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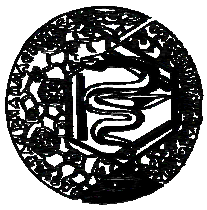

SIMULTÁNNÍ STANOVENÍ KOVOVÝCH KATIONŮ (HOŘČÍKU, VÁPNÍKU A HLINÍKU) V LÉČIVÝCH PŘÍPRAVCÍCH VYUŽITÍM IONTOVĚ VÝMĚNNÉ KAPALINOVÉ CHROMATOGRAFIE A ELS DETEKCE

DIPLOMOVÁ PRÁCE

2006 Zdeněk Spáčil

CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRALOVE
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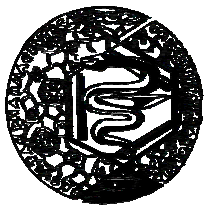

SIMULTANEOUS KATION EXCHANGE LIQUID CHROMATOGRAPHY DETERMINATION OF METALS (MAGNESIUM, CALCIUM AND ALUMINIUM) IN PHARMACEUTICAL FORMULATIONS USING EVAPORATIVE LIGHT SCATTERING DETECTION

GRADUATION THESIS

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CONTENTS:

ABBREVIATION LIST

AAS	Atomic Absorption Spectrometry
AES	Atomic Emission Spectrometry
AFS	Atomic Fluorescence Spectrometry
CCC	Countercurrent Chromatography
CE	Capillary Electrophoresis
CZE	Capillary Zone Electrophoresis
DNA	deoxyribonucleoic acid
ELSD	Evaporative Light Scattering Detector
ET-AAS	Electro-Thermal Atomic Absorption Spectrometry
FIA	Flown Injection Analysis
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry

LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometry
NFPA	nonafluoropentanoic acid
RID	Refractive Index Detector
RNA	ribonucleoic acid
RSD	Relative Standard Deviation
SFC	Supercritical Fluid Chromatography
SIA	Sequential Injection Analysis
TFA	trifluoracetic acid
UV-VIS	Ultraviolet – Visible

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1. INTRODUCTION

Magnesium and calcium play an essential role in human and animal health and are provided in the case of deficiency syndromes and other diseases. Also aluminium (in the form of various salts, complexes or hydroxides) is commonly used as pharmaceutical substance. Their compounds are described in pharmacopoeias and are provided in pharmaceutical or/and food supplement formulations (mainly tablets, chewing tablets, suspension or solutions), alone or in combination, in relatively high amounts. In several cases a combination of these official compounds is formulated, for example magnesia, alumina and calcium carbonate tablets and suspensions.

Evaporative Light Scattering Detection (ELSD) has been recently used in many chromatographic applications as a quasi-universal detector, especially in the case of the absence of chromophoric groups in the analytes molecules. Despite the wide use of ELSD in organic analysis (drugs, natural products, polymers), very few inorganic analytes have been determined using LC-ELSD methods [sulfate (as counter-ion of aminoglycoside antibiotics), sodium carbonate (in a drug substance)].

In this paper an LC-ELSD method has been developed and validated for the simultaneous, simple, low operational costs and reliable determination of the main metals of pharmaceutical use (magnesium, calcium and aluminium) and its application in pharmaceutical and food supplement formulations. Using ion-exchange column and volatile acids as mobile phase the separation of the three metal ions is successful and the detection by ELSD is achieved at the $\mu g\ ml^{-1}$ concentration level.

2. THEORETICAL PART

2.1. MAGNESIUM, CALCIUM AND ALUMINIUM

2.1.1. Magnesium

Magnesium is an essential mineral for human nutrition mainly found in foods like cereals, nuts, cacao, meat, milk and vegetables. Magnesium has several important functions. It is involved in energy metabolism, acting as a metal activator or co-factor for enzymes requiring adenosine triphosphate (ATP), in replication of DNA and in the synthesis of RNA and proteins; it appears to be essential for all phosphate transferring systems. Together with calcium, magnesium is involved in muscle contraction and blood clotting [1][2]. Its deficiency occurs, in general as complications of other diseases like alcoholism, diabetes, and kidney failure and in some post-operative periods. Magnesium deficiency can be treated by oral or parental administration of some magnesium salts (magnesium supplement tablets). Oversupply in severe cases lead to coma and death [1].

Pharmaceutically is magnesium used in the form of acetate, aspartate, carbonate, chloride, citrate, gluconate, glycerophosphate, hydroxide, oxide, phosphate, pidolate, salicylate, stearate, sulfate, trisilicate, etc. [3][4][5].

2.1.2. Calcium

Calcium is the most common mineral in the human body (approximately 99% of total body calcium is in the skeleton and teeth and 1% in blood and soft tissues) where it is present in almost the same relative abundance as in the earth's crust. Dairy products are the most concentrated, well absorbed sources of calcium. Other foods which can contribute to dietary calcium include firm tofu (chemically set with calcium), dried beans, kale, broccoli, and bok choy. Calcium has four major biological functions: (1) structural as stores in the skeleton, (2) electrophysiological - carries charge during an action potential across membranes, (3) intracellular regulator, and (4) as a cofactor for extracellular enzymes and regulatory proteins. In this way it regulates heart rhythm; eases insomnia; helps regulate the

passage of nutrients in & out of the cell walls; assists in normal blood clotting; helps maintain proper nerve and muscle function; lowers blood pressure; important to normal kidney function [6] and in current medical research reduces the incidence of colon cancer [7], and reduces blood cholesterol levels [8]. Deficiency syndromes may result in arm and leg muscles spasms, softening of bones, back and leg cramps, brittle bones, rickets, poor growth, osteoporosis, tooth decay, depression. Dietary calcium deficiency also has been associated with increased risk of hypertension, preeclampsia, and colon cancer [6].

Pharmaceutically is calcium used in the form of carbonate, chloride, glubionate, gluceptate, gluconate, hydroxide, lactate, lactobionate, levulinate, pantothenate, phosphate, saccharate, etc. [3][4][5].

2.1.3. Aluminium

Aluminium is a trivalent cation found in its ionic form in most kinds of animal and plant tissues and in natural waters everywhere. It is the third most prevalent element and the most abundant metal in the earth's crust. Dietary aluminium is ubiquitous, but in such small quantities that it is not a significant source of concern in persons with normal elimination capacity. If a significant load exceeds the body's excretory capacity, the excess is deposited in various tissues, including bone, brain, liver, heart, spleen, and muscle. Lactate, citrate, and ascorbate all facilitate gastrointestinal absorption. No known physiologic need exists for aluminum; however, it is sometimes a competitive inhibitor of several essential elements of similar characteristics, such as magnesium, calcium, and iron. Mechanisms of toxicity include inhibition of enzyme activity and protein synthesis, alterations in nucleic acid function, and changes in cell membrane permeability. Aluminium toxicity is usually found in patients with impaired renal function. In aluminium-related disease, the predominant features are defective mineralization and osteomalacia with a closely associated dialysis encephalopathy, which is thought to be caused by aluminium deposition in the brain. Aluminium causes an oxidative stress within brain tissue, leading to the formation of Alzheimerlike neurofibrillary tangles [9][10]. Aluminium also has a direct effect on hematopoiesis [11].

Pharmaceutically is aluminium used in the form of hydroxide, acetate, chlorohydrate, phosphate, subacetate, etc. [3][4][5].

2.2. DETERMINATION OF MAGNESIUM, CALCIUM AND ALUMINIUM

The pharmacopoeial analytical methods [3][4][5] for the assay of the aforementioned metals in starting materials and formulations are complexometric titrations with disodium edetate (direct or back-titrations). In the case of combination of two metal compounds (e.g. magnesium and aluminium) different pH adjustments are used in separate titrations to obtain specificity. Atomic absorption spectrometry (AAS) is also widely used. For example in the alumina, magnesia and calcium carbonate combination, aluminium is determined with edetate by back-titration at acidic buffer, calcium with edetate at very alkaline buffer and magnesium with AAS at wavelength of 282.5 nm.

From the above described official methods it is clear that in the case of metal combinations separate assay experiments are required, very often using different analytical techniques (titrimetry and AAS). Therefore an analytical method enabling the simultaneous determination of all metals in the formulation is very desirable.

