume of the MP which had passed through the column while analyte molecules were retained by the packing material. The SP retards substance B so that its migration speed is slowed down, and the drug substance B takes longer to migrate through the column than substance A. Substance C is even more retarded than B and migrates at the slowest speed through the column. When the differences in retention are sufficiently large, the components elute from the column at different times and are separated.



**Figure III.** Chromatographic separation of components A, B, and C [1]

Differential migration of individual components through the column depends on the equilibrium distribution of each component between the SP and the MP. Therefore, differential migration is determined by those experimental variables that affect this distribution, such as the composition of the MP, the composition of the SP and the temperature. If we want to alter migration to improve separation, we must change one of these variables. The equilibrium distribution is given by the **distribution constant**:

$$K_C = \frac{C_s}{C_m}$$

*Equation 1. The distribution constant [1]*

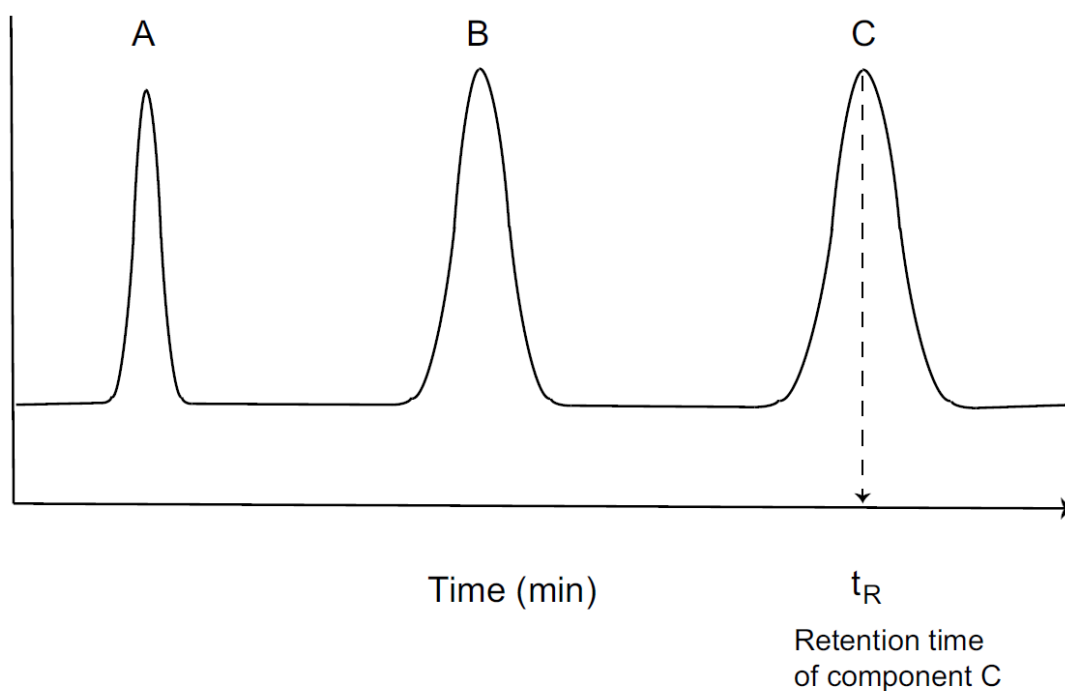
where  $C_s$  is the concentration of component in the SP and  $C_m$  is the concentration of component in the MP. The distribution constant is also called the equilibrium distribution coefficient. The speed by which each component migrates through the column is determined by the number of molecules of that component which is in the MP at any instant, since sample molecules do not move through the column while they are in the SP. Retention of a component is therefore determined by its distribution constant. Components with a large distribution constant have a large portion of its molecules in the SP and these components are strongly retained in the column. Components with a small distribution constant have a small portion of its molecules in the SP and these are less retained. In a LC system the sample solution is introduced into the flow of MP using an injector, which is placed in front of the column. A detector detects compounds that are eluted from the column. Such a configuration is shown in Figure IV.



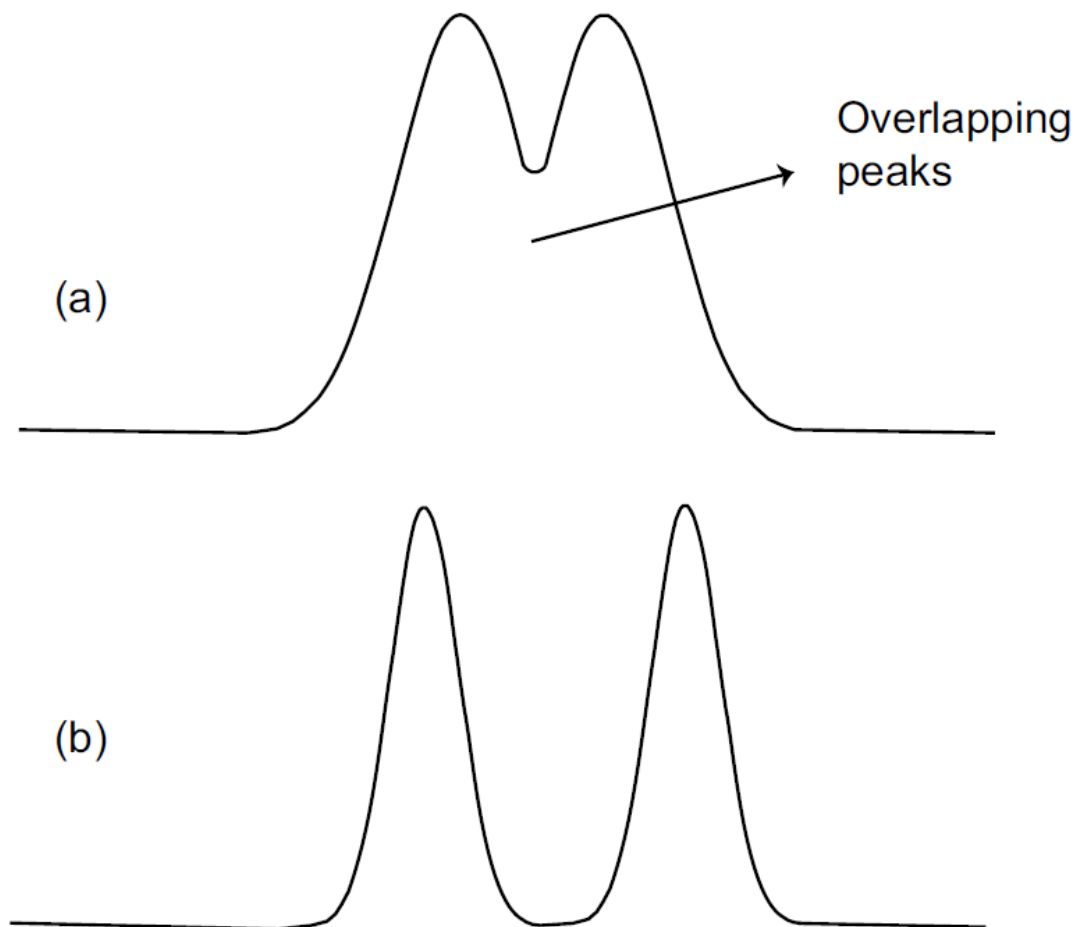
*Figure IV. Typical configuration of LC system [1]*

In a mentioned setup with an injector, a column and a detector, it is possible to measure the detector response of a component eluting from the column as a function of time. If the detector response to compounds eluted from the column is recorded as a function of time after injection, the visual output of the separation is called a **chromatogram**. The idealized chromatogram is a sequence of Gaussian peaks on a baseline. The time from injection of

the sample to maximum detector response of a substance is called the **retention time**  $t_R$  (Figure V). In a given chromatographic system the retention time of a substance is characteristic for its physicochemical and physical properties and is used for **identification**. For the substances to be separated completely the detector response must reach a baseline between the chromatographic peaks, otherwise the peaks are overlapping (Figure VI).[1, 5]



**Figure V.** Chromatogram of the sample containing components A, B, and C [1]



**Figure VI.** Chromatogram of (a) two overlapping peaks and (b) two completely separated peaks [1]

### 1.4.1 Retention

To provide a measure of the substance's retention it is common to use retention times. The retention times are affected, however, by several factors such as the speed of the MP and the column length. A parameter that is independent of these factors is the **retention factor  $k$** . The retention factor shows how the amount of the drug substance is distributed between the SP and the MP. When the retention factor is equal to 1, the compound will be distributed equally between the SP and the MP. When the retention factor is 5, there will be five times more of the compound in the SP than in the MP.

The retention factor of a component may be determined from the chromatogram using the following equation:

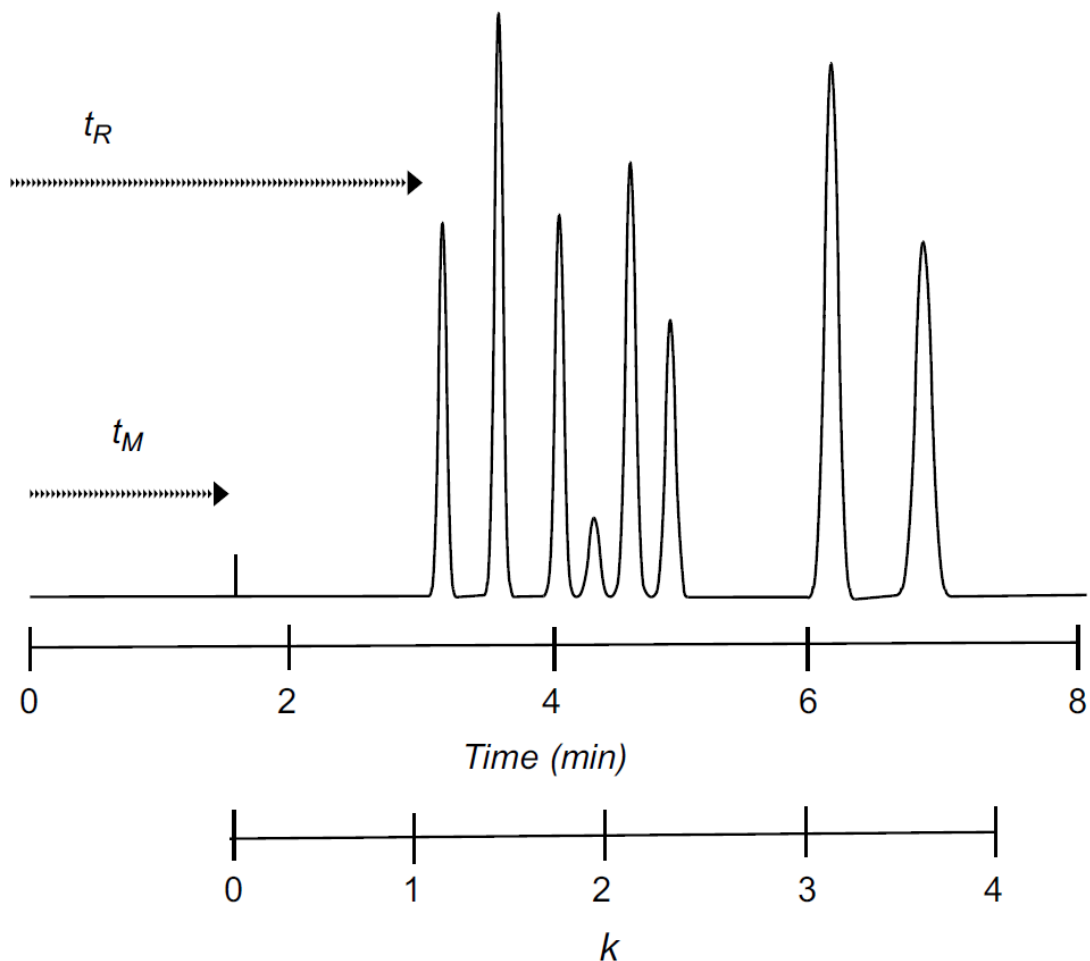
$$k = \frac{t_R - t_M}{t_M}$$

**Equation II. Retention factor ( $k$ ) [1]**

The expression  $t_R - t_M$  is the time the compound stays in the SP, while  $t_M$  is the time the compound stays in the MP or in other words  $t_M$  is the retention time of component that migrates through the column with the same speed as the MP and it is the time taken to pass through the void volume of the column.

Assume that a compound has a retention time  $t_R$  of 6 min and that  $t_M$  is 1 min. The retention factor  $k$  is then  $(6 - 1)/1 = 5$ . Of the total time the molecules spend in the column (6 min) they will move with the MP for one-sixth of the time and be temporally stagnant on the SP for five-sixths of the time.

Figure VII shows a chromatogram in which both the retention time and retention factor are shown. The substances have retention times between 3.2 and 7.0 min and retention factors between 1.3 and 3.8.[1]



**Figure VII.** Chromatogram showing retention times ( $t_R$ ) and retention factors ( $k$ ) [1]

### 1.4.2 Selectivity

The relative retention of two adjacent peaks in the chromatogram is described by the separation factor  $\alpha$  given by the equation:

$$\alpha = \frac{k_2}{k_1}$$

**Equation III.** Separation factor ( $\alpha$ ) [1]

where  $k_2$  is the retention factor of the later of the two eluting peaks, and  $k_1$  is the retention factor of the first eluting peak. The **separation factor is a measure of the selectivity** of a chromatographic system. It is a constant for a given set of analytical conditions and is independent of the column type and column length. The separation factor has values greater or equal to 1.0. When  $\alpha = 1$ , separation is not possible.[1]



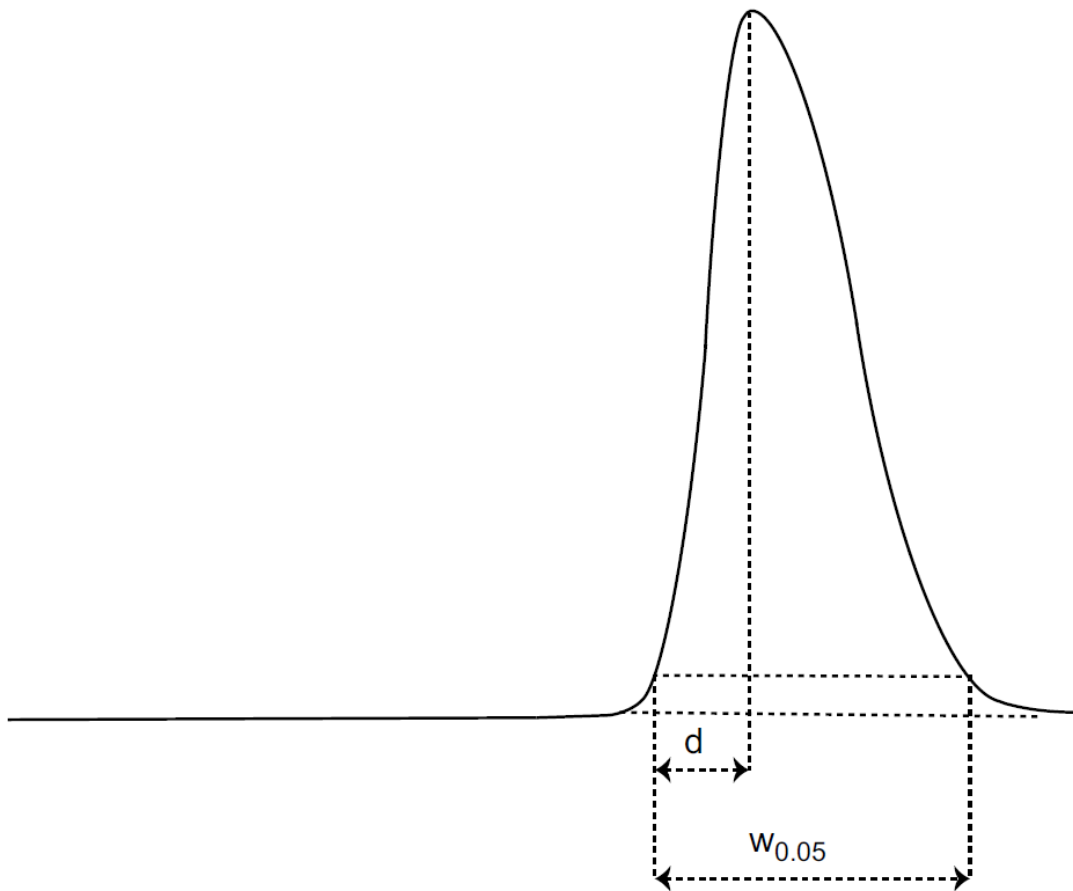
### 1.4.3 Peak Symmetry

The **symmetry factor**  $A_s$  is used to signify peak symmetry. The symmetry factor is calculated using the following equation:

$$A_s = \frac{w_{0.05}}{2d}$$

*Equation IV. The symmetry factor ( $A_s$ ) [1]*

where  $w_{0.05}$  is the width of the peak at one-twentieth of the peak height and  $d$  is the distance between the perpendicular dropping from the peak maximum and the leading edge of the peak at one-twentieth of the peak height. An  $A_s$  value of 1.0 signifies symmetry. When  $A_s > 1.0$ , the peak is **tailing**. When  $A_s < 1.0$ , the peak is **fronting**. The peak shown in Figure VIII is a tailing peak.[1]



*Figure VIII. Parameters used to calculate peak symmetry ( $A_s$ ) [1]*

### 1.4.4 Resolution

The degree of separation between two peaks is defined as the **resolution**,  $R_s$ :

$$R_s = 1.18 \frac{t_{R_2} - t_{R_1}}{w_{h_1} + w_{h_2}}$$

*Equation V. Resolution,  $R_s$  (Ph. Eur.) [1]*

where  $t_{R_2}$  and  $t_{R_1}$  are the retention times of peaks 2 and 1 and  $w_{h_2}$  and  $w_{h_1}$  are the corresponding peak widths at half peak height. The European Pharmacopoeia (Ph. Eur.) uses the equation above.

When the peak width is measured between tangents drawn through the inflection points, the equation is then:

$$R_s = \frac{2(t_{R_2} - t_{R_1})}{w_1 + w_2}$$

*Equation VI. Resolution,  $R_s$  [1]*

A value of  $R_s = 1.0$  corresponds to a peak separation of 94% and **baseline separation corresponds to an  $R_s$  value of 1.5**.

The resolution increases with the square root of the plate number, thus the **plate number must increase fourfold to increase resolution by a factor of 2**. Resolution is strongly influenced by the separation factor and increases with increasing values of selectivity  $\alpha$ . [1]

### 1.4.5 The van Deemter Equation

The molecules in a sample are exposed to a number of physical actions on their transport through the chromatographic system. The molecules are introduced in the MP and interact with the SP during the chromatographic process. One of the important parameters for the result of the separation is the flow rate of the MP. In the 1950s van Deemter and coworkers were already studying the effect of the MP on the efficiency of the GC separation and they could express the efficiency in this formula – **the van Deemter equation**:

$$H = A + \frac{B}{u} + Cu$$

*Equation VII. The van Deemter equation [1]*

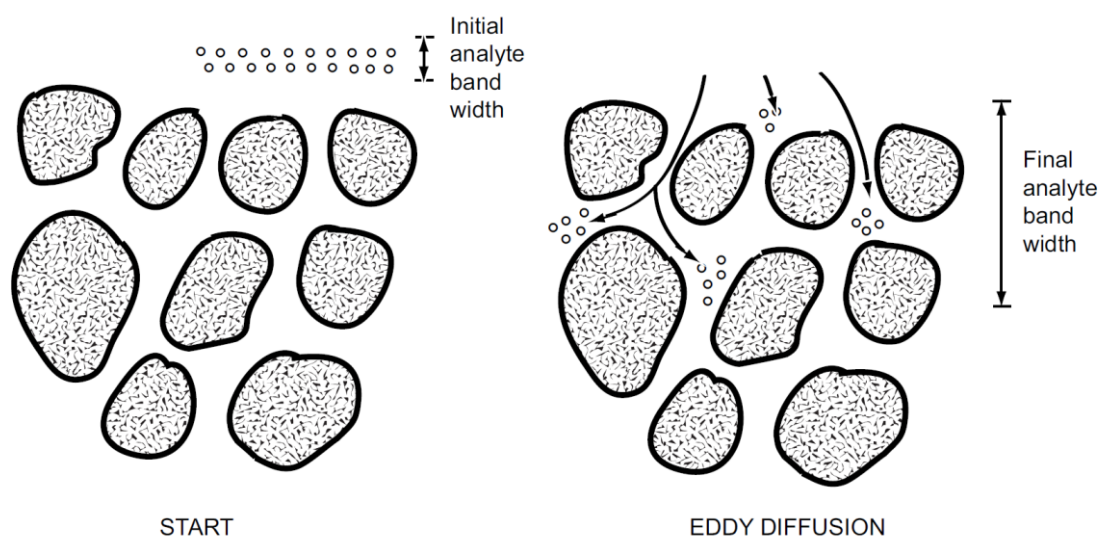
This formula expresses the correlation between the efficiency given by  $H$ , the height equivalent to a theoretical plate (HETP), and the band broadening phenomena  $A$ ,  $B$  and  $C$  as a function of the flow rate,  $u$ .

$A$  – represents multipath effect or eddy diffusion

$B$  – represents molecular diffusion

$C$  – represents mass transfer

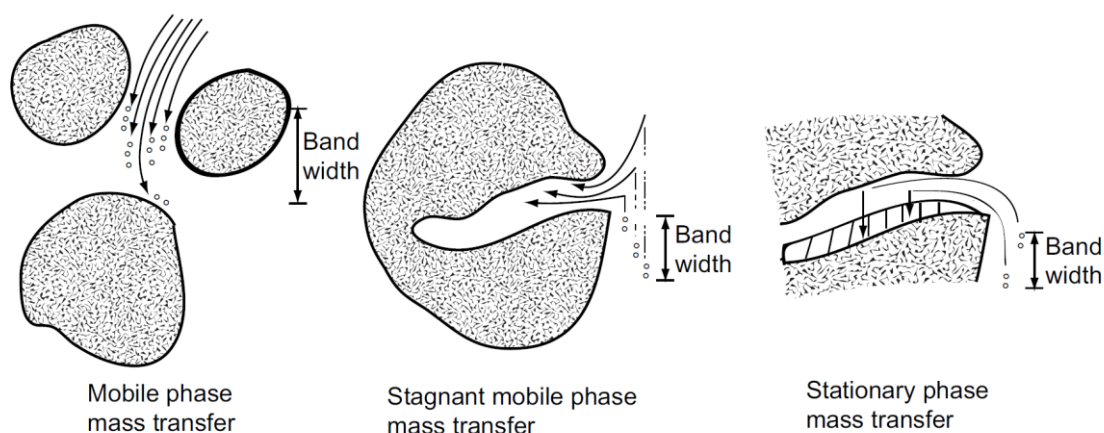
The contribution to the **A term** comes from the fact that identical molecules travel different distances on their way through the chromatographic system due to the **non-uniform packing** of the particles of the SP (Figure IX). The contribution of the A term to  $H$  for a given chromatographic system is more or less independent of the flow rate.



**Figure IX.** Peak broadening due to eddy diffusion – **A term** [1]

The **B term** takes account of the diffusion of the molecule (Brownian movements) in the MP. Drug molecules dissolved in a liquid always diffuse away from areas in the liquid with a high drug concentration towards areas of lower concentration until the concentration of the substance is the same throughout the fluid volume. The analyte molecules diffuse in all directions and the radial diffusion does not affect the efficiency. But the diffusion in the direction of the length of the column, the **longitudinal diffusion, results in band broadening especially if the flow rate is low**. The B term becomes insignificant at higher flow rates. The B term is of higher significance in GC than in HPLC due to higher diffusion at higher temperatures and to less hindrance of diffusion from other MP molecules.

The **C term** covers the mass transfer between the two phases (Figure X). This involves the direct back and forth transfer of molecules between the mobile and the stationary phases. A peak broadening results as the drug molecules at a given time period retarded in the SP are stationary while the drug molecules in the MP are not retarded and thus pushed ahead by the MP. Mobile mass transfer takes place as the flow rate through channels is different in the middle compared to close to the side walls of the channels where the flow rate approaches zero. Both phenomena give rise to a broadening of the analyte into a larger volume. Mass transfer in the SP also involves the transfer to stagnant pools of SP in closed pores of the porous particles.



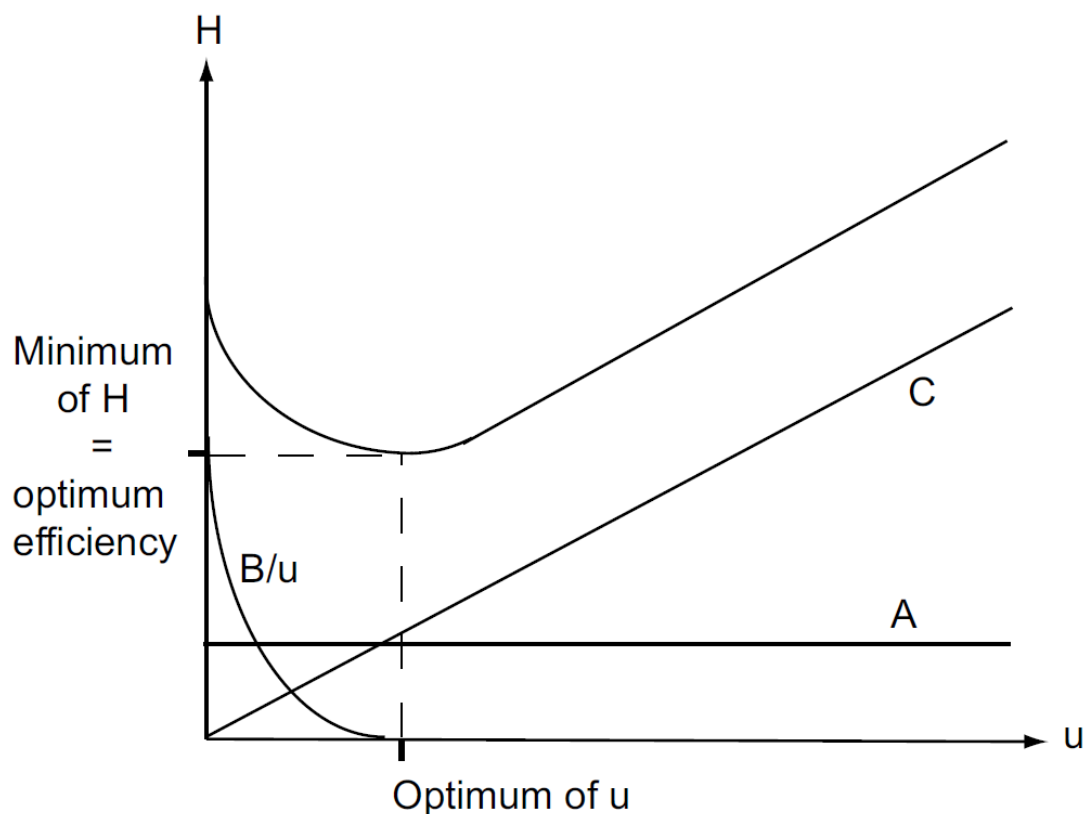
**Figure X.** Mass transfer in mobile and in stationary phase – **C term** [1]

A more detailed equation used for HPLC could be:

$$H = C_e dp + \frac{C_m dp^2 u}{D_m} + \frac{C_d D_m}{u} + \frac{C_{sm} dp^2 u}{D_m} + \frac{C_s d_f^2 u}{D_s}$$

**Equation VIII.** The van Deemter equation – reworked and more detailed [1]

where  $D_m$  and  $D_s$  are the diffusion in the MP and in the SP, respectively,  $dp$  is the particle diameter of the particles constituting the chromatographic bed and  $d_f$  is the layer thickness of the SP.  $C_e$ ,  $C_m$ ,  $C_d$ ,  $C_{sm}$  and  $C_s$  are constants. In this equation special consideration has been given to diffusion and mass transfer in the mobile as well as in the stationary phases. From this it is obvious that the **particle diameter,  $dp$ , is of major importance and the plate height decreases when the particle diameter decreases**. A smaller plate height gives room for more plates in a given column length and the efficiency thus increases. The van Deemter equation can also be visualized as shown in Figure XI.[1, 5]



**Figure XI.** The van Deemter plot showing the optimum efficiency (at minimum  $H$ ) and the optimum flow rate [1]

### 1.4.6 Pressure Drop

The ability to increase the MP linear velocity,  $u$ , depends on the pressure capabilities of the instrument, since pressure is directly proportional to velocity:

$$\Delta P = \frac{uL\eta\phi}{dp^2}$$

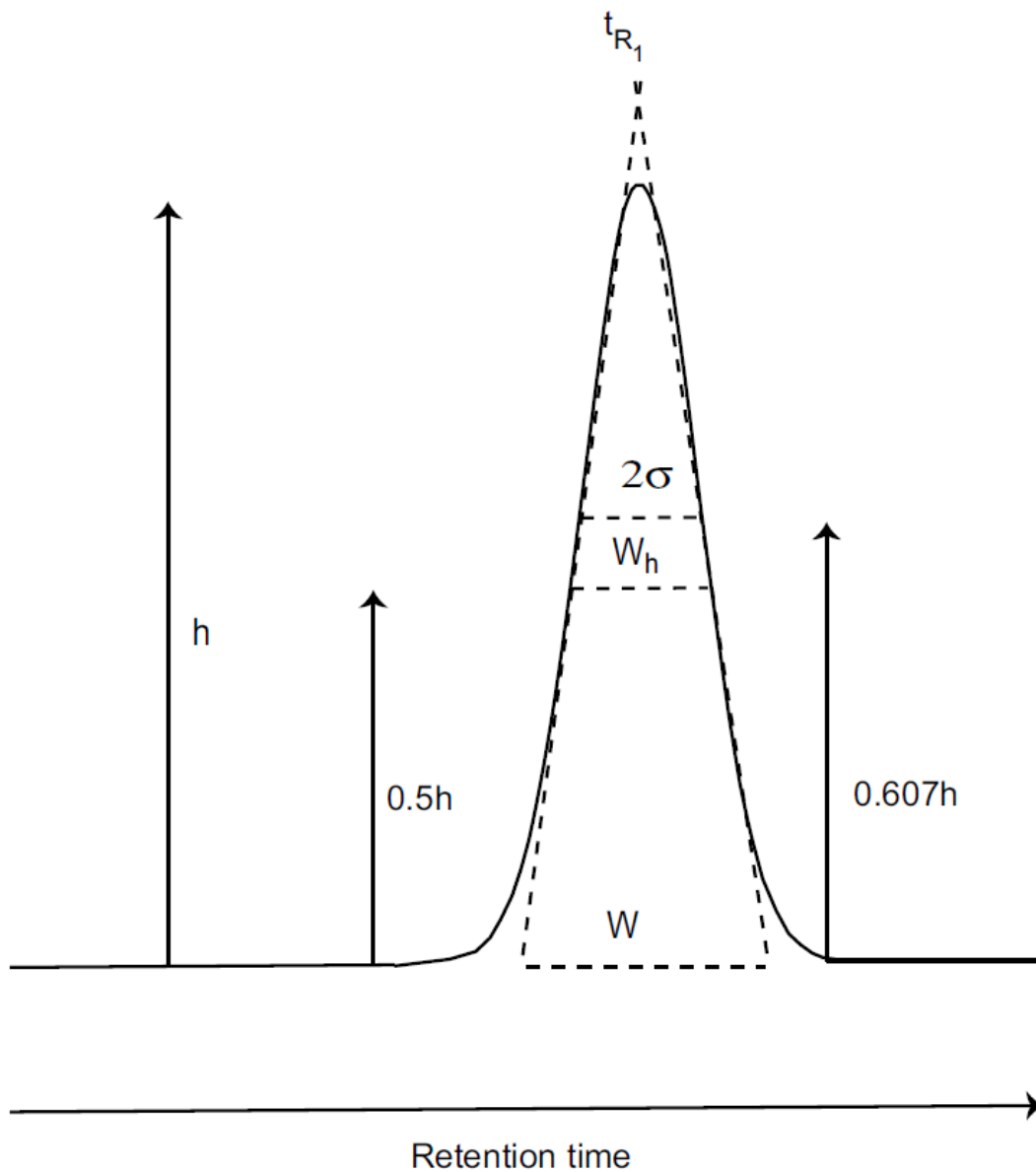
**Equation IX.** Pressure drop across the column [5]

where  $\Delta P$  is the **pressure drop across the column**,  $\eta$  is viscosity,  $L$  is column length, and  $\phi$  is the flow resistance factor. Thus the speed of analysis is limited by the maximum pressure capability of the instrument. As a result, the most should be made of the pressure available by reducing the pressure drop across the column as much as possible. **Decreasing the column length** lowers the pressure requirement proportionally, allowing use of the available pressure to gain an advantage in speed. Column efficiency, however, drops with use of a shorter column and at faster velocities. Care must therefore be taken to ensure that

resolution between peaks is not lost when decreasing analysis time in this manner. **Lowering the viscosity of the MP** is another way to lessen the required pressure. This may be accomplished by **raising the column temperature**. Increasing temperature has the double advantage of allowing use of a higher flow velocity and speeding up diffusion. This is a strong motivator for the use of temperature above ambient conditions in order to speed up the separation. Of course, sample degradation, the boiling point of our MP, stability of the SP, and the capability of the column heater limit the maximum temperature that can be used. Temperatures up to about 70°C are considered routine; beyond that, columns and heaters specifically designed for high-temperature chromatography are needed. Much research has been done in the area of elevated-temperature chromatography, where interesting possibilities arise, such as the use of temperature gradients and purely aqueous mobile phases.[5]

### 1.4.7 Column Efficiency

The peak width expresses how efficiently a compound is transported through the column. If the molecules to a small extent are spread throughout the column, peaks widths are small. The narrower the peaks are in relation to their retention times, the more effective is the column. Peak broadening throughout the column is expressed by the parameter  $N$ , called **the number of theoretical plates or plate number**. A Gaussian peak as shown in Figure XII is characterized by its retention time  $t_R$  and the standard deviation  $\sigma$ .