Several chromatographic techniques have been developed for the simultaneous determination of trace metal elements, metals of specific groups and metal speciation. These include gas chromatography (GC), HPLC interfaced to AAS, atomic emission (AES) and atomic fluorescence (AFS) spectrometry. Recent tandem analytical systems are based on HPLC – inductively coupled plasma mass spectrometry (ICP-MS) and HPLC – thermochemical hydride generation – AAS system [12].

Several reviews have been published on ion-pair chromatography of metal ions [13], directly coupled chromatography – atomic spectroscopy [14], ion-exchange HPLC of metal complexes [15], determination of metal ions by HPLC-photometry [16], ion-pair reversed-phase chromatography of metal chelates [17], metal determination and metal speciation by LC [18], analysis of metal ions by HPLC [19], HPLC-AAS hybrid technique for the speciation of trace metals in biological fluids [20], trace-elemental speciation by HPLC using microbore columns hyphenated to AAS [21], chromatographic and hyphenated methods for elemental speciation analysis in environmental media [22], the coupling of size-exclusion HPLC with ICP MS in bio-inorganic analysis [23], chemical modification of analytes in speciation analysis by CE, LC and GC [24], HPLC-isotope dilution ICP-MS for speciation

studies [25], bio-inorganic speciation analysis by hyphenated techniques [26], and separation and quantisation of low-molecular mass organic acid – metal complexes using HPLC and CZE for speciation purposes [27].

2.2.1. Determination of Magnesium

Out of Czech pharmacopoeia analytical method (complexometric titrations with disodium edetate at alkalic buffer [5]), several articles describing alternative determinations of magnesium in pharmaceutical preparations were published. These include AAS [1] sequential injection analysis (SIA) [1] and recently a multi-commutation-based flow system for multi-element analysis also suitable for determination of calcium [28].

A variety of other instrumental methods is used for magnesium analysis in non-pharmaceutical samples as beverages, food or body fluids. These include UV-VIS spectrophotometry [29], ion chromatography with a piezoelectric detector [30], ICP-AES [31], ICP-MS [32] and ion selective electrode [33]. Other methods used for magnesium analysis are based on flow based procedures, e.g. continuous on-line feedback based flow titration [34], FIA based on magnesium ion-selective electrode [35] and a multi-component flow injection based analysis with diode array detection [36]. Nevertheless, these methods have not been applied on determination of magnesium in pharmaceuticals.

2.2.2. Determination of Calcium

Czech officinal analytical method is complexometric titrations with disodium edetate in strongly alkalic contitions [5].

Other methods used for determination of calcium in pharmaceutical formulations and/or pharmaceutical raw materials ICP-MS and electrothermal atomic absorption spectrometry (ET AAS) [37], SIA [1], multi-commutation-based flow system for multi-element analysis [28][38] and capillary isotachophoresis [39].

2.2.3. Determination of Aluminium

Czech pharmacopoeia determination of Aluminium is based on back-titration of disodium edentate at acidic conditions [5].

The commonly used analytical methods for the quantitative determination of aluminium are fluorimetry, AAS, AES, GC and induced plasma atomic emission spectrometry (ICP-AES). Although most of these are high precision methods, they necessitate pre-treatment steps, special and expensive instruments, large volumes of sample solution and a long period of analysis. Electrogravimetry and coulometric methods generally have moderate selectivity, sensitivity and speed. Strong buffering of some fixed pH is necessary to obtain reproducible data in polarography and voltammetric methods. Since the reduction of aluminium at the electrode in aqueous solutions is difficult, it cannot be easily determined by conventional voltammetry. Thermo gravimetric methods are largely limited to decomposition and oxidation reactions and such physical processes as vaporization, sublimation, and desorption. UV–VIS spectrophotometry is used due to its accuracy and good precision, associated with the low cost and widespread diffusion of equipment [40].

2.3. EVAPORATIVE LIGHT SCATTERING DETECTION

2.3.1. Introduction

About twenty years have been past since the introduction of the first commercially available Evaporative Light Scattering Detector (ELSD) in early 1980s (Mass Detector, Applied Chromatography System Limited, Macclesfield, Cheshire, UK), and nowadays ELSDs have moved into the mainstream of HPLC detection techniques. The inherent advantage of ELSD to detect any analyte, regardless of the optical (i.e. UV absorptivity), electrochemical or other analyte properties, is the main reason for ELSD expanded applicability. ELSD is considered to be a quasi-universal rather than a fully universal detector, since analytes with higher volatility than the mobile phase can not be detected. It is mainly considered to be an LC detector. However it also appears compatibility with countercurrent (CCC) and supercritical fluid chromatography (SFC) [41].

Beyond the common usefulness, which any universal detector appear [e.g. refractive index detector (RID) and mass spectrometers (MS)], the increasing interest for ELSD is additionally attributed to some special characteristics: (a) compatibility with gradient elution and insensibility to temperature variation (unlike RID), (b) much better detectability than RID for most molecular classes, similar to conventional LC detectors (regular detection limit is in the nanogram range, depending on analyte volatility and relative molecular mass) and (c)

low cost and easy operation (unlike MS). However, it should be clarified that until now, ELSD is mainly considered to be a good alternative or supplemental detector rather than a substitute for the conventional HPLC detectors (UV-VIS, fluorimeters etc.), while it lacks the huge identification potential of the wide range of LC-MS techniques [41].

The operation principle of ELSD mainly consists of three successive processes depicted in Figure 2.1 (a) nebulization of the chromatographic effluent, (b) evaporation of the mobile phase, and (c) detection of the non-volatile residual particles, by means of the measurement of the scattered light [41].

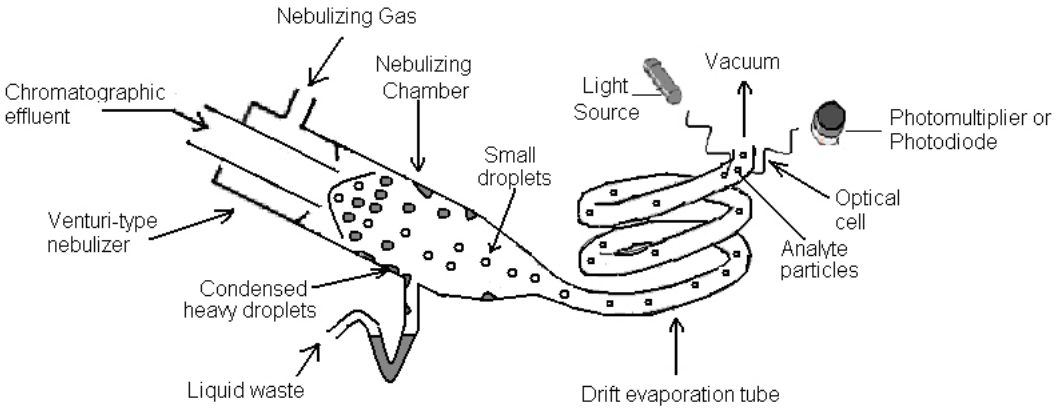

Figure 2.1 Depiction of the main steps of the ELSD design type B operation.

2.3.2. Nebulization

In the first step of ELSD detection mechanism, the effluent from a chromatographic column enters a Venturi-type nebulizer, where it is transformed into an aerosol. These nebulizers create a high flow of carrier gas (air or inert gas, such as nitrogen, carbon dioxide, argon or helium) over the liquid surface producing a high amount of droplets with remarkably uniform size [41].

Distribution and mean values of droplets diameter are considered to be very critical parameters, which strongly influence the analytical characteristics (i.e. detectability, sensitivity and repeatability) of the ELSD methods. The formation of uniform, reproducible and stable aerosols depends on the relation of the nozzle diameter and the flow rates of mobile phase and nebulizing gas. For constant diameter of the nozzle, stable aerosols are formed only for a

limited range of flow rates, and further, the flow rate of the nebulizer gas must be adjusted in relation to the flow rate of the mobile phase. For a mobile phase flow rate of 1 ml min^{-1}, the usual consumption of the nebulizing gas must be in the range of 2-5 l min^{-1} [41].

Furthermore, it has been indicated that mean diameter of aerosol droplets strongly influences ELSD response and in fact an increase of the mean diameter of aerosol droplets results in ELSD response enhancement [41].

If the gas flow rate is too low, mobile phase would not be completely nebulized and/or it would not be completely vaporized, which would result in an excessive noise or baseline with spiked sharp peaks [41].

ELSDs are classified in two types according to their structure after the nebulization unit. In ELSD of type A (non aerosol splitting), the entire aerosol immediately enters the heated evaporation tube (drift tube), where the evaporation process begins. In ELSD of type B (aerosol splitting, Figure 2.1), the aerosol, before the evaporation step, enters a glass chamber or a focusing cone (nebulization chamber), in which the droplets of high size are condensed on the walls of the chamber and diverted to waste. The proportion of the wasted aerosol depends on mobile phase volatility and varies from > 90% (aqueous mobile phase) to < 10% (highly volatile organic solvents). Each type appears its own benefits, while the appropriate choice depends on the nature of the analyte and the composition of the mobile phase. ELSD of type B requires lower evaporation temperature than type A and thus it is more sensitive for volatile, semi-volatile or thermo-sensitive analytes. On the other hand, for non-volatile analytes, ELSD of type A appears to be more sensitive, since the entire quantity of analyte reaches the optical cell. Considering the composition and flow rate of the mobile phase, ELSD of type A is incompatible with gradient elution and requires low flow rates and highly volatile mobile phases (non-aqueous or low water portion), while ELSD of type B appears wider compatibility [41].

2.3.3. Evaporation of Mobile Phase

In this stage, the size of the aerosol droplets is reduced, due to the evaporation of the mobile phase, which is performed in a heated drift tube. Ideally, the purpose of this stage is to completely vaporise the mobile phase, without any analyte loss (due to evaporation or

thermal decomposition). The completeness of the mobile phase evaporation and the extent of loss of analyte is mainly determined by the evaporation temperature, which should be selected in accordance to the mobile phase and analyte volatility, to the mobile phase flow rate and to the ELSD type (A or B). Inappropriate selection of the evaporation temperature results, in case of low temperature, in an excessive noise or baseline with spiked sharp peaks, or in case of high temperature, in reduced sensitivity. Apart from the analyte loss, high evaporation temperature causes rigorous solvent evaporation, which destroys uniformity of particle size, and favours the formation of liquid rather than solid particles. Both effects result in decrease of ELSD sensitivity. The evaporation temperature is usually set between 30 and 100°C. Decrease of the required evaporation temperature can be obtained with nebulizing gas of high thermal conductivity (helium was found to require at least 30°C lower evaporation temperature than carbon dioxide), which in cases of volatile and thermosensitive compounds results in enhancement of detector sensitivity. On the other hand, for stable and non volatile compounds, the ELSD response factor has been found to be independent of the nature of the nebulizing gas [41].

2.3.4. Light Scattering

The aerosol, after the evaporation process, ideally composed by solid particles of analyte, enters the optical cell and passes through a light beam. The scattered light is measured by a photomultiplier or a photo diode, providing the output signal [41].

Light scattering processes are classified in two types: elastic scattering, in which the scattered radiation is of the same frequency as the incident radiation, and inelastic scattering, in which the scattered radiation is of a different frequency. In ELSDs, inelastic scattering is considered to be negligible and it is not further examined. Elastic scattering is classified in three types: Rayleigh, Debye and Mie. Refraction-reflection mechanism, which has its origin in the induced secondary emission of particles in the path of the incident beam, has also been reported as a potential mechanism of scattering in the ELSD optical cell [41].

Since scattering and not absorbing phenomenon is intended to occur when the light interacts with the analyte particles, a tungsten filament or halogen lamp that produces a continuous spectrum of wavelengths, rather than a monochromatic laser-emitting diode, is favoured as a light source. In some instruments a secondary gas, independent of the

nebulizing gas, is used to concentrate the particles in the centre of the detection cell and to prevent the deposition on the cell inner surfaces [41].

The power of scattered light is controlled by the particle diameter, the light wavelength and the angle of scattered light. It has been observed that the ELSDs sensitivity is higher, but the dynamic range narrower, for low detection angle, with wide angular acceptance and the use of vertically polarized or unpolarized light [41].

2.3.5. Limitations

Apart from the fact that ELSDs appear nearly no selectivity, an inherent characteristic of most 'universal' detectors, some additional requirements may limit their applicability. The main difficulty for the development of LC-ELSD analytical methods is the restriction on the mobile phase volatility. Non-volatile modifiers, ion-pairing reagents, acids, bases and buffers cannot be used with ELSD. Therefore, a very useful part of the mobile phase chemistry is not compatible with ELSD, making quite difficult to convert an LC-UV method to an LC-ELSD method or to achieve efficient chromatographic separations for some type of analytes. Some acceptable volatile reagents are trifluoroacetic (TFA), heptafluorobutyric, nonafluoropentanoic (NFPA), acetic and formic acid and their ammonium salts in low concentrations (< 0.1 M) [41].

ELSD is a destructive detector, therefore it must be last in line if it is used in series with other detectors. In cases that it is used in line with another destructive detector (e.g. MS), a line splitter should be added and the flow rate of the nebulizating gas should be accordingly adjusted [41].

Generally, it appears relatively low detectability, inadequate for the direct analysis of compounds (e.g. impurities, residues) at ng ml^{-1} concentration level (quantitation limit is usually above 0.1 μg ml^{-1}). In these cases, preconcentration steps should be developed, in order to enrich the under analysis samples. However, development of a preconcentration procedure is quite difficult, mainly for two reasons: firstly, non-volatile reagents can not be applied (e.g. precipitation reagents and buffers) and secondly, preconcentration procedure may simultaneously enrich some of the matrix components resulting in excessive interferences due to the low ELSD selectivity [41].

2.3.6. Applications

ELSD has been effectively used for the determination of a wide variety of compounds in various synthetic or natural matrices. The main application areas of ELSD concern pharmaceuticals, foods and beverages, natural products and biological samples and polymers. A wide range of column types and mobile phase polarity have been utilized and various procedures for sample preparation have been developed depending on the analyte nature and the sample matrix. Beyond the differences of analyte and matrix nature, a common characteristic of all ELSD methods is the conformity with the following rule: "non volatile analytes are determined utilizing a volatile mobile phase" [41].

3. EXPERIMENTAL PART

3.1. CHEMICALS AND REAGENTS

All chemicals were of analytical reagent grade.

3.1.1. Water

HPLC grade water was prepared in three steps: (a) deionization, (b) distillation and (c) purification of HPLC grade. Tap water passed deionizating column Zalion 2.004 purchased from IONEL A.E.B.E. company (N. Irakleio Attikis, Greece), then it was distilled in "Mega-Pure automatic" distilling instrument manufactured by CORNING (New York, USA). Purification of HPLC grade was performed by Milli-Q R6 system made by MILLIPORE Corporation (Billerica, MA, USA). HPLC water was prepared within 15 days before use.

3.1.2. Mobile Phase

Trifluoracetic acid (TFA) in purity > 98% (Sigma-Aldrich, St. Louis, USA) and nonafluoropentanoic acid (NFPA) in purity > 97% (Aldrich, Steinheim, Germany) were used for preparation of aqueous mobile phase.

The examined mobile phases were prepared directly in HLPC system trough the medium of flown-channel selection valve diluting stock solutions by HPLC grade water in appropriate ratio. The following stock solutions were commonly used TFA 3.1 ml l^{-1} (isocratic elution) 8.0 ml l^{-1} (gradient elution) and NFPA 6.2 ml l^{-1}.

For the reason that TFA and NFPA are highly volatile substances, their stock solutions were prepared by adding appropriate volume of acid to the volumetric flask (250 ml) almost full of already degassed HPLC grade water and filled with the same diluent to the punch mark. All stock solutions were kept in refrigerator in airtight container.

3.1.3. Standards

The standard solutions of metals were prepared from Magnesium acetate tetrahydrate in purity > 99% (Hopkin&Williams LTD, Chadwell Health, Essex, England),

Calcium hydroxide in purity > 96% and Aluminium Nitrate nonahydrat in purity > 98.5% (both from Merck, Darmstadt, Germany). Organic anion standards were prepared from L-Ascorbic acid in purity > 99%; DL-Aspartic acid in purity > 99% and Citric acid, monohydrate in purity > 98% (all three by Sigma-Aldrich, Steinheim, Germany).