**Figure XII.** Parameters used to measure peak widths [1]

The plate number is defined as:

$$N = \left( \frac{t_R}{\sigma} \right)^2$$

**Equation X.** Plate number  $N$  [1]

In Gaussian peaks the peak width is  $2\sigma$  at the points of inflection, which are at  $0.607 h$ . In practice it is difficult to measure peak widths accurately at  $0.607 h$ , and in the Ph. Eur. the

**peak width  $W_h$  at half-height**,  $0.5 h$ , is measured instead. The relationship between  $W_h$  and  $\sigma$  is given by:

$$W_h = 2.354\sigma$$

*Equation XI. The relationship between  $W_h$  and  $\sigma$  [1]*

and  $N$  is estimated by the following equation:

$$N = 5.54 \left( \frac{t_R}{W_h} \right)^2$$

*Equation XII. The relationship between  $N$ ,  $t_R$  and  $W_h$  [1]*

Another way to estimate  $N$  is to measure **peak width at the baseline,  $W$** , defined as the width between tangents drawn through the inflection points as shown in Figure XII. The relationship between  $W$  and  $\sigma$  is:

$$W = 4\sigma$$

*Equation XIII. The relationship between  $W$  and  $\sigma$  [1]*

and the plate number  $N$  is calculated by the equation:

$$N = 16 \left( \frac{t_R}{W} \right)^2$$

*Equation XIV. The relationship between  $N$ ,  $t_R$  and  $W$  [1]*

The narrower peaks are in relation to the retention time the greater  $N$  will be. The greater  $N$  is, the more efficient is the column to give narrow peaks in relation to the retention time,  $t_R$ . The quantity  $N$  is proportional to column length  $L$ , so that when other factors are equal, **an increase in  $L$  results in an increase in  $N$** . The proportionality of  $N$  and  $L$  can be expressed as:

$$H = \frac{L}{N}$$

*Equation XV. The relationship between  $H$ ,  $L$  and  $N$  [1]*



where ***H*** is the height of a single theoretical plate, also called as **the height equivalent to a theoretical plate (HETP)**. Small values of *H* mean large *N* values and efficient columns. By using *H* instead of *N*, one can compare the efficiency of columns with different length.

Since it is not valid to calculate theoretical plates under MP gradient conditions, **peak capacity** ( $n_p, P_c$ ) is used as an alternative measure of the separating power of a system. Peak capacity is defined as the total number of peaks separable with unit resolution in a given separation space and it is suitable for the column efficiency evaluation both in gradient and isocratic elution. Peak capacity can be estimated by the following equation:

$$n_p = 1 + \frac{t_g}{W}$$

*Equation XVI. Peak capacity  $n_p$  [21]*

where  $n_p$  is the number of peaks,  $t_g$  is the gradient time and  $W$  is the peak width at the baseline value. [1, 5, 21-22]

## 1.5 Chromatographic Separation Principles

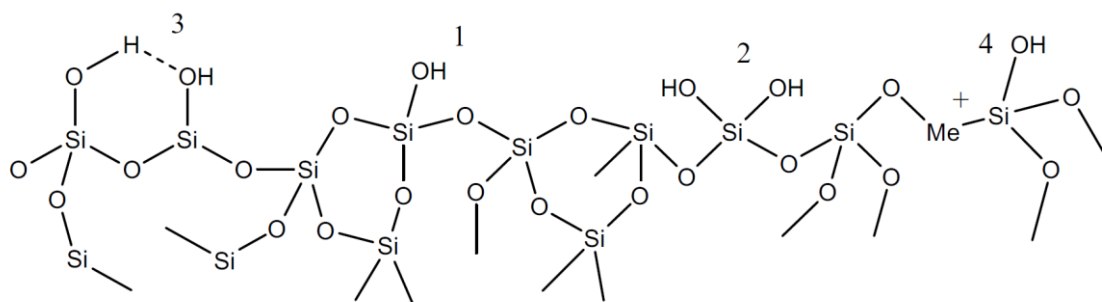
### 1.5.1 Normal Phase Chromatography

Normal phase chromatography is the most common separation principle in thin-layer chromatography (TLC) but can also be performed in HPLC/UHPLC mode. A **polar SP** is used along with a **more nonpolar MP**. A typical choice could be the use of silica as the SP and a heptane-ethyl acetate or a heptane-propanol mixture as the MP. When analytes enter such a system the analytes interact with both the stationary and the mobile phase. Analytes with no affinity to (no interaction with) the polar stationary silica are not retained and travel with the speed of the MP. Analytes having polar functional groups have a higher affinity to the polar silica and show retention in the system. Adsorption onto the stationary silica is a reversible interaction, and an increase in the polar component of the MP increases the competition for the adsorption sites on the surface, thus weakening the interaction of the analyte with the silica. When the elution strength of the MP is increased, the retention of analytes decreases.

Silica, or silica gel, is the most important SP in normal phase chromatography. Silica has strong adsorption characteristics and is among other things used as a desiccant and many substances can be adsorbed onto silica. Normal phase chromatography is also called adsorption chromatography. Silica has typically a large surface area in the range 200–800 m<sup>2</sup>/g. The large surface area is due to the structure of the silica being a porous material similar to a sponge. When the MP flows through the silica, it enters the entire volume between the particles and the whole pore volume inside the particles. This provides a tremendous network of contacts between the stationary and the mobile phase and analytes are exposed to this surface area of SP. The silica is covered with silanol groups on the surface (–Si–OH) which are the adsorptive groups (Figure XIII). The silanol groups make the surface polar and behave as weak acids. The structure differences between silanol groups mean that they have different activity. If a silanol group forms a hydrogen bond to a neighboring group it will be less active in adsorption processes than an isolated one.

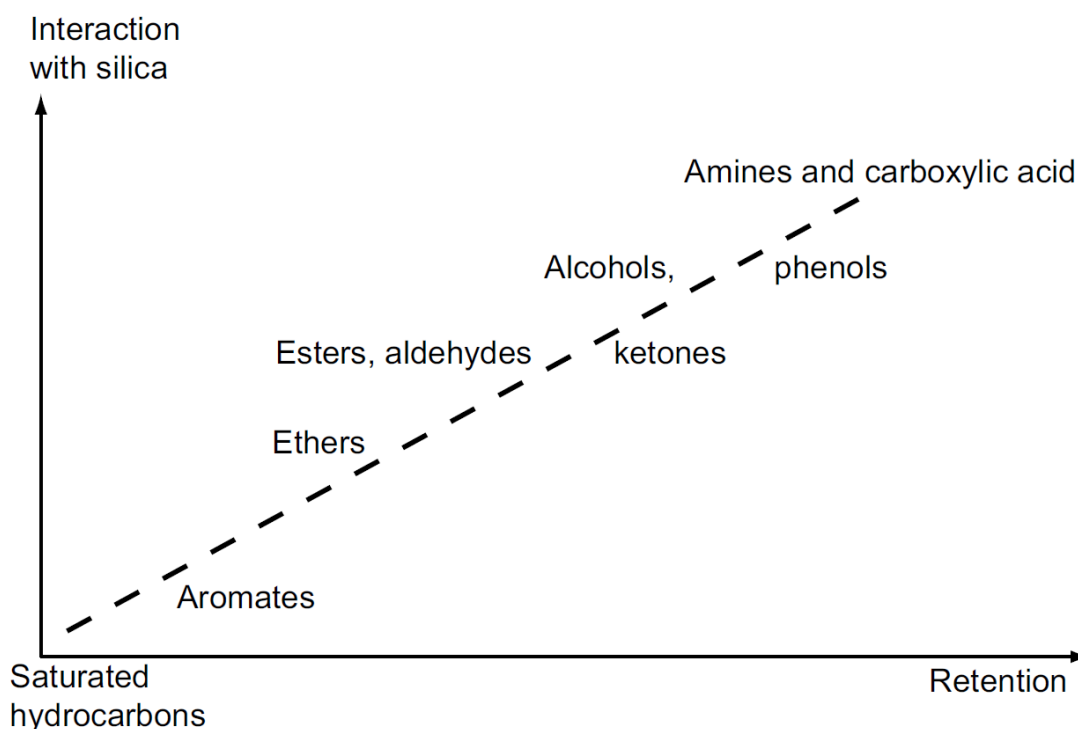
The interactions between silanol groups on silica and functional groups on the substances to be separated are called polar interactions and are divided into four main groups:

- 1) Dispersion interactions – weak; aromatics
- 2) Dipole–dipole interactions – stronger than 1);  $CN > NO_2 > C=O, CHO > COOR > \text{halogen} > OH > COOH > -O- > NH_2$
- 3) Hydrogen bonding interactions – stronger than 1) and 2); carboxylic acids and phenols; amines
- 4) Ionic interactions – undesirable; very strong; can be avoided, *e.g.*, by masking the most acidic silanol groups



**Figure XIII.** Silanol groups: (1) free (isolated) silanol; (2) geminal silanols; (3) associated silanols; (4) metal activated silanol on the surface of silica [1]

Saturated hydrocarbons do not have any interactions with silica and thus have no retention, on the opposite end stand primary amines and carboxylic acids that provide the strongest hydrogen bonding interactions and are retarded strongly by silica (Figure XIV). Retention increases with the number of functional groups. A substance with two hydroxyl groups is retarded more than a drug with only one hydroxyl group.



**Figure XIV.** Retention of different functional groups as a function of the interaction with silica [1]

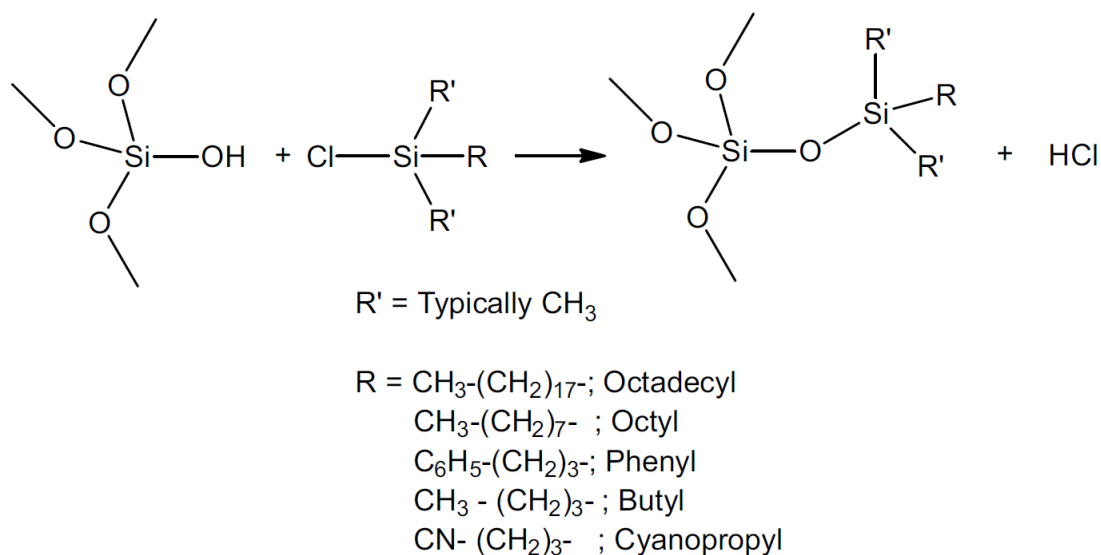
In normal phase chromatography the MP is more nonpolar than the SP and therefore made of organic solvents. Solvent strength increases with increasing polarity. Increased solvent strength of MP decreases the retention of the substances.[1]

### 1.5.2 Reversed Phase Chromatography

Reversed phase (**RP**) chromatography is the most important separation principle in LC. In RP chromatography the **SP is hydrophobic**, and the **MP is a more polar aqueous solution**. The SP consists of hydrophobic groups placed on the surface of a solid matrix. The solid matrix is most often silica onto which the SP is chemically bonded. The MP is typically an aqueous buffer solution mixed with an organic modifier such as methanol (MeOH) or acetonitrile (ACN). Retention of neutral compounds is not affected by pH, while the retention of ionic compounds increases when the ionization of the analytes decreases. Silica-based stationary phases can normally be used in the pH range 2–8. If satisfactory separations are not achieved in this pH range, organic polymers as stationary phases or other more pH-stable column packing materials can be used. Alternatively, ion-pair chromatography may solve the problem.[1]

## Stationary Phases

Column packing materials used as stationary phases for RP chromatography are typically made of silica derivatized with reagents to form a more or less hydrophobic surface on the silica particle. They are typically obtained by binding hydrophobic groups to the silanol groups using chlorosilanes or other organic silane reagents. The examples of stationary phases shown in Figure XV are ranked after declining hydrophobicity.

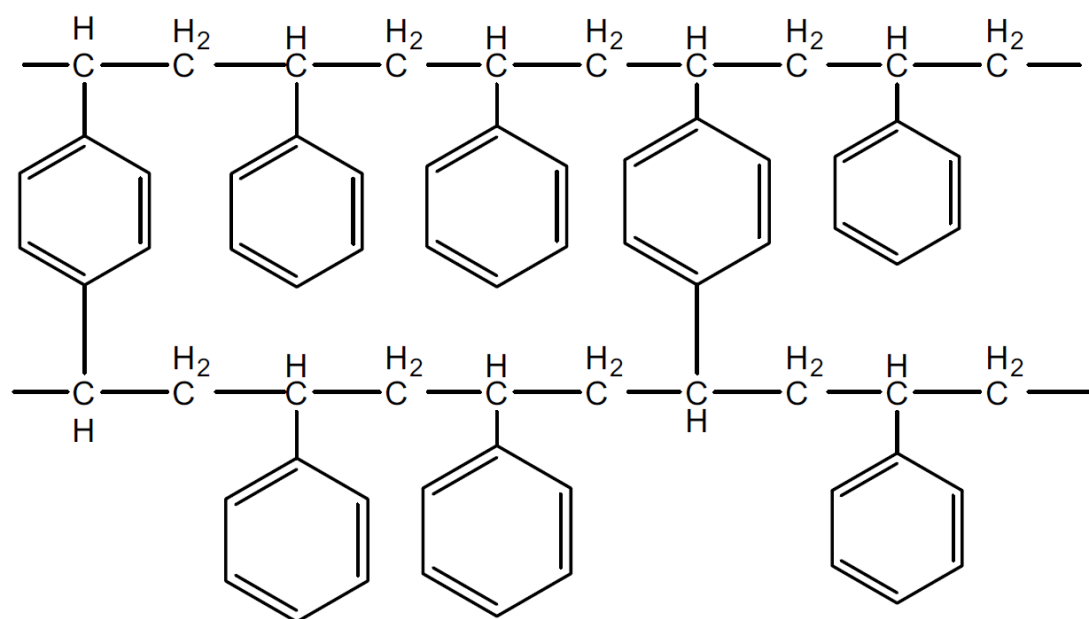


**Figure XV.** Derivatization of silica with a chlorosilane reagent. Varying the substituents  $R'$  and  $R$  produces stationary phases with different properties [1]

**Octadecyl (C18)** is the most hydrophobic phase and cyanopropyl (CN) is the least hydrophobic of these phases. By far, most of the chromatography performed in RP mode makes use of C18 materials, which are also known as octadecyl or **ODS** (octadecylsilane) materials. The surface of silica is covered with silanol groups placed fairly close together. The derivatizing silane reagent has three alkyl groups connected to the silicium atom and is therefore a bulky group. Due to steric hindrance it is not possible to react all silanol groups with the reagent. A significant percentage of the silanol group can therefore still be present after derivatization and in order to minimize the number of free silanol groups the material can then be treated with trimethylchlorosilane. This process is named **endcapping**. However even after endcapping some free silanol groups will still be present and accessible for polar interactions, for example, ionic interactions with amines.

Silica-based stationary phases can be used with mobile phases with pH in the range 2–8. Silica dissolves in the alkaline environment above pH 8 and also to some extent in an

acidic environment with  $\text{pH} < 2$ . It is the siloxane bonds that are cleaved. However, it is now possible to obtain silica-based polymers stable in the whole pH range by including ethane bridges in the silica polymer. These materials can be derivatized in the same manner as described above. When selecting a C18 material for chromatography it is important to be aware of the pH interval in which it can be used. When performing chromatography outside the pH range 2–8 another possibility is to use pH stable organic polymers like **polystyrene–divinylbenzene** (PS–DVB). The structure of polystyrene–divinylbenzene copolymer is shown in Figure XVI.

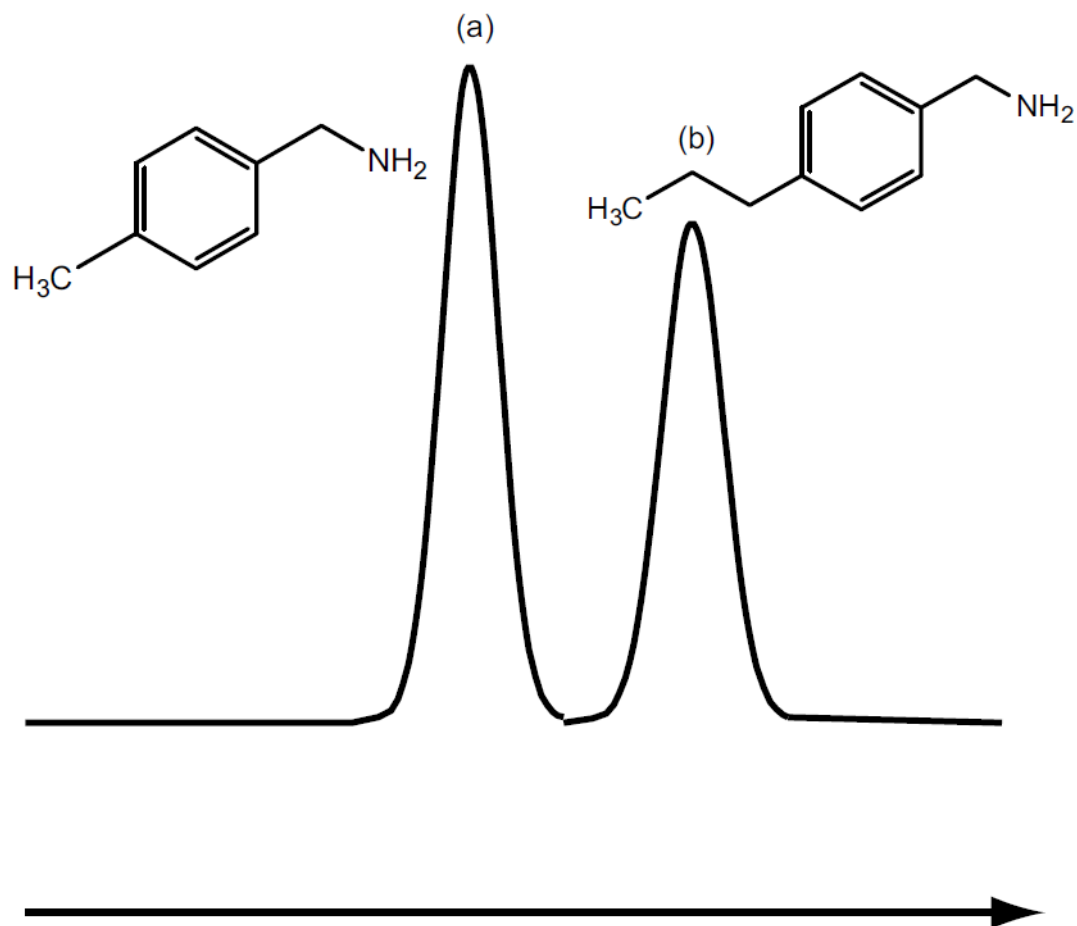


**Figure XVI.** Polystyrene–divinylbenzene copolymer [1]

PS–DVB can be used in the pH range 1–13 and it often provides a stronger retention of analytes compared to silica-based C18 materials, but the column efficiency of the organic polymeric stationary phases is less than for silica-based materials. Furthermore, the organic polymeric stationary phases are less rigid than silica and only highly crosslinked materials are suitable for HPLC. **Activated carbon** is an example of another nonpolar adsorbent. Hydrophobic substances in an aqueous environment adsorb onto the hydrophobic surface of the carbon. Recently also diamond, which is also made of carbon, has been introduced as a column packing material for reversed phase chromatography. This latter material is however not often used in pharmaceutical analysis.[1]

### ***Retention Mechanisms***

The main separation mechanism in RP chromatography is **hydrophobic interaction**. **Nonpolar analytes are therefore retained strongly** while more polar analytes elute earlier. Octadecylsilane (C18) column packing materials are the most hydrophobic of the usual commercial stationary phases available. The main forces of interaction are **van der Waal's forces**, which are relatively weak forces, but are present in a high number per molecule, and interaction thus increases with molecular size. The interactions take place between the hydrophobic hydrocarbon chains of the SP and the hydrophobic parts on the analyte molecule. Even highly polar analytes have a small retention due to the presence of minor hydrophobic parts in the molecule. Organic analytes having the same functional group are separated according to the size of the hydrophobic moiety, thus an additional CH<sub>2</sub> group increases retention. This is illustrated in Figure XVII. Analyte (b) is retarded more strongly than analyte (a) because it has a longer hydrophobic side chain.



**Figure XVII.** Chromatogram of two analytes with a different size of side chain (RP chromatography) [1]

Besides the hydrophobic interactions with the hydrocarbon chains a secondary retention mechanism can be displayed by the remaining silanol groups. Polar analytes especially amines can have a high affinity for the silanol groups besides the hydrophobic interactions. It is therefore of importance how many remaining silanol groups are present and how well they are covered.

For substances with ionizable functional groups, retention is dependent on whether these groups are ionized or not. Retention decreases with increasing ionization and this is of course a function of the change in the mass distribution ratio for such analytes at different pH values.[1]



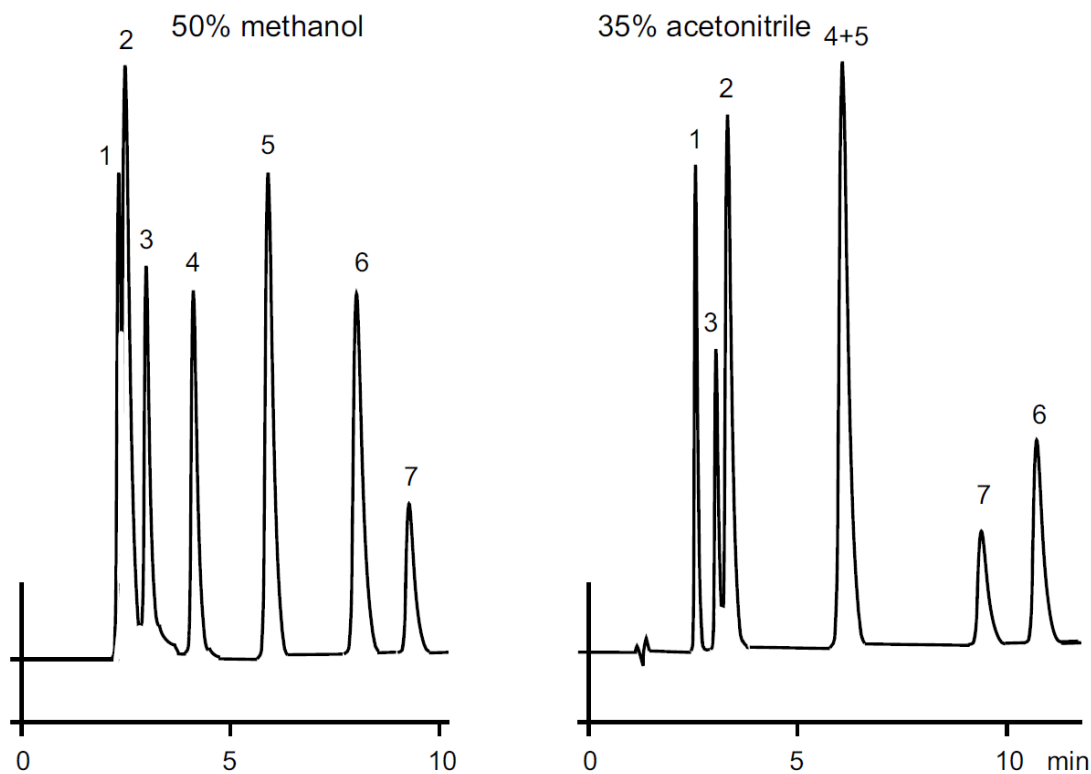
## **Mobile Phases**

Mobile phases for RP chromatography consist of mixtures of water and one or more organic solvents that must be miscible with water. The organic solvents used are called **organic modifiers** as they modify the strength of the mobile phase. **Increased content of organic modifier increases the strength of the mobile phase and retention of analytes decreases.** The solvent strength of MeOH is somewhat weaker than that of ACN which is weaker than tetrahydrofuran (THF). Mixtures A, B, and C shown in Table I have about the same solvent strength, they are **isoeluotropic**. A mobile phase of 60% MeOH in water have about the same eluting strength as 46% ACN in water or 37% THF in water.

	Mobile phase		
	A	B	C
MeOH	60		
ACN		46	
THF			37
Water	40	54	63

**Table I.** Mobile phases with similar eluting strengths [1]

When performing a separation of a mixture of compounds using the isoeluotropic phases A–C, the average retention of the compounds is about the same magnitude for the three phases. However, the order of elution between the substances can be somewhat different because the selectivity in the three systems can be different. **Changing the organic modifier can thus be used to change the separation selectivity** (Figure XVIII).



**Figure XVIII.** Example of solvent selectivity. Separation of seven test solutes on a C18 reversed phase HPLC column using MeOH or ACN as organic modifier: (1) benzylalcohol, (2) acetophenone, (3) phenylethanol, (4) propiophenone, (5) anisole, (6) toluene, (7) p-cresole [1]

**MeOH** is cheaper and less toxic than ACN. The main drawback when using MeOH as modifier is the formation of more **viscous mixtures with water creating an increase in back pressure** in the HPLC system. **ACN** may be better suited for UV detection at low wavelength and it **does not form viscous mixtures with water**.

Retention of neutral substances is only controlled by the content of organic modifier in the MP and is not affected by pH. For substances with ionizable functional groups retention are dependent on both the content of organic modifier in the MP and pH.

pH in the MP is controlled by the addition of buffers. The buffer concentration in the final mobile phase is typically in the range from 0.01 to 0.05 M. **Phosphate buffers** have for many years been the first choice because phosphate buffers have good buffering properties and low UV absorbance. A problem with phosphate buffers is that they have poor solubility in organic solvents and thus can precipitate at high concentration of organic modifier in the mobile phase. After the introduction of **mass spectrometers** as routine detectors in LC there was a change towards **volatile buffers** of organic acids such as acetic

acid or formic acid and their ammonium salts. These buffer substances also have a better solubility in organic solvents.

For the **separation of acids or bases**, changing the pH of the MP can cause large changes in separation selectivity. Retention decreases with increasing ionization. If an acid in the sample has a pKa value of 4.5 it is 50% ionized at pH 4.5 and fully ionized at pH > 6.5. The partition between the mobile and stationary phases is shifted at high pH towards the MP, resulting in a low retention. Retention varies greatly in the range around the pKa value, where small changes in pH provide major changes in ionization and retention. The weak acid is retarded most strongly when ionization is suppressed at low pH. The same consideration is true for a weak base at high pH. In this example, it has a pKa value of 9.5 Retention is strongest when ionization is suppressed, at pH 9.5 half of the molecules are ionized, and pH < 7.5 it is fully ionized. Many silica-based column packing materials cannot be operated above pH 8.0 and therefore amines are often chromatographed at about pH 3–4. Retention is then unaffected by pH as long as the base is fully ionized. In the pH range where drugs are either fully ionized or where the ionization is completely suppressed, the retention is not affected by minor changes in pH. To obtain robust analytical methods, it is important to choose a pH where retention only varies little with small changes in the composition of the mobile phase. **It is unfavorable to select a pH near the pKa value**, unless it is a question of the separation of two closely related substances with a small difference in pKa values. Typically, acids are separated by a pH that suppresses ionization and bases at a pH where they are ionized. When using column packing materials stable in the entire pH range it is of course possible to chromatograph bases in the ion suppressed mode.[1]

### ***Ion-Pair Chromatography***

This technique is particularly useful for the separation of basic compounds in RP chromatography, because they are completely or partially ionized in pH range (2–8) of generic silica-based stationary phases.

In ion-pair chromatography the analytes have to be ionized and therefore pH has to be controlled. The MP typically has a pH of 7 where both carboxylic acids and aliphatic amines are ionized. All ions in the system are surrounded by ions of opposite charge in order to keep electro neutrality in the system. Buffer ions are normally very polar and have only little interaction with the analytes. But when larger ions are added to the MP they can

form ion pairs with other ions of opposite charge in the system. In ion-pair chromatography larger hydrophobic ions are added to the MP. The formed hydrophobic ion pairs appear neutral to the surrounding. The ion pair is hydrophobic and has a retention depending on the nature and concentration of the counter ion. When the molecular size or the concentration of the counter ion increases the retention increases. However, the effect of increasing concentration of the counter ion decreases at higher concentrations and therefore concentrations in the range 1–5mM is recommended. Sulfonic acids and perfluorocarboxylic acids (*e.g.*, octanesulfonate and heptafluorobutyrate, respectively) are common counter ions for protonated bases, and quaternary ammonium compounds (*e.g.*, tetrabutylammonium) are commonly used for ionized acids. Octanesulfonic acid has a pKa below 1, is negatively charged throughout the pH range used and forms ion pairs with positively charged bases. Heptafluorobutyrate behaves similarly and can also be considered negatively charged throughout the pH range. Tetrabutylammonium is a quaternary ammonium compound that is positively charged in the full pH range and its positively charged group provides ion pairs with negatively charged acid groups. Octanesulfonate provides larger retention of bases than pentanesulfonate, and tetrabutylammonium ions provide larger retention of acids than tetramethylammonium ions.

Retention in ion-pair chromatography can be increased by reducing the concentration of organic modifier; increasing the concentration of the counter ion; or increasing the molecular size of the counter ion.[1]

### 1.5.3 Hydrophilic Interaction Chromatography

Hydrophilic interaction chromatography (**HILIC**) is a chromatographic principle used for the separation of relatively **polar analytes** where the RP mode does not provide sufficient retention. The mobile phases used in HILIC often have a content of organic solvent (*e.g.*, ACN) between 80 and 100%. **The stationary phases are very polar**, *e.g.*, silica or silica derivatized with polar groups (diols, twitter ions, *etc.*). The systems can therefore be considered as highly deactivated normal phase systems, where a hydrophilic SP is present. The less water present in the MP the stronger is the retention of the analytes. Thus, **water is the strongest solvent in HILIC**. If gradient elution is applied in HILIC it is initiated using a high content of organic solvent and progresses with increasing amounts of water. The HILIC mode has become very popular for **analysis of polar substances** where RP chromatography has been less successful. The high content of organic solvent eases the

spray in electrospray mass spectrometry (MS) and thus **improves sensitivity in LC-MS** methods, and although HILIC is a normal phase mode the content of water in the MP still makes it possible to use this mode for bioanalysis, which is more problematic when using non-aqueous normal phase chromatography.[1]

#### 1.5.4 Chiral Separations

It is a challenge to separate pairs of enantiomers as they have the same physicochemical characteristics apart from their ability to rotate the plane-polarized light. If two substances have the same distribution constants (Equation I) they cannot be separated in a chromatographic system. Two enantiomers can therefore only be separated on chromatographic systems if it is possible to introduce a difference in their distribution constants. This can in principle be done in two ways: an indirect way and a direct way.

In the **indirect** way the enantiomers are **derivatized with an optically active reagent** to form diastereomers. Diastereomers have more than one chiral center and have different physicochemical characteristics and thus also different distribution constants. It is therefore possible to separate enantiomers after derivatization to diastereomers in a standard chromatographic system.

When using **direct** separation of enantiomers they are injected directly into the chromatographic system similar to what is used in other chromatographic methods. The direct chiral separation (without derivatization) of enantiomers is only possible if chirality is introduced into the chromatographic system. Chirality of chromatographic systems is achieved using **chiral mobile or stationary phases**. In LC chirality can be introduced in the MP or SP and both modes have been applied. A chiral reagent is added to the MP, and due to the different complex constants of the diastereomeric complexes formed with the enantiomers to be separated, they can be separated on an achiral SP. But more often chiral stationary phases have been used and a huge number are available on the market. The phases consist of a polymer (silica, cellulose, methacrylate) to which a chiral molecule is attached. The chiral molecules used are very different in nature (proteins, polysaccharides, cyclodextrins, antibiotics, helical methacrylates, *etc.*) and therefore also provide different selectivity. Unfortunately, the achievement of chiral separation is still much of a “trial and error” and therefore it is important to consult literature and vendors of stationary phases before starting the experiments.[1]

### 1.5.5 Size Exclusion Chromatography

Size exclusion chromatography (SEC) is used for the **separation of substances by their size** in solution. The SP consists of porous particles with a controlled pore size. Large molecules are excluded from the pores and the volume of MP transporting them out of the column is called the exclusion volume. Small molecules penetrate the pores and are retarded on the column. The volume of the MP transporting them out of the column is called the total permeation volume. SEC can be used for separation of substances with molecular sizes corresponding to elution volumes in the window between the exclusion volume and the permeation volume. Peak capacity is limited compared to other separation techniques. For HPLC rigid packing materials that resist the higher pressure are used. Rigid packaging materials are made of silica or a highly crosslinked organic polymer like polystyrene-divinylbenzene copolymer. Silica is used with aqueous mobile phases, while polystyrene-divinylbenzene often is used with organic solvents as mobile phases.[1]

### 1.5.6 Ion Exchange Chromatography

Ion exchange chromatography (IEC) is a technique that allows the **separation of ions based on their charge**. It can be used for almost any kind of charged molecules, including large proteins, nucleotides and amino acids. The ionic analytes are retained by ionic interaction between the analytes and ionic sites of the opposite charge placed on the SP. Ionic groups like  $-\text{SO}_3^{2-}$ ,  $-\text{COO}^-$ ,  $-\text{NH}_3^+$  or  $-\text{NR}_3^+$  are placed on the matrix of the particles. The charges are neutralized by counter ions which can exchange with analytes. The positive cations have an affinity to negatively charged ion exchangers and these ion exchangers are therefore called **cation exchangers**, and positively charged ion exchangers are likewise pronounced **anion exchangers**. In daily work a strong cation exchanger (*e.g.*,  $-\text{SO}_3^{2-}$ ) is abbreviated **SCX**, and a strong anion exchanger (*e.g.*  $-\text{NR}_3^+$ ) is abbreviated **SAX**. Similarly weak ion exchangers are pronounced **WCX** (*e.g.*  $-\text{COO}^-$ ) or **WAX** (*e.g.*  $-\text{NH}_3^+$ ). The strong ion exchangers are charged throughout the usable pH range while the weak exchangers are only charged within a minor range of the pH interval depending on the pKa value of the functional group.

Retention of analytes on an ion exchanger requires that the analyte as well as the ion exchanger are charged. In general the affinity of analytes depends on their charge and

size. More charges and a smaller hydrated size increase the affinity for the ion exchanger and thus retention.