Magnesium (isocratic elution)

Accurately weighted amount 0.0892 g of Magnesium acetate chemical was dissolved in mobile phase (TFA 11 mM l^{-1}) and sonicated few minutes till complete dissolution, forming 100.1 μg ml^{-1} solution of Mg^{2+}. From this stock solution, volumes of 0.8 ml, 1.0 ml, 1.4 ml, 1.6 ml and 2.4 ml were taken by automatic pipette to 10 ml volumetric flasks, in order to gain working standard solutions of 8.0 μg ml^{-1}; 10.0 μg ml^{-1}; 14.0 μg ml^{-1}; 16.0 μg ml^{-1} and 24.0 μg ml^{-1} of magnesium. From these five dilutions the calibration curve for isocratic elution was constructed.

Calcium

The precise amount of Calcium hydroxide substance (0.1847 g) dissolved by mobile phase (TFA 11 mM l^{-1}) in 100 ml volumetric flask and sonicated few minutes till complete dissolution for 959.2 μg ml^{-1} solution of Ca^{2+}. From this stock solution, volumes of 0.375 ml, 0.8 ml, 1.125 ml, 1.5 ml were taken by automatic pipette to 25 ml volumetric flasks and 0.75 ml was taken by automatic pipette to 10 ml volumetric flasks, in order to gain working standard solutions of 14.4 μg ml^{-1}; 28.8 μg ml^{-1}; 43.2 μg ml^{-1}; 57.6 μg ml^{-1} and 71.9 μg ml^{-1} of calcium cations. From these five dilutions the calibration curve was constructed.

Magnesium and Aluminium (linear gradient elution)

Standards substances of Magnesium acetate (0.0886 g) and Aluminium nitrate (0.2084 g) were weighted into two different 100 ml volumetric flasks and completely dissolved in mobile phase (TFA 12 mM l^{-1}), using sonication, forming 99.4 μg ml^{-1} of Mg^{2+} and 147.6 μg ml^{-1} of Al^{3+} stock solutions. From magnesium stock solution volumes of 0.6 ml, 1.2 ml, 1.8 ml, 2.4 ml; 3.0 ml were taken by automatic pipette into 10 ml volumetric flasks and from aluminium stock solution volumes of 0.67 ml; 1.3 ml; 2 ml; 2.67 ml; 3.33 ml were taken by automatic pipette to the same 10 ml volumetric flasks, in order to gain mixed

working standard solutions of magnesium 6.0 μg ml^{-1}; 11.9 μg ml^{-1}; 17.9 μg ml^{-1}; 23.9 μg ml^{-1}; 29.8 μg ml^{-1} and aluminium 9.9 μg ml^{-1}; 19.6 μg ml^{-1}; 29.5 μg ml^{-1}; 39.4 μg ml^{-1}; 49.2 μg ml^{-1}. From these five dilutions the calibration curve for linear gradient elution was constructed.

Organic acids

Accurate amounts of organic acids (0.0149 g of DL-Aspartic acid; 0.0148 g of L-Ascorbic acid; 0.0168 g of Citric acid) were dissolved in HPLC grade water separately in three 100 ml volumetric flasks forming three stock solutions in concentrations 147.5 μg ml^{-1} of Aspartic acid; 146.5 μg ml^{-1} of Ascorbic acid and 150.5 μg ml^{-1} of Citric acid. From stock solution of each acid was taken volume of 3.3 ml by automatic pipette into 10 ml volumetric flask, in order to gain mixed working standard solutions of Aspartic acid 49.2 μg ml^{-1}; Ascorbic acid 48.8 μg ml^{-1} and Citric acid 50.2 μg ml^{-1}. These solutions were used to observe retention times and separation of organic acids.

3.1.4. Other Chemicals

Calcium lactate (Mallinckrodt Chemical Works) during development of method. Hydrochloric acid fuming 37%; Formic acid 98 - 100% and Acetic acid in purity > 99.5% (all three from Merck, Darmstadt, Germany) were used to improve separation of metals from drug matrix.

3.2. INSTRUMENTATION

3.2.1. HPLC System

Modular Shimadzu HPLC system (Tokyo, Japan) consisting of a LC-10AD VP pump; a FCV-10AD VP flow-channel selection valve and a RHEODYNE 7725i by Perkin Elmer (Wellesley, MA, USA) manual injector with a built-in position sensing switch, which provided the chromatograph start signal. The complete filling method with 20 μl loop was used. Syringe containing excess of sample was required to completely flush mobile phase from the loop and the volume of the loop was injected.

3.2.2. Chromatographic Column

IONPAC® CS – 14 analytical column by DIONEX® is a moderately hydrophilic, carboxylate-functionalized cation exchanger. Its packing is an 8.0 μm diameter macroporous particle consisting of ethylvinylbenzene crosslinked with 55% divinylbenzene. The substrate is functionalized with hydrophilic carboxylic acid, which permits the elution of monovalent and divalent cations. Dimensions are 4 × 250 mm with 1300 μeq capacity.

3.2.3. ELSD Detector

Evaporative Light Scattering Detector (ELSD) was SEDEX 75 (S.E.D.E.R.E., Alfortville Cedex, France). The nebulizing gas was nitrogen. Appropriate pressure drop was applied at the end of the flow line in order to ensure the complete removal of the gas wastes.

3.2.4. Software

The data from HPLC system were compiled by Class VP Chromatography Data System, version 4.3; (Shimadzu, Germany).

Statistics were performed by StatMost Analysis and Graphics, version 2.50 (DataMost Corporation, Dataxiom Software Inc., LA, USA).

3.3. PROCEDURES

3.3.1. General Procedures

Analytical column, which was utilized, is polymeric one, resistant to strongly acidic environment. After the measuring day column was carefully washed with mobile phase for 20 minutes, flow rate 1.5 ml min^{-1} and it was stored in the same medium, since the applied mobile phase contained a strong acid (TFA). Before measurements, flow path was rinsed with mobile phase for about 15 min, until baseline noise became negligible (less than 5 mV at detector gain 11).

In case of linear gradient elution, equilibration of the analytical column was required between runs. Equilibration was performed at a flow rate of 2.0 ml min^{-1} in two successive steps: (a) HPLC grade water for 15 minutes and (b) mobile phase for 15 minutes with composition identical to the composition of gradient program start.

Degassing was carried using Helium air-leak tube of another HPLC system for five minutes.

The detector was necessary to switch on at least two hours before measurements, due to lamp aging, in order to stabilise the light intensity. When measuring sooner, there was a higher response of the detector which caused bigger areas of peaks and significant inaccuracy of results.

3.3.2. Optimal Mobile Phase and Chromatographic Parameters

The optimum composition of mobile phase was aqueous solution of 0.85 ml l^{-1} TFA for the analysis of Milk of Magnesia® and Tums® by the means of isocratic elution. For the analysis of Aludrox® linear gradient elution was used; from 0 min to 6th using TFA 0.96 ml l^{-1} as mobile phase, from 6th min to 7th min linear gradient to aqueous TFA 6.4 ml l^{-1}.

Flow rate of mobile phase during the analyses was 1.0 ml min^{-1}, corresponding to a back pressure of 1330 psi. Column was operating at laboratory temperature.

Gain of the detector was set on 11. It was the best compromise between sensitivity and baseline noise occurrence. Its working temperature was 70 centigrade for the reason that mobile phase used contains a high portion of water which is not as volatile as organic solvents and analytes were not declinable to decomposing. Pressure of the nebulizing gas was 3.5 Bars.

3.3.3. Validation Procedures

The LC-ELSD analytical method was validated in terms of resolution and symmetry of chromatographic peaks, precision (repeatability and reproducibility), concentration range, correlation coefficient of calibration curve, detectability and accuracy.

Since ELSD is not a linear detector, double logarithmic relations were established and correlation coefficients were determined for magnesium, calcium and aluminium by triplicate measurements of the corresponding chromatographic peaks of five standard solutions.

Repeatability of the method was evaluated by replicate measurements of standard solutions (n=3) and repeated analyses of pharmaceutical formulations (3 independent sub-samples × 3 measurements).

Reproducibility of the method was evaluated by the estimation of %RSD of the peak area of standard solutions and the slope of calibration curves obtained at three different days within a week, with 3 replicates per day.