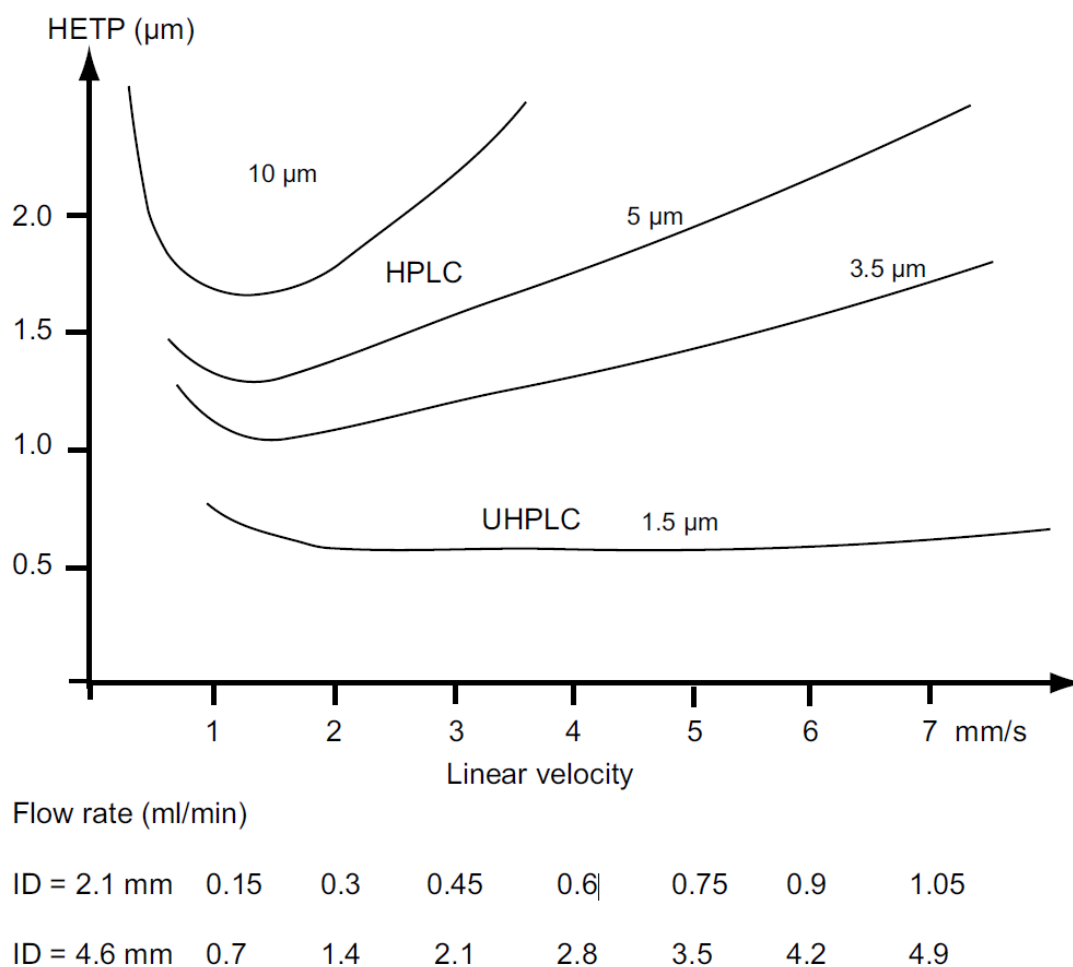
The retention can be changed by modifying the MP either by the addition of an organic solvent or more effectively by changing the concentration or nature of the buffer. The buffer ions in the MP compete with the analyte ions for the ionic sites on the SP. When the buffer concentration is increased or buffer ions with a stronger affinity to the ionic sites are used the retention of the analytes decreases. It is common to perform gradient elution in ion exchange chromatography by increasing the ionic strength of the buffer during chromatography.[1]

# 2 HPLC/UHPLC Instrumentation



## 2.1 Columns

The column is the heart of the separation process. The column should have a high number of theoretical plates,  $N$ , and as known from the van Deemter equation (Equation VII; Equation VIII), this depends on the particle size of the column packing material. Decreasing particle size provides a larger number of theoretical plates,  $N$ . This is illustrated in Figure XIX where the HETP is plotted versus particle sizes of 1.5; 3.5; 5 and 10  $\mu\text{m}$ .



**Figure XIX.** Height equivalent to a theoretical plate as a function of the flow rate of the mobile phase for 1.5; 3.5; 5; and 10  $\mu\text{m}$  particles [1]

The optimum efficiency is at the smaller particle sizes shifted towards higher linear flow rates. This indicates that higher flow rates can be used without losing efficiency. However, the small particle size also provides a very high back pressure and thus shorter column lengths have to be used. The practical implication of this is that short columns with

particles of about 2  $\mu\text{m}$  can give efficient separations in a very short time. Depending on the back pressure it can be necessary to use UHPLC equipment. The typical analytical HPLC column has been a 15–25 cm long steel tube packed with 5  $\mu\text{m}$  particles. Inner diameter of the tube has been 4.6 mm. With the introduction of MS as a routine LC detection technique and the improvement of column packings for HPLC, column dimensions of 10–15 cm with 2 mm internal diameter have become the common standard. For fast analysis smaller particles between 1.5 and 2.0  $\mu\text{m}$  are used in columns of 3–5 cm in length, and this is designated as UHPLC. Using shorter columns with smaller internal diameters results in major savings in the consumption of MP. In Table II the reduction in MP consumption is calculated for columns of equal length with different internal diameters for a constant flow rate of MP through the columns.

Internal diameter (mm)	Flow rate (mL/min)	Reduction in mobile phase (%)
4.6	1.00	–
4.0	0.69	31
3.0	0.43	57
2.0	0.19	81
1.0	0.05	95

**Table II.** Saving in mobile phase as a result of the reduction in internal column diameter keeping the linear flow rate (1.33 mm/s) in the column constant [1]

A reduction in column length also results in a similar reduction in analysis time. The column packing material is held in place in the column by a metal filter at each end. The metal filter is either a porous frit or a net so that the MP can pass through. The pore size of the filter has to be smaller than the diameter of the packing material in order to prevent particles from leaking from the column.[1]

### 2.1.1 Type of Packing Material (Porous, Nonporous, Monolithic)

Majority of packing materials used in HPLC are **porous** particles with average diameters between 3 and 10  $\mu\text{m}$ . For most pharmaceutical applications, 3- $\mu\text{m}$  particle sizes are recommended. Porosity provides the surface area necessary for the analyte retention (usually between 100 and 400  $\text{m}^2/\text{g}$ ). Interparticle space is large enough to allow up to 1–3 mL/min flow within acceptable pressure range (however, the pressure drop across the

column depends on the particle size, length of column, temperature of separation, and MP composition).

Introduction of small **nonporous** spherical particles in the mid-1990s was an attempt to increase efficiency by eliminating dual column porosity. In the column packed with porous particles, interparticle space is about 100-fold larger than pores inside the particles, and liquid flow around the particles is also faster; this leads to the significant band broadening. Unfortunately, elimination of particle porosity dramatically decreases adsorbent surface area, thereby decreasing the column loading capacity. Columns packed with small (1.5  $\mu\text{m}$ ) nonporous particles also require ultra-microinjection volumes and a corresponding increase of detector sensitivity.

The introduction of **monolithic columns** in the 1990s was another and more successful attempt to increase column permeability while decreasing the gap in column dual porosity. **Macropores** in the monolith are between 4000 and 6000  $\text{\AA}$  in diameter, and they occupy almost 80% of the column volume. Compared to the conventional packed column with 5- or even 3- $\mu\text{m}$  particles, the silica skeleton in monolith is only approximately 1 $\mu\text{m}$  thick, which facilitates accessibility of the adsorbent surface inside the **mesopores** of the skeleton (pores between 20 and 500  $\text{\AA}$  in diameter are usually called mesopores).[5]

### 2.1.2 Base Material (Silica, Zirconia, Alumina, Polymers)

In modern LC, almost all RP separations are performed on chemically modified adsorbents. Analyte interactions with the SP surface are the primary factor for successful separations. Most commercial adsorbents reflect their surface chemistry in their names (*e.g.*, C18, C8, Phenyl, *etc.*) while the base material used usually is not specified, although its properties are very important. Specific parameters of the base of packing material are:

- Surface area
- Pore size
- Pore volume
- Pore size distribution
- Particle shape
- Particle size
- Particle size distribution

- Structural rigidity
- Chemical stability
- Surface reactivity
- Density and distribution of the surface reactive centers

Surface area is directly related to the analyte retention. Generally, the higher the surface area, the greater the retention. Pore size is a critical parameter for the surface accessibility. Molecules of different size could have different accessible surface area due to the steric hindrance effect (bigger molecules might not be able to penetrate into all pores). Pore size is also related to the surface area.

The most commonly used base material is **silica (SiO<sub>2</sub>)**, the most common substance on the Earth and thoroughly studied in the last two centuries. Development of modern HPLC techniques promoted advancement in porous silica technology. Almost all silica-based HPLC packings manufactured in the twenty-first century are very uniform spherical porous particles with narrow particle and pore size distribution. Silica has one significant drawback: It is **soluble at high pH**, although chemical modification with high bonding density of attached alkylsilanes extends its stability range to over pH 10.

Another porous base material suggested in the last decade as an alternative to silica is zirconia. **Zirconia** is stable in a very wide pH range (**pH 1–14**), but zirconia surface has relatively low reactivity (more difficult to bond different functional groups to the surface), which significantly limits a selection of available stationary phases.

**Polymer-based** materials have been on the market for more than 30 years. Crosslinked styrene-divinylbenzene and methylmethacrylate copolymers are the most widely used. These materials show high pH stability and chemical inertness. Their rigidity and resistance to the swelling in different mobile phases is dependent on the degree of crosslinkage. Practical application of these materials for the separation of small molecules are somewhat limited due to the presence of microporosity. Gaps between cross-linked polymer chains are on the level of molecular size of low- molecular-weight analytes. These analytes could diffuse inside the body of a polymer-based packing material, which produce drastically different retention of a small portion of injected sample than the rest of it. At the same time, polymers are the main packing material for SEC.[5]

### 2.1.3 New Generation 1.3 $\mu\text{m}$ Core-Shell Particles Column

Performance of new generation narrow-bore columns packed with 1.3  $\mu\text{m}$  core-shell particles (Kinetex® 1.3  $\mu\text{m}$  C18, 50  $\times$  2.1 mm, by Phenomenex®) was evaluated by Fekete and Guillaume in 2013. This particular column, compared to fully porous particle columns, benefits both from smaller particle size and high particle density that helps create optimal bed structure which reduces band broadening effects of Eddy Diffusion. The data clearly demonstrate the kinetic advantages of this new generation of SP. Using van Deemter representation,  $H_{min}$  value of only 1.95  $\mu\text{m}$  was achieved, corresponding to efficiency of more than 500,000 plates/m and about 25,000 plates for a 50 mm column length. For comparison purpose, a good column packed with fully porous sub-2  $\mu\text{m}$  particles is able to produce “only” 300,000 plates/m. To illustrate the gain in performance afforded by 1.3  $\mu\text{m}$  core-shell particles, two real-life separations were carried out on columns packed with 1.3; 1.7 and 2.6  $\mu\text{m}$  core-shell particles. As expected, the attained plate count was in average 60% higher on the 1.3  $\mu\text{m}$  material compared to the 1.7 and 2.6  $\mu\text{m}$  counterparts. However, **the performance achieved with this new material was limited by the possibilities of current instrumentation.** Indeed, the best UHPLC systems on the market offer an extra-column variance of about 2  $\mu\text{L}^2$  and upper pressure limit of 120 MPa (17,400 psi; 1200 bar). It was demonstrated that the pressure limit was too restrictive to attain the optimal linear velocity. This is of course related to the low permeability of this packing. To overcome this issue, it could be beneficial to extend the column mechanical stability and the instrument capability. Indeed, it was demonstrated that 1.3  $\mu\text{m}$  material would outperform the other core-shell material up to  $N = 60,000$  and 85,000 plates, if the  $\Delta P_{max}$  of columns and system was extended to 150 and 200 MPa (21,800 and 29,000 psi; 1500 and 2000 bar), respectively. Second, because of the very high efficiency produced by this short narrow bore columns packed with 1.3  $\mu\text{m}$  core-shell particles, the extracolumn variance could have a major impact on kinetic performance. This study confirms that this new generation of columns packed with 1.3  $\mu\text{m}$  core-shell particles should exclusively be used on powerful state-of-the-art UHPLC system possessing an upper pressure limit of at least 100 MPa (15,000 psi; 1000 bar) and a system variance of less than 10  $\mu\text{L}^2$ , ideally down to only a few  $\mu\text{L}^2$ . Under such conditions, the possibilities offered by this column technology are impressive, particularly for high throughput separation with elevated plate count. In 2013 Sanchez *et al.* similarly concluded that column technology is evolving faster than the LC instrumentation and there is a need to develop instruments having reduced extra-

column variance (down to only 1  $\mu\text{L}^2$  or less), and higher upper pressure limit (in the range 150–200 MPa; 21,800–29,000 psi; 1500–2000 bar).[23-25]

### 2.1.4 Monolithic Columns

Both the speed and efficiency attainable by an HPLC column are ultimately limited by the maximum pressure capabilities of the instrument. In the case of particle-packed columns, decreasing the particle diameter leads to improved efficiency and speed; however, because the pressure drop across the column,  $\Delta P$ , is proportional to  $1/d_p^2$  (Equation IX), the price paid in pressure will always be proportionally greater than the gain in column performance.

Monolithic columns are a viable alternative to particle-packed columns as a means to achieving efficient separations while overcoming the pressure limitation due to their comparably higher permeabilities (lower flow resistances). High efficiencies together with lower pressure drop make monolithic columns attractive options for fast HPLC. Monolithic columns are generally prepared by the in situ polymerization of either organic or inorganic monomers to form the skeletal support. Control of the polymerization kinetics determine the size of the macro- and mesopores. A main drawback of polymer monoliths, however, is that polymers tend to swell or shrink in the presence of organic solvent, which leads to poor chromatographic performance and a lack of mechanical stability under pressure-driven flow. Monolithic silica columns are prepared using a sol-gel method by hydrolytic polymerization of alkoxy silanes to form the skeleton. Physical features such as through-pore size and skeletal size can be more precisely controlled in the preparation of silica monoliths. In addition, the chemical and mechanical stability of silica monoliths is better than polymeric columns. However, due to shrinkage during solidification, silica monoliths cannot be prepared in situ, but must first be prepared in a mold, and then removed and encased in PEEK tubing before bonding of SP takes place.

Sklenarova *et al.* compared the new generation of HPLC monolithic column, Merck Chromolith® HighResolution, with common monolithic columns with different inner diameters (4.6 and 3 mm). The new type of monolithic column showed good peak symmetry, the highest column efficiency [number of theoretical plates 13,137 and HETP 7.61  $\mu\text{m}$  – twice the number of theoretical plates compared with the “classic” monolithic column (CMC) Onyx™ monolithic C18, 100 mm×4.6 mm (Phenomenex) and three times greater compared with the “narrow” monolithic column (NMC) Chromolith® Performance RP-18e, 100 mm×3 mm (Merck)], high resolution, but also higher working pressure (still

under 5 MPa) compared with the CMC. The best symmetry factor, the lowest consumption of the mobile phase, the shortest analysis time and the highest working pressure were achieved with the NMC. The CMC permits application of higher flow rates owing to a lower working pressure and good separation efficiency. The new Chromolith® HighResolution column showed the best blend of all properties for the separation under typical HPLC conditions; according to the manufacturer, prolonged lifetime compared with other monolithic column sorbents can be expected even for a matrix of biological samples. The broadened choice of monolithic columns enables to use the optimal one in separations with various requirements, not only in HPLC systems but also for lower pressure separation methods such as sequential injection chromatography, where the working pressure was still found to be acceptable.[5, 26]

### **2.1.5 Column Backpressure**

Column backpressure is limiting factor for using very small particles with high flow rates, on the other hand it gives us a good indication of how the column and/or system are operating. The initial backpressure of the column should be checked prior to running a method. The backpressure with column attached at initial run conditions should be stated in the method. If high column back- pressure is observed, the column should be taken off and a zero dead volume should be installed and system backpressure recorded and compared to known system backpressure for that particular system under a certain set of MP conditions. Note that the system backpressure will be different from instrument to instrument due to the different types of tubing that are employed. This depends on the tubing diameters and total tubing length. The system backpressure is also dependent on the MP composition, the type of organic modifier, the temperature, and the flow rate. If the system backpressure value is known on that particular system with a certain set of eluent and column conditions and the backpressure value is higher than normal, then the cause of this increased backpressure needs to be investigated.[5]

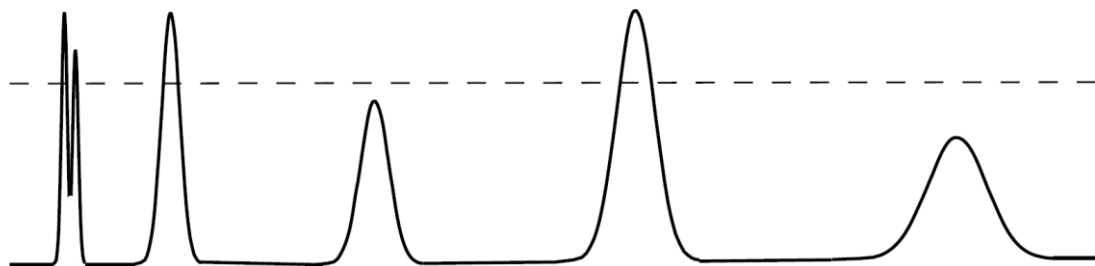
## 2.2 Pumps

The pumps deliver the MP at a constant flow rate through the column. The pumps can be constructed in different ways, but a piston pump is the most common. The piston pump consists of a small steel cylinder with a volume of approximately 100  $\mu\text{L}$ . A piston is moved back and forth in the cylinder by means of a motor. There are two ball valves attached to the cylinder so that the MP can only flow in one direction, into the cylinder from the reservoir or out to the column. When the piston is moved back the lower ball valve opens while the upper ball valve closes, dragging the MP into the cylinder. When the piston again is moved forward into the cylinder the bottom ball valve closes, while the top valve opens. The MP is then forced out of the cylinder through the injector into the column.

Since the MP is forced into the column only when the plunger is pushed into the cylinder, the fluid flow pulsates. This pulsation introduces extra noise in the detector signal and should be eliminated if possible. A pulse dampener is therefore included in the system to ensure a smooth flow of the MP. Other pump systems ensure a smooth flow by linking together two piston heads into a double piston pump, where one piston head delivers MP to the column while the second is filled up with MP. When the pumping system delivers a MP with a constant composition to the column it is called **isocratic elution**. However, it is also possible to have a system where liquid is pumped from two or more reservoirs and then to mix them during chromatography. This can be done using a single pump equipped with a **low pressure mixing valve** connected to up to four different reservoirs containing liquids. The mixing valve opens for only one pipeline at a time and in this way the solvent mixture can be controlled. It is also possible to use more than one pump where each pump delivers a controlled amount of each liquid. The mixing is then performed at the high pressure side, but the two principles are of similar performance. The change of the composition of the MP during chromatography is called **gradient elution** and this technique can be compared to temperature programming in GC. Gradient elution is used to separate samples containing compounds with large differences in retention. When big differences in retention of analytes are current, isocratic elution results in unnecessary long analysis time. The least retarded substances elute early as partially overlapping peaks, while the most retarded substances elute as broad peaks with a long retention time. This is

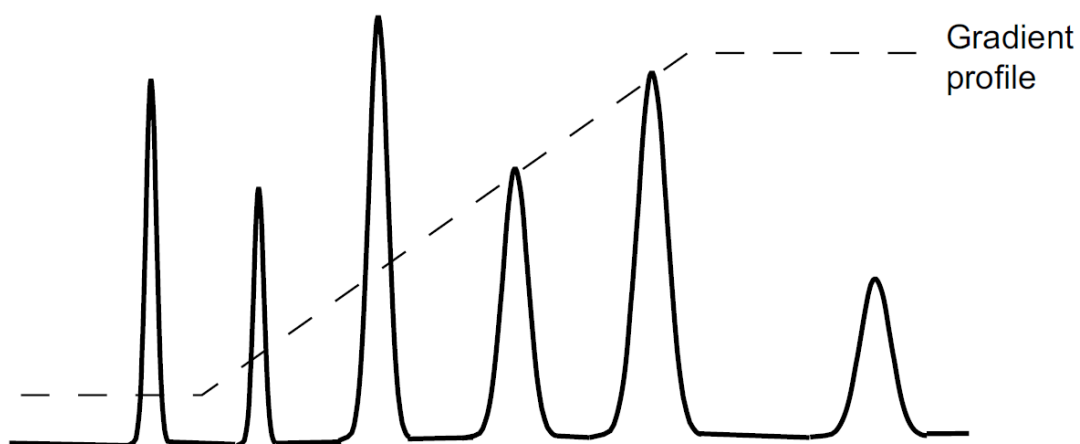


illustrated in Figure XX. It is only the analytes in the middle of the chromatogram that elute satisfactory.



**Figure XX.** Isocratic elution of a sample containing analytes with large differences in retention [1]

Using gradient elution the composition of MP is changed during chromatography, starting with a weaker eluting composition of the MP. In this way the least retarded substances obtain sufficient retention and separation. The strength of the MP is then increased during chromatography and the late elution peaks are now eluted faster and with a better sensitivity. This is illustrated in Figure XXI.[1]



**Figure XXI.** Gradient elution of a sample containing analytes with large differences in retention [1]

## 2.3 Injectors

The purpose of the injector is to bring a certain volume of sample solution into the flow of the MP just before it enters the top of the column. The high back pressure of the column necessitates a special device for this purpose. An injector that is leak proof in the whole pressure range of the system is needed. For manual injections a simple loop injector can be used. The loop injector is a six-port valve. In the load position the MP from the pump passes through the valve directly to the column. In this position it is possible to inject the sample into the loop using a syringe. When the valve then is switched to the inject position, the MP from the pump passes through the loop and in this way brings the sample to the column. The loop is a piece of tubing with a total volume which should not exceed the capacity of the column used. In standard analytical work it is common to use a loop of 20  $\mu\text{L}$  together with column of 4.6 mm internal diameter.

The injection process can be automated using an **autosampler**. Samples from sample vials placed in the autosampler are injected into the HPLC according to the pre-programmed volumes and time intervals. The process can be controlled by the HPLC software so that analysis can take place without supervision, running for 24 h or more. Autosamplers can also be equipped with a refrigerator for the cooling of sensitive samples so that they do not decompose before analysis. Modern autosampler such as Acquity® UPLC® Sample Manager (Waters Corporation) is offering injection modes that are matching various needs:

- 1) Partial-loop with needle overfill (PLNO) mode is default injection mode suitable for a wide range of samples.
- 2) Partial-loop pressure assisted (PLPA) mode offers the lowest cycle time and the lowest sample consumption and thus it is suitable for applications when high throughput is needed, or if the sample is precious.
- 3) Full-loop (FL) mode offers the best precision and accuracy; the lowest dispersion and best resolution and therefore it is suitable for method development projects or quality control (QC) assays.[1, 27]

## 2.4 Detectors

The LC detector gives a response for the analyte that is converted into an electrical signal. The response should be proportional to the concentration of either substance in the MP or with the mass of the substance in the MP, so quantitative analysis can be carried out based on the measurement of peak areas or peak heights. The detectors can be divided into two types:

- 1) General detectors that measure any change in the MP.
- 2) Specific detectors that respond only to substances with specific properties.

The main detectors used in pharmaceutical analysis are given in Table III. For the QC of pharmaceutical preparations and drug substances the UV detector is the standard detector. Fluorescence detectors and electrochemical detectors have for selected analytes a much lower limit of detection than the UV detector. The mass spectrometer provides additional information on the molecular structure. A refractive index detector is used in quality control in the case of substances that do not have an adequate UV response. It is considerably less sensitive and it is not applicable to methods using gradient elution.[1]

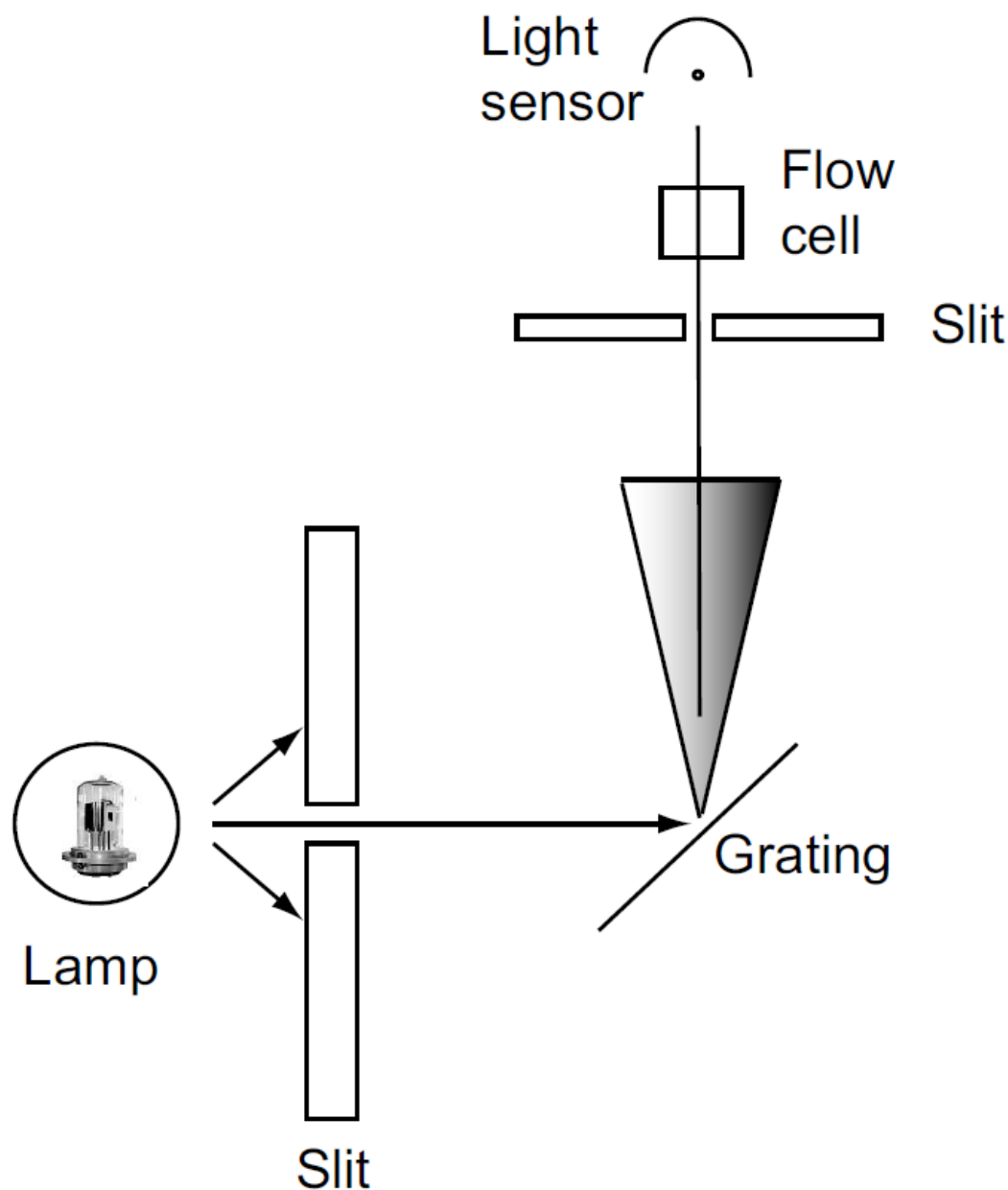
Detector	Lower limit of detection [ng]	Gradient elution?
Ultraviolet (UV)	0.1–1.0	Yes
Fluorescence	0.001–0.01	Yes
Electrochemical (ECD)	0.01–1.0	No
Mass spectrometry (MS)	0.001–0.01	Yes
Refractive index (RI)	100–1000	No
Evaporative light scattering (ELSD)	0.1–1.0	Yes
Charged aerosol (CAD)	0.1–1.0	Yes

*Table III. LC detectors and their typical performance [1]*

### 2.4.1 UV Detectors

The UV detector is based on the analytes' absorption of UV light. Analytes capable of absorbing UV radiation can be detected. This requires the analyte to contain a **chromophore**, being at least one double bond in the molecule. The wavelength range is

from 190 to about 400 nm, and at higher wavelengths the visible range up to about 800 nm can be used. Colored analytes are more selectively detected in the visible region although they also absorb light in the UV region. It is first and foremost the high operational stability and ease that makes the UV detector the preferred detector for QC. It is also very good when using gradient elution. The lower limit of detection is adequate for most analysis, but it is not sufficiently sensitive to be used for the analysis of low concentrations of drugs in biological material. Figure XXII shows a sketch of a UV detector.



*Figure XXII. Schematic diagram of an UV detector [1]*

The eluent from the column is directed to a flow cell through which also UV light of a defined wavelength is directed. The radiation which passes through the flow cell is detected by a light sensor. It is common to use a deuterium lamp as radiation source. A continuum of light is emitted in the whole UV range and the detector can therefore be used continuously variable in the range 200–400 nm. For optimal detection sensitivity of the substances they should be measured at their maximum UV absorbance. A monochromator ensures that UV radiation of the correct wavelength is directed through the flow cell. According to **Beer's law** (also called Beer-Lambert law or Beer-Lambert-Bouguer law), the absorbance ( $A$ ) is proportional to the concentration of the substance in the MP ( $c$ ), to the path length of radiation through the flow cell ( $l$ ), and to the molar absorptivity ( $\epsilon$ ), which is constant for that particular substance:

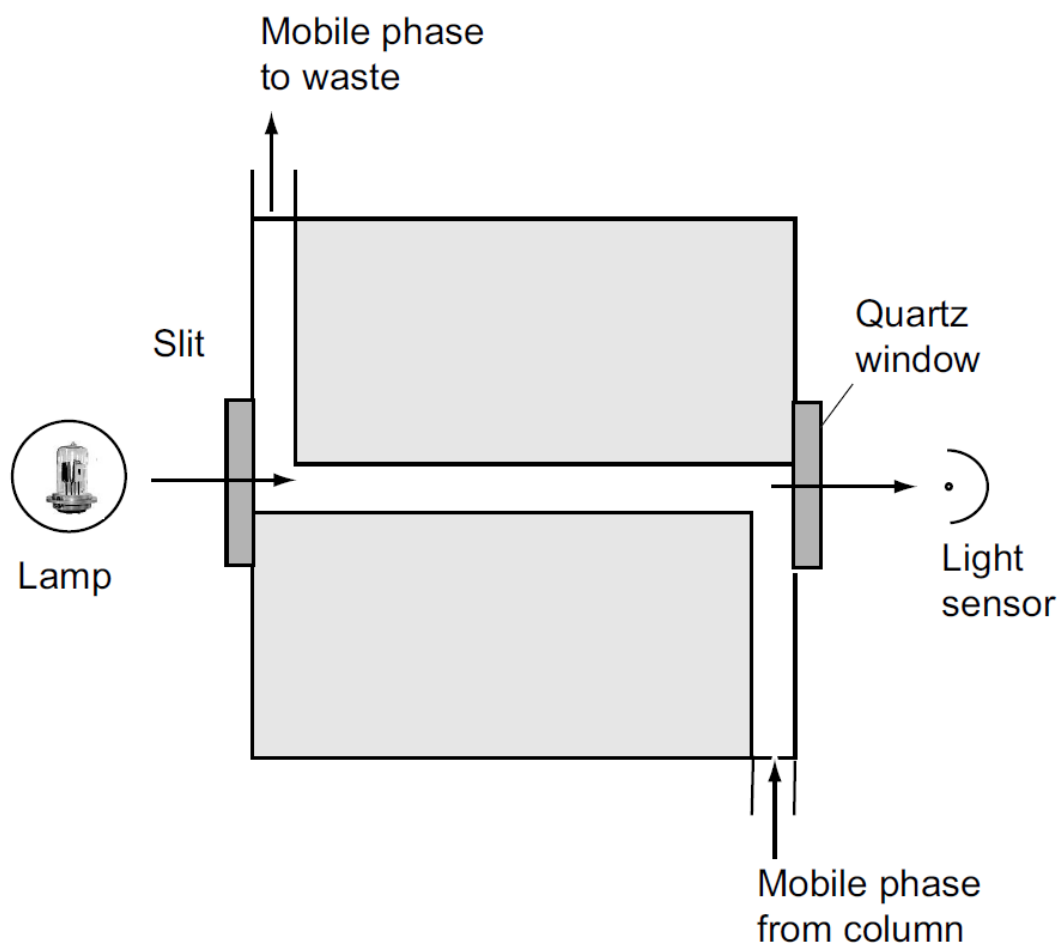
$$A = \epsilon cl$$

*Equation XVII. Beer's (Beer-Lambert or Beer-Lambert-Bouguer) law [1, 28]*

Figure XXIII shows a scheme of a flow cell. The eluent from the column flows through a Z-shaped channel in the cell. The UV radiation passes the flow cell through two quartz windows that do not absorb UV radiation. The path length ( $l$ ) of the flow cell is in the range from 6 to 10 mm and the volume for a standard cell is in the order of 10  $\mu$ L. When very efficient separation is obtained it can be necessary to use a flow cell with a smaller internal volume in order to avoid mixing of the separated peaks in the cell. In the diagram of the UV detector shown in Figure XXII, the monochromatic radiation passes through the flow cell and is directed towards the detection unit.

In another type of UV detector called a **diode array detector (DAD)** the polychromatic radiation is passed through the flow cell. After the flow cell the transmitted light is split in an optical lattice into the individual wavelengths, and the intensity of each of these is measured by a number of (array of) photodiodes. There may be up to several hundred diodes in series to measure the intensity of the array of UV radiation. Diode array detectors offer several possibilities. A full UV spectrum of the peak can be recorded “on the fly”, which can be used to identify the substance. It is also possible to choose a few selected wavelengths, so that each substance in a sample can be detected at the optimal wavelength.

Note: Expression “DAD detector” is redundant (= “Diode Array Detector detector”). DAD (e.g., Thermo Fisher Scientific/Dionex) is also called Photodiode Array (PDA) detector (e.g., Shimadzu or Waters).[1, 28-31]



*Figure XXIII. Scheme of flow cell in a UV detector [1]*

## 2.4.2 Combination of Detectors

It is possible to combine detectors in series and the combination of a UV detector, a fluorescence detector and an electrochemical detector in series is a powerful tool. The detectors have to be coupled in the order given above. The fluorescence detector cell does not withstand higher backpressures and the electrochemical detector has to be placed last in the series as no backpressure is allowed. The major advantage of this set-up is that the relative detector signals achieved for a given compound in the three detectors should be constant for a reference standard and for the similar peak in a complex sample. If this is not the case there probably is some interference in the sample that biases the analytical result.

When also a MS detector is to be included in such a series it is necessary to split the eluent after the UV detector with one part going to the MS and the other part to the fluorescence and electrochemical detectors.[1]

## 2.5 HPLC and Health & Safety

Since MP used in HPLC contains typically several volatile components (*e.g.*, ACN, MeOH, THF, isopropyl alcohol, acetic acid or formic acid), HPLC instrument should be equipped with filters and valves that prevent evaporation of chemicals outside the instrument into the lab environment. Despite relatively high costs of HPLC/UHPLC instrumentation, vendors are typically not providing their products with such equipment, and thus laboratory workers should take care of making their instruments more safe on their own.

### 2.5.1 Human Hazard Potential of Selected Mobile Phase Components

#### *ACN*

*Odor threshold:* 98 ppm.