Detection limit was determined as the analyte concentration for which the area of the chromatographic peak was equal to 3.3 times the standard deviation of the most dilute standard and was practically equal to the concentration having S/N ratio equal to 3.3.

Quantification limit was determined as the analyte concentration for which the area of the chromatographic peak was equal to 10 times the standard deviation of the most dilute standard and was practically equal to the concentration having S/N ratio equal to 10.

Accuracy was evaluated by recovery experiments. Formulation samples were fortified by standard solutions at three concentration levels, with three samples being prepared at each level and measured in triplicate. Mean recovery and range of recovery values were calculated. Accuracy was also evaluated by comparison of the results for magnesium, calcium and aluminium analyzed by the Pharmacopoeia [3][5] and the proposed method.

3.3.4. Pharmaceutical Formulations

Following pharmaceutical formulations were submitted to trials; Milk of Magnesia® and Tums® (both by GlaxoSmithKline) and Aludrox® (Wyeth Hellas AEBE). All three pharmaceutical formulations have registration in Greece.

Milk of Magnesia®

active substance:	Magnesium Hydroxide 425 mg in 5 ml
excipients:	Water
	Calcium Hypochloride
manufacturer:	GlaxoSmithKline
dosage form:	Suspension

Since this is higher density suspension, the representative volume (15.0 ml) was required for sampling. This volume, containing 531.3 mg of Mg^{2+} (according to label content), was added by pipette into 250 ml volumetric flask with mobile phase in addition acidified by 3.5 ml of TFA in order to neutralize concentrated solution of Magnesium Hydroxide. Volumetric flask was put into ultrasonic bath for five minutes and after filled to punch mark in with mobile phase setting in 2125.1 $\mu g\ ml^{-1}$ solution of Mg^{2+}. Still very concentrated solution had to been diluted by mobile phase again. Volume of 10.0 ml was pipetted into 100 ml volumetric flask and was prepared 212.5 $\mu g\ ml^{-1}$ solution of Mg^{2+} used for further particular runs dilutions. The volumes of 0.375 ml; 0.47 ml and 0.75 ml were taken by automatic pipette into 10 ml volumetric flasks, in order to gain run solutions of Mg^{2+} in concentrations 8.0 $\mu g\ ml^{-1}$; 10.0 $\mu g\ ml^{-1}$ and 15.9 $\mu g\ ml^{-1}$.

For every day, a new two-point calibration curve was constructed

Tums®

active substance:	Calcium Carbonate 600 mg in 1 tablet
	Magnesium Carbonate 125 mg in 1 tablet
excipients:	Sodium Bicarbonate
	Malic Acid
	Microtal DCE Sugar
	Saccharin Sodium
	Magnesium Stearate
	Orange Flavour
	Talc
manufacturer:	GlaxoSmithKline
dosage form:	chewing tablets

Pre-treatment of sample started with weighting of 10 tablets. From this quantity, mean tablet was found (1.4974 g), after that were tablets finely pulverized in ceramic bowl. Calcium and magnesium were quantified separately in different days, because of greatly different content in formulation. 0.9996 g (for calcium trial) and 1.0004 g (for magnesium trial) of powder was dissolved in 100 ml volumetric flask by mobile phase (TFA 10 mM l^{-1}) and additionally acidified by 0.80 ml of TFA according to calculations. This extra amount of acid was used in order to neutralize basic solution of carbonates. Generated stock solutions in concentration of 1603.9 $\mu g\ ml^{-1}$ Ca^{2+} and 240.8 $\mu g\ ml^{-1}$ Mg^{2+} (according to label content)

were sonicated for 15 min to release ions from insoluble excipients into solution. Turbidity of insoluble excipients was removed by centrifuging for 15 min with consequent membrane filtration (Millipore, 0.45 μm). Further dilutions of filtrated solutions were made taking 0.1 ml; 0.25 ml; 0.4 ml for calcium and 0.4 ml; 0.65 ml; 0.9 ml for magnesium into 10 ml volumetric flasks for individual runs. Concentrations of 16.0 μg ml^{-1}; 40.1 μg ml^{-1}; 64.2 μg ml^{-1} for calcium and 9.6 μg ml^{-1}; 15.7 μg ml^{-1}; 21.7 μg ml^{-1} for magnesium.

For every day, a new two-point calibration curve was constructed.

Aludrox®

active substance:	Aluminium Hydroxide 233.00mg in 1 tablet
	Magnesium Hydroxide 83.46mg in 1 tablet
excipients:	Confectioner's Sugar
	Starch Maze
	Talc
	Calcium Stearate
	Saccharin Sodium
	Hydrogenated Vegetable Oil
	Peppermint Oil
	Flavour compound
manufacturer:	Wyeth Hellas AEBE
dosage form:	chewing tablets

Ten tablets were weighed on analytical scales, mean tablet weight was found (0.9170 g) and after that were tablets finely pulverized in a ceramic bowl. Accurately 0.7036 g and 0.7005 g of the powder was added into 100 ml volumetric flasks for pre-treatment of two samples in different days. The tablet powder was dissolved in HPLC grade water with addition of 3.0 ml of hydrochloric acid 37%, in order to obtain entire quantity of ions from matrix. Gained solutions in concentration of 618.4 μg ml^{-1} respectively 615.7 μg ml^{-1} of Al^{3+} and 376.1 μg ml^{-1} respectively 374.5 μg ml^{-1} of Mg^{2+} were sonicated in ultrasonic bath for 15 min in order to discorporate the aggregates. The volumetric flasks were filled with HPLC grade water up to the punch mark. The suspension was filtered through a membrane filter (Millipore, 0.45 μm) in order to get rid of insoluble particles of excipients and to gain the clear stock solutions of ions. The filtrate volume of 0.5 ml for each sample was taken by automatic pipette into 10 ml volumetric flask and diluted by mobile phase, in order to gain

solutions of 30.9 μg ml^{-1} and 30.8 μg ml^{-1} for aluminium; 18.8 μg ml^{-1} and 18.7 μg ml^{-1} for magnesium.

For every day, a new two-point calibration curve was constructed.

4. RESULTS AND DISCUSSION

4.1. METHOD OPTIMALIZATION

4.1.1. Separation of Mg^{2+} and Ca^{2+}

Standard solutions of magnesium acetate and calcium lactate were analysed in order to determinate the retention time of magnesium and calcium and their corresponding resolution in various TFA mobile phases (Table 4.1). The best result was achieved with TFA 0.085 % (v/v) aqueous mobile phase, resulting sufficient difference in retention times (magnesium 7.0 min, calcium 7.9 min), good resolution (1.7) and symmetry (magnesium 0.9, calcium 0.9) was obtained. Lower concentration of TFA in mobile phase means extension of retention time, cut-down of peak area and downgrade of peak symmetry. Reversely higher concentration gives shorter time and much superior response with better symmetry, but unfortunately defies coincidental magnesium and calcium quantification.

Improvement of separation was examined using an eluting ion of higher molecular weight (NFPA). In this case, a double separation mechanism was able to be performed: (a) cation exchange between metal cations and protons and (b) reversed phase chromatography due to the adsorption of the lipophilic complexes of $[metal(NFPA)_n]^{2-n}$ to the polymeric stationary phase. Addition of NFPA increased retention time, but had undesirable influence on the peak symmetry (Table 4.1). Best results were obtained with TFA-NFPA mixed mobile phase, TFA 0.100 % (v/v) and NFPA 0.015 % (v/v), in which the NFPA was responsible for the adsorption procedure, while TFA provided the adequate mobile phase acidity for the cation exchange mechanism.