(Odor thresholds vary greatly. Do not rely on odor alone to determine potentially hazardous exposures.)

*Symptoms and effects (both acute and delayed):* irritant effects, nausea, vomiting, convulsions, shortness of breath, unconsciousness, respiratory arrest, cardiac arrest, headache, dizziness.

ACN is readily absorbed in the respiratory tract and distributes throughout the body, where it is metabolized in the liver. Metabolism is known to result in formation of cyanide and thiocyanate, with formaldehyde and formic acid as additional postulated metabolites. Hydrocyanic acid has been detected in various organs (*e.g.*, brain and heart) of the rat upon inhalation. There is little direct information concerning the nature of effects that ACN causes in humans. Case reports of adults or children who ingested ACN indicate that symptoms included respiratory distress (*e.g.*, pulmonary edema), vomiting, confusion, convulsions, gastric erosion, and seizures. Case reports involving occupational exposure involved a similar spectrum of symptoms. **Autopsy findings from an occupationally exposed individual revealed brain, kidney, thyroid, and liver involvement.** From these



reports alone, **it is clear that the respiratory tract, the central nervous system, and other organs can be adversely affected.**

Because the carcinogenic potential in both the rat and mouse is low, coupled with an overall lack of genotoxicity potential, the carcinogenic potential in humans is expected to be low. However, there is uncertainty in this area in as much as there was a **positive trend for hepatocellular adenomas/carcinomas (combined) in the male rat as well as an increase in the incidence of basophilic liver foci**, of which the latter may represent a preneoplastic effect.[32-35]

### ***MeOH***

*Odor threshold:* 100 to 1,500 ppm.

(Odor thresholds vary greatly. Do not rely on odor alone to determine potentially hazardous exposures.)

*Symptoms and effects (both acute and delayed):* irritant effects, drowsiness, dizziness, narcosis, agitation, spasms, inebriation, nausea, vomiting, headache, blindness, impairment of vision, coma, drying-out effect resulting in rough and chapped skin.

In humans, acute central nervous system (CNS) toxicity can result from relatively low ingested doses (as low as 3-20 mL of MeOH), which can metabolize to formic acid and lead to metabolic acidosis. The resulting acidosis can potentially cause lasting nervous system effects such as blindness, Parkinson-like symptoms, and cognitive impairment. These effects have been observed in humans with blood methanol levels as low as 200 mg/L (Adanir *et al.*, 2005).

There is no evidence that MeOH causes cancer in animals. This is based on test results presently available to the New Jersey Department of Health from published studies. MeOH may be a **teratogen** in humans since it is a teratogen in animals. MeOH **may damage the liver, kidneys and nervous system.**[36-39]

### ***THF***

Tetrahydrofuran should be handled as a **carcinogen – with extreme caution.**

*Odor threshold:* 31 ppm.

(The range of accepted odor threshold values is quite broad. Caution should be used in relying on odor alone as a warning of potentially hazardous exposures.)

*Symptoms and effects (both acute and delayed):* irritant effects, cough, shortness of breath, narcosis, somnolence.

Although no epidemiological studies of THF have been conducted, several occupational exposure case studies in humans suggest that **target organs in humans are the CNS, respiratory tract, liver, and kidney** (Garnier *et al.*, 1989; Albrecht *et al.*, 1987; Juntunen *et al.*, 1984; Edling, 1982; Emmett, 1976). The major uncertainty associated with all of the reported human case studies is the fact that workers were exposed to other solvents and chemicals in addition to THF, so it is not possible to conclusively attribute the observed effects to THF exposure.

The weight of evidence suggests that THF is **carcinogenic** for more than one species, sex, and site; therefore, there is suggestive evidence of carcinogenic potential following exposure to THF in humans (U.S. EPA, 2005).[40-48]

### ***Isopropyl Alcohol***

*Odor threshold:* 22 ppm.

(Odor thresholds vary greatly. Do not rely on odor alone to determine potentially hazardous exposures.)

*Symptoms and effects (both acute and delayed):* irritant effects, respiratory paralysis, drowsiness, dizziness, unconsciousness, narcosis, inebriation, headache, somnolence, coma, drying-out effect resulting in rough and chapped skin.

While isopropyl alcohol (IPA) has been tested, it is not classifiable as to its potential to cause cancer. There is **limited evidence that IPA may damage the developing fetus in animals**. This chemical has not been adequately evaluated to determine whether repeated exposure can cause brain or other nerve damage. However, many solvents and other petroleum-based chemicals have been shown to cause such damage. Effects may include reduced memory and concentration, personality changes (withdrawal, irritability), fatigue, sleep disturbances, reduced coordination, and/or effects on nerves supplying internal organs (autonomic nerves) and/or nerves to the arms and legs (weakness, “pins and needles”). **IPA may affect the liver and kidneys.**[49-50]

## ***Acetic Acid***

*Odor threshold:* 0.48 to 1.0 ppm.

(The range of accepted odor threshold values is quite broad. Caution should be used in relying on odor alone as a warning of potentially hazardous exposures.)

*Symptoms and effects (both acute and delayed):* irritation and corrosion, bronchitis, shortness of breath, gastric spasms, nausea, vomiting, circulatory collapse, shock, risk of corneal clouding, risk of blindness.

According to the information presently available to the New Jersey Department of Health, acetic acid (AcA) has been tested and has not been shown to cause cancer in animals and has not been shown to affect reproduction.

Repeated exposure can cause thickening and cracking of the skin, particularly the skin of the hands.[51-52]

## ***Formic Acid***

*Odor threshold:* 49 ppm.

(Odor thresholds vary greatly. Do not rely on odor alone to determine potentially hazardous exposures.)

*Symptoms and effects (both acute and delayed):* conjunctivitis, dermatitis, irritation and corrosion, cough, shortness of breath, risk of blindness.

According to the information presently available to the New Jersey Department of Health, formic acid (FA) has not been tested for its ability to cause cancer in animals. There is no evidence that FA affects reproduction.

Exposure to high concentrations, particularly in susceptible individuals, may cause a **skin allergy**. If allergy develops, very low future exposure can cause itching and a skin rash. **FA may damage the kidneys.**[53-54]

## **HPLC Safety Accessories**

*HPLC instrument should be if possible equipped with the following accessories:*

1. Safety caps onto MP bottles – apart from increasing safety for lab workers, it also prevents evaporation of highly volatile components of MP mixtures and thus prevents changing of MP elution strength and also avoid dust particles entering the MP bottle:
  - a. Cheaper, simple, but not “100%” solution (air valve not included) – safety caps with adapters for generic disposable membrane filters (typical pore size 0.45 or better 0.2  $\mu\text{m}$ ; *e.g.*, Duran<sup>TM</sup>/ Fisher Scientific).
  - b. More expensive and more advanced solution is represented by safety caps with disposable air check valve (filter incl.) that avoids evaporation of organic solvents and other volatile MP components even during the time when the pumps are off (*e.g.*, S.C.A.T. Europe).
2. Waste container should be properly sealed to avoid evaporation of volatiles and there should be installed also some apparatus to prevent overpressure in waste container, *e.g.*:
  - a. Simple and cheap – exhaust tube connected into fume hood.
  - b. When the fume hood solution is not possible for any reason, the exhaust filter that cleans solvent vapors from the exhaust air can be used. It is based on active charcoal and contains additional components which prevent sticking or clump formation and so prevents restriction of the filter performance. 99 % of the volatile substances are captured by the exhaust filter according to S.C.A.T. Europe producer.[55-57]

# 3 Validation

In pharmaceutical analysis the recommendations on analytical validation are based on the work of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The document **Validation of Analytical Procedures: Text and Methodology Q2(R1)** is the core document on analytical validation. According to the document the validation process, which should demonstrate that an analytical procedure is suitable for its intended purpose, consists of a series of performance characteristics.[1, 58]

## **3.1 Analytical Procedure**

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include the sample, the reference standard and the reagents, use of apparatus, generation of calibration curve and use of the formulae for calculation.[1]

## 3.2 Accuracy

The accuracy of an analytical procedure is defined as the closeness of the test results obtained by the procedure to the true value. In the case of the assay of a drug substance, accuracy may be determined by application of the analytical procedure to an analyte of known purity, for example, a certified reference substance or a substance with a similar high quality. In the determination of a drug substance in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the formulated product (drug-free) to which known amounts of the drug substance (*e.g.*, a reference substance) have been added. Accuracy should be reported in either of two ways:

- The percentage of recovery by the assay of the known added amount of analyte in the sample.
- The difference between the mean and the accepted true value.

The accuracy of the analytical procedure should be established across its entire range. The ICH document recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range. This is three concentrations and three replicates of each concentration.[1]

### 3.3 Precision

The precision of an analytical procedure expresses the closeness of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample. Precision is usually expressed as the standard deviation ( $s$ ) or the relative standard deviation (% *RSD*) of the mean ( $m$ ) of a series of measurements:

$$\%RSD = \frac{s}{m} \times 100\%$$

*Equation XVIII. The relative standard deviation [1]*

According to the ICH document, precision may be considered at three levels:

- Repeatability
- Intermediate precision
- Reproducibility

The **repeatability** expresses the precision under the same operating conditions over a short interval of time. Normally, the same analyst with the same equipment carries out the analysis within one day within the same laboratory.

**Intermediate precision** expresses within-laboratories variations. Normally different analysts with different equipment carry out the analysis on different days. The intermediate precision is **also called ruggedness**.

**Reproducibility** expresses the precision of a procedure between different laboratories in a collaborative study.

The precision of the analytical procedure is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation. The assays are independent analysis of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

According to the ICH document repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure. This is three



concentrations and three replicates of each concentration. Repeatability can also be assessed using a minimum of six determinations at 100% of the test concentration.

The acceptance criteria for precision depend very much on the type of analysis. Pharmaceutical QC precision of better than 1% RSD is easily achieved for compound analysis, but the precision for biological samples is more like 20% at the concentration limits and 15% at other concentration levels.[1]

## 3.4 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of compounds that may be expected to be present. Typically these might include impurities, degradation products and matrix components. Lack of specificity of an individual procedure may be compensated by other supporting analytical procedures. The ICH document states that when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of **selectivity**. **Peak purity tests** may be used to show that the analyte chromatographic peak is not attributed to more than one component. Peak purity tests are based on spectra, such as UV/visible spectra, recorded by diode array detectors. If all the individual spectra recorded during elution of a peak are identical, the peak is considered pure. Selectivity studies should also assess interferences that may be caused by matrix components. In bioanalysis, interferences caused by biological fluids must be examined. The absence of matrix interferences for quantitative methods should be demonstrated by the analysis of drug-free samples from at least five independent sources of control matrix.[1]

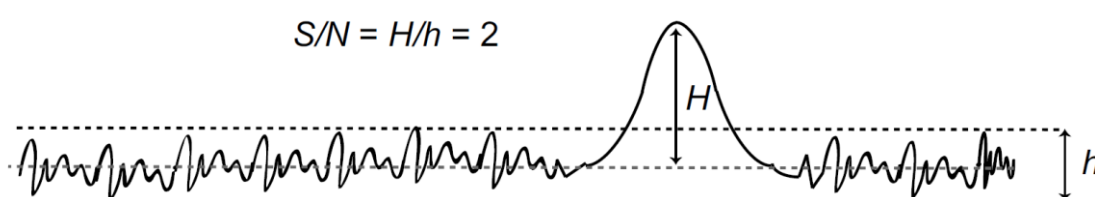
## 3.5 Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessary quantified as an exact value. The test is a limit test, which substantiate that the amount of an analyte is above or below a certain level. The detection limit is usually expressed as the concentration of an analyte in the sample.

For instrumental methods, such as HPLC/UHPLC, which exhibit baseline noise, the detection limit is based on signal to noise (S/N) ratios. Determination of the S/N ratio is performed by measuring signals from samples with known concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A **S/N level between 3 : 1 or 2 : 1 is generally considered acceptable for estimating the detection limit.**

In LC the detection limit is the injected amount that results in a peak with a peak height ( $H$ ) at least two or three times as high as the baseline noise level ( $h$ ). This is illustrated in Figure XXIV, where  $H$  is the height of the peak and  $h$  is the range of the noise. The detection limit is at a S/N level of 2.

Other approaches described by the ICH documents depend on the determination of the slope of the calibration curve and the standard deviation of the response. [1]

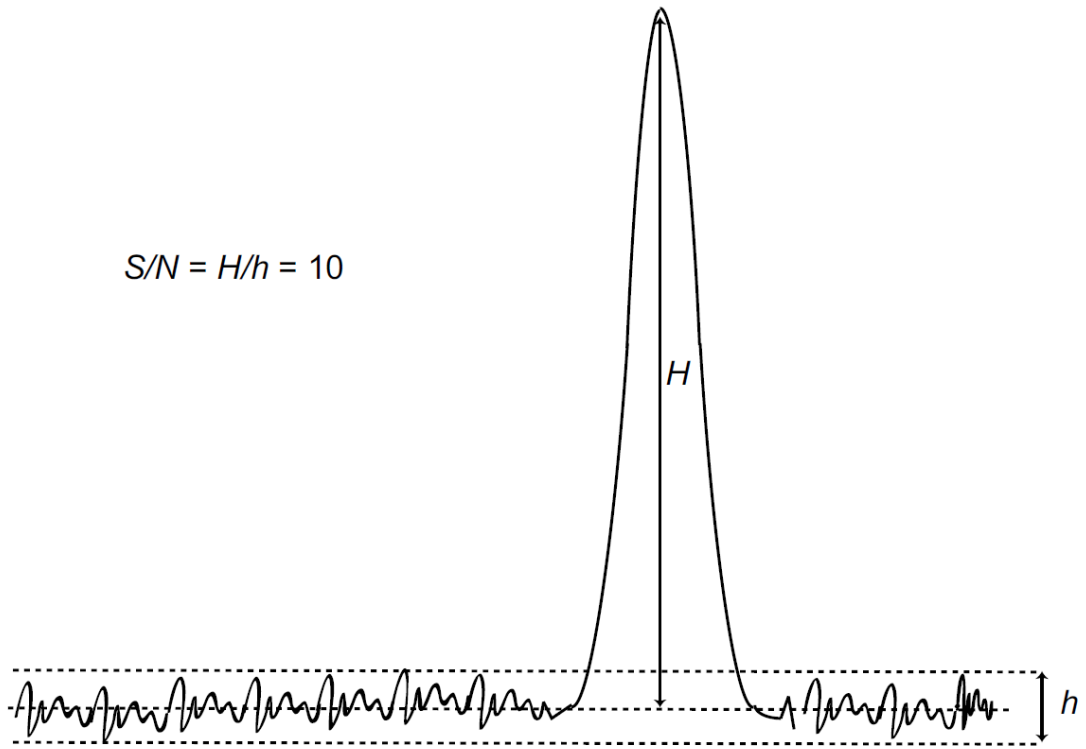


**Figure XXIV.** Limit of detection at  $S/N=2$  [1]

## 3.6 Quantification Limit

The quantification limit of an individual analytical procedure is the lowest amount of an analyte in a sample, which can be quantitatively determined with suitable accuracy and precision. The quantification limit is a parameter of quantitative determinations of low levels of compounds in sample matrices. It is used particularly for the **determination of impurities or degradation products** of active ingredients and formulated products and in bioanalysis for quantitative determinations of **low levels of drug substances in biological matrices**.

For instrumental methods that exhibit baseline noise the S/N ratio may be applied. A **typical S/N ratio is 10 : 1**. In LC the quantification limit is the injected amount that results in a peak with a peak height ( $H$ ) ten times as high as the baseline noise level ( $h$ ). This is illustrated in Figure XXV. If the required precision of a method has been specified, the quantification limit of an analyte can be determined by injecting a number of samples with decreasing amounts of analyte. The calculated %RSD is then plotted against the analyte concentration. The RSD increases when the concentration decreases and the quantification limit can be read on the plot. Normally, the RSD criterion for defining the quantification limit is set to 20%. [1]



**Figure XXV.** Limit of quantification at  $S/N = 10$  [1]

## 3.7 Linearity and Range

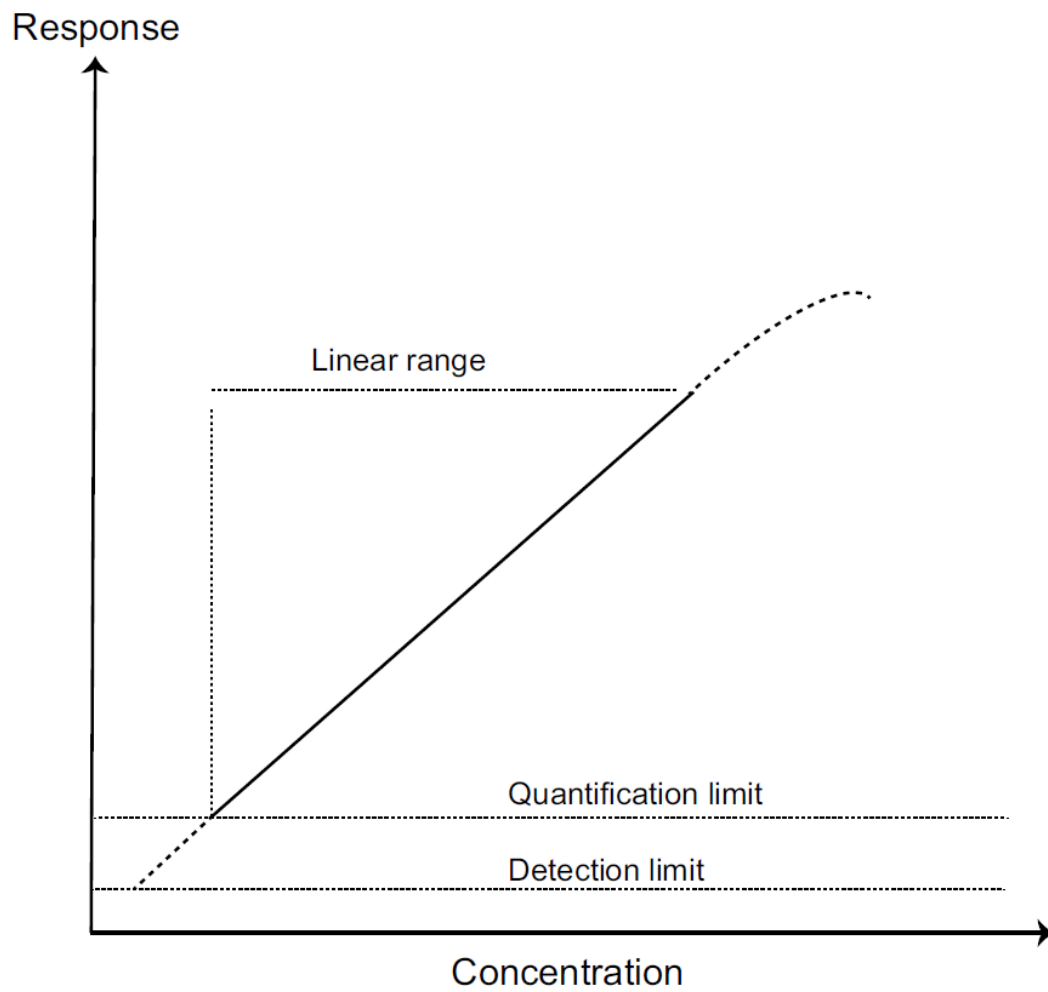
The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample as shown in Figure XXVI. Linearity should be established across the range of the analytical procedure. It should be evaluated by inspection of a plot of signals as a function of analyte concentration or content. The signal can be peak area, peak height or peak area or peak height ratios in a chromatographic procedure. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. The square of the correlation coefficient and the regression line should be reported.

The ICH recommends that, for establishing linearity a minimum of five concentrations should normally be used. It is also recommended that the following minimum specified ranges should be considered:

- *Assay of an active substance or a finished product:* 80–120% of the test concentration.
- *For content uniformity:* a minimum of 70–130% of the test concentration.
- *For determination of an impurity:* 50–120% of the acceptance criteria.

For bioanalysis, the range is normally established from the quantification limit and to somewhat above the highest expected concentration found in the samples.

The range of an analytical procedure is the interval between the upper and lower concentration (amount) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable precision, accuracy and linearity. The range of the procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy and linearity when applied to samples containing analyte at the extreme range as well as within the range. [1]



*Figure XXVI. Linear range [1]*

## 3.8 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. In the case of LC, examples of typical variations are:

- Influence of variations of pH in the MP
- Influence of variations in MP composition
- Different columns (different lots/or suppliers)
- Temperature
- Flow rate

### 3.8.1 System Suitability

One consequence of the evaluation of robustness should be that a series of system suitability parameters is established to ensure that the validity of the procedure is maintained whenever used. Typical variations are the stability of analytical solutions, different equipment and different analysts. For chromatographic methods the variations listed under robustness are crucial.

System suitability is an integrated part of many analytical procedures. The tests are based on the concept that equipment; electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability tests parameters to be established for a particular procedure depend on the type of procedure being validated. In Ph. Eur. system suitability tests are listed in the monographs. For a chromatographic procedure the parameters that are usually employed in assessing the performance of the columns are:

- Number of theoretical plates
- Retention factor
- Resolution
- Relative retention



- Symmetry factor

In Ph. Eur. and the United States Pharmacopeia (USP) the requirements for system suitability testing of HPLC methods are typically:

- The relative standard deviation of peak areas or peak heights are less than 1% (for drug substance assay)
- The resolution ( $R_s$ ) is greater than 2
- The symmetry factor is in the range 0.8–1.5
- The number of theoretical plates are larger than 2000 (for HPLC)
- The retention factor ( $k$ ) is larger than 2 [1]

# 4 Stability Testing

Although some drugs (*e.g.*, tablets) often have a stability that may last for many years there is a maximum life time for all drugs and drug substances of five years. This is to avoid any discussion on how long drugs and drug substances may be stored. However, drug substances should always comply with the monographs in the pharmacopoeias or similar standards.

Drug preparations in general should at the time of production at the most deviate 5% from the declared content. On the basis of stability testing a larger deviation of the lower limit of up to 10% within the life time of the drug is accepted. The **life time** is also named the **shelf-life** of the drug.

Guideline **Stability Testing of New Drug Substances and Products Q1A(R2)** has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. This Q1A(R2) guideline defines the stability data package for a new drug substance or drug product that is sufficient for a registration application within the three regions of the European Union, Japan, and the United States. Q1A(R2) guideline has been adopted, *e.g.*, by the European Medicines Agency.[1, 59-60]

## 4.1 Stability Testing of Drug Substance

Information on the stability of the drug substance is an integral part of the systematic approach to stability evaluation.[59-60]

### 4.1.1 Stress Testing

Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved.

Stress testing is likely to be carried out on a single batch of the drug substance. It should include the effect of temperatures (in 10°C increments (*e.g.*, 50°C, 60°C, *etc.*) above that for accelerated testing), humidity [*e.g.*, 75% relative humidity (RH) or greater] where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photostability testing should be an integral part of stress testing. The standard conditions for photostability testing are described in ICH Q1B.

Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical procedures. However, it may not be necessary to examine specifically for certain degradation products if it has been demonstrated that they are not formed under accelerated or long term storage conditions.

Results from these studies will form an integral part of the information provided to regulatory authorities.[59-60]

### 4.1.2 Selection of Batches

Data from formal stability studies should be provided on at least three primary batches of the drug substance. The batches should be manufactured to a minimum of pilot scale by the same synthetic route as, and using a method of manufacture and procedure that simulates the final process to be used for, production batches. The overall quality of the batches of

drug substance placed on formal stability studies should be representative of the quality of the material to be made on a production scale. Other supporting data can be provided.[59-60]

### **4.1.3 Container Closure System**

The stability studies should be conducted on the drug substance packaged in a container closure system that is the same as or simulates the packaging proposed for storage and distribution.[59-60]

### **4.1.4 Specification**

Specification, which is a list of tests, reference to analytical procedures, and proposed acceptance criteria, is addressed in ICH Q6A and Q6B. In addition, specification for degradation products in a drug substance is discussed in Q3A.

Stability studies should include testing of those attributes of the drug substance that are susceptible to change during storage and are likely to influence quality, safety, and/or efficacy. The testing should cover, as appropriate, the physical, chemical, biological, and microbiological attributes. Validated stability-indicating analytical procedures should be applied.[59-60]

### **4.1.5 Testing Frequency**

For long term studies, frequency of testing should be sufficient to establish the stability profile of the drug substance. For drug substances with a proposed re-test period of at least 12 months, the frequency of testing at the long term storage condition should normally be every 3 months over the first year, every 6 months over the second year, and annually thereafter through the proposed re-test period.

At the accelerated storage condition, a minimum of three time points, including the initial and final time points (*e.g.*, 0; 3; and 6 months), from a 6-month study is recommended. Where an expectation (based on development experience) exists that results from accelerated studies are likely to approach significant change criteria, increased testing should be conducted either by adding samples at the final time point or by including a fourth time point in the study design.

When testing at the intermediate storage condition is called for as a result of significant change at the accelerated storage condition, a minimum of four time points, including the initial and final time points (*e.g.*, 0; 6; 9; and 12 months), from a 12-month study is recommended.[59-60]

#### **4.1.6 Storage Conditions**

In general, a drug substance should be evaluated under storage conditions (with appropriate tolerances) that test its thermal stability and, if applicable, its sensitivity to moisture. The storage conditions and the lengths of studies chosen should be sufficient to cover storage, shipment, and subsequent use.

The long term testing should cover a minimum of 12 months' duration on at least three primary batches at the time of submission and should be continued for a period of time sufficient to cover the proposed re-test period. Additional data accumulated during the assessment period of the registration application should be submitted to the authorities if requested. Data from the accelerated storage condition and, if appropriate, from the intermediate storage condition can be used to evaluate the effect of short term excursions outside the label storage conditions (such as might occur during shipping).

Long term, accelerated, and, where appropriate, intermediate storage conditions for drug substances are detailed in the sections below. The general case applies if the drug substance is not specifically covered by a subsequent section. Alternative storage conditions can be used if justified.

### **General case storage conditions**

Study	Storage condition	Minimum time period covered by data at submission
Long term*	25°C ± 2°C / 60% RH ± 5% RH	12 months
	or 30°C ± 2°C / 65% RH ± 5% RH	
Intermediate**	30°C ± 2°C / 65% RH ± 5% RH	6 months
Accelerated	40°C ± 2°C / 75% RH ± 5% RH	6 months

\* It is up to the applicant to decide whether long term stability studies are performed at 25 ± 2°C / 60% RH ± 5% RH or 30°C ± 2°C / 65% RH ± 5% RH.

\*\* If 30°C ± 2°C / 65% RH ± 5% RH is the long-term condition, there is no intermediate condition.

**Table IV.** Storage conditions – general case [59-60]

If long-term studies are conducted at 25°C ± 2°C / 60% RH ± 5% RH and “significant change” occurs at any time during 6 months’ testing at the accelerated storage condition, additional testing at the intermediate storage condition should be conducted and evaluated against significant change criteria. Testing at the intermediate storage condition should include all tests, unless otherwise justified. The initial application should include a minimum of 6 months’ data from a 12-month study at the intermediate storage condition. “Significant change” for a drug substance is defined as failure to meet its specification. [59-60]

### **Drug substances intended for storage in a refrigerator**

Study	Storage condition	Minimum time period covered by data at submission
Long term	5°C ± 3°C	12 months
Accelerated	25°C ± 2°C / 60% RH ± 5% RH	6 months

**Table V.** Storage conditions – drug substances intended for storage in a refrigerator [59-60]

Data from refrigerated storage should be assessed according to the 4.1.7 Evaluation section, except where explicitly noted below.

If significant change occurs between 3 and 6 months' testing at the accelerated storage condition, the proposed re-test period should be based on the real time data available at the long term storage condition.

If significant change occurs within the first 3 months' testing at the accelerated storage condition, a discussion should be provided to address the effect of short term excursions outside the label storage condition, *e.g.*, during shipping or handling. This discussion can be supported, if appropriate, by further testing on a single batch of the drug substance for a period shorter than 3 months but with more frequent testing than usual. It is considered unnecessary to continue to test a drug substance through 6 months when a significant change has occurred within the first 3 months.[59-60]

***Drug substances intended for storage in a freezer***

Study	Storage condition	Minimum time period covered by data at submission
Long term	- 20°C ± 5°C	12 months

***Table VI. Storage conditions – drug substances intended for storage in a freezer [59-60]***

For drug substances intended for storage in a freezer, the re-test period should be based on the real time data obtained at the long term storage condition. In the absence of an accelerated storage condition for drug substances intended to be stored in a freezer, testing on a single batch at an elevated temperature (*e.g.*, 5°C ± 3°C or 25°C ± 2°C) for an appropriate time period should be conducted to address the effect of short term excursions outside the proposed label storage condition, *e.g.*, during shipping or handling.[59-60]

***Drug substances intended for storage below -20°C***

Drug substances intended for storage below -20°C should be treated on a case-by-case basis.[59-60]

**4.1.7 Evaluation**

The purpose of the stability study is to establish, based on testing a minimum of three batches of the drug substance and evaluating the stability information (including, as appropriate, results of the physical, chemical, biological, and microbiological tests), a re-

test period applicable to all future batches of the drug substance manufactured under similar circumstances. The degree of variability of individual batches affects the confidence that a future production batch will remain within specification throughout the assigned re-test period.

The data may show so little degradation and so little variability that it is apparent from looking at the data that the requested re-test period will be granted. Under these circumstances, it is normally unnecessary to go through the formal statistical analysis; providing a justification for the omission should be sufficient.

The nature of any degradation relationship will determine whether the data should be transformed for linear regression analysis. Usually the relationship can be represented by a linear, quadratic, or cubic function on an arithmetic or logarithmic scale. Statistical methods should be employed to test the goodness of fit of the data on all batches and combined batches (where appropriate) to the assumed degradation line or curve.

Limited extrapolation of the real time data from the long term storage condition beyond the observed range to extend the re-test period can be undertaken at approval time, if justified. This justification should be based on what is known about the mechanism of degradation, the results of testing under accelerated conditions, the goodness of fit of any mathematical model, batch size, existence of supporting stability data, *etc.* However, this extrapolation assumes that the same degradation relationship will continue to apply beyond the observed data.

Any evaluation should cover not only the assay, but also the levels of degradation products and other appropriate attributes.[59-60]

#### **4.1.8 Statements/Labeling**

A storage statement should be established for the labeling in accordance with relevant national/regional requirements. The statement should be based on the stability evaluation of the drug substance. Where applicable, specific instructions should be provided, particularly for drug substances that cannot tolerate freezing. Terms such as “ambient conditions” or “room temperature” should be avoided.

A re-test period should be derived from the stability information, and a retest date should be displayed on the container label if appropriate.[59-60]



## **4.2 Stability Testing of Drug Product**

The design of the formal stability studies for the drug product should be based on knowledge of the behavior and properties of the drug substance and from stability studies on the drug substance and on experience gained from clinical formulation studies. The likely changes on storage and the rationale for the selection of attributes to be tested in the formal stability studies should be stated.[59-60]

### **4.2.1 Photostability Testing**

Photostability testing should be conducted on at least one primary batch of the drug product if appropriate. The standard conditions for photostability testing are described in guideline ICH Q1B.[59-60]

### **4.2.2 Selection of Batches**

Data from stability studies should be provided on at least three primary batches of the drug product. The primary batches should be of the same formulation and packaged in the same container closure system as proposed for marketing. The manufacturing process used for primary batches should simulate that to be applied to production batches and should provide product of the same quality and meeting the same specification as that intended for marketing. Two of the three batches should be at least pilot scale batches and the third one can be smaller, if justified. Where possible, batches of the drug product should be manufactured by using different batches of the drug substance. Stability studies should be performed on each individual strength and container size of the drug product unless bracketing or matrixing is applied.[59-60]

### **4.2.3 Container Closure System**

Stability testing should be conducted on the dosage form packaged in the container closure system proposed for marketing (including, as appropriate, any secondary packaging and container label). Any available studies carried out on the drug product outside its immediate container or in other packaging materials can form a useful part of the stress

testing of the dosage form or can be considered as supporting information, respectively.[59-60]

#### **4.2.4 Specification**

Specification, which is a list of tests, reference to analytical procedures, and proposed acceptance criteria, including the concept of different acceptance criteria for release and shelf life specifications, is addressed in ICH Q6A and Q6B. In addition, specification for degradation products in a drug product is addressed in Q3B.

Stability studies should include testing of those attributes of the drug product that are susceptible to change during storage and are likely to influence quality, safety, and/or efficacy. The testing should cover, as appropriate, the physical, chemical, biological, and microbiological attributes, preservative content (*e.g.*, antioxidant, antimicrobial preservative), and functionality tests (*e.g.*, for a dose delivery system). Analytical procedures should be fully validated and stability indicating. Whether and to what extent replication should be performed will depend on the results of validation studies.

Shelf life acceptance criteria should be derived from consideration of all available stability information. It may be appropriate to have justifiable differences between the shelf life and release acceptance criteria based on the stability evaluation and the changes observed on storage. Any differences between the release and shelf life acceptance criteria for antimicrobial preservative content should be supported by a validated correlation of chemical content and preservative effectiveness demonstrated during drug development on the product in its final formulation (except for preservative concentration) intended for marketing. A single primary stability batch of the drug product should be tested for antimicrobial preservative effectiveness (in addition to preservative content) at the proposed shelf life for verification purposes, regardless of whether there is a difference between the release and shelf life acceptance criteria for preservative content. [59-60]

#### **4.2.5 Testing Frequency**

For long term studies, frequency of testing should be sufficient to establish the stability profile of the drug product. For products with a proposed shelf life of at least 12 months, the frequency of testing at the long term storage condition should normally be every 3 months over the first year, every 6 months over the second year, and annually thereafter through the proposed shelf life.

At the accelerated storage condition, a minimum of three time points, including the initial and final time points (e.g., 0; 3; and 6 months), from a 6-month study is recommended. Where an expectation (based on development experience) exists that results from accelerated testing are likely to approach significant change criteria, increased testing should be conducted either by adding samples at the final time point or by including a fourth time point in the study design.

When testing at the intermediate storage condition is called for as a result of significant change at the accelerated storage condition, a minimum of four time points, including the initial and final time points (e.g., 0; 6; 9; and 12 months), from a 12-month study is recommended.

Reduced designs, *i.e.*, matrixing or bracketing, where the testing frequency is reduced or certain factor combinations are not tested at all, can be applied, if justified. [59-60]

#### **4.2.6 Storage Conditions**

In general, a drug product should be evaluated under storage conditions (with appropriate tolerances) that test its thermal stability and, if applicable, its sensitivity to moisture or potential for solvent loss. The storage conditions and the lengths of studies chosen should be sufficient to cover storage, shipment, and subsequent use.

Stability testing of the drug product after constitution or dilution, if applicable, should be conducted to provide information for the labeling on the preparation, storage condition, and in-use period of the constituted or diluted product. This testing should be performed on the constituted or diluted product through the proposed in-use period on primary batches as part of the formal stability studies at initial and final time points and, if full shelf life long term data will not be available before submission, at 12 months or the last time point for which data will be available. In general, this testing need not be repeated on commitment batches.