Table 4.1 Influence of mobile phase composition on retention time, resolution and asymmetry factor for magnesium and calcium

Mobile Phase Composition (% v/v)		Retention time (min)		Resolution	Asymmetry Factor	
		Magnesium	Calcium		Magnesium	Calcium
TFA	0.077	7.2	8.3	1.9	0.9	1.0
	0.085	7.0	7.9	1.7	0.9	0.9
	0.100	6.4	7.2	1.6	0.9	1.0
TFA NFPA	0.100 0.015	6.8	7.7	1.8	1.0	1.0

Mobile Phase Composition (% v/v)		Retention time (min)		Resolution	Asymmetry Factor	
		Magnesium	Calcium		Magnesium	Calcium
TFA	0.100	9.2	10.7	1.7	0.8	1.0
NFPA	0.031					

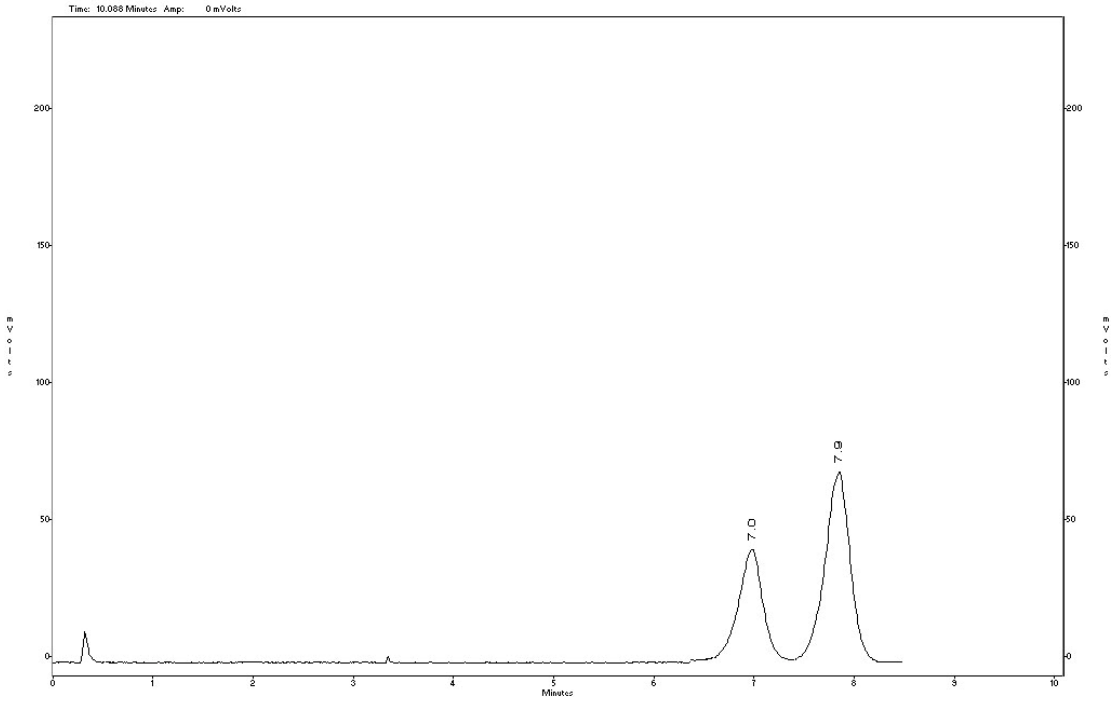

Figure 4.1 Typical chromatogram of magnesium (Rt=7.0 min); calcium (Rt=7.9 min) and noise peak in void volume using DIONEX® IONPAC® CS – 14 analytical column and aqueous mobile phase containing 0.085% (v/v) TFA.

4.1.2. Separation of Organic Anions (citric, ascorbic, aspartic)

Since occurrence of organic anions in pharmaceutical formulations is common, their separation was examined, by analysing standard stock solutions of ascorbic, aspartic and citric acid. Different concentrations of TFA, acetic acid and formic acid were examined, but separation of organic anions peaks was never adequate for ascorbate and aspartate. On the other hand, citrate was completely separated from ascorbate and aspartate. Organic anions are keeping similar retention time, practically independently on concentration of TFA, due to cation-exchange character of the column. Best separation was observed for mobile phase containing acetic acid 0.077 % (v/v).

Table 4.2 Influence of mobile phase composition on retention time for ascorbic, aspartic and citric acid

Mobile Phase Composition (% v/v)		Retention time (min)		
		Ascorbate	Aspartate	Citrate
TFA	0.062	2.5	2.9	3.2
	0.077	2.5	2.8	3.2
	0.087	2.6	2.8	3.4
	0.092	2.5	2.7	3.2
Formic Acid	0.077	2.6	2.9	3.5
Acetic Acid	0.077	2.1	2.4	3.3
	0.092	2.2	2.4	3.4

Table 4.3 Influence of mobile phase composition on resolution and asymmetry factor for ascorbic, aspartic and citric acid

Mobile Phase Composition (% v/v)		Resolution		Asymmetry Factor		
		Asc/Asp	Asp/Cit	Ascorbate	Aspartate	Citrate
TFA	0.062	1.3	1.1	0.9	0.9	1.3
	0.077	1.2	1.3	1.0	0.9	1.1
	0.087	1.1	1.8	0.8	1.3	1.2
	0.092	0.7	1.9	1.3	-	1.1
Formic Acid	0.077	1.1	1.7	0.9	1.2	1.1
Acetic Acid	0.077	1.2	3.6	1.0	1.4	1.0
	0.092	1.1	3.8	1.0	1.3	1.1

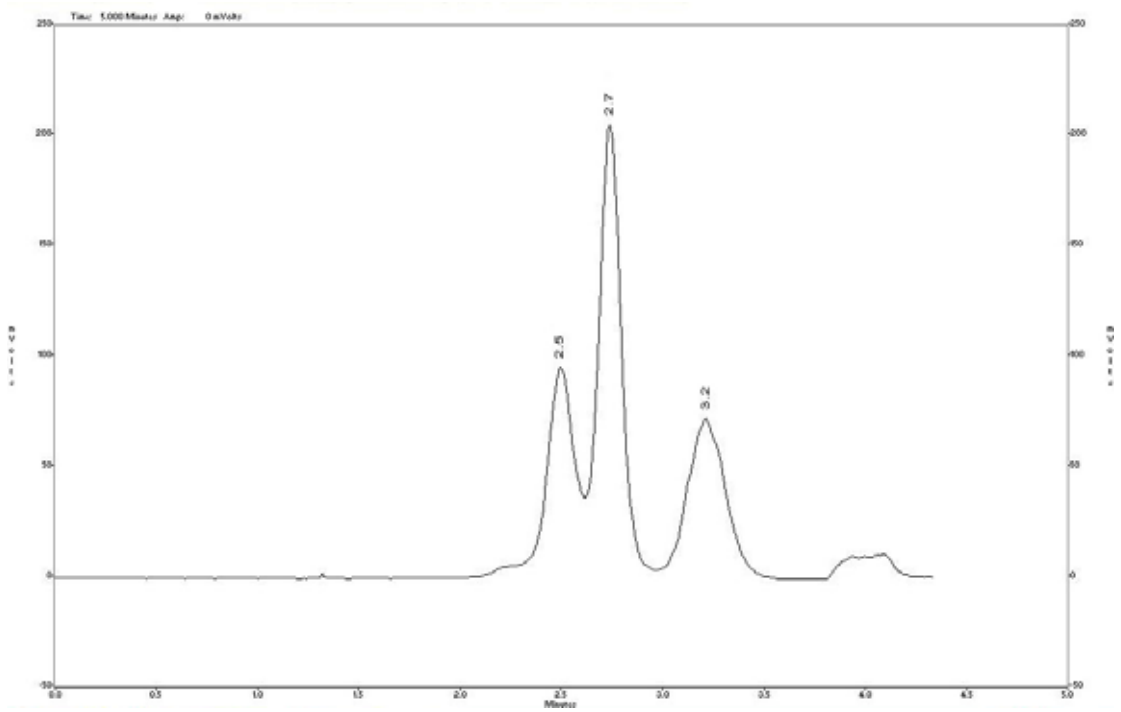

Figure 4.2 Typical chromatogram of ascorbic acid (Rt=2.5 min), aspartic acid (Rt=2.7 min) and citric acid (Rt=3.2 min) using DIONEX® IONPAC® CS – 14 analytical column and aqueous

mobile phase containing 0.092% v/v TFA. There is not analyzed noise peak after the peaks of organic acid.

4.1.3. Separation of Mg^{2+}; Ca^{2+} and Al^{3+} by Linear Gradient Elution

The standard solutions of Magnesium acetate, Calcium hydroxide and Aluminium nitrate, dissolved in TFA 0.085 % (v/v) mobile phase, were used for experiments. Mixed run dilution of standards was injected in conditions, which were searched out as ideal for magnesium and calcium separation, aqueous TFA around 0.085 % (v/v), to observe separation of aluminium, but it was not eluted from the column. It was needed a gradient elution using high concentration of TFA, in order to suppress retention of aluminium in the column. Examination of several linear gradients (Table 4.4 and Table 4.5) led to optimal one: from 0 min to 6th min aqueous TFA 0.096 % (v/v) mobile phase, between 6th min and 7th min was performed the linear gradient change to aqueous TFA 0.640 % (v/v). Resulting retention times were 6.2 min for magnesium, 7.1 min for calcium and 11.4 min for aluminium. Resolution was magnesium/calcium (1.8), calcium/aluminium (8.8) and asymmetry factors were magnesium (1.0), calcium (1.2) and aluminium (1.6). This linear gradient setting had sufficiently separated three metals in one run. Disadvantage was high retention time instability of magnesium and calcium, in the course of even slightly modification of TFA concentration. On the other hand retention time of aluminium was robust and method provided good detector response for all metals.

Table 4.4 Influence of mobile phase composition and different linear gradient settings on retention times for magnesium, calcium and aluminium

Time (min)	Mobile Phase Composition (% v/v)	Retention time (min)		
		Magnesium	Calcium	Aluminium
0-6 6-7	TFA 0.085 TFA 0.310	6.9	7.8	13.7
0-5.5 5.5-7	TFA 0.085 TFA 0.462	7.9	9.1	11.7
0-6.5 6.5-8	TFA 0.085 TFA 0.462	7.2	8.3	12.7
0-6 6-7	TFA 0.096 TFA 0.640	6.2	7.1	11.4

Table 4.5 Influence of mobile phase composition and different linear gradient settings on resolution and asymmetry factor for magnesium, calcium and aluminium

Time (min)	Mobile Phase Composition (% v/v)	Resolution		Asymmetry Factor		
		Mg^{2+}/Ca^{2+}	Ca^{2+}/Al^{3+}	Mg^{2+}	Ca^{2+}	Al^{3+}
0-6 6-7	TFA 0.085 TFA 0.310	1.7	5.4	0.9	1.2	1.4
0-5.5 5.5-7	TFA 0.085 TFA 0.462	1.9	3.6	1.0	1.1	1.5
0-6.5 6.5-8	TFA 0.085 TFA 0.462	1.9	6.2	1.0	1.1	1.3
0-6 6-7	TFA 0.096 TFA 0.640	1.8	8.8	1.0	1.2	1.6

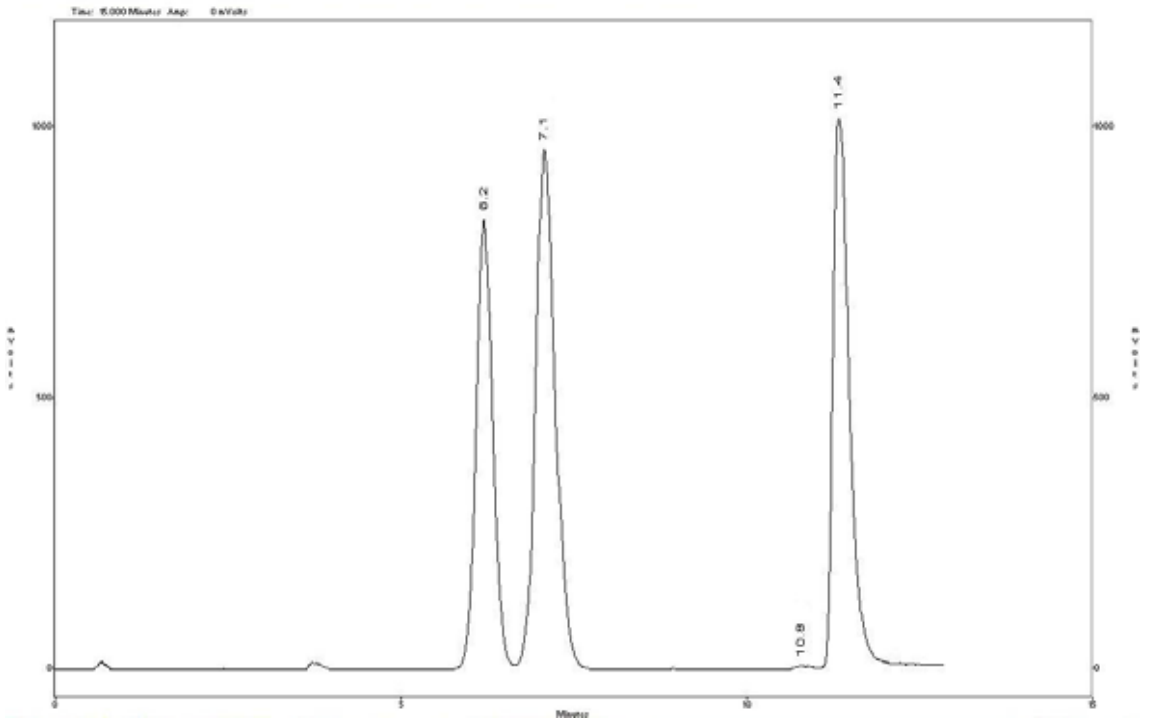

Figure 4.3 Typical chromatogram of magnesium(Rt=6.2 min), calcium (Rt=7.1 min) and aluminium (Rt=11.4 min) using DIONEX® IONPAC® CS – 14 analytical column and gradient elution program: from 0 min to 6 min 0.096% (v/v) TFA aqueous mobile phase and from 6 min to 7 min linear gradient to aqueous 0.640% (v/v) TFA aqueous mobile phase. Small peak of TFA is visible (Rt=10.8 min) due to the gradient procedure.

4.1.4. Inner Standard Method

Addition of calcium inner standard was used for Aludrox® formulation, since difficulties with quantification of magnesium appeared. It was used to prove that magnesium complexes with TFA are not adsorbing on lipophilic excipients of formulation, because

calcium forms similar complexes and should be adsorbed by the same mechanism. Recovery of calcium standard addition was 100%.

4.2. VALIDATION

4.2.1. Resolution and Asymmetry

Resolution (R) of two neighbouring peaks was studied on standard solutions, according to the Equation 4.1,

Equation 4.1 $$R = \frac{2\,(t_{R2} - t_{R1})}{w_1 + w_2}$$

where t_{R1} and t_{R2} are the retention time of the peaks and w_1 and w_2 the corresponding peak width at 10% of the peak height.

Asymmetry factor (A_s) determined according to the Equation 4.2 in 0.1 high (h) of the peak,

Equation 4.2 $$A_s = \frac{a}{b}$$

where a is the front part and b is the tail path of the peak width in 0.1 high, splitted by the middle line of the peak.

Results are presented on Table 4.6 and Table 4.7 There is a lower resolution, than optimal 2.0, between magnesium and calcium. However the time difference is still sufficient for simultaneous quantification of both metals. The standards for isocratic elution were measured in different days, that causing nuances in chromatographic conditions (especially composition of mobile phase). Asymmetry factors values are in the optimal range (0.9 – 1.2) except of aluminium (1.6). This is probably caused by the aging of chromatographic column.

Table 4.6 Resolution and asymmetry for magnesium and calcium; isocratic elution

	Magnesium	**Calcium**
Concentration (μg ml^{-1})	7.8	14.4

	Magnesium	Calcium
Mobile phase (% v/v)	TFA 0.085	TFA 0.085
Retention time (min)	7.0	7.9
Width 10%	0.52	0.53
Resolution	**1.7**	
Asymmetry	**0.9**	**0.9**

Table 4.7 Resolution and asymmetry factors for magnesium, calcium and aluminium; linear gradient elution

	Magnesium	Calcium	Aluminium
Concentration (μg ml^{-1})	9.9	14.4	14.9
Mobile phase (% v/v)	*	*	*
Retention time (min)	6.2	7.1	11.4
Width 10%	0.46	0.53	0.44
Resolution	**1.8**	**8.8**	
Asymmetry	**1.0**	**1.2**	**1.6**

* gradient elution program: from 0 min to 6 min 0.096% (v/v) TFA aqueous mobile phase and from 6 min to 7 min linear gradient to aqueous 0.640% (v/v) TFA aqueous mobile phase

4.2.2. Linearity and Range

Very good correlation was achieved with the well-established exponential relationship between peak area (A) and analyte mass (m) (Equation 4.3). The linearity of the method was calculated by using the linear least squares regression technique of the logarithm of peak area (A) versus the logarithm of analyte concentration (c) (Equation 4.4),

Equation 4.3 $$A = a \times m^b$$

Equation 4.4 $$\log A = b \log c + \log a$$

where a and b are coefficients depending on the ELSD instrumentation and on nebulizative and evaporative processes (flow rates of the nebulization gas, flow rate of mobile phase, composition of the mobile phase, evaporation temperature, etc.).