The long term testing should cover a minimum of 12 months' duration on at least three primary batches at the time of submission and should be continued for a period of time sufficient to cover the proposed shelf life. Additional data accumulated during the assessment period of the registration application should be submitted to the authorities if requested. Data from the accelerated storage condition and, if appropriate, from the

intermediate storage condition can be used to evaluate the effect of short term excursions outside the label storage conditions (such as might occur during shipping).

Long term, accelerated, and, where appropriate, intermediate storage conditions for drug products are detailed in the sections below. The general case applies if the drug product is not specifically covered by a subsequent section. Alternative storage conditions can be used, if justified.[59-60]

### ***General case storage conditions***

Recommended storage conditions of drug substance shown in **Table IV** are also valid for drug products.

If long-term studies are conducted at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  /  $60\% \text{ RH} \pm 5\% \text{ RH}$  and “significant change” occurs at any time during 6 months’ testing at the accelerated storage condition, additional testing at the intermediate storage condition should be conducted and evaluated against significant change criteria. The initial application should include a minimum of 6 months’ data from a 12-month study at the intermediate storage condition.

In general, “significant change” for a drug product is defined as:

1. A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures.
2. Any degradation product’s exceeding its acceptance criterion.
3. Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (*e.g.*, color, phase separation, resuspendibility, caking, hardness, dose delivery per actuation); however, some changes in physical attributes (*e.g.*, softening of suppositories, melting of creams) may be expected under accelerated conditions.

and, as appropriate for the dosage form:

4. Failure to meet the acceptance criterion for pH.
5. Failure to meet the acceptance criteria for dissolution for 12 dosage units.[59-60]

### ***Drug products packaged in impermeable containers***

Sensitivity to moisture or potential for solvent loss is not a concern for drug products packaged in impermeable containers that provide a permanent barrier to passage of

moisture or solvent. Thus, stability studies for products stored in impermeable containers can be conducted under any controlled or ambient humidity condition (e.g., glass vials/bottles containing oral liquid preparations).[59-60]

***Drug products packaged in semi-permeable containers***

Aqueous-based products packaged in semi-permeable containers should be evaluated for potential water loss in addition to physical, chemical, biological, and microbiological stability. This evaluation can be carried out under conditions of low relative humidity, as discussed below. Ultimately, it should be demonstrated that aqueous-based drug products stored in semi-permeable containers can withstand low relative humidity environments.

Other comparable approaches can be developed and reported for non-aqueous, solvent-based products.

Study	Storage condition	Minimum time period covered by data at submission
Long term*	25°C ± 2°C / 40% RH ± 5% RH	12 months
	or 30°C ± 2°C / 35% RH ± 5% RH	
Intermediate**	30°C ± 2°C / 65% RH ± 5% RH	6 months
Accelerated	40°C ± 2°C / not more than (NMT) 25% RH	6 months

\* It is up to the applicant to decide whether long term stability studies are performed at 25 ± 2°C / 40% RH ± 5% RH or 30°C ± 2°C / 35% RH ± 5% RH.

\*\* If 30°C ± 2°C / 35% RH ± 5% RH is the long-term condition, there is no intermediate condition.

***Table VII. Storage conditions – drug products packaged in semi-permeable containers [59-60]***

For long-term studies conducted at 25°C ± 2°C / 40% RH ± 5% RH, additional testing at the intermediate storage condition should be performed as described under the general case to evaluate the temperature effect at 30°C if significant change other than water loss occurs during the 6 months’ testing at the accelerated storage condition. A significant change in water loss alone at the accelerated storage condition does not necessitate testing at the intermediate storage condition. However, data should be provided to demonstrate that the

drug product will not have significant water loss throughout the proposed shelf life if stored at 25°C and the reference relative humidity of 40% RH.

A 5% loss in water from its initial value is considered a significant change for a product packaged in a semi-permeable container after an equivalent of 3 months' storage at 40°C / NMT 25% RH. However, for small containers (1 mL or less) or unit-dose products, a water loss of 5% or more after an equivalent of 3 months' storage at 40°C / NMT 25% RH may be appropriate, if justified.

An alternative approach to studying at the reference relative humidity as recommended in the table above (for either long term or accelerated testing) is performing the stability studies under higher relative humidity and deriving the water loss at the reference relative humidity through calculation. This can be achieved by experimentally determining the permeation coefficient for the container closure system or, as shown in the example below, using the calculated ratio of water loss rates between the two humidity conditions at the same temperature. The permeation coefficient for a container closure system can be experimentally determined by using the worst case scenario (*e.g.*, the most diluted of a series of concentrations) for the proposed drug product.[59-60]

### ***Drug products intended for storage in a refrigerator***

Recommended storage conditions of drug substances intended for storage in a refrigerator shown in **Table V** are also valid for drug products intended for storage in a refrigerator.

If the drug product is packaged in a semi-permeable container, appropriate information should be provided to assess the extent of water loss.

If significant change occurs between 3 and 6 months' testing at the accelerated storage condition, the proposed shelf life should be based on the real time data available from the long term storage condition.

If significant change occurs within the first 3 months' testing at the accelerated storage condition, a discussion should be provided to address the effect of short term excursions outside the label storage condition, *e.g.*, during shipment and handling. This discussion can be supported, if appropriate, by further testing on a single batch of the drug product for a period shorter than 3 months but with more frequent testing than usual. It is considered unnecessary to continue to test a product through 6 months when a significant change has occurred within the first 3 months.[59-60]

### ***Drug products intended for storage in a freezer***

Recommended storage conditions of drug substances intended for storage in a freezer shown in **Table VI** are also valid for drug products intended for storage in a freezer.

For drug products intended for storage in a freezer, the shelf life should be based on the real time data obtained at the long term storage condition. In the absence of an accelerated storage condition for drug products intended to be stored in a freezer, testing on a single batch at an elevated temperature (*e.g.*,  $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$  or  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) for an appropriate time period should be conducted to address the effect of short term excursions outside the proposed label storage condition.[59-60]

### ***Drug products intended for storage below -20°C***

Drug products intended for storage below  $-20^{\circ}\text{C}$  should be treated on a case-by-case basis.[59-60]

## **4.2.7 Evaluation**

See section 4.1.7 Evaluation.

## **4.2.8 Statements/Labeling**

There should be a direct link between the label storage statement and the demonstrated stability of the drug product. An expiration date should be displayed on the container label. See also section 4.1.8 Statements/Labeling.[59-60]

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# 6 Abbreviations

AcA	Acetic acid
ACN	Acetonitrile
CAD	Charged aerosol detector
CMC	Classic monolithic column
CN	Cyanopropyl
CNS	Central nervous system
CZK	Czech koruna
DAD	Diode array detector
ECD	Electrochemical detector
ELSD	Evaporative light scattering detector
EPA	Environmental protection agency
EUR	euro
FA	Formic acid
FL	Full-loop
GC	Gas chromatography
HETP	Height equivalent to a theoretical plate
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
IEC	Ion exchange chromatography
IF	Impact factor
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
IPA	Isopropyl alcohol
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography or High performance liquid chromatography
LSC	Liquid-solid chromatography
MeOH	Methanol
MP	Mobile phase
MS	Mass spectrometry
NMC	Narrow monolithic column

NMT	Not more than
ODS	Octadecylsilane
PDA	Photodiode array
Ph. Eur.	The European Pharmacopoeia
PLNO	Partial-loop with needle overfill
PLPA	Partial-loop pressure assisted
PS–DVB	Polystyrene–divinylbenzene
QC	Quality control
RH	Relative humidity
RI	Refractive index
RP	Reversed phase
RSD	Relative standard deviation
S/N	Signal to noise
SAX	Strong anion exchanger
SCX	Strong cation exchanger
SEC	Size exclusion chromatography
SP	Stationary phase
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
U.S.	United States
UHPLC	Ultra-high performance liquid chromatography
USD	United States dollar
USP	United States Pharmacopeia
UV	Ultraviolet
WAX	Weak anion exchanger
WCX	Weak cation exchanger

# 7 Aims

Aims of presented dissertation were the following ones:

- 1) Development and validation of HPLC method for the simultaneous determination of propranolol hydrochloride and sodium benzoate in presence of various excipients and evaluation of stability of propranolol hydrochloride and sodium benzoate in extemporaneous oral liquid preparations by using the developed HPLC method mentioned above and by measuring pH of solutions. (Publication *No. I*; Publication *No. II*).
- 2) Development and validation of HPLC method for the simultaneous determination of sotalol hydrochloride and potassium sorbate in presence of various excipients and evaluation of stability of sotalol hydrochloride and potassium sorbate in extemporaneous oral liquid preparations using the mentioned HPLC method and by measuring pH of solutions. (Publication *No. III*; Publication *No. IV*).
- 3) Evaluation of stability of furosemide and methylparaben in extemporaneous oral liquid preparations by means of HPLC and by measuring pH of solutions. (Publication *No. V*).

# 8 List of Publications & Author's Contribution

## The dissertation is based on the following articles:

- I. **Zahalka, L.**; Matysova, L.; Sklubalova, Z.; Klovrzova, S.; Solich, P., Simultaneous Determination of Propranolol Hydrochloride and Sodium Benzoate in Oral Liquid Preparations by HPLC. *Chromatographia* **2013**, 76 (21-22), 1553-1558. (IF<sub>2016</sub> = **1.402**) – design of experiments, HPLC method development and validation, summarization of results, draft preparation and manuscript finalization
- II. Klovrzova, S.; **Zahalka, L.**; Matysova, L.; Horak, P.; Sklubalova, Z., Pediatric oral solutions with propranolol hydrochloride for extemporaneous compounding: the formulation and stability study. *Česká a slovenská farmacie* **2013**, 62 (1), 35-9. (peer reviewed journal without IF) – participation in design of experiments, conducting stability study (HPLC and pH), summarization of stability study results, participation in draft preparation
- III. Matysova, L.; Zahalkova, O.; Klovrzova, S.; Sklubalova, Z.; Solich, P.; **Zahalka, L.**, Development of a Gradient HPLC Method for the Simultaneous Determination of Sotalol and Sorbate in Oral Liquid Preparations Using Solid Core Stationary Phase. *Journal of Analytical Methods in Chemistry* **2015**, 6. (IF<sub>2016</sub> = **1.801**) – design of experiments, HPLC method development and validation, summarization of results, draft preparation and manuscript finalization
- IV. Klovrzova, S.; **Zahalka, L.**; Kriz, T.; Zahalkova, O.; Matysova, L.; Sklubalova, Z.; Horak, P., Extemporaneous sotalol hydrochloride oral solutions for use in paediatric cardiology: formulation and stability study. *European Journal of Hospital Pharmacy* **2016**, 23 (1), 33-37. (IF<sub>2016</sub> = **0.718**) – conducting stability study (HPLC and pH), summarization of stability study results, participation in draft preparation
- V. **Zahalka, L.**; Klovrzova, S.; Matysova, L.; Sklubalova, Z.; Solich, P., Furosemide Ethanol-Free Oral Solutions for Paediatric Use – Formulation, HPLC Method, and Stability Study. *European Journal of Hospital Pharmacy* **2017**, DOI: 10.1136/ejhpharm-2017-001264. (IF<sub>2016</sub> = **0.718**) – participating in conducting

stability study (HPLC and pH), summarization of stability study results, draft preparation and manuscript finalization

**Publication not directly related to the presented dissertation:**

- VI. Sklenářová, H.; Chocholouš, P.; Kobllová, P.; **Zahálka, L.**; Šatínský, D.; Matysová, L.; Solich, P., High-resolution monolithic columns—a new tool for effective and quick separation. *Analytical and Bioanalytical Chemistry* **2013**, 405 (7), 2255-63. (IF<sub>2016</sub> = **3.431**) – participating in conducting experiments and in data processing



# 9 Articles Related to the Dissertation

## – Commentary

## 9.1 Publication No. I

**Zahalka, L.;** Matysova, L.; Sklubalova, Z.; Klovrzova, S.; Solich, P., Simultaneous Determination of Propranolol Hydrochloride and Sodium Benzoate in Oral Liquid Preparations by HPLC. *Chromatographia* **2013**, 76 (21-22), 1553-1558. (IF<sub>2016</sub> = **1.402**)

### 9.1.1 Commentary *ad* Publication No. I

Infantile hemangiomas are the most common soft-tissue tumors of infancy. Corticosteroids had been the main treatment for complicated infantile hemangiomas, with interferon or vincristine as second- or third-line treatment. Because of the partial efficacy and side effects of these drugs, new treatments were needed. Propranolol is a nonselective beta blocker. Its main indication has changed from therapy of cardiovascular diseases (such as hypertension) to therapy of infantile hemangiomas in pediatric patients during last few years.

HPLC–UV method for quantification of propranolol hydrochloride and preservative sodium benzoate in oral liquid preparations was developed and fully validated. Separation was performed by Supelco Discovery® C18 (25 cm × 4.6 mm, particles 5 μm) column. UV/VIS absorbance detector was set at wavelength 230 nm. Column oven was conditioned to 25°C. Mobile phase was prepared by dissolving 1.6 g of sodium dodecyl sulfate and 0.31 g of tetrabutylammonium dihydrogen phosphate in 450 mL of ultrapure water; 1 mL of sulfuric acid (95–97 %) and 550 mL of acetonitrile were added. Sodium hydroxide solution (2.1 M) was used for adjusting pH to value 3.3 (±0.05). Retention times of sodium benzoate, propranolol hydrochloride and butylparaben (internal standard) were 2.2; 3.3; and 4.1 min, respectively.

Developed method is suitable for simultaneous determination of propranolol hydrochloride and sodium benzoate in oral liquid preparations which are used for the therapy of hemangiomas in pediatric patients. Method has been applied for stability testing of newly developed extemporaneous pediatric oral liquid preparations (Publication No. II).

## 9.2 Publication No. II

Klovřzova, S.; **Zahalka, L.**; Matysova, L.; Horak, P.; Sklubalova, Z., Pediatric oral solutions with propranolol hydrochloride for extemporaneous compounding: the formulation and stability study. *Česká a slovenská farmacie* **2013**, 62 (1), 35-9. (peer reviewed journal without IF)

### 9.2.1 Commentary ad Publication No. II

The aim of the study was to find an optimal vehicle for pediatric oral solution of non-cardio selective beta blocker propranolol hydrochloride and to verify its stability at two temperatures of storage. The proposed oral aqueous solutions (formulations F1 and F2) for extemporaneous compounding were stable at room temperature and/or refrigerator for 180 days. In accordance with the European Pharmacopoeia 7.0 (5.1.3 Efficacy of antimicrobial preservation), the efficacy of the antimicrobial preservative, sodium benzoate 0.05 % w/v, was demonstrated by an accredited laboratory. A labelled shelf-life of 3 months, storage in a refrigerator at  $5 \pm 3^\circ\text{C}$ , and protection from light can be recommended. The formulation F1 consisting of citrate-phosphate buffer mixed with sugar syrup has been considered better than F2 for a sweet and sour taste, particularly in the therapy of older children. Formulation F3 represents the composition formulated with a minimal content of excipients and is preservative-free. It must, therefore, be prepared under aseptic conditions. It can be expected for use in the therapy of neonates under supervision of a caregiver. A labelled shelf-life of 7 days can be recommended for extemporaneous compounding in real-life situations if stored in a refrigerator at  $5 \pm 3^\circ\text{C}$ . To protect from microbial contamination and to allow easy administration, preparations should be packaged in a glass container with a screw cap suitable for administration using a syringe for oral use.

Since January 2016 propranolol oral liquid preparation **Hemangiol®** has been according to the Czech State Institute for Drug Control (Medicinal products database) available on the Czech market. Hemangiol® is registered by Pierre Fabre Company and in July 2017 its prize was almost 6 thousand CZK (= approx. 260 USD or 230 EUR) for one package (= 120 mL).[61]

## 9.3 Publication No. III

Matysova, L.; Zahalkova, O.; Klovrczova, S.; Sklubalova, Z.; Solich, P.; **Zahalka, L.**, Development of a Gradient HPLC Method for the Simultaneous Determination of Sotalol and Sorbate in Oral Liquid Preparations Using Solid Core Stationary Phase. *Journal of Analytical Methods in Chemistry* **2015**, 6. (IF<sub>2016</sub> = **1.801**)

### 9.3.1 Commentary *ad* Publication No. III

Sotalol is a Class III antiarrhythmic agent that prolongs the QT interval and exhibits beta-adrenergic blocking properties. Sotalol has been widely used in the management of atrial arrhythmias for several decades including patients in the pediatric age group and those with congenital heart disease. In pediatric patients, sotalol has proven efficacy in suppressing supraventricular arrhythmias and maintaining a sinus rhythm and has also been used in the management of ventricular arrhythmias with more modest efficacy.

A gradient HPLC-UV method for quantification of sotalol hydrochloride and potassium sorbate in five types of oral liquid preparations was developed and fully validated. The separation of an active substance sotalol hydrochloride, preservative potassium sorbate, and other substances (for taste and smell correction, *etc.*) was performed using an Ascentis® Express C18 (100 × 4.6 mm, solid core particles 2.7 μm) HPLC column. Linear gradient elution mode with a flow rate of 1.3 mL/min was used, and the sample injection volume was 5 μL. The UV/Vis absorbance detector was set to a wavelength of 237 nm, and the column oven was conditioned at 25°C. A sodium dihydrogen phosphate dihydrate solution (pH 2.5; 17.7mM) was used as the mobile phase buffer. The total analysis time was 4.5 min (+ 2.5 min for reequilibration).

The method was successfully employed in a stability evaluation (Publication No. IV) of newly developed sotalol oral liquid formulations, which are now already being used in the therapy of arrhythmias in pediatric patients; the method is also suitable for general quality control, that is, not only just for extemporaneous preparations containing the mentioned substances.

## 9.4 Publication No. IV

Klovřzova, S.; **Zahalka, L.**; Kriz, T.; Zahalkova, O.; Matysova, L.; Sklubalova, Z.; Horak, P., Extemporaneous sotalol hydrochloride oral solutions for use in paediatric cardiology: formulation and stability study. *European Journal of Hospital Pharmacy* **2016**, 23 (1), 33-37. (IF<sub>2016</sub> = **0.718**)

### 9.4.1 Commentary *ad* Publication No. IV

Sotalol is an anti-arrhythmic  $\beta$ -blocker which is well tolerated and highly effective for the treatment of ventricular and supraventricular tachycardia in children.

Stability of three extemporaneous oral liquid formulations containing sotalol hydrochloride was evaluated by using developed HPLC method mentioned above (Publication No. III). HPLC analysis demonstrated that the concentration of sotalol hydrochloride in the formulations was in accordance with the criterion that at least 95% of the initial content should remain during storage at cold or room temperature throughout the 180-day study period. Preparations should be stored in a brown glass container with a screw cap suitable for use with a graduated pipette for accurate oral dosing. Storage in a refrigerator is preferred, particularly in case of presence of an artificial sweetener sodium saccharin. It was evaluated by HPLC that the additive-free solution of sotalol hydrochloride can be autoclaved to ensure microbiological stability and it can be used particularly for neonates and in emergency situations.

**In July 2017** there was according to the Czech State Institute for Drug Control (Medicinal products database) **no registered oral liquid dosage form of sotalol** available on the Czech market.[61]

## 9.5 Publication No. V

**Zahalka, L.;** Klovrczova, S.; Matysova, L.; Sklubalova, Z.; Solich, P., Furosemide Ethanol-Free Oral Solutions for Paediatric Use – Formulation, HPLC Method, and Stability Study. *European Journal of Hospital Pharmacy* **2017**, DOI: 10.1136/ejhpharm-2017-001264. (IF<sub>2016</sub> = 0.718)

### 9.5.1 Commentary *ad* Publication No. V

Oral liquid solutions of the diuretic active ingredient furosemide marketed across Europe do not comply with recent requirements for pediatric preparation owing to their ethanol content and, moreover, in some countries (including the Czech Republic) only tablet or injection dosage forms of furosemide are available.

Two aqueous, ethanol-free oral solutions containing furosemide in the concentration 2 mg/mL were developed in accordance with the recent requirements of the safety of pediatric drugs. The preparations formulated for easy extemporaneous compounding in a pharmacy are suitable for the edema therapy of various origins as well as hypertension in pediatric age groups above 1 month of age. The excipients used ensured stable pH, antimicrobial stability, and pleasant taste. A 9-month stability study performed by validated HPLC analysis demonstrated that the concentration of furosemide in both F1 and F2 formulations was in accordance with the criterion that at least 90 % of the initial content should remain during storage at 25°C or 40°C. Nevertheless, preparation F1 having a worse, slightly burning taste caused by the presence of sodium hydroxide, although an artificial sweetener sodium saccharin 0.1 % w/v was added, is less preferable when compared to F2 containing disodium hydrogen phosphate dodecahydrate. Formulation F2 represents a compromise between good furosemide solubility in water, taste acceptance in pediatric patients, and fast compounding procedure. For long stability at room temperature, the stock F2 solution could be prepared in advance in the pharmacy and be available until needed. Methylparaben 0.1 % w/v in preparation F2 stored at room temperature fully satisfied the recommended criteria for preservative efficacy in oral preparations according to the European Pharmacopoeia 9.0 (5.1.3 Efficacy of antimicrobial preservation).

**In July 2017** there was according to the Czech State Institute for Drug Control (Medicinal products database) **no registered oral liquid dosage form of furosemide** available on the Czech market.[61]

# 10 Conclusion

Extemporaneous oral liquid preparations containing active substances mentioned below were developed and their stability under various storage conditions was evaluated using developed and validated HPLC methods.

## 1) Propranolol oral liquid solutions

- Nonselective  $\beta$ -blocker preparations for the treatment of infantile hemangiomas.
- Isocratic HPLC method for the determination of pharmaceutical active ingredient propranolol hydrochloride and preservative sodium benzoate in presence of various excipients using Supelco Discovery® C18 (250 × 4.6 mm, particles 5  $\mu$ m) column was developed and validated for ten oral liquid formulations. (Publication No. I)
- Concentration of propranolol hydrochloride and sodium benzoate; and pH of solutions, at time intervals of 0–180 days were evaluated in three selected formulations under various storage conditions. (Publication No. II)
- Two monographs in the national (Czech) part of the European Pharmacopoeia entitled “*PROPRANOLOLI HYDROCHLORIDI SOLUTIO CUM ACIDO CITRICO*” and “*PROPRANOLOLI HYDROCHLORIDI SOLUTIO CUM NATRII HYDROGENOPHOSPHATE*” were created based upon the research project results.[62]
- *Since January 2016 registered propranolol oral liquid preparation Hemangiol® has been available on the Czech market.*

## 2) Sotalol oral liquid solutions

- Anti-arrhythmic  $\beta$ -blocker preparations for the treatment of ventricular and supraventricular tachycardia particularly in children.
- Gradient HPLC method for the determination of sotalol hydrochloride and preservative potassium sorbate using Ascentis® Express C18 (100 × 4.6 mm, solid core particles 2.7  $\mu$ m) column was developed and validated for five various formulations. (Publication No. III)



- Concentration of sotalol hydrochloride and potassium sorbate; and pH of solutions, at time intervals of 0–180 days were evaluated in three selected formulations under various storage conditions. (Publication No. IV)
- *In July 2017 there was **no registered oral liquid dosage form of sotalol** on the Czech market.*

### **3) Furosemide oral liquid solutions**

- Diuretic preparations for the treatment of hypertension and edema associated with heart failure including pulmonary edema particularly in children.
- Isocratic HPLC method using Supelco Discovery® HS C18 (150 × 4.6 mm, particles 5 µm) column was used for conducting stability study. The method was developed in cooperation with Mgr. Tereza Břežná during preparation of her diploma thesis.[63]
- Concentration of furosemide and preservative methylparaben; and pH of solutions, at time intervals of 0–270 days were evaluated in two various formulations under various storage conditions and the influence of autoclaving on preparation stability was evaluated. (Publication No. V)
- *In July 2017 there was **no registered oral liquid dosage form of furosemide** on the Czech market.*

# 11 Full-texts of Research Articles Related to the Dissertation

Note: The last article entitled “*Furosemide ethanol-free oral solutions for paediatric use: formulation, HPLC method and stability study*”, was accepted for publication on 3<sup>rd</sup> July 2017 (DOI: 10.1136/ejhpharm-2017-001264) and the presented article below is the final version before typesetting and editing by BMJ publisher.

# Simultaneous Determination of Propranolol Hydrochloride and Sodium Benzoate in Oral Liquid Preparations by HPLC

L. Zahálka · L. Matysová · Z. Šklubalová ·  
S. Klovrzová · P. Solich

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**Abstract** A simple, selective and sensitive HPLC–UV method for quantification of propranolol hydrochloride and sodium benzoate in oral liquid preparations was developed and fully validated. Separation was performed by Supelco Discovery® C18 (25 cm × 4.6 mm, particles 5 µm) column. UV/VIS absorbance detector was set at wavelength 230 nm. Column oven was conditioned to 25 °C. Mobile phase was prepared by dissolving 1.6 g of sodium dodecyl sulphate and 0.31 g of tetrabutylammonium dihydrogen phosphate in 450 mL of ultrapure water; 1 mL of sulphuric acid (95–97 %) and 550 mL of acetonitrile were added. Sodium hydroxide solution (2.1 M) was used for adjusting pH to value 3.3 (±0.05). Retention times of sodium benzoate, propranolol hydrochloride and butylparaben (internal standard) were 2.2, 3.3 and 4.1 min, respectively. Newly developed method is suitable for simultaneous determination of propranolol hydrochloride and sodium benzoate in oral liquid preparations which are used for therapy of haemangiomas in paediatric patients. Method

has been applied for stability testing of extemporaneous paediatric oral formulations containing propranolol hydrochloride.

**Keywords** HPLC · Propranolol hydrochloride · Sodium benzoate · Paediatric oral formulations

## Introduction

Infantile haemangiomas (IHs) are the most common soft-tissue tumours of infancy. Corticosteroids are currently the main treatment for complicated IHs, with interferon or vincristine as second- or third-line treatment. Because of the partial efficacy and side effects of these drugs, new treatments are needed [1]. Propranolol (PRO) is a non-selective beta blocker. Its main indication has changed from therapy of cardiovascular diseases (such as hypertension) to therapy of IHs in paediatric patients during last few years. Propranolol administered orally at 2–3 mg kg<sup>-1</sup> per day has a consistent, rapid, therapeutic effect, leading to considerable shortening of the natural course of IHs, with good clinical tolerance [1]. Sodium benzoate (BEN) is used as an antimicrobial preservative in cosmetics, foods, and pharmaceuticals. BEN is used to prolong microbial stability in preparations dedicated for children over 3 years. There is no registered medicinal product containing propranolol available in the Czech Republic [2]. Paediatric formulations have many specificities. The most important are the ability to dose variable amount of active substance according to the weight of the children and dosage form has to be easy to swallow [3, 4]. The most suitable way is using oral liquid extemporaneous preparations with antimicrobial agent for older children and with no antimicrobial agent for infants. The cooperation of

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L. Zahálka · L. Matysová (✉) · P. Solich  
Department of Analytical Chemistry, Charles University  
in Prague, Faculty of Pharmacy in Hradec Králové,  
Heyrovského 1203, 500 05 Hradec Králové, Czech Republic  
e-mail: ludmila.matysova@faf.cuni.cz

Z. Šklubalová · S. Klovrzová  
Department of Pharmaceutical Technology,  
Charles University in Prague, Faculty of Pharmacy,  
Hradec Králové, Czech Republic

S. Klovrzová  
Hospital Pharmacy, General Hospital in Motol,  
Prague, Czech Republic

Department of Pharmaceutical Technology (Hradec Králové) and Hospital Pharmacy of General Hospital in Motol (Prague) led to development of ten versions of oral liquid preparations with PRO as an active substance and BEN as a preservative. Determination of PRO by means of HPLC has been already mentioned in literature [5–8], but simultaneous determination of PRO and BEN in various matrices of liquid dosage forms has not been published yet. The aim of this study was to develop and validate selective, simple and rapid HPLC method for the determination of active substance propranolol hydrochloride and antimicrobial agent sodium benzoate in newly developed paediatric oral preparations.

## Materials and Methods

### Materials and Reagents

Propranolol hydrochloride (Sigma-Aldrich, Steinheim, Germany) and sodium benzoate (Dr. Kulich Pharma, Hradec Králové, Czech Republic) were used as standards. Labetalol hydrochloride (Sigma-Aldrich, Steinheim, Germany), acebutolol hydrochloride (Sigma-Aldrich, Steinheim, Germany), pindolol (Sigma-Aldrich, Steinheim, Germany), metoprolol tartrate (Sigma-Aldrich, Steinheim, Germany), methylparaben (Fluka, Buchs, Germany), ethylparaben (Fluka, Buchs, Germany), propylparaben (Fluka, Buchs, Germany) and butylparaben (BP) (Fluka, Buchs, Germany) were used as internal standards. Sodium dodecyl sulphate (Sigma-Aldrich, Steinheim, Germany), tetrabutylammonium dihydrogen phosphate (Sigma-Aldrich, Steinheim, Germany), sulphuric acid (Sigma-Aldrich, Steinheim, Germany), sodium hydroxide (Penta, Chrudim, Czech Republic), acetonitrile (ACN) gradient grade (Sigma-Aldrich, Steinheim, Germany) and methanol (MeOH) gradient grade (Sigma-Aldrich, Steinheim, Germany) were used for the preparation of the mobile phase. Water for sample and mobile phase preparation was purified by Milli-Q Integral 15 system with 0.22 µm output filter. 0.45 µm Nylon membrane filters were used for mobile phase filtration (Merck Millipore, Billerica, USA). 0.45 µm Nylon membrane filters (Fisher Scientific, Pardubice, Czech Republic) and 2 mL syringes (Chirana T. Injecta, Stará Tůra, Slovak Republic) were used for filtration of the solutions before injection. Micropipette Transferpette 1,000 µL (Brand, Wertheim, Germany) was used. Formulations ST1–ST10 (incl. blank solutions) that contain propranolol hydrochloride (2 or 8 mg mL<sup>-1</sup>), sodium benzoate (0.5 mg mL<sup>-1</sup>) and excipients (e.g. water for injection, sirupus simplex, sodium saccharin, citrate–phosphate buffer pH 3 and citric acid) were obtained from Hospital Pharmacy of General Hospital in Motol, Prague, Czech Republic.

### Instrumentation and Chromatographic Conditions

Chromatographic analysis was performed on integral HPLC system Shimadzu LC-2010C (Shimadzu, Kyoto, Japan). Supelco Discovery<sup>®</sup> C18 (25 cm × 4.6 mm, particles 5 µm) column was used. Dual absorbance UV/VIS detector was set at wavelength 230 nm. Isocratic elution with flow 1.8 mL min<sup>-1</sup> was used. Column temperature was conditioned to 25 °C. Injection volume was 5 µL and time of analysis was 5 min.

### Preparation of Mobile Phase (~1 L)

1.6 g of sodium dodecyl sulphate and 0.31 g of tetrabutylammonium dihydrogen phosphate were dissolved in 450 mL of ultrapure water, 1 mL of sulphuric acid (95–97 %) and 550 mL of acetonitrile were added. Sodium hydroxide solution (2.1 M) was used for adjusting pH to value 3.3 (±0.05). Mobile phase was filtered through nylon membrane filter (0.45 µm) by using Millipore Glass Filter Holder and degassed in ultrasonic bath. Mobile phase was stored in the refrigerator in closed glass bottles.

### Preparation of Stock, Standard, Sample and Blank Solutions

Preparation of stock, standard, sample and blank solutions is described in Table 1.

## Results and Discussion

### Method Development

Composition of mobile phase, column type and flow rate used in monograph Propranolol hydrochloride (Related substances) in European Pharmacopoeia 7.0 [9] provided total separation of PRO and BEN. Pharmacopoeia uses mobile phase with two ion-pair reagents, sodium dodecyl sulphate (forms ion pairs with cations–propranolol cations) and tetrabutylammonium dihydrogen phosphate (forms ion pairs with anions–benzoate anions). It was observed that addition of sulphuric acid according to the Pharmacopoeia method [9] helps in dissolving sodium dodecyl sulphate in water and prevents the formation of bubbles in mobile phase caused by this surfactant. Optimization steps in changing mobile phase pH and water component:ACN ratio were performed to find out the best separation performance. Mobile phase pH (at water component:ACN = 45:55) was tested in the range of 3.3 ± 0.2 and it was observed that even little changes in pH led to undesirable increase in retention time of analytes (up to 112 % relatively). Water

**Table 1** Sample, standard and blank solutions preparation

Composition and process	Stock solution of standards	Stock solution of IS	Standard solution	Sample solution 1	Sample solution 2	Blank solution 1	Blank solution 2
Propranolol hydrochloride (PRO)	~40.00 mg	–	–	–	–	–	–
Sodium benzoate (BEN)	~10.00 mg	–	–	–	–	–	–
Butylparaben (BP)	–	~1200.00 mg	–	–	–	–	–
Stock solution of standards	–	–	1.000 mL	–	–	–	–
Stock solution of internal standard	–	–	1.000 mL	1.000 mL	1.000 mL	–	–
Oral preparation (PRO 2 mg mL <sup>-1</sup> )	–	–	–	1.000 mL	–	–	–
Oral preparation (PRO 8 mg mL <sup>-1</sup> )	–	–	–	–	0.500 mL	–	–
Placebo of preparation (PRO 2 mg mL <sup>-1</sup> )	–	–	–	–	–	1.000 mL	–
Placebo of preparation (PRO 8 mg mL <sup>-1</sup> )	–	–	–	–	–	–	0.500 mL
Dissolvent water:ACN	50:50 (v/v)	50:50 (v/v)	50:50 (v/v)	50:50 (v/v)	50:50 (v/v)	50:50 (v/v)	50:50 (v/v)
Total volume	20.00 mL	100.00 mL	25.00 mL	25.00 mL	25.00 mL	25.00 mL	25.00 mL
Membrane filtration 0.45 µm	–	–	Yes	Yes	Yes	Yes	Yes
Injection to the column	–	–	Yes (5 µL)	Yes (5 µL)	Yes (5 µL)	Yes (5 µL)	Yes (5 µL)

component:ACN ratio (at optimal pH 3.3) was tested from values 35:65–55:45. Increasing of water component amount led to prolongation of retention times and 55:45 ratio did not ensure total separation of PRO and BP. Mobile phase with increased proportion of ACN than original (45:55) might be used to shorten analysis time, but it also causes significant decrease of BEN and PRO peaks resolution, and reduces method robustness. Simple mobile phases containing only water:MeOH = 20:80–30:70 or water:ACN = 40:60–60:40 were also tested, but peak shapes of analytes and resolution were not acceptable. Various injection volumes (1–10 µL) were tested and concentrations of analytes and internal standard (IS) both in standard and sample solutions were adjusted to ensure suitable tailing factor, sufficient response (absorbance) and similar absorbance level of determined analytes. Labetalol, acebutolol, pindolol, metoprolol, methylparaben, ethylparaben, propylparaben and BP were tested as the possible ISs. BP was chosen as IS, because the other mentioned substances were not totally separated from substances of pharmaceutical formulation or were eluted with insufficient tailing factor. Wavelength of UV/VIS absorbance detector was tested at 200–300 nm range. PRO maximum absorbance was at wavelength 214 nm, BEN at 224 nm and BP at 256 nm. It was observed that measuring at wavelength lower than 220 nm raises significantly baseline noise. Wavelength was set to 230 nm according to good sensitivity of PRO and BEN and minimum baseline noise. All optimal conditions, mobile phase composition and preparation of solutions are mentioned in “[Instrumentation and Chromatographic Conditions](#)”, “[Preparation of Mobile Phase \(~1 L\)](#)”, and [Preparation of Stock, Standard, Sample and Blank Solutions](#).

### Sample Preparation Development

Simple method “dilute and shoot” was used for sample preparation. Water:ACN = 50:50 (v/v) solution was chosen to ensure good solubility of tested compounds. 50:50 = water:ACN ratio is also similar to the water component:ACN ratio used in mobile phase to avoid worsening of peak shapes. It was observed that insufficient dilution (e.g. 10) led to recovery of determined substances significantly over 100 %. Dilution 25 (i.e. 1.000 mL of preparation was diluted into 25 mL of mixture water:ACN = 50:50 v/v) contributed to elimination of matrix effects and recovery of all formulations resulted in range of 100 ± 2 %. Sample solution was prepared by the same way as standard solution. Concentration of PRO, BEN and BP was selected to ensure the same concentration level both in sample and standard solution.

### Method Validation

Presented method was validated according to ICH Q2(R1) [10] guideline. System suitability test (repeatability of retention times and areas, number of theoretical plates, resolution, tailing factor), precision, linearity, accuracy, selectivity and robustness were evaluated during method validation. Validation results are summarized in [Tables 2 and 3](#).

### System Suitability Test (SST)

SST was performed on standard solution which was injected into the column six times. Presented values are arithmetic means of six injections.

**Table 2** Validation parameters of formulations ST1–ST5

Parameter	Formulations										Criteria
	ST1		ST2		ST3		ST4		ST5		
SST	PRO	BEN	PRO	BEN	PRO	BEN	PRO	BEN	PRO	BEN	
Repeatability $t_R$ RSD (%) <sup>a</sup>	0.16	0.24	0.16	0.24	0.16	0.24	0.16	0.24	0.16	0.24	$X < 1 \%$
Repeatability area RSD (%) <sup>a</sup>	0.09	0.08	0.09	0.08	0.09	0.08	0.09	0.08	0.09	0.08	$X < 1 \%$
Theoretical plates <sup>a</sup>	8,441	6,408	8,441	6,408	8,441	6,408	8,441	6,408	8,441	6,408	$N > 1,500$
Resolution <sup>a</sup>	8.82	–	8.82	–	8.82	–	8.82	–	8.82	–	$R_{ij} > 1.5$
Tailing factor <sup>a</sup>	1.19	1.23	1.19	1.23	1.19	1.23	1.19	1.23	1.19	1.23	$T = 0.8–1.5$
Validation											
Precision RSD (%) <sup>b</sup>	0.20	–	0.44	0.59	0.28	0.39	0.25	0.16	0.21	0.19	$X < 5 \%$
Linearity (correlation coefficient) <sup>c</sup>	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	$R > 0.9990$
Accuracy recovery (%) <sup>b</sup>	100.49	–	101.49	101.11	99.42	98.14	100.24	100.15	99.55	99.09	$X = 100 \pm 5 \%$
Accuracy RSD (%) <sup>b</sup>	0.24	–	0.80	0.78	0.76	0.78	0.55	0.64	0.30	0.29	$X < 5 \%$
Selectivity	No interference		No interference		No interference		No interference		No interference		No interference

PRO propranolol hydrochloride, BEN sodium benzoate, STx formulations with various excipients

<sup>a</sup> Six injections

<sup>b</sup> Six samples, three injections of each sample

<sup>c</sup> At 50, 75, 100, 135, 170, 200 % levels

**Table 3** Validation parameters of formulations ST6–ST10

Parameter	Formulations										Criteria
	ST6		ST7		ST8		ST9		ST10		
SST	PRO	BEN	PRO	BEN	PRO	BEN	PRO	BEN	PRO	BEN	
Repeatability $t_R$ RSD (%) <sup>a</sup>	0.16	0.24	0.16	0.24	0.16	0.24	0.16	0.24	0.16	0.24	$X < 1 \%$
Repeatability area RSD (%) <sup>a</sup>	0.09	0.08	0.09	0.08	0.09	0.08	0.09	0.08	0.09	0.08	$X < 1 \%$
Theoretical plates <sup>a</sup>	8,441	6,408	8,441	6,408	8,441	6,408	8,441	6,408	8,441	6,408	$N > 1,500$
Resolution <sup>a</sup>	8.82	–	8.82	–	8.82	–	8.82	–	8.82	–	$R_{ij} > 1.5$
Tailing factor <sup>a</sup>	1.19	1.23	1.19	1.23	1.19	1.23	1.19	1.23	1.19	1.23	$T = 0.8–1.5$
Validation											
Precision RSD (%) <sup>b</sup>	0.16	0.18	0.49	0.41	0.63	0.67	0.34	0.18	0.33	–	$X < 5 \%$
Linearity (correlation coefficient) <sup>c</sup>	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	0.9997	$R > 0.9990$
Accuracy recovery (%) <sup>b</sup>	99.99	99.73	99.49	99.16	100.20	100.11	100.11	100.02	99.51	–	$X = 100 \pm 5 \%$
Accuracy RSD (%) <sup>b</sup>	0.28	0.29	0.42	0.37	0.23	0.22	0.37	0.36	0.23	–	$X < 5 \%$
Selectivity	No interference		No interference		No interference		No interference		No interference		No interference

PRO propranolol hydrochloride, BEN sodium benzoate, STx formulations with various excipients

<sup>a</sup> Six injections

<sup>b</sup> Six samples, three injections of each sample

<sup>c</sup> At 50, 75, 100, 135, 170, 200 % levels

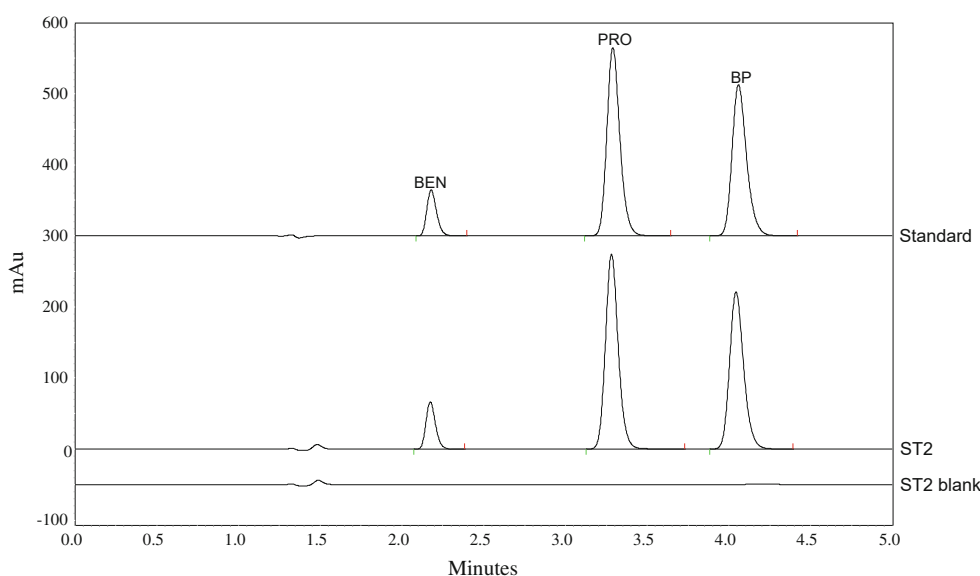
### Precision

Six sample solutions were prepared from each of ten preparations. Each sample was injected three times. Final results are presented as relative standard deviations (RSD) of BEN/BP and PRO/BP ratios.

### Linearity

Calibration curve was created by six points which cover the concentration range of PRO from 0.04 to 0.16 mg mL<sup>-1</sup> and of BEN from 0.01 to 0.04 mg mL<sup>-1</sup>. Linear regression was used for processing of calibration data. Correlation

**Fig. 1** Chromatogram of standard solution (BEN 0.02 mg mL<sup>-1</sup>, PRO 0.08 mg mL<sup>-1</sup>, BP 0.48 mg mL<sup>-1</sup>), sample solution and its blank solution (ST2: 1.000 mL of formulation ST2 and 1.000 mL of stock solution of internal standard BP in 25 mL; ST2 blank: 1.000 mL of placebo ST2 in 25 mL); injection volume 5  $\mu$ L, mobile phase flow 1.8 mL min<sup>-1</sup>, wavelength 230 nm, 25 °C



coefficient of linearity was 0.9997 for PRO and 0.9997 for BEN which means good correlation between peak areas and concentrations.

#### Accuracy

Solutions for injection were prepared by using placebo and stock solution of standards instead of oral preparation. Six solutions were prepared from each of ten preparations. Each solution was injected into the column three times. Accuracy is presented as a recovery parameter with relative standard deviations.

#### Selectivity

Selectivity was observed by comparing chromatograms of sample solutions, standard solution and blank solutions. It is obvious that active substance PRO, antimicrobial agent BEN and internal standard BP are all completely separated both in standard solution and in sample solution (Fig. 1). No interference was found (Fig. 1).

#### Robustness

Various pH and composition of mobile phase were tested. It is possible to use mobile phase with pH range from 3.1 to 3.5 without remarkable changes of accuracy (98.60–100.24 %). It is possible to use water component:ACN ratio from 35:65 to 50:50 (v/v) without remarkable changes of accuracy (99.59–100.00 %). Last tested mobile phase ratio 55:45 (v/v) is not suitable because peaks of PRO and BP were not completely separated to baseline. Stability of standard solution was tested at room temperature and at 4 °C after 24, 48 and 72 h from preparation. Accuracy range was 99.67–100.50 %.

#### Conclusion

Optimal chromatographic conditions cover using Supelco Discovery<sup>®</sup> C18 (25 cm  $\times$  4.6 mm, particles 5  $\mu$ m) column, isocratic elution mode with flow rate 1.8 mL min<sup>-1</sup>. Mobile phase contains ion-pair reagents sodium dodecyl sulphate and tetrabutylammonium dihydrogen phosphate, water component:ACN = 45:55 and pH is adjusted to 3.3. Dual absorbance UV/VIS detector was used for detection and was set at wavelength 230 nm. Column temperature was conditioned to 25 °C. Injection volume was 5  $\mu$ L. Method is fast with total analysis time of 5 min. Sample preparation is simple “dilute and shoot” with using of internal standard BP. Validation results show that newly developed HPLC method is selective, precise and accurate (Tables 2, 3) and is suitable for identification and quantification of liquid preparations containing PRO and BEN. Presented method has been already used for stability testing of ten variants of paediatric oral preparations and is suitable for evaluating content of PRO and BEN in these preparations.

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## ORIGINAL ARTICLE

# Pediatric oral solutions with propranolol hydrochloride for extemporaneous compounding: the formulation and stability study

## Pediatrické perorální roztoky s propranolol-hydrochloridem pro magistraliter přípravu: formulace a hodnocení stability

Sylva Klovrzová • Lukáš Zahálka • Ludmila Matysová • Petr Horák • Zdenka Šklubalová

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### Summary

The aim of this study is to formulate an extemporaneous pediatric oral solution of propranolol hydrochloride (PRO) 2 mg/ml for the therapy of infantile haemangioma or hypertension in a target age group of 1 month to school children and to evaluate its stability. A citric acid solution and/or a citrate-phosphate buffer solution, respectively, were used as the vehicles to achieve pH value of about 3, optimal for the stability of PRO. In order to mask the bitter taste of PRO, simple syrup was used as the sweetener. All solutions were stored in tightly closed brown glass bottles at  $5 \pm 3$  °C and/or  $25 \pm 3$  °C, respectively. The validated HPLC method was used to evaluate the concentration of PRO and a preservative, sodium benzoate, at time intervals of 0–180 days. All preparations were stable at both storage temperatures with pH values in the range of 2.8–3.2. According to pharmacopoeial requirements, the efficacy of sodium benzoate 0.05 % w/v was proved (Ph.Eur., 5.1.3). The preparation formulated with the citrate-phosphate buffer, in our experience, had better palatability than that formulated with the citric acid solution.

**Keywords:** propranolol hydrochloride • pediatric preparation • extemporaneous preparation • solution • stability testing • HPLC

### Souhrn

Cílem práce je formulace pediatrického perorálního přípravku s propranolol-hydrochloridem (PRO) 2 mg/ml pro magistraliter přípravu, určeného k terapii infantilního hemangiomu nebo hypertenze u cílové skupiny dětí od 1 měsíce do školního věku, a hodnocení jeho stability. K dosažení pH okolo 3, optimálního pro PRO, byl jako vehikulum využito roztok kyseliny citronové nebo citráto-fosfátový pufr. K maskování hořké chuti PRO byl použit prostý sirup. Všechny roztoky byly uchovávány v dobře uzavřené hnědé lékovce při  $5 \pm 3$  °C a/nebo  $25 \pm 3$  °C. V časových intervalech 0–180 dní byla hodnocena koncentrace PRO a protimikrobní látky, benzoanu sodného, validovanou HPLC metodou. Všechny přípravky byly stabilní při obou teplotách s hodnotou pH v rozmezí 2,8–3,2. V souladu s požadavky lékopisu byla prokázána účinnost protimikrobní látky, benzoanu sodného (Ph. Eur., 5.1.3). Podle našich zkušeností má přípravek s citráto-fosfátovým pufrům lepší chuť než přípravek s kyselinou citronovou.

**Klíčová slova:** propranolol-hydrochlorid • pediatrický přípravek • magistraliter přípravek • roztok • testování stability • HPLC

### Introduction

Propranolol hydrochloride (PRO) is a non-cardio selective beta blocker. It is usually administered in the form of tablets or capsules in therapy of cardiovascular diseases, to control symptoms of hyperthyroidism, the prophylaxis of migraine, and many other indications<sup>1</sup>. A successful treatment of infantile hemangioma has been observed recently; PRO is orally administered from newborns to school children at an initial dose of 2 to 3 mg/kg daily in two or three divided doses<sup>1–3</sup>.

A liquid preparation is the best dosage form for paediatric patients as young children are simply unable to swallow conventionally sized tablets or capsules. Unfortunately, no pediatric oral liquid dosage form is on the market until now.

S. Klovrzová • P. Horák  
University Hospital Motol, Hospital Pharmacy, Prague,  
Czech Republic

doc. PharmDr. Zdenka Šklubalová, Ph.D. (✉)  
Charles University in Prague, Faculty of Pharmacy,  
Department of Pharmaceutical Technology, Hradec Králové  
Heyrovského 1203, 500 05 Hradec Králové, Czech Republic  
e-mail: zdenka.skclubalova@faf.cuni.cz

L. Zahálka • L. Matysová  
Charles University in Prague, Faculty of Pharmacy,  
Department of Analytical Chemistry, Hradec Králové, Czech Republic

Under these circumstances, the pharmacist needs to compound such a preparation extemporaneously. When formulating a pediatric preparation in a hospital pharmacy, the pharmacist should attend to the stability of the active pharmaceutical substance for a labelled time period, the suitability and safety of excipients for children in the indicated target age groups, and expected duration of treatment<sup>4, 5</sup>. A simple way of preparing an oral liquid preparation is to crush commercial tablets to make a fine powder and mix it with a suitable vehicle.

Many empirical formulations prepared that way have been published for PRO<sup>6–8</sup>. Unfortunately, some authors of the earlier publications have used excipients which are not suitable for paediatric patients; a commercial suspending vehicle consisting of ethanol 1%, saccharin 0.05%, and cherry-flavoured 33% polyethylene glycol 8000 base, is an example<sup>7</sup>. The lack of valid stability data is the second common disadvantage of earlier publications.

This study was focused on the formulation of an extemporaneous solution containing PRO 2 mg/ml, suitable for therapy of infantile hemangioma in a target group of children from 1 month to approximately 6 years for hospital and/or home care. The stability of PRO was evaluated under two different conditions of storage within a shelf life of 180 days using high performance liquid chromatography (HPLC).

## Experimental part

### Materials

Citric acid monohydrate, sodium phosphate dibasic dodecahydrate, sodium benzoate (SB), and propranolol hydrochloride (PRO) of pharmaceutical quality were used. Simple sucrose syrup (64% w/w) was obtained from Fagron (Czech Republic). Water for injection (WFI) was used throughout the study as the solvent in the preparation of the vehicles and solutions.

### Analytical reagents

The following reagents of analytical grade were used: acetonitrile, sulphuric acid ( $\geq 95$ – $97\%$ ), and sodium dodecyl sulphate ( $\geq 98.5\%$ ) (all obtained from Sigma-Aldrich, Germany), butylparaben and tetrabutylammonium

dihydrogenphosphate ( $\geq 97.0\%$ ) (both from Fluka, Germany), and sodium hydroxide (Penta, Czech Republic).

### Methods

#### Compounding of buffer solution

To prepare a citrate-phosphate buffer solution of pH 3 (CPB), 1.67 g of citric acid and 1.47 g of dibasic sodium phosphate were dissolved in WFI and made up to 100.0 ml of a solution with WFI. The stock solution was stored in a tightly closed brown glass bottle, protected from light, and refrigerated ( $5 \pm 3$  °C).

#### Compounding of solutions of PRO

The composition of all prepared solutions **F1–F3** is shown in Table 1.

The **F1** solution of PRO 2 mg/ml was prepared by dissolution of 0.20 g of the substance and 0.05 g of sodium benzoate in an appropriate volume of CPB, then filled with buffer solution up to 50 ml and made up to the total volume of 100.0 ml with Simple Sucrose Syrup.

In the formulation **F2**, 0.2 g of propranolol hydrochloride, 0.05 g of sodium benzoate, and 0.2 g of citric acid were dissolved in an appropriate volume of WFI, made up to 50 ml with WFI and then filled up to a total volume of 100.0 ml with Simple Sucrose Syrup.

The solution **F3** was prepared by dissolution of 0.20 g of propranolol hydrochloride and 0.05 g of citric acid that way as the previous one. This solution was preservative-free.

#### Measurement of pH

The pH value was measured under stabilized conditions using a pH meter (pH 212 Microprocessor pH Meter, Hanna instruments, Germany) with a combined pH electrode. The pH meter was calibrated at pH 4.01 and 7.00 at 20 °C using standard buffer solutions (WTW, Germany). The results obtained at the time intervals chosen in the stability study are presented in Table 2.

#### Instrumentation and analytical conditions

A stability indicating HPLC assay was developed for PRO and sodium benzoate, using butylparaben as an internal standard. The HPLC system consisted of a Shimadzu LC-2010C (CLASS-VP Software, Shimadzu, Japan) with a Dual – Absorbance UV Detector. Separation was achieved using a Supelco Discovery<sup>®</sup> C18 column

Table 1. Composition of the evaluated propranolol hydrochloride solutions

Composition	F1	F2	F3
PRO	0.20 g	0.20 g	0.20 g
Citric acid	–	0.20 g	0.05
CPB	50 ml	–	–
Sodium benzoate	0.05 g	0.05 g	–
Simple syrup	to 100 ml	50 ml	50 ml
WFI	–	to 100 ml	to 100 ml
Taste	sweet&sour	sweet slightly bitter	sweet slightly bitter

Table 2. The results of pH measurement during the stability study at room temperature (room) and/or in a refrigerator (cold)

Time (days)	F1		F2		F3	
	Room	Cold	Room	Cold	Room	Cold
t <sub>0</sub>	3.14	3.14	2.89	2.89	2.87	2.88
t <sub>1</sub>	3.14	3.16	2.89	2.90	2.86	2.88
t <sub>3</sub>	3.15	3.14	2.90	2.88	2.87	2.87
t <sub>7</sub>	3.16	3.15	2.90	2.90	2.89	2.89
t <sub>14</sub>	3.15	3.15	2.90	2.87	2.92	2.89
t <sub>30</sub>	3.16	3.16	2.91	2.90	2.86	2.87
t <sub>60</sub>	3.13	3.13	2.88	2.87	–	–
t <sub>90</sub>	3.08	3.11	2.82	2.84	–	–
t <sub>120</sub>	3.09	3.08	2.82	2.82	–	–
t <sub>180</sub>	3.12	3.13	2.89	2.90	–	–

Table 3. System suitability parameters of HPLC method for determination of propranolol hydrochloride (PRO) and sodium benzoate (SB)

System suitability parameters	F1		F2		F3	
	PRO	SB	PRO	SB	PRO	SB
Repeatability t <sub>R</sub> RSD (%)	0.16	0.24	0.16	0.24	0.16	–
Repeatability Area	0.09	0.08	0.09	0.08	0.09	–
Theoretical Plates	8441	6408	8441	6408	8441	–
Resolution	8.82	–	8.82	–	8.82	–
Tailing factor	1.19	1.23	1.19	1.23	1.19	–

Table 4. Validation data of HPLC method for determination of propranolol hydrochloride (PRO) and sodium benzoate (SB)

Validation criteria	F1		F2		F3	
	PRO	SB	PRO	SB	PRO	SB
Precision RSD (%) <sup>a</sup>	0.44	0.59	0.21	0.19	0.33	–
Linearity (R) <sup>b</sup>	0.9997	0.9997	0.9997	0.9997	0.9997	–
Accuracy Recovery (%) <sup>a</sup>	101.49	101.11	99.55	99.09	99.51	–
Accuracy RSD (%) <sup>a</sup>	0.80	0.78	0.30	0.29	0.23	–
Selectivity	No interference		No interference		No interference	

<sup>a</sup> six samples, three injections of each sample<sup>b</sup> at 50, 75, 100, 135, 170, 200 % levels

Table 5. The percentage content of propranolol hydrochloride during the stability study at room temperature (room) and/or in a refrigerator (cold). RSD (%) in brackets

Time (days)	F1		F2		F3	
	Room	Cold	Room	Cold	Room	Cold
t <sub>0</sub>	100.00 (0.34)	100.00 (0.68)	100.00 (0.11)	100.00 (0.49)	100.00 (0.40)	100.00 (0.40)
t <sub>1</sub>	98.82 (0.06)	99.01 (0.86)	100.17 (0.14)	98.65 (1.03)	100.17 (0.27)	100.34 (0.07)
t <sub>3</sub>	100.60 (0.14)	100.14 (0.18)	103.24 (0.08)	101.13 (2.06)	100.39 (0.30)	100.14 (0.31)
t <sub>7</sub>	99.57 (0.16)	100.15 (0.09)	99.94 (0.35)	101.23 (0.65)	99.87 (0.23)	100.37 (0.13)
t <sub>14</sub>	101.99 (0.16)	100.25 (0.45)	101.89 (0.46)	100.83 (0.77)	100.97 (0.11)	101.30 (0.15)
t <sub>30</sub>	102.31 (0.13)	102.51 (0.39)	102.96 (0.75)	102.47 (0.23)	99.87 (0.18)	99.80 (0.09)
t <sub>60</sub>	99.14 (0.51)	98.20 (0.11)	98.96 (0.24)	97.87 (0.04)	–	–
t <sub>90</sub>	100.40 (0.07)	100.77 (0.41)	100.79 (0.14)	100.34 (0.26)	–	–
t <sub>120</sub>	101.18 (0.34)	100.91 (0.04)	102.32 (0.62)	101.09 (0.50)	–	–
t <sub>180</sub>	101.82 (0.14)	100.86 (0.17)	101.71 (0.28)	101.63 (0.09)	–	–

(25 cm × 4.6 mm × 5 μm) (Supelco, USA). The isocratic flow rate was 1.8 ml/min and the UV detector was set at a wavelength of 230 nm.

The mobile phase consisted of 1.6 g of sodium dodecyl sulphate, 0.31 g tetrabutylammonium dihydrogenphosphate, 1 ml of sulphuric acid, 450 ml of HPLC grade water, and

550 ml of acetonitrile, and was adjusted to the pH value of 3.3 using sodium hydroxide solution. The mobile phase solution was filtrated through a 0.45 μm filter (Glass Microfiber Filters, Whatman, UK) and then was sonicated for a few minutes (Sonorex Digitec, Bandelin, Germany) before HPLC analysis.

Table 6. The percentage content of sodium benzoate during the stability study at room temperature (room) and/or in a refrigerator (cold). RSD (%) in brackets

Time (days)	F1		F2	
	Room	Cold	Room	Cold
t <sub>0</sub>	100.00 (0.37)	100.00 (0.74)	100.00 (0.21)	100.00 (0.47)
t <sub>1</sub>	98.15 (0.65)	97.67 (1.15)	97.52 (0.26)	97.40 (0.30)
t <sub>3</sub>	99.91 (0.60)	99.14 (0.59)	99.55 (0.19)	99.83 (0.91)
t <sub>7</sub>	99.42 (0.23)	99.71 (0.35)	99.35 (0.18)	99.76 (0.23)
t <sub>14</sub>	100.82 (0.19)	99.46 (0.14)	100.48 (0.21)	99.43 (0.14)
t <sub>30</sub>	102.76 (0.13)	102.89 (0.17)	102.96 (0.60)	102.69 (0.19)
t <sub>60</sub>	98.54 (0.51)	97.67 (0.10)	98.42 (0.28)	97.47 (0.10)
t <sub>90</sub>	99.83 (0.16)	100.40 (0.33)	100.00 (0.16)	99.42 (0.27)
t <sub>120</sub>	99.48 (0.35)	99.28 (0.64)	99.89 (0.52)	99.02 (0.25)
t <sub>180</sub>	101.08 (0.23)	99.71 (0.19)	100.37 (0.08)	100.17 (0.10)

The HPLC method for the analysis of the proposed oral solution was successfully and completely validated by following the Q2(R1) ICH guideline (1997). System suitability parameters (n = 6) and validation data are summarized in Tables 3 and/or 4, respectively.

#### Stability study

The batch of the preparation was divided into two separate samples and stored in a tightly closed brown glass bottle at room temperature (25 ± 3 °C) and in a refrigerator (5 ± 3 °C). The concentration of propranolol hydrochloride and the preservative, sodium benzoate, in the preparations **F1** and **F2** were evaluated at the beginning of the stability assay (t<sub>0</sub>, a content of 100 %) and thereafter at time intervals of 1 – 3 – 7 – 14 – 30 – 60 – 90 – 120 – 180 days. The concentration of propranolol hydrochloride in solution **F3** was evaluated the same way but only at the time interval up to 30 days. Stability limit of maximum 5% degradation of the drug and the preservative contents were the basic criteria.

Each sample was measured in triplicate. The average values of the percentage content (n = 6) of propranolol hydrochloride with relative standard deviations (RSD, %) in brackets are summarized in Table 5. Similarly, the results for sodium benzoate are shown in Table 6.

## Results and Discussion

In an aqueous vehicle, PRO has good solubility (50 mg/mL). Solutions are stable at about pH 2.8 – 4 with the best at pH 3<sup>9)</sup>. A disadvantage of PRO is a bitter taste leading to the necessity of the addition of a sweetener.

In this study, three formulations of PRO solution were compounded (Table 1). The citric acid and/or the citrate-phosphate buffer solution, respectively, were used as the vehicles to achieve pH value of about 3. Generally, a multi-dose preparation needs an addition of a preservative. Since there are some references indicating possible incompatibility between PRO and parabens resulting in the degradation of the parabens<sup>6)</sup>,

sodium benzoate was used as an alternative<sup>8, 10)</sup> assuming the use in a children target group of 1 month and older (the formulations **F1** and **F2**). Simple Sucrose Syrup is added to improve palatability of the solutions. The preparation **F3** was formulated preservative-free assuming the use for neonates below 1 month.

According to the analytical procedures validation ICH guidelines (Q2(R1)), the HPLC method was completely validated. In Tables 3 and 4, system suitability parameters (n = 6) and validation data are presented.

All solutions were stored in tightly closed brown glass bottles at 5 ± 3 °C and/or 25 ± 3 °C, respectively. At time intervals mentioned in the experimental section, samples were withdrawn and used to estimate pH value and the content of PRO and SB (preserved preparations **F1** and **F2**). The results in Table 2 show good consistency in pH value during the stability study. This is important particularly in the case of the preserved solutions **F1** and **F2** as sodium benzoate has an alkaline effect on pH value, which might lead to degradation of PRO<sup>9)</sup>.

The percentage content of PRO and SB content estimated using HPLC during the stability study at room temperature and/or refrigerator are summarized in Table 5 and/or Table 6, respectively. As **F3** did not contain sodium benzoate, only the results for **F1** and **F2** are shown in Table 6. In all cases, the concentration of drug and/or preservative, respectively, was within recommended limits of ± 5% of the initial concentration at the beginning of the stability assay (t<sub>0</sub>)<sup>11)</sup>. Based on the results, the estimated shelf-life<sup>12)</sup> of 180 days was proved at both temperatures of storage for **F1** and **F2** formulations when stored in a tightly closed brown glass bottle.

## Conclusions

The aim of the study was to find an optimal vehicle for paediatric oral solution of PRO and to verify its stability at two temperatures of storage. The proposed oral aqueous solutions **F1** and **F2** for extemporaneous compounding

were stable at room temperature and/or refrigerator for 180 days. In accordance with the European Pharmacopoeia (Ph.Eur. 7.0, 5.1.3 Efficacy of antimicrobial preservation), the efficacy of the antimicrobial preservative, sodium benzoate 0.05 % w/v, was demonstrated by an accredited laboratory. A labelled shelf-life of 3 months, storage in a refrigerator at  $5 \pm 3$  °C, and protection from light can be recommended. The formulation **F1** consisting of citrate-phosphate buffer mixed with sugar syrup we considered better than **F2** for a sweet and sour taste, particularly in the therapy of older children. Formulation **F3** represents the composition formulated with a minimal content of excipients and is preservative-free. It must, therefore, be prepared under aseptic conditions. It can be expected for use in the therapy of neonates under supervision of a caregiver. A labelled shelf-life of 7 days can be recommended for extemporaneous compounding in real-life situations if stored in a refrigerator at  $5 \pm 3$  °C. To protect from microbial contamination and to allow easy administration, preparations should be packaged in a glass container with a screw cap suitable for administration using a syringe for oral use.

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## Research Article

# Development of a Gradient HPLC Method for the Simultaneous Determination of Sotalol and Sorbate in Oral Liquid Preparations Using Solid Core Stationary Phase

Ludmila Matysova,<sup>1</sup> Oxana Zahalkova,<sup>1</sup> Sylva Klovrzova,<sup>2,3</sup> Zdenka Sklupalova,<sup>2</sup> Petr Solich,<sup>1</sup> and Lukas Zahalka<sup>1</sup>

<sup>1</sup>Department of Analytical Chemistry, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, 500 05 Hradec Kralove, Czech Republic

<sup>2</sup>Department of Pharmaceutical Technology, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, 500 05 Hradec Kralove, Czech Republic

<sup>3</sup>Hospital Pharmacy, University Hospital in Motol, 150 06 Prague 5, Czech Republic

Correspondence should be addressed to Petr Solich; [solich@faf.cuni.cz](mailto:solich@faf.cuni.cz)

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A selective and sensitive gradient HPLC-UV method for quantification of sotalol hydrochloride and potassium sorbate in five types of oral liquid preparations was developed and fully validated. The separation of an active substance sotalol hydrochloride, potassium sorbate (antimicrobial agent), and other substances (for taste and smell correction, etc.) was performed using an Ascentis Express C18 (100 × 4.6 mm, particles 2.7 μm) solid core HPLC column. Linear gradient elution mode with a flow rate of 1.3 mL min<sup>-1</sup> was used, and the injection volume was 5 μL. The UV/Vis absorbance detector was set to a wavelength of 237 nm, and the column oven was conditioned at 25°C. A sodium dihydrogen phosphate dihydrate solution (pH 2.5; 17.7 mM) was used as the mobile phase buffer. The total analysis time was 4.5 min (+2.5 min for reequilibration). The method was successfully employed in a stability evaluation of the developed formulations, which are now already being used in the therapy of arrhythmias in pediatric patients; the method is also suitable for general quality control, that is, not only just for extemporaneous preparations containing the mentioned substances.

## 1. Introduction

Sotalol (SOT) is a Class III antiarrhythmic agent that prolongs the QT interval and exhibits beta-adrenergic blocking properties. SOT has been widely used in the management of atrial arrhythmias for several decades including patients in the pediatric age group and those with congenital heart disease. In pediatric patients, SOT has proven efficacy in suppressing supraventricular arrhythmias and maintaining a sinus rhythm with recurrence-free intervals of >80% and has also been used in the management of ventricular arrhythmias with more modest efficacy [1].

Potassium sorbate (SORB) is an antimicrobial preservative with antibacterial and antifungal properties and is used in pharmaceuticals, foods, enteral preparations, and cosmetics.

In general, SORB is used at concentrations of 0.1–0.2% in oral and topical formulations. Potassium sorbate is used in approximately twice as many pharmaceutical formulations as sorbic acid due to its greater solubility and stability in water. As with sorbic acid, potassium sorbate exhibits minimal antibacterial properties in formulations with pH values higher than 6 [2].

There are no registered medicinal products containing sotalol suitable for administration in pediatric patients and available in the European Union (EU) member states and selected non-EU countries (Supplement) (see Supplementary Material available online at <http://dx.doi.org/10.1155/2015/806736>) [3]. Pediatric formulations have many specific characteristics. The most important one is the ability to administer dosages of an active substance in variable and precise

amounts according to the actual weight of a child. In addition, the dosage form has to be easily swallowed [4, 5]. When no appropriate dosage form is commercially available, the most suitable alternative is the use of oral liquid extemporaneous preparations with an antimicrobial agent for older children and with no antimicrobial agent for infants. The cooperation between the Department of Pharmaceutical Technology (Charles University, Faculty of Pharmacy, Hradec Kralove) and the Hospital Pharmacy (University Hospital in Motol, Prague) has led to the development of five versions of oral liquid preparations with sotalol hydrochloride as the active substance and potassium sorbate as the preservative. The HPLC determination of sotalol has been previously reported [6–10], but the simultaneous determination of sotalol and sorbate in various matrices (e.g., sirupus simplex~sucrose syrup, saccharine, and citric acid) in a liquid dosage has not been previously published. The aim of this study was to develop and validate a selective and rapid method using standard HPLC system for the determination of sotalol hydrochloride (i.e., the active substance) and potassium sorbate (i.e., the antimicrobial agent) and their separation from other present substances in newly developed pediatric oral preparations and its application for stability study. In order to achieve total separation of sotalol, sorbate, and other analytes that possess different chromatographic properties at the lowest possible analysis time at standard HPLC system, modern solid core columns and gradient elution were adopted during method development.

Columns of solid core particles exhibit unusual chromatographic efficiency. Presumably, this is due to the ability to form very homogeneous packed beds as a result of an extremely narrow particle size distribution and higher particle density. Solid core particles exhibit highly improved mass transfer (kinetic) effects because of the thin porous shell surrounding a solid core, allowing solutes to rapidly diffuse in and out of the porous structure containing the stationary phase for interaction. Columns of the solid core particles (2.7  $\mu\text{m}$ ) exhibit theoretical plates nearly comparable to those of sub-2-micron totally porous particles, but with much reduced pressure requirements and thus it is possible to use them at standard HPLC systems [11].

## 2. Materials and Methods

**2.1. Materials and Chemicals.** Sotalol hydrochloride (Fagron, Olomouc, Czech Republic) and potassium sorbate (Dr. Kulich Pharma, Hradec Kralove, Czech Republic) were used as the standards. Ethylparaben (Sigma-Aldrich, Steinheim, Germany) was used as an internal standard (IS). Sodium dihydrogen phosphate dihydrate (Sigma-Aldrich, Steinheim, Germany), orthophosphoric acid (Merck, Darmstadt, Germany), acetonitrile (ACN) gradient grade (Sigma-Aldrich, Steinheim, Germany), methanol (MeOH) gradient grade (Sigma-Aldrich, Steinheim, Germany), and tetrahydrofuran (THF) Chromasolv (Sigma-Aldrich, Steinheim, Germany) were used to prepare the mobile phase. Water for the sample and mobile phase preparation was purified by Milli-Q Integral 15 system with 0.22  $\mu\text{m}$  output filter. Nylon membrane filters

TABLE 1: Linear gradient.

$T$ [min]	% A (buffer)	% B (ACN)
0.00	90	10
4.00	40	60
4.49	40	60
4.50	90	10
7.00	90	10

(0.20  $\mu\text{m}$ ) were used for mobile phase filtration (Albet, Dassel, Germany). Nylon membrane filters (0.22  $\mu\text{m}$ ) (Vitrum, Prague, Czech Republic) and 2 mL syringes (Chirana T. Injecta, Stara Tura, Slovak Republic) were used to filter the samples. A 1000  $\mu\text{L}$  Transferpette micropipette (Brand, Wertheim, Germany) was used. Formulations F1–F5 (and respective blank solutions), which contain sotalol hydrochloride (5 mg mL<sup>-1</sup>), potassium sorbate (1 mg mL<sup>-1</sup>), and excipients (e.g., water for injection, sirupus simplex, citric acid, disodium hydrogen phosphate dodecahydrate, and sodium saccharine), were obtained as extemporaneous preparations from the Hospital Pharmacy at the University Hospital in Motol, Prague, Czech Republic.

**2.2. Instrumentation and Chromatographic Conditions.** The chromatographic analysis was performed on an integral system Shimadzu LC-2010C (Shimadzu, Kyoto, Japan). The following chromatographic columns were tested during method development: Ascentis Express C18 (150  $\times$  4.6 mm, particles 2.7  $\mu\text{m}$ ), Ascentis Express C18 (100  $\times$  4.6 mm, particles 2.7  $\mu\text{m}$ ), and Ascentis Express Phenyl-Hexyl (100  $\times$  4.6 mm, particles 5  $\mu\text{m}$ ). An Ascentis Express C18 (100  $\times$  4.6 mm, particles 2.7  $\mu\text{m}$ ) column was finally chosen for the method validation and stability testing. The dual absorbance UV/Vis detector was set to a wavelength of 237 nm. Linear gradient elution (Table 1) with a flow rate of 1.3 mL min<sup>-1</sup> was used. A column oven was conditioned at 25°C. The injection volume was 5  $\mu\text{L}$  and analysis time was 4.5 minutes (7 minutes with reequilibration time incl.).

**2.3. Preparation of Buffer Component of Mobile Phase (Approximately 1L).** 2.76 g of sodium dihydrogen phosphate dihydrate was dissolved in 1L of ultrapure water. An orthophosphoric acid solution (6%) was used to adjust the pH to 2.5 ( $\pm 0.05$ ). The mobile phase buffer was filtered through a nylon membrane filter (0.20  $\mu\text{m}$ ) using a Millipore glass filter holder. The mobile phase buffer was used immediately after preparation or stored in the refrigerator in closed borosilicate glass bottles for a maximum of 24 hours.

**2.4. Preparation of Stock, IS, Standard, Sample, and Blank Solutions.** The preparation of the stock, IS, standard, sample, and blank solutions is described in Table 2.

## 3. Results and Discussion

**3.1. Method Development.** The initial chromatographic conditions and mobile phase composition were chosen to be

TABLE 2: Stock, IS, standard, sample, and blank solutions preparation.

Composition and process	Stock solution of standards	Stock solution of IS	Standard solution	Sample solution	Blank solution
Sotalol hydrochloride	~100.00 mg	—	—	—	—
Potassium sorbate	~20.00 mg	—	—	—	—
Ethylparaben	—	~100.00 mg	—	—	—
Stock solution of standards	—	—	1.000 mL	—	—
Stock solution of IS	—	—	1.000 mL	1.000 mL	—
Oral preparation (SOT 5 mg mL <sup>-1</sup> )	—	—	—	1.000 mL	—
Placebo of preparation (neither SOT nor SORB)	—	—	—	—	1.000 mL
Dissolvent ACN : water	50 : 50 (v/v)	50 : 50 (v/v)	30 : 70 (v/v)	30 : 70 (v/v)	30 : 70 (v/v)
Total volume	20.00 mL	50.00 mL	25.00 mL	25.00 mL	25.00 mL
Membrane filtration 0.22 $\mu$ m	—	—	Yes	Yes	Yes
Injection to the column	—	—	Yes (5 $\mu$ L)	Yes (5 $\mu$ L)	Yes (5 $\mu$ L)

similar to those used by Delamoye et al. for the separation of thirteen  $\beta$ -blockers [12]. C18 stationary phase column with solid core 2.7  $\mu$ m particles, 4.6 mm i.d., and 100 mm length was initially tested. A mobile phase consisting of sodium dihydrogen phosphate dihydrate (pH 3.8; 17.7 mM)-ACN (65 : 35, v/v) did not provide separation of the sotalol peak from the dead volume peak. An increase in the phosphate buffer (pH 3.8; 17.7 mM) component led to a desirable increase in the sotalol retention. Phosphate buffer (pH 3.8; 17.7 mM)-ACN (80 : 20 and 85 : 15, v/v) only provided partial separation of sotalol from the dead volume peak. Phosphate buffer (pH 3.8; 17.7 mM)-ACN (90 : 10, v/v) was sufficient for proper sotalol retention. The addition of THF was tested to observe possible positive effects on peak shape. Unfortunately, THF addition did not provide any advantages and led to a rapid increase in the baseline noise and drift. The use of MeOH instead of acetonitrile also led to a less stable baseline as well as an undesirable increase in the system back pressure. Avoiding the phosphate buffer and using only ACN-water mobile phases caused unacceptable peak fronting and tailing. Therefore, the buffer is necessary for maintaining good peak shapes and separation. Isocratic elution with the phosphate buffer (pH 3.8; 17.7 mM)-ACN (90 : 10, v/v) mobile phase cannot be used due to a significant increase in the analysis time caused by different retention properties of sotalol (base) and sorbate (acid). Under acidic conditions sotalol is in ionized form and thus it is not well retained on the stationary phase; opposite sorbate is in nonionized form and it is therefore significantly retained on the column. Different gradient curve profiles were tested, and a linear gradient was chosen because it resulted in the lowest baseline drift. A terminal gradient concentration of ACN was tested up to 70%, but a maximum usable concentration of 60% was required to maintain a straight baseline. Gradient elution with initial phosphate buffer (pH 3.8; 17.7 mM)-ACN (from 90 : 10 to 40 : 60, v/v) could be used for the separation of sotalol and sorbate. Unfortunately, these conditions cannot be used for analysis of preparations containing the artificial sweetener saccharine (SACC) due to its coelution with

the sotalol peak. An increase in the temperature up to 60°C or the use of an Ascentis Express Phenyl-Hexyl column did not provide any favorable changes in the selectivity and using of Ascentis Express C18 (150  $\times$  4.6 mm, 2.7  $\mu$ m particles) also did not provide sufficient SOT-SACC separation. An elevated temperature resulted even in a decreased resolution of the SOT and SACC peaks. Several buffer pH values (4.6; 3.8; 3.0; 2.5; and 2.0) were tested (Figure 1). Using 4.6 or 3.8 pH buffers caused coelution of SOT/SACC, pH 3.0 buffer provided reasonable separation of the SOT/SACC peaks (resolution = 1.44), and finally the 2.5 buffer led to complete separation of the mentioned analytes to the baseline (resolution > 1.5). The pH 2.0 buffer also provided total SOT/SACC separation but it is not recommended due to an expected decrease in the column lifetime. These experimental results correspond to the theoretical useful pH range of phosphate buffer which is 2.1–3.1 [13]. Methylparaben, ethylparaben, propylparaben, butylparaben, paracetamol, and salicylic acid were tested as possible internal standards (IS). Paracetamol was coeluted with the dead volume peak, methylparaben and salicylic acid were not sufficiently separated from the sorbate peak, and propylparaben with butylparaben was eluted with unfavorable long retention times. Ethylparaben was finally chosen as the IS because it is stable in solution, inexpensive, and well separated from all of the analytes in the oral preparations. In addition, ethylparaben exhibits good UV absorption in UV. Various concentrations of the sample solution and injection volumes were tested to ensure a suitable tailing factor and sufficient response (absorbance). The UV spectra of sotalol and sorbate were obtained with a UV/Vis DAD spectrophotometer, and the tested wavelengths of the UV/Vis absorbance HPLC detector ranged from 200 to 300 nm. Finally, the wavelength was set to 237 nm to ensure good sensitivity, as well as low baseline noise.

**3.2. Sample Preparation Development.** The simple method known as “dilute and shoot” was used for sample preparation. The pharmaceutical preparation was diluted 25 times (i.e., 1.000 mL of the preparation with 1.000 mL of the IS stock



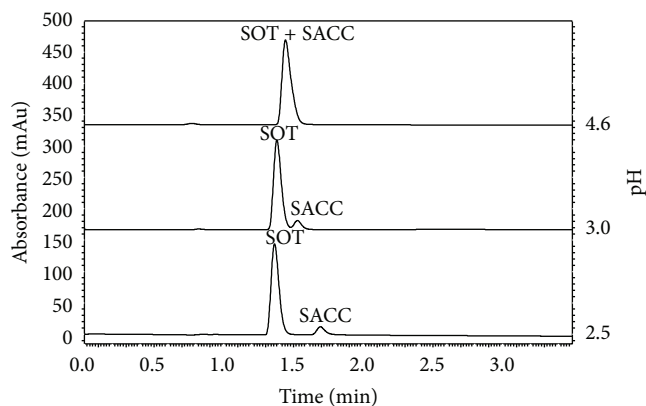


FIGURE 1: Significance of mobile phase buffer pH controlling in SOT/SACC separation; pharmaceutical formulation F4 (1.000 mL of pharmaceutical preparation diluted to 25.00 mL); injection volume 5  $\mu$ L; mobile phase flow 1.3 mL  $\text{min}^{-1}$ ; linear gradient (ACN: 10% to 60% in 4 minutes); UV/Vis detector wavelength 237 nm; column oven 25°C.

solution was diluted to 25.00 mL with a mixture of ACN-water (30:70, v/v) to avoid previously reported matrix effects of the liquid pharmaceutical formulations [14]. The standard solution was prepared in the same way as the sample solution using a stock solution of the standards. The concentration of SOT, SORB, and EP was selected to ensure the same concentration level in the sample and standard solutions. An increase in the ACN component (e.g., to 50%) led to rapid deterioration of the peak shapes, especially significant fronting of the SOT and SACC peaks. Therefore, an ACN concentration higher than 30% is not practical.

**3.3. Method Validation.** The method was validated according to ICH Q2 (R1) guidelines [15]. The system suitability (i.e., repeatability of retention times and areas, number of theoretical plates, resolution, and tailing factor), precision, linearity, accuracy, selectivity, and robustness were evaluated during method validation (Table 3). The parameters accuracy, precision, and selectivity were performed and evaluated for all five pharmaceutical formulations.

**3.3.1. System Suitability Test (SST).** SST was performed on a standard solution that was injected into the column six times. The reported values are arithmetic means of six injections.

**3.3.2. Precision.** Six sample solutions were prepared from each of the five preparations. Each sample was injected three times. The final results are reported as relative standard deviations (R.S.D.) of the SOT/EP and SORB/EP ratios of the peak areas.

**3.3.3. Linearity.** A calibration curve was created using 6 points that covered the concentration range of sotalol hydrochloride from 0.1  $\text{mg mL}^{-1}$  to 0.4  $\text{mg mL}^{-1}$  and potassium sorbate from 0.02  $\text{mg mL}^{-1}$  to 0.08  $\text{mg mL}^{-1}$ . Linear regression was used to process the calibration data. The correlation

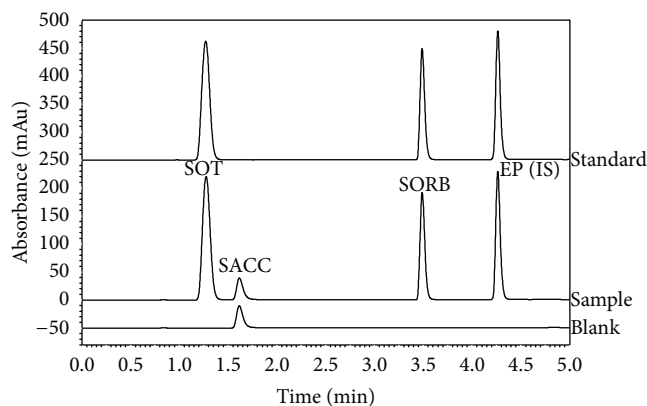


FIGURE 2: Chromatograms of the standard solution (SOT 0.2  $\text{mg mL}^{-1}$ , SORB 0.04  $\text{mg mL}^{-1}$ , and EP 0.08  $\text{mg mL}^{-1}$ ), sample solution (1.000 mL of pharmaceutical preparation and 1.000 mL of stock solution of internal standard EP diluted to 25.00 mL), and blank solution (1.000 mL of placebo diluted to 25.00 mL); injection volume 5  $\mu$ L; mobile phase flow 1.3 mL  $\text{min}^{-1}$ ; linear gradient (ACN: 10% to 60% in 4 minutes); UV/Vis detector wavelength 237 nm; column oven 25°C.

coefficients of linearity were 0.9995 for sotalol hydrochloride and 0.9995 for potassium sorbate, which indicate good correlation between the peak areas and the range of concentrations studied.

**3.3.4. Accuracy.** The solutions for injection were prepared using a placebo and stock solution of standards instead of the oral preparation. Six solutions were prepared from each of the five preparations. Each solution was injected onto the column three times. Accuracy is reported as a parameter recovery with relative standard deviations.

**3.3.5. Selectivity.** The selectivity was determined by comparing the chromatograms of sample solutions, standard solution, and blank solutions. Figure 2 shows that sotalol hydrochloride (i.e., the active substance), potassium sorbate (i.e., antimicrobial agent), and ethylparaben (i.e., internal standard) are all completely separated from each other and from the saccharine peak both in the standard solution and in the sample solution. No interference was observed.

**3.3.6. Robustness.** Various buffer pH values and compositions of the mobile phase were tested. A mobile phase buffer with a pH ranging from 2.3 to 2.7 was used without remarkable changes in the accuracy (98.99–100.37%). A sodium dihydrogen phosphate dihydrate (pH 2.5; 17.7 mM)-ACN initial gradient ratio ranging from 92:8 to 89:11 (v/v) was used without remarkable changes in the accuracy (97.42–100.70%). However, the 88:12 ratio led to higher fluctuations in the retention times, and, therefore, this ratio is not recommended. All of the tested ratios ensured complete separation to the baseline for all of these compounds. The stability of the standard solution was tested at room temperature without light protection and at  $5 \pm 3^\circ\text{C}$  light protected 24, 48, and 72 hours after its preparation. The accuracy of the peak areas for

TABLE 3: Validation parameters of formulations F1–F5.

	F1		F2		F3		F4		F5		Criterion
	SOT	SORB	SOT	SORB	SOT	SORB	SOT	SORB	SOT	SORB	
Repeatability $t_R$ (% R.S.D.) <sup>a</sup>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	$X < 1\%$
Repeatability area (% R.S.D.) <sup>a</sup>	0.22	0.57	0.22	0.57	0.22	0.57	0.22	0.57	0.22	0.57	$X < 1\%$
Theoretical plates per meter	11,810	282,650	11,810	282,650	11,810	282,650	11,810	282,650	11,810	282,650	—
Resolution <sup>a</sup>	—	18.39	—	18.39	—	18.39	—	18.39	—	18.39	$R_{ij} > 1.5$
Tailing factor <sup>a</sup>	1.10	1.23	1.10	1.23	1.10	1.23	1.10	1.23	1.10	1.23	$T = 0.8–1.5$
Precision (% R.S.D.) <sup>b</sup>	0.43	—	0.16	—	0.49	0.98	0.26	1.51	0.21	1.11	$X < 5\%$
Linearity (correlation coefficient) <sup>c</sup>	0.9995	0.9995	0.9995	0.9995	0.9995	0.9995	0.9995	0.9995	0.9995	0.9995	$R > 0.9990$
Accuracy recovery (%) <sup>b</sup>	101.09	—	99.57	—	99.59	98.26	99.93	98.70	99.35	98.57	$X = 100 \pm 5\%$
Accuracy (% R.S.D.) <sup>b</sup>	0.58	—	0.85	—	1.44	2.27	0.71	2.07	0.57	1.14	$X < 5\%$
Selectivity	No interference		No interference		No interference		No interference		No interference		No interference

<sup>a</sup>Six injections.

<sup>b</sup>Six samples, three injections of each sample.

<sup>c</sup>At 50, 75, 100, 134, 166, and 200% concentration levels.

SOT: sotalol hydrochloride.

SORB: potassium sorbate.

Fx: formulations with various excipients.

% R.S.D.: relative standard deviation in %.

both storage conditions during the entire 72 hours was 99.32–100.62%.

## 4. Conclusions

The optimal chromatographic conditions for separation of an active substance sotalol hydrochloride, potassium sorbate, and other substances were achieved on an Ascentis Express C18 (100 × 4.6 mm, particles 2.7 μm) solid core particles column and with a linear gradient elution at a flow rate of 1.3 mL min<sup>-1</sup>, using pH 2.5 phosphate buffer-ACN mixture (ACN~10–60%) as mobile phase and detection set to a wavelength of 237 nm. The method is rapid with a total analysis time of 4.5 minutes (+2.5 minutes of reequilibration). The sample preparation is a simple “dilute and shoot” method using an internal standard (ethylparaben). All measured parameters of the validation demonstrate the suitability of this new HPLC method for the analysis of oral liquid pharmaceutical preparations containing the above substances. The method was successfully employed in a stability evaluation of the four developed formulations with different composition, which are now already being used in the therapy of arrhythmias in pediatric patients. The method is also suitable for general quality control, that is, not only just for extemporaneous preparations containing the mentioned substances.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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# Extemporaneous sotalol hydrochloride oral solutions for use in paediatric cardiology: formulation and stability study

Sylva Klovřová,<sup>1,2</sup> Lukáš Zahálka,<sup>3</sup> Tomáš Kříž,<sup>2</sup> Oxana Zahálková,<sup>3</sup> Ludmila Matysová,<sup>3</sup> Zdenka Šklubalová,<sup>2</sup> Petr Horák<sup>1</sup>

<sup>1</sup>Hospital Pharmacy, University Hospital Motol, Prague, Czech Republic

<sup>2</sup>Faculty of Pharmacy, Department of Pharmaceutical Technology, Charles University in Prague, Hradec Králové, Czech Republic

<sup>3</sup>Faculty of Pharmacy, Department of Analytical Chemistry, Charles University in Prague, Hradec Králové, Czech Republic

## Correspondence to

Dr Zdenka Šklubalová, Faculty of Pharmacy, Department of Pharmaceutical Technology, Charles University in Prague, Heyrovského 1203, Hradec Králové 500 05, Czech Republic; [zdenka.sklubalova@faf.cuni.cz](mailto:zdenka.sklubalova@faf.cuni.cz)

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## ABSTRACT

**Objectives** Sotalol hydrochloride (SOT) is an antiarrhythmic  $\beta$ -blocker which is highly effective for the treatment of supraventricular tachycardia in children. However, a licensed paediatric dosage form with sotalol is not currently available in Europe. The aim of this work was to formulate paediatric oral solutions with SOT 5 mg/mL for extemporaneous preparation in a hospital pharmacy with the lowest possible amount of excipients and to determine their stability.

**Methods** Three aqueous solutions were formulated. One preparation without any additives for neonates and two preparations for children from 1 month of age were compounded using citric acid to stabilise the pH value, potassium sorbate 0.1% w/v as a preservative, and simple syrup or sodium saccharin as a sweetener. The samples were stored at room temperature and in a refrigerator, respectively, and the content of SOT and potassium sorbate was determined simultaneously using a validated high performance liquid chromatography method at different time points over 180 days.

**Results** At least 95% of the initial sotalol concentration remained throughout the 180-day study period in all three preparations at both temperatures. The content of potassium sorbate decreased by 17% with sodium saccharin stored at room temperature.

**Conclusions** The three proposed oral aqueous solutions of SOT for neonates and infants were stable for 180 days. Storage in a refrigerator is preferred, particularly with sodium saccharin. The additive-free solution of SOT can be autoclaved to ensure microbiological stability and used particularly for neonates and in emergency situations.

## INTRODUCTION

Sotalol hydrochloride (SOT) is an anti-arrhythmic  $\beta$ -blocker which is well tolerated and highly effective for the treatment of ventricular and supraventricular tachycardia in children.<sup>1</sup> The British National Formulary recommends sotalol should be administered to children in an initial oral dose of 1 mg/kg twice daily, increased as necessary every 3–4 days to a maximum of 4 mg/kg twice daily.<sup>2</sup> Recently, age-specific dosage guidelines for sotalol were developed by Læer *et al*<sup>3</sup> to ensure safe and effective anti-arrhythmic therapy in children, especially neonates and infants.

Sotalol is commercially available in tablet dosage forms for adults in four strengths: 80, 120, 160 and 240 mg.<sup>1</sup> However, the lack of marketed low-dose

paediatric products means extemporaneous preparation is often necessary. Extemporaneous preparations for paediatric use must be formulated in accordance with the guidelines of the European Medicines Agency.<sup>4 5</sup> Compounding should be restricted to an approved institution, for example, a hospital pharmacy.

In general, there are three basic approaches to the pharmacy preparation of paediatric dosage forms.

1. *The preparation of capsules from licensed tablets or from the active substance* is time-consuming for pharmacists and inconvenient for caregivers. As a small child is unable to swallow capsules, they should be opened and mixed with baby food or a beverage before administration. The advantage of this method is relatively good chemical and microbiological stability without the need to add preservatives.
2. *The preparation of a suspension from licensed tablets or a solution from licensed injection* is a simple way to prepare an oral liquid preparation. Commercial tablets should be crushed to a fine powder and mixed with a suitable vehicle; commercial injections could be diluted with water. Excipients improving stability and palatability should be added. However, the stability of the final product is not ensured due to the presence of other excipients in licensed medicines and their potential interactions with vehicles. Above all, there is a high risk of an inaccurate dose in the case of suspensions and drugs with a narrow therapeutic range, particularly in children.<sup>6</sup>
3. *The preparation of an aqueous oral solution from the active substance* is the best method if the active ingredient is of the required pharmacopoeial quality and soluble in water.

In all these circumstances, the pharmacist should pay attention to the stability of the active pharmaceutical substance for the labelled time period, excipient safety and tolerability, particularly for very young children, and expected duration of treatment.<sup>7</sup> Special attention must be given to formulations for neonates to whom no preservatives, antioxidants or hyperosmotic solutions should be administered.<sup>5</sup>

Regarding the paediatric use of SOT, some suspensions prepared from commercial tablets are referred to in the literature as being stable for a maximum of 90 days.<sup>8–11</sup> The presence of many different additives in tablets as well as in commercial vehicles (ORA-Sweet, ORA-Plus), sedimentation



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and possible dose inaccuracy make suspensions a less suitable dosage form for infants.

The aim of our research was to formulate extemporaneous paediatric solutions of SOT 5 mg/mL for two different paediatric groups: neonates to 1 month of age (without any additives) and infants (with the lowest possible amount of excipients) and to evaluate their stability under two different conditions of storage (refrigerated and room temperature) throughout the 180-day study period. In the unpreserved solution, the influence of autoclaving on the stability of SOT was also investigated. High performance liquid chromatography (HPLC) was used to simultaneously estimate the concentrations of SOT and potassium sorbate (PS) in the preserved preparations.

## MATERIALS AND METHODS

### Materials

SOT, PS, citric acid monohydrate, sodium saccharin and simple syrup (64% w/w, preservative-free) of pharmaceutical quality were used. Water for injection (WFI) was used throughout the study as a solvent.

### Methods

#### Sample preparation

Sotalol samples were carefully prepared in University Hospital Motol in Prague.

Solution S1 5 mg/mL was prepared by dissolving 0.50 g of SOT in WFI and made up to 100 mL under aseptic conditions. One sample (S1<sub>aut</sub>) was filled into a infusion glass bottle, stoppered and crimped, and sterilised in a laboratory autoclave at 121°C for 20 min.

Solution S2 5 mg/mL was made by dissolving 0.50 g of SOT, 0.10 g of PS and 0.08 g of citric acid in an appropriate amount of WFI. Then, 20 g of simple sucrose syrup (64% w/w) was added and the solution was made up to 100 mL (ie, 105 g) with WFI.

Solution S3 5 mg/mL was prepared by dissolving 0.50 g of SOT, 0.10 g of PS, 0.08 g of citric acid and 0.10 g of sodium saccharin in WFI and made up to 100 mL.

The composition of solutions S1, S2 and S3 is shown in [table 1](#).

#### Measurement of density, osmolality and pH value

The density of the preparations was measured at 20±0.1°C using a DMA 4100M density meter (Anton Paar, Austria). The

osmolality of the solutions was measured using an automatic semi-micro osmometer (Knauer, Germany) calibrated in accordance with Ph. Eur. 8.0 (2.2.35. Osmolality). Density and osmolality were measured five times in each formulation.

pH was measured under stabilised conditions using a pH metre (pH 212 meter, Hanna instruments, Germany) with a combined pH electrode. Samples were measured at 7, 14, 30, 60, 90, 120, 150 and 180 days.

#### Instrumentation and analytical conditions

A stability-indicating HPLC assay was developed for simultaneous determination of SOT and PS by Matysova *et al.*<sup>12</sup> Briefly, determination of SOT and PS was performed on an HPLC system with an absorbance UV detector. Separation was achieved using an Ascentis Express C18 (100×4.6 mm, particles 2.7 µm; Supelco, USA) column. Linear gradient elution was used.

#### Stability method and sample analysis

All preparations (S1, S2 and S3) were prepared in duplicate with the same composition. Each solution was divided into four amber glass bottles (50 mL). Samples were stored at room temperature (25±2°C) or in a refrigerator (5±3°C) and protected from light; that is, two samples from each batch were stored at each of the experimental conditions (n=4).

The concentration of SOT in all preparations and of the preservative, PS, in preparations S2 and S3 was evaluated at the beginning of the stability assay (t<sub>0</sub>, an initial content of 100%) and at the time points mentioned above. Each sample was measured in triplicate.

Samples of solution S1<sub>aut</sub> were stored in an autoclave bottle under the same storage conditions as above. The concentration of SOT was evaluated before sterilisation in an autoclave, after sterilisation (t<sub>0</sub>) and then at 7, 14 and 30 days.

#### Data analysis

At each time point, the percentage of the actual initial concentration remaining was calculated for sotalol and PS (n=4). Stability was defined as the retention of at least 95% of the initial concentration of sotalol and 90% of PS.

## RESULTS

[Table 1](#) shows the composition and the properties: the average of five measurements of density and osmolality, the relative SD of which was less than 1%, and the taste of the prepared solutions. In our opinion, both solutions formulated with a sweetener tasted sweet, while solution S3 containing sodium saccharin had a slightly bitter aftertaste. [Table 1](#) also gives the pH values measured at the stability study time points. The pH of the aqueous solution of sotalol S1 without additives varied between 5.43 and 5.87; the average pH value of 4.15 in the buffered solutions with preservative (S2, S3) remained practically unchanged throughout the stability study.

In [figure 1](#), the HPLC chromatograms of sample S1<sub>aut</sub> before (A) and after (B) autoclaving are compared. The lack of change in the retention time of sotalol demonstrated that autoclaving did not influence SOT stability. The concentration of sotalol before and after autoclaving was unchanged at 5.17±0.11 mg/mL and therefore taken to be equal to the initial value (t<sub>0</sub>).

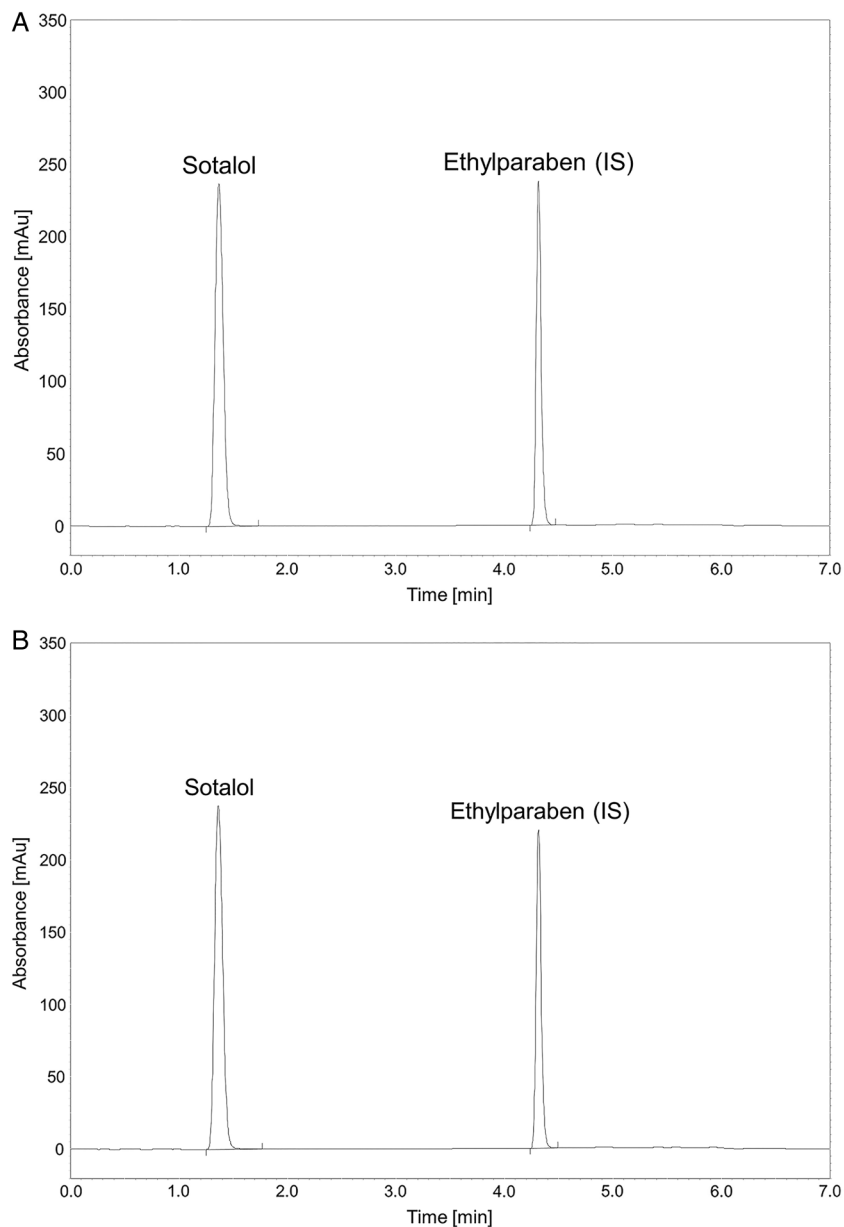
[Table 2](#) shows the percentage±SD of the initial concentration of SOT in solutions S1, S2 and S3 (n=4) stored under various conditions as mentioned above. The first row gives the amount of SOT in milligrams per millilitre at the beginning of the study (t<sub>0</sub>=100%). SOT demonstrated good stability in the

**Table 1** Composition and properties of sotalol hydrochloride solutions

	S1 (g)	S2 (g)	S3 (g)
Sotalol hydrochloride	0.50	0.50	0.50
Citric acid	–	0.08	0.08
Potassium sorbate	–	0.10	0.10
Simple syrup	–	20.0	–
Sodium saccharin	–	–	0.10
Water for injection to	100.0 mL	100.0 mL (=105.0 g)	100.0 mL
Density* (g/mL)	0.9997	1.0500	1.0008
Osmolality (mOsmol/kg)	49	497	60
pH	5.43–5.87	4.16–4.19	4.14–4.19
Taste	Slightly bitter	Sweet	Sweet, slightly bitter

\*At 20±0.1°C.

**Figure 1** (A) High performance liquid chromatography chromatogram of sotalol hydrochloride in sample S1<sub>aut</sub> before sterilisation. (B) HPLC chromatogram of sotalol hydrochloride in sample S1<sub>aut</sub> after sterilisation.



preparations, with final content being within  $\pm 5\%$  of the initial concentration after 180 days of storage at cold or room temperature. Chromatograms showed no evidence of degradation products throughout 6-month stability study.

The results for PS are presented in [table 3](#). The remaining percentage content of PS was within  $\pm 5\%$  of the initial PS concentration for solutions S2 and S3 stored in a refrigerator for 180 days. At room temperature, the percentage of PS declined

**Table 2** The percentage content of sotalol hydrochloride during the stability study at cold and room temperature\*

Time point (day)	Cold ( $5\pm 3^{\circ}\text{C}$ )			Room ( $25\pm 2^{\circ}\text{C}$ )		
	S1	S2	S3	S1	S2	S3
0 (100%)	5.17 $\pm$ 0.11 mg/mL	5.19 $\pm$ 0.03 mg/mL	5.19 $\pm$ 0.05 mg/mL	5.17 $\pm$ 0.11 mg/mL	5.19 $\pm$ 0.03 mg/mL	5.19 $\pm$ 0.05 mg/mL
7	101.10 $\pm$ 1.37	99.03 $\pm$ 0.93	100.89 $\pm$ 0.88	100.37 $\pm$ 0.89	99.52 $\pm$ 0.74	100.19 $\pm$ 0.93
14	96.72 $\pm$ 0.48	99.58 $\pm$ 1.29	98.48 $\pm$ 0.23	98.32 $\pm$ 0.73	100.22 $\pm$ 0.69	98.12 $\pm$ 0.34
30	100.65 $\pm$ 0.66	100.55 $\pm$ 1.16	101.20 $\pm$ 0.33	98.91 $\pm$ 1.01	99.69 $\pm$ 1.18	99.0 $\pm$ 0.39
60	98.41 $\pm$ 0.32	98.75 $\pm$ 1.12	99.36 $\pm$ 0.80	98.78 $\pm$ 0.59	99.29 $\pm$ 0.69	98.79 $\pm$ 0.84
90	98.75 $\pm$ 0.28	99.02 $\pm$ 0.89	98.84 $\pm$ 0.63	99.04 $\pm$ 0.30	99.54 $\pm$ 0.29	99.27 $\pm$ 0.40
120	98.58 $\pm$ 0.97	98.99 $\pm$ 0.66	99.22 $\pm$ 0.56	98.39 $\pm$ 0.60	98.89 $\pm$ 0.39	98.27 $\pm$ 0.92
150	97.33 $\pm$ 0.67	99.17 $\pm$ 0.83	98.62 $\pm$ 0.74	97.84 $\pm$ 0.34	99.23 $\pm$ 0.70	98.27 $\pm$ 0.25
180	99.29 $\pm$ 0.83	98.85 $\pm$ 0.91	101.14 $\pm$ 0.91	100.07 $\pm$ 0.52	99.27 $\pm$ 0.81	98.97 $\pm$ 1.06

\*Mean $\pm$ SD of determinations for four samples (n=4).

**Table 3** The percentage content of potassium sorbate during the stability study at cold and room temperature\*

Time point (day)	Cold (5±3°C)		Room (25±2°C)	
	S2	S3	S2	S3
0 (100%)	1.03±0.02 mg/mL	1.04±0.01 mg/mL	1.03±0.02 mg/mL	1.04±0.01 mg/mL
7	98.65±1.99	102.55±1.90	99.34±1.82	101.42±1.92
14	99.04±2.02	99.49±1.46	99.39±0.81	98.24±1.92
30	99.17±2.25	99.94±1.64	99.56±0.84	97.69±1.38
60	98.20±1.31	98.54±2.45	97.45±0.99	95.34±0.89
90	97.46±1.65	98.83±0.89	97.99±0.51	94.28±0.67
120	98.53±1.42	98.09±1.58	96.13±0.68	89.94±1.34
150	97.95±0.58	99.34±1.73	94.34±0.79	86.76±1.51
180	98.37±0.32	98.78±1.19	92.60±0.75	83.42±0.75

\*Mean±SD of determinations for four samples (n=4).

slowly, remaining within ±5% for 60 days, within ±10% for 90 days, and then decreasing further, particularly for S3. Nevertheless, no detectable changes in colour, odour or taste were observed in any formulation.

## DISCUSSION

Approximately 14 000 capsules containing 5–30 mg of SOT were prepared in the hospital pharmacy of the University Hospital Motol in Prague in 2014 for paediatric patients. In cooperation with the children's heart centre at the same hospital, the aim of this work was to replace the preparation of SOT-containing capsules with extemporaneous 5 mg/mL oral solutions which would cover most paediatric needs in the hospital.

Oral paediatric solutions provide many benefits including easy and faster preparation in a hospital pharmacy and more flexible and accurate dosing. Unfortunately, aqueous solutions often have less stability and a short shelf-life, so preservatives must generally be added to multi-dose preparations. In addition, the pharmacist is responsible for the selection of suitable excipients safe for children in the targeted age groups. Adequate palatability also plays an important role in patient acceptability, with flavours or sweeteners often added to improve taste.<sup>7</sup>

SOT is a white powder, freely soluble in water and chemically stable at pH 4–5.<sup>9–13</sup> PS is believed to be a safe antimicrobial preservative, is freely soluble in water and is generally used at 0.1–0.2% concentration in oral formulations.<sup>14</sup> PS (in the form sorbic acid) displays highest antimicrobial efficacy at pH 4–5, the same pH as sotalol.<sup>15</sup> Because SOT has a slightly bitter taste, sucrose syrup and/or sodium saccharin were used to improve the palatability of the S2 and S3 preparations, respectively.

A simple aqueous solution of SOT without any additives is proposed for neonates. Microbiological stability is ensured by the aseptic technique and final sterilisation of the product. Bacteria retention using a 0.22 µm membrane filter, sterilisation after compounding in an autoclave or a combination of both are the most common sterilisation methods employed in hospital pharmacies. The stability of solution S1 is documented in [table 2](#). The effect of sterilisation in an autoclave at 121°C for 20 min on the concentration of SOT is shown in [figure 1](#), where the HPLC sample chromatograms are compared before (A) and after (B) autoclaving. As can be seen, autoclaving did not influence the retention time of sotalol. The concentration of sotalol (5.17±0.11 mg/mL) before autoclaving was the same as that after autoclaving and therefore considered to be the initial value (t<sub>0</sub>). The percentage content of SOT remained within ±5% of

the initial concentration during 30 days of storage at both cold and room temperature.

Solutions S2 and S3 were formulated with an antimicrobial agent and are proposed for children above 1 month of age. The results in [table 2](#) document the good stability of SOT in all preparations tested. As can be seen in [table 3](#), the concentration of PS remained within ±5% of its initial concentration for solutions S2 and S3 stored in a refrigerator for 6 months. However, the percentage content of PS decreased slowly at room temperature, declining finally below 90% of the original concentration after 90 days. This was noted particularly for solution S3.

## CONCLUSIONS

Three aqueous oral solutions of SOT 5 mg/mL for antiarrhythmic therapy in children were formulated for extemporaneous preparation in a hospital pharmacy. Validated HPLC analysis demonstrated that the concentration of SOT in the formulations was in accordance with the criterion that at least 95% of the initial content should remain during storage at cold or room temperature throughout the 180-day study period.

The used excipients ensured stable pH and a more pleasant taste, while the preservative afforded sufficient antimicrobial stability in solutions S2 and S3 targeted at children aged 1 month and over. Storage in a refrigerator is preferred, and the solutions

## Key messages

### What is already known on this subject

- ▶ Most sotalol hydrochloride preparations on the market are not suitable for small children.
- ▶ Good stability of sotalol hydrochloride in an aqueous preparation has been shown.
- ▶ Extemporaneous preparation of stable pharmaceutical products in pharmacies is essential if marketed paediatric products are lacking.

### What this study adds

- ▶ Three oral aqueous solutions of sotalol hydrochloride for neonates and infants were formulated and stability for 180 days was demonstrated in a validated high performance liquid chromatography assay.
- ▶ Autoclaving had no effect on the stability of an additive-free aqueous solution of sotalol hydrochloride which can be used in particular for neonates and in emergency situations.

were stable for 180 days under this condition. Preparations should be stored in a brown glass container with a screw cap suitable for use with a graduated pipette for accurate oral dosing. The efficacy of PS 0.1% w/v in formulation S2, which is a better candidate for microbial contamination due to the content of sucrose syrup, was demonstrated by an accredited laboratory (Ph. Eur., 5.1.3 Efficacy of antimicrobial preservation).

In formulation S1<sub>auts</sub> no effect of autoclaving on the stability of SOT was observed; the solution was stable for 30 days regardless of storage conditions. Although this preservative-free solution is particularly targeted at neonates, it could be prepared in advance in the pharmacy and stored until needed.

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ORIGINAL ARTICLE

**FUROSEMIDE ETHANOL-FREE ORAL SOLUTIONS FOR PAEDIATRIC USE –  
FORMULATION, HPLC METHOD, AND STABILITY STUDY**

Lukáš Zahálka<sup>1</sup>, Sylva Klovrzová<sup>2, 3</sup>, Ludmila Matysová<sup>1</sup>, Zdenka Šklubalová<sup>\*,2</sup>, Petr Solich<sup>1</sup>

<sup>1</sup> Charles University, Faculty of Pharmacy, Department of Analytical Chemistry, Hradec Kralove, Czech Republic

<sup>2</sup> Charles University, Faculty of Pharmacy, Department of Pharmaceutical Technology, Hradec Kralove, Czech Republic

<sup>3</sup> Hospital Pharmacy, University Hospital Motol, Prague, Czech Republic

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\* Address correspondence to Zdenka Šklubalová, Charles University, Faculty of Pharmacy, Department of Pharmaceutical Technology, Ak. Heyrovského 1203, 500 05 Hradec Králové, Czech Republic, Fax: +420 495 518 002, E-mail: [zdenka.skubalova@faf.cuni.cz](mailto:zdenka.skubalova@faf.cuni.cz)

## **KEY MESSAGES**

### *What is already known about this subject*

- Ethanol is widely used in registered furosemide oral preparations to improve its solubility. However, ethanol is not a suitable excipient for preparations intended for use in paediatrics.
- If marketed paediatric product is not available, extemporaneous preparation of a stable pharmaceutical product in a pharmacy has an essential role in the therapy of children.
- The stability of furosemide in disodium hydrogen phosphate dodecahydrate aqueous solution in the presence of methylparaben is not known.

### **What this study adds**

- Two developed formulations of furosemide ethanol-free oral solution targeted for infants were proposed for easy extemporaneous compounding in pharmacies. Stability for 270 days under room storage temperature was demonstrated by HPLC analytical assay and pH measurement.
- The preparation containing disodium hydrogen phosphate dodecahydrate to reach the alkaline pH necessary for FUR dissolution in water is easier to prepare in routine practice and has a more pleasant taste than that one prepared with sodium hydroxide.
- The preparations proposed offer personalisation of child therapy reflecting the actual need.

## **ABSTRACT**

**Objectives.** Oral liquid solutions of diuretic active ingredient furosemide (**FUR**) marketed across Europe do not comply with the recent requirements for paediatric preparation due to their ethanol content and, moreover, in some countries only tablet or injection dosage forms of furosemide are available.

**Methods.** Our work presents two developed formulations of furosemide ethanol-free paediatric oral solutions 2 mg mL<sup>-1</sup> for easy extemporaneous compounding in a pharmacy. Furosemide solubility avoiding the use of ethanol was achieved by using sodium hydroxide (formulation F1) or disodium hydrogen phosphate dodecahydrate (formulation F2). The preparations were stored at 25 ± 3°C or at 40 ± 0.5°C, protected from light. For FUR and preservative, methylparaben (MP), a stability assay was conducted by high performance liquid chromatography validated method and pH stability.

**Results.** The remaining furosemide concentration was higher than 90 % of the initial concentration after 270 days in both formulations at both storage conditions, 25°C and 40°C. The concentration of methylparaben decreased significantly in the formulation F2 stored at 40°C.

**Conclusions.** Both formulations were stable when stored at room temperature for up to 9 months; the formulation F1 was stable even at 40°C. Methylparaben used as an antimicrobial agent fully satisfied the recommended criteria for preservative efficacy in oral preparations according to Ph. Eur. 9.0 (5.1.3).

**KEY WORDS:** furosemide, extemporaneous preparation, oral solution, ethanol-free, stability, HPLC

## INTRODUCTION

Furosemide (FUR) is a traditional diuretic widely used in adults and in paediatric patients; it is generally administered intravenously or orally. FUR is used in the treatment of hypertension and oedema associated with heart failure including pulmonary oedema.[1] Usually, the oral dose for neonates is 0.5 to 2 mg per kilogram of weight every 12 to 24 hours, for children aged from 1 month to 12 years the same dose 2–3 times daily is used, and for children 12 years and above 20 to 40 mg daily is administered. In resistant oedema, the higher dose can be permitted.[1]

However, the registered tablets contain at least 40 mg of FUR in one tablet. To achieve the required paediatric dose, it is necessary to crush commercially available tablets, mix the powder with a filler, and prepare capsules extemporaneously in a pharmacy. Afterwards, the capsule has to be opened prior to use and mixed with baby food or liquid before administration. In the Hospital Pharmacy Motol in Prague, the usually prepared dose for therapy of paediatric patients is 3 to 5 mg per capsule in agreement with the doctor's prescription.

A liquid preparation represents a better way with the advantages of more flexible dosing, improved patient as well as caregiver compliance, and, moreover, it is also easier for compounding in a pharmacy.[2-4] Registered oral liquid preparations containing FUR cannot generally be recommended for administration in children because of the high-concentration ethanol (EtOH) vehicle used. As examples: Frusol 20mg/5ml Oral Solution (Rosemont Pharmaceuticals Ltd; registered in UK) contains 10 % EtOH, Impugan 10 mg/ml oral drops (Actavis Group hf.; registered in Sweden) contains 9.8 % EtOH, and finally LasixR liquid 10 mg/ml (Sanofi-Aventis Deutschland GmbH, Germany) contains

even 11.9 % EtOH [5-7]. Using ethanol as the excipient in paediatric drugs does not comply with the general requirements for paediatric preparations[1] and it is considered unsuitable for use in paediatric patients by paediatric drug committees, drug agencies, and papers.[8-13]

One, although not optimal, way of preparing furosemide oral solutions in a pharmacy is simply diluting a commercially available registered aqueous injection of FUR with water. The absence of preservatives and the unpleasant taste of the active ingredient are limiting factors for use in oral multi-dose liquid preparations. If the active pharmaceutical ingredient (API) is available on the market and it is freely soluble in water, the preparation of an aqueous solution could be considered as the best way for extemporaneous compounding in the pharmacy. However, lower stability of API and excipients could occur in water solution and a shorter shelf life of the aqueous preparation over the capsules is expected. Therefore, a stability assay of each drug composition should be conducted prior to administering the preparation to the patients. FUR occurs as a white to slightly yellow, odourless, light sensitive, crystalline powder with a  $pK_a$  value of 3.9. It is sparingly soluble in ethanol, freely soluble in solutions of alkali hydroxides ( $pH > 8.0$ ) but, unfortunately, practically insoluble in water or dilute acids.[14]

The aim of our work was to formulate extemporaneous paediatric ethanol-free solutions of FUR ( $2 \text{ mg mL}^{-1}$ ) with a suitable solubility of FUR in the aqueous vehicle as well as an acceptable taste for use in paediatric cardiology and to evaluate their stability under two different storage conditions during a 9-month study period. A high performance liquid chromatography (HPLC) method was developed, validated, and used to determine the concentration of furosemide and the antimicrobial agent methylparaben (MP) throughout

the stability period as well. The main criterion of stability was defined as the retention of at least 90 % of initial concentration of furosemide and at least 80 % of initial concentration of methylparaben.

## **MATERIALS AND METHODS**

### **Materials and chemicals**

Furosemide (FUR), methylparaben (MP), disodium hydrogen phosphate dodecahydrate, and sodium hydroxide were obtained from Fagron, Czech Republic; sodium saccharine was obtained from Dr. Kulich Pharma, Czech Republic. Water for injection (WFI) was used for the preparation of the extemporaneous oral solutions and their blank solutions; it was obtained from the Hospital Pharmacy of the University Hospital in Motol, Prague, Czech Republic.

In an analytical study, the following substances were used for preparing the mobile phase and samples: methanol (MeOH) CHROMASOLV<sup>®</sup> gradient grade, acetonitrile (ACN) CHROMASOLV<sup>®</sup> gradient grade, formic acid 95 % and triethylamine (TEA) 99.5 % were obtained from Sigma-Aldrich, Czech Republic; and 18 M $\Omega$ .cm ultrapure water from Milli-Q<sup>®</sup> Integral water purification system with 0.22  $\mu$ m Millipak<sup>®</sup> output filter (Millipore, USA).

### **Methods**

#### ***Sample preparation***

2 mg mL<sup>-1</sup> furosemide solutions F1 and F2 were prepared from the furosemide substance and excipients (Table 1).

Formulation 1 (F1) was prepared by dissolving FUR in approximately 2.4 mL of 1 % w/v sodium hydroxide solution (60°C, freshly prepared from NaOH and WFI). Sodium

saccharine and 50 mL of 0.2 % w/v MP solution (prepared by dissolving MP in WFI at 100°C and cooled down) were added and the solution was made up by adding WFI to the final volume of 100.0 mL and transferred to a 100 mL amber glass vial with a syringe adapter.

In formulation 2 (F2), FUR was dissolved in approximately 20 mL of disodium hydrogen phosphate dodecahydrate solution freshly prepared from 1.5 g of disodium hydrogen phosphate dodecahydrate and WFI. Sodium saccharine and 50 mL of 0.2 % w/v MP solution (prepared by dissolving MP in WFI at 100°C and cooled down) were added and the solution was made up by adding WFI to the final volume of 100.0 mL and transferred to a 100 mL amber glass vial with a syringe adapter.

### ***Instrumentation and analytical assay***

#### ***Liquid chromatography***

A HPLC method for the determination of active pharmaceutical ingredient FUR and the antimicrobial preservative MP in presence of FUR impurity A (mentioned in the European Pharmacopoeia)[15] and pharmaceutical excipients used was developed and validated. Integral HPLC system Shimadzu LC-2010C (SW Class VP, ver. 6.13; Shimadzu Corp.) with a octadecyl (C18) silica gel HPLC column (Supelco Discovery<sup>®</sup> HS C18, 150 x 4.6 mm, 5 µm; Sigma-Aldrich) was used for the chromatographic analysis. The mobile phase consisted of the buffer (1000 mL of Milli-Q<sup>®</sup> water, 250 mL of formic acid, and 750 µL of triethylamine; adjusted to the pH value of 5.75) and acetonitrile in the ratio 65: 35 (v/v); the mobile phase was filtered by 0.45 µm nylon membrane filter before use. The isocratic flow rate was 1.5 mL/min and the dual absorbance UV detector was set at a wavelength of

270 nm. Chromatograms of standard solution and selected formulation (injection volume 5  $\mu\text{L}$ ) are shown in Figure 1, and method validation results are presented in Table 2.

#### *Reference standard solution preparation*

A standard solution was prepared by dissolving the active substance and impurity A in methanol. The final concentrations of the reference standards were 50  $\mu\text{g mL}^{-1}$  of furosemide and 10  $\mu\text{g mL}^{-1}$  of impurity A.

#### *Sample preparation*

An accurately weighed portion of pharmaceutical formulation corresponding to 2.5 mg of furosemide (about 1.25 g) was transferred into a 50 ml volumetric flask and methanol was added to 50.00 mL. The solution was mixed and after filtration (0.45  $\mu\text{m}$ -pore filter) was injected into the column and analysed by HPLC.

#### *Method validation*

The method was validated according to ICH Q2 (R1) guidelines. [16] The system suitability (i.e., repeatability of retention times and areas, number of theoretical plates, resolution, tailing factor), precision, linearity, accuracy, selectivity and robustness were evaluated during method validation (Table 2). The parameters of accuracy, precision, and selectivity were performed and evaluated for both pharmaceutical formulations.

#### *System suitability test (SST)*

SST was performed on a standard solution that was injected into the column six times. The reported values are the arithmetic means of six injections.

#### *Precision*



Six sample solutions were prepared from each of the preparations. Each sample was injected three times. The final results are reported as relative standard deviations (R.S.D.) of the FUR and MP peak areas.

#### *Linearity*

A calibration curve was created using 6 points that covered the concentration range of furosemide from 0.02 mg mL<sup>-1</sup> to 0.8 mg mL<sup>-1</sup> and methylparaben from 0.01 mg mL<sup>-1</sup> to 0.04 mg mL<sup>-1</sup>. Linear regression was used to process the calibration data.

#### *Accuracy*

The solutions for injection were prepared using a placebo and stock solution of standards instead of the oral preparation. Six solutions were prepared from both preparations. Each solution was injected into the column three times. Accuracy is reported as a parameter recovery with relative standard deviations.

#### *Selectivity*

The selectivity was determined by comparing the chromatograms of sample solution, and standard solution. Figure 1 shows that furosemide (i.e., the active substance), methylparaben (i.e., the antimicrobial agent) and the impurity A are all completely separated from each other and from the saccharine peak in the standard solution as well as in the sample solution. No interference was observed.

#### *Robustness*

Various buffer pH values and compositions of the mobile phase were tested. A mobile phase buffer with a pH 5.6 was used without a remarkable change in the accuracy (98.50 %). The mobile phases from ratio 55:45 (buffer:acetonitrile) are not recommended, because the peaks of furosemide and impurity A are not separated. The stability of the standard

solution was tested at room temperature without light protection and at  $5\pm 3$  °C light protected 24, 48 and 72 hours after its preparation. The accuracy of the peak areas for storage at room temperature without light protection was higher than 1 %, and therefore the storage at  $5\pm 3$  °C light protected condition is recommended.

### ***Stability assay and sample analysis***

Two batches were prepared for each out of two formulations and each batch solution was divided into four 100-mL amber glass bottles; two of them for storage at room temperature ( $25 \pm 3^\circ\text{C}$ ), and two for storage at  $40 \pm 0.5^\circ\text{C}$  (i.e.  $n = 4$  in each of the experimental conditions). The samples were protected from light.

The concentration of FUR and MP in the samples was estimated at the beginning of the stability study ( $c_0$  = day of solution preparation, an initial content of 100 %) and then at the time intervals of 7, 30, 90, 180, and 270 days. Each sample was measured in triplicate.

### ***Measurement of pH value***

The pH value was measured under stabilized conditions using a pH meter (pH 212 Microprocessor pH Meter, Hanna instruments, Germany) with a combined pH electrode. Each sample was measured at the time intervals mentioned above.

### ***Efficacy of antimicrobial preservation***

The test of the antimicrobial activity of the preservative methylparaben 0.1 % w/v (Ph.Eur., 5.1.3) which consists of challenging the preparation with a prescribed inoculum of microorganisms was carried out with the accredited laboratory (ITEST plus, Hradec Kralove, Czech Republic).

### ***Data analysis***

At each time interval, the percentage of the actual initial concentration remaining was calculated for FUR and MP (n = 4). Stability was defined as the retention of at least 90 % and/or 80 % of initial concentration of furosemide and/or methylparaben, respectively.

## RESULTS

The compositions of the preparations F1 and F2 are shown in Table 1. Both formulations contained saccharine sodium 0.1 % w/v as a taste modifier. They were prepared as quickly as possible in order to prevent decomposition of FUR by light.

In Figure 1, the HPLC chromatogram showing the separation of standard solution  $50 \mu\text{g mL}^{-1}$  of furosemide and formulation F2 is illustrated; the results of method validation are summarized in Table 2.

In Tables 3 and 4, the mean value of percentage concentration  $\pm$  SD of the initial furosemide and the antimicrobial agent methylparaben, respectively, in preparations F1 and F2 (n = 4) are shown for the stability time points and conditions mentioned in the Methods section. The amount of FUR and MP in milligrams per millilitre at the beginning of the study ( $c_0 = 100 \%$ ) is listed in the first row.

As illustrated in Table 3, the FUR concentration remaining was higher than 91 % after 270 days in both formulations F1 and F2 stored at both storage conditions (25°C and 40°C). The remaining MP concentration was higher than 80 % after 270 days in both formulations stored at 25°C as well as in the formulation F1 stored even at 40°C as shown in Table 4. In all cases, the chromatograms showed no evidence of product degradation throughout the 9-month stability study. No detectable changes in colour, odour or taste were observed in either furosemide formulations.

In contrast, a significant decrease in MP concentration in the formulation F2 stored at 40°C was observed. The percentage of MP remained within  $\pm 11\%$  for 30 days, decreasing to

approximately 70 % of the initial content after 90 days. At the end of the stability study (270 days), only approximately 40 % of MP was found (Table 4). Nevertheless, no apparent changes in colour, odour or taste were observed.

The value of pH for formulations F1 and F2 under conditions of the stability testing mentioned above was measured. The pH 6.6 and 7.5 for F1 and F2, respectively, remained practically unchanged throughout the storage at room temperature as well as for F2 at 40°C; in the preparation F1, the pH value slightly declined to 6.1 after 270 days when stored at 40°C.

## **DISCUSSION**

Furosemide is an active compound traditionally used in paediatric cardiology. In paediatrics, oral liquid preparations, particularly solutions, are the best dosage forms for flexible and accurate dosing and compliance of the patients. However, there is no commercially available liquid preparation that follows the latest recommendations on safety of paediatric drugs in terms of excipients used. Furosemide is practically insoluble in water, which is the main complication when preparing aqueous solutions. To increase the solubility of FUR in water, ethanol is often used in commercial preparations. [5-7, 17]. Unfortunately, preparations containing ethanol cannot be recommended for use in paediatric age group patients. The formation of FUR sodium salt by adjusting the alkaline pH is another method of making FUR soluble in water. In general, an aqueous solution of sodium hydroxide is used to achieve this. A furosemide injection solution whose pH value is approximately 9 is the example. In pharmacy, the commercially available aqueous injection can be simply diluted by WFI to achieve the paediatric suitable FUR concentration, e.g. 2 mg mL<sup>-1</sup>. Apart from the mentioned high pH value, moreover, such

an extemporaneously prepared oral solution has an unpleasant taste due to the presence of sodium hydroxide.

According to Pharmacopoeial requirements, multi-dose liquid preparations must be protected from microbial contamination by an addition of a suitable preservative.[15]

Unfortunately, widely used preservatives such as sodium benzoate or potassium sorbate show practically no antibacterial activity in the alkaline pH value. On the other hand, the paraben group of preservatives is effective over a wide pH range of 4–8 having a broad spectrum of antimicrobial activity. The activity of the parabens increases with increasing chain length of the alkyl moiety, but solubility decreases. [18] However, the reproductive toxicity of parabens appears to increase with increasing length of the alkyl chain, and there are specific data showing adverse reproductive effects in male rats of propyl and butyl parabens. In view of this and the fact that propyl and butyl parabens were not included in the acceptable daily intake (ADI) group for parabens, the World Health Organization committee concluded that the propyl and butyl paraben specifications for their use as a food additive should be withdrawn. In contrast to propyl and butyl parabens, neither methylparaben nor ethylparaben showed any effects on male reproductive organs, sperm parameters or sex hormones in juvenile rats. [19] Therefore, and also due to better solubility, methylparaben was finally chosen as a preservative.

To improve palatability of the oral solution, the addition of a suitable sweetener is usually necessary. Sucrose is often used in most paediatric liquid preparations and it was also tested during furosemide formulation development. Unfortunately, we observed two main disadvantages. The first one, the decrease in the pH value to approximately 6 leading to the risk of furosemide decomposition and/or precipitation. [14] The second, the change in

the solution colour to yellow or light brown during storage. The stability of sucrose containing solutions was determined by HPLC to only 90 days at room temperature (data not shown in this article). Finally, sodium saccharine 0.1 % w/v was used in both formulations presented in this work (F1, F2) due to its better stability.

### ***Developed paediatric formulations***

Two preparations of FUR (Table 1) were formulated for extemporaneous preparation in a hospital or community pharmacy. The composition F1 was prepared by dissolving FUR in an appropriate volume of 1 % sodium hydroxide solution similarly to the large scale manufacturing of FUR injections. The accurate added volume of hydroxide solution was determined by observing the dissolution visually. The final pH value of F1 preparation was 6.6. The preparation is similar to the simple dilution of parenteral injection; of course, the content of hydroxide makes its taste unpleasant for oral administration in children and a sweetener (sodium saccharine 0.1 % w/v) was therefore used to improve palatability.

In the formulation F2, the alkaline pH necessary for FUR dissolution in water was reached by the addition of disodium hydrogen phosphate dodecahydrate which was successfully used also in the previous paediatric propranolol and sotalol liquid formulations. [20-22] An appropriate amount of disodium hydrogen phosphate dodecahydrate was adjusted experimentally during the preparation development. In our experience, this formulation possesses more pleasant taste properties compared to the F1 formulation.

In the stability study, two batches of the formulated FUR aqueous solutions F1 and F2 were prepared in the Motol hospital pharmacy and stored in tightly closed amber glass bottles at  $25 \pm 3^{\circ}\text{C}$  and  $40 \pm 0.5^{\circ}\text{C}$ . The concentration of the FUR and the preservative MP was estimated throughout the time interval of 0 – 270 days using HPLC method. The content

of the furosemide in mg per mL at time of preparation was considered to be the actual initial one ( $c_0 = 100\%$ ). As can be seen in Table 3, the FUR percentage content remained within the targeted limit of the initial concentration in both formulations throughout the 270-day storage period at room temperature. Both preparations had suitable pH for maintaining FUR solubility.

The concentration of methylparaben remained within  $\pm 20\%$  of the initial concentration for both solutions stored at room temperature as well as at  $40^\circ\text{C}$  for F1 for 9 months. On the contrary, a significant decrease in concentration was observed for methylparaben in formulation F2 stored at  $40^\circ\text{C}$ , probably due to its decomposition. As can be seen in Table 4, the targeted remaining concentration  $\geq 80\%$  was maintained only up to 30 days.

Preparations F1 and F2 should be packaged in a brown glass container to protect from light. A screw cap suitable for use with a graduated pipette for oral use to achieve accurate dosing is recommended.

## **CONCLUSIONS**

Two aqueous, ethanol-free oral solutions containing furosemide in the concentration  $2\text{ mg mL}^{-1}$  were developed in accordance with the recent requirements of the safety of paediatric drugs. The preparations formulated for easy extemporaneous compounding in a pharmacy are suitable for the oedema therapy of various origins as well as hypertension in paediatric age groups above 1 month of age. The excipients used ensured stable pH, antimicrobial stability, and pleasant taste. A 9-month stability study performed by validated HPLC analysis demonstrated that the concentration of FUR in both F1 and F2 formulations was in accordance with the criterion that at least  $90\%$  of the initial content should remain during storage at  $25^\circ\text{C}$  or  $40^\circ\text{C}$ . Nevertheless, preparation F1 having a worse, slightly burning

taste caused by the presence of sodium hydroxide, although a sweetener sodium saccharine 0.1 % w/v was added, is less preferable when compared to F2 containing disodium hydrogen phosphate dodecahydrate. Moreover, sodium hydroxide is a highly caustic base which readily absorbs moisture and carbon dioxide from the air. This makes its manipulation problematic and the routine preparation of its solution quite inconvenient in a pharmacy. On the other hand, the preparation F2 has a more pleasant taste and is easier to prepare in a pharmacy as disodium hydrogen phosphate is easier to manipulate and weigh than sodium hydroxide. Formulation F2 therefore represents a compromise between good FUR solubility in water, taste acceptance in paediatric patients, and fast compounding procedure. For long stability at room temperature, the stock F2 solution could be prepared in advance in the pharmacy and be available until needed. Methylparaben 0.1 % w/v in preparation F2 stored at room temperature fully satisfied the recommended criteria for preservative efficacy in oral preparations according to Ph. Eur. 9.0 (5.1.3 Efficacy of antimicrobial preservation).

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## FIGURE LEGEND

**Figure 1.** Liquid chromatography separation of standard  $50 \mu\text{g mL}^{-1}$  solution of furosemide and formulation F2 ( $2 \text{ mg mL}^{-1}$  of furosemide)

SACC (saccharine), IMP A (FUR impurity A), FUR (furosemide), MP (methylparaben).

**Table 1.** Composition of formulations

	<b>F1</b>	<b>F2</b>
Furosemide	0.2 g	0.2 g
Methylparaben	0.1 g	0.1 g
Sodium hydroxide	0.024 g	–
Disodium hydrogen phosphate dodecahydrate	–	1.5 g
Saccharine sodium	0.1 g	0.1 g
Water for injections	to 100.0 mL	to 100.0 mL

**Table 2.** Validation results of pharmaceutical formulation F1 and F2

	F1		F2		Criterion
	FUR	MP	FUR	MP	
Repeatability tR (%R.S.D.) <sup>a</sup>	0.26	0.12	0.26	0.12	X < 1 %
Repeatability Area (%R.S.D.) <sup>a</sup>	0.16	0.32	0.16	0.32	X < 1 %
Number of theoretical plates	2 499	7 892	2 499	7 892	-
Resolution <sup>a</sup>	1.57	12.49	1.57	12.49	R <sub>ij</sub> > 1.5
Tailing factor <sup>a</sup>	1.18	1.09	1.18	1.09	T = 0.8–1.5
Precision (%R.S.D.) <sup>b</sup>	3.55	3.54	2.13	1.52	X < 5 %
Linearity (Correlation coefficient) <sup>c</sup>	0.9990	1.0000	0.9990	1.0000	R ≥ 0.9990
Accuracy Recovery (%) <sup>b</sup>	103.48	104.35	100.83	102.56	X = 100 ± 5 %
Accuracy (%R.S.D.) <sup>b</sup>	0.61	0.36	1.75	1.84	X < 5 %
Selectivity	No interference		No interference		No interference

*a**six injections**b**six samples, three injections of each sample**c**at 40, 50, 80, 100, 120 and 150 % concentration levels**FUR**furosemide**MP**methylparaben**FUR X**formulations with various excipients**%R.S.D.**Relative Standard Deviation in %*

**Table 3.** Stability of furosemide in formulations F1 and F2 stored at 25 °C and at 40 °C.\*

<b>Time (days)</b>	<b>25 ± 3°C</b>		<b>40 ± 0.5°C</b>	
	<b>F1</b>	<b>F2</b>	<b>F1</b>	<b>F2</b>
0 (100 %)	2.15 ± 0.02 mg/mL	2.12 ± 0.05 mg/mL	2.15 ± 0.02 mg/mL	2.12 ± 0.05 mg/mL
7	102.33 ± 1.39	98.14 ± 2.10	99.06 ± 2.35	92.89 ± 2.62
30	98.60 ± 1.30	97.67 ± 0.12	100.00 ± 1.50	94.34 ± 1.77
90	98.14 ± 1.48	96.28 ± 0.78	98.58 ± 0.99	94.81 ± 1.27
180	99.07 ± 0.61	91.63 ± 0.66	100.00 ± 0.61	95.75 ± 1.05
270	93.95 ± 0.56	92.56 ± 1.05	95.28 ± 2.33	91.98 ± 0.50

\*Mean ± SD of determinations for four samples (n = 4)

**Table 4.** Stability of methylparaben in formulations F1 and F2 stored at 25 °C and at 40°C.\*

Time (days)	25 ± 3°C		40 ± 0.5°C	
	F1	F2	F1	F2
0 (100 %)	1.03 ± 0.03 mg/mL	1.04 ± 0.02 mg/mL	1.03 ± 0.03 mg/mL	1.04 ± 0.02 mg/mL
7	100.00 ± 0.15	98.06 ± 2.80	95.14 ± 2.75	89.52 ± 2.26
30	99.03 ± 1.91	98.06 ± 0.25	97.08 ± 0.81	83.50 ± 1.50
90	99.03 ± 1.06	96.11 ± 0.47	95.14 ± 0.80	69.90 ± 1.37
180	98.06 ± 1.41	92.29 ± 2.00	90.29 ± 0.59	52.43 ± 1.06
270	97.12 ± 1.10	91.98 ± 0.30	89.42 ± 2.21	41.75 ± 0.53

\*Mean ± SD of determinations for four samples (n = 4)