Standard solutions were measured in triplicate. The regression data are presented in Table 4.8. The calibration curve of magnesium using linear gradient elution is depicted in Figure 4.4.

Table 4.8 Regression data of magnesium, calcium and aluminium

	Concentration Range ($\mu g\ ml^{-1}$)	Intercept (±SD)	Slope (±SD)	Correlation Coefficient
Magnesium (isocratic elution)	8.0 - 24.0	4.922 (±0.035)	1.470 (±0.031)	**0.9993**
Magnesium (gradient elution)	6.0 - 29.8	4.855 (±0.032)	1.554 (±0.026)	**0.9995**
Calcium	14.4 - 71.9	4.291 (±0.053)	1.584 (±0.033)	**0.9993**
Aluminium	9.9 - 49.2	5.247 (±0.027)	1.148 (±0.019)	**0.9996**

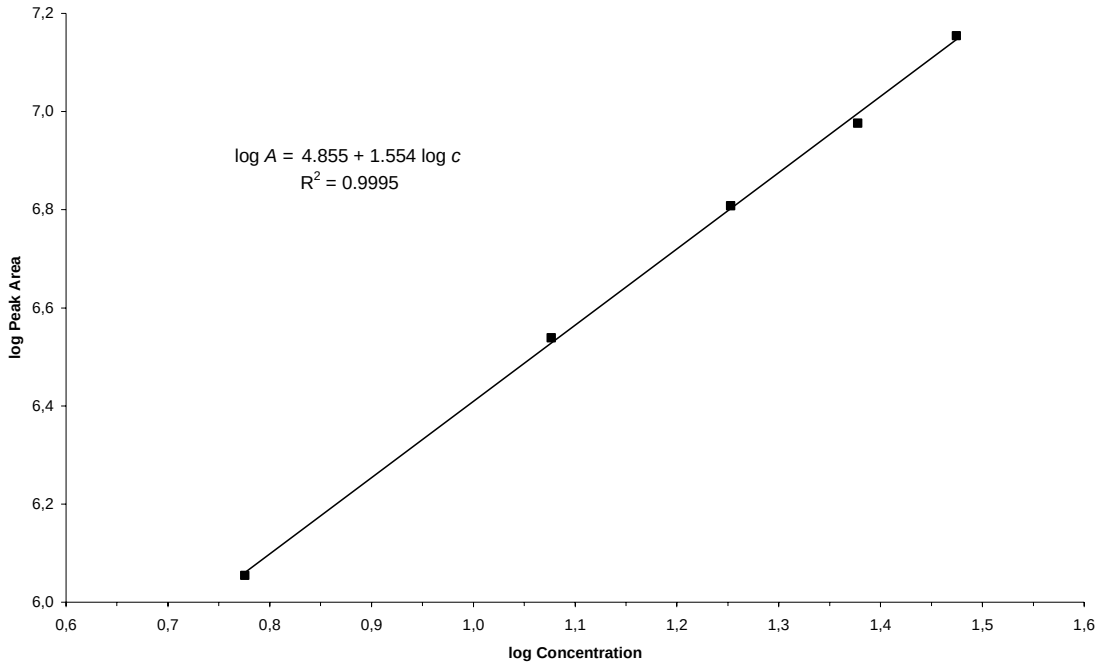

Figure 4.4 Calibration curve of magnesium; linear gradient elution

4.2.3. Precision

Repeatability of the method was evaluated by replicate measurements of standard solutions, n=3-5 (Table 4.9). Reproducibility of the method was evaluated by the estimation of % RSD of the slope of calibration curves obtained at three different days within a week, with 3 replicates per day (Table 4.10).

Table 4.9 Precision of HPLC-ELSD determination of magnesium, calcium and aluminium

	Concentration Level (μg ml^{-1})	% RSD	n
Magnesium	8.0	**2.01**	4
Calcium	14.4	**2.42**	5
Aluminium	9.9	**5.73**	3

Table 4.10 Slope % RSD of HPLC-ELSD calibration curves of magnesium, calcium and aluminium

	Day 1	Day 2	Day 3	% RSD
Magnesium (isocratic elution)	1.466	1.596	1.605	**4.99**
Magnesium (gradient elution)	1.554	1.646	1.544	**3.55**
Calcium	1.585	1.601	1.499	**3.50**
Aluminium	1.148	1.259	1.197	**4.65**

4.2.4. Detectability

Detection and quantification limits, with statement about number of replication, are presented in Table 4.11.

Table 4.11 Detectability of HPLC-ELSD determination of magnesium, calcium and aluminium

	LOD (μg ml^{-1})	LOQ (μg ml^{-1})	n
Magnesium (isocratic elution)	1.25	2.65	4
Magnesium (gradient elution)	0.58	1.18	3
Calcium	2.86	5.76	5
Aluminium	2.03	5.45	3

Owing to good properties of the detector, all of the assessed ions can be determined very sensitively with ELS detection.

4.3. RESULTS OF ANALYSES

4.3.1. Milk of Magnesia®

The formulation was analysed according to the new HPLC-ELSD method. The results obtained from the assay of magnesium revealed conformance to the European Pharmacopoeia [3]. The general chapter of pharmacopoeia is requiring the active substance content in formulation within the range of 95.0 – 105.0 % of the labelled content. Individual values are presented Table 4.12. Typical chromatogram of the analysis is depicted on Figure 4.7.

Table 4.12 Comparison of the HPLC-ELSD assay results to the label content; Milk of Magnesia®

Analyte Content in Formulation	Sample Run Concentration ($\mu g\ ml^{-1}$)	Content in Five ml (mg)	Percentage of Label Concentration (%)	n
Magnesium	8.0	169.2	**95.55**	3
(177.1 mg in 5 ml)	10.0	177.9	**100.48**	3
	15.9	179.8	**101.53**	3

The accuracy of the new HPLC-ELSD method was evaluated by recovery experiments. Samples were fortified by adding known amounts of magnesium standard. Five spiked samples were prepared. The good accuracy of the proposed method was confirmed since the individual recovery values are within the range of 95 – 105 % (except of one measurement, which is minutely outstanding) (Table 4.13).

Table 4.13 Spiked samples recovery; Milk of Magnesia®

	Formulation Concentration ($\mu g\ ml^{-1}$)	Standard Concentration ($\mu g\ ml^{-1}$)	Recovery (%)	Mean Recovery (%)	n
Magnesium	4.0	4.0	**98.68**		3
	10.0	4.0	**101.03**		3
	18.0	4.0	**100.25**	101.68	3
	4.0	10.0	**105.01**		3
	4.0	18.0	**103.41**		2

Further study of the matrix effect on the determination was carried out by dilution experiments (determination of magnesium content using a varying dilution factor D

($V_{initial}/V_{final}$) at three different levels). The correlation curve of the concentration found (in the diluted solution) versus D was linear ($r > 0.95$) with a slope equal to the content of the formulation and a statistically (proven by t-test) zero intercept. Similarly, the correlation curve of formulation content found versus D was very linear with statistically (proven by t-test) zero slopes. These results confirmed the absence of any constant or proportional determinate error due to matrix (excipients) effect (Table 4.14; Table 4.15 and Table 4.16).

Table 4.14 Dependence of run concentration and formulation concentration on dilution factor; Milk of Magnesia®

	1/Dilution Factor	Run Concentration ($\mu g\ ml^{-1}$)	Formulation Concentration ($\mu g\ ml^{-1}$)
Magnesium	0.00375	7.6	33842.5
	0.00470	10.0	35588.3
	0.00750	16.2	35961.0

Table 4.15 Dependence of run concentration on dilution factor; Milk of Magnesia®

	Parameter a	Std.Dev. a	t_a^*	Parameter b	Std.Dev. b	Correlation Coefficient
Magnesium	0.76	0.41	**1.862**	2264.33	73.75	**0.9994**

* For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95%.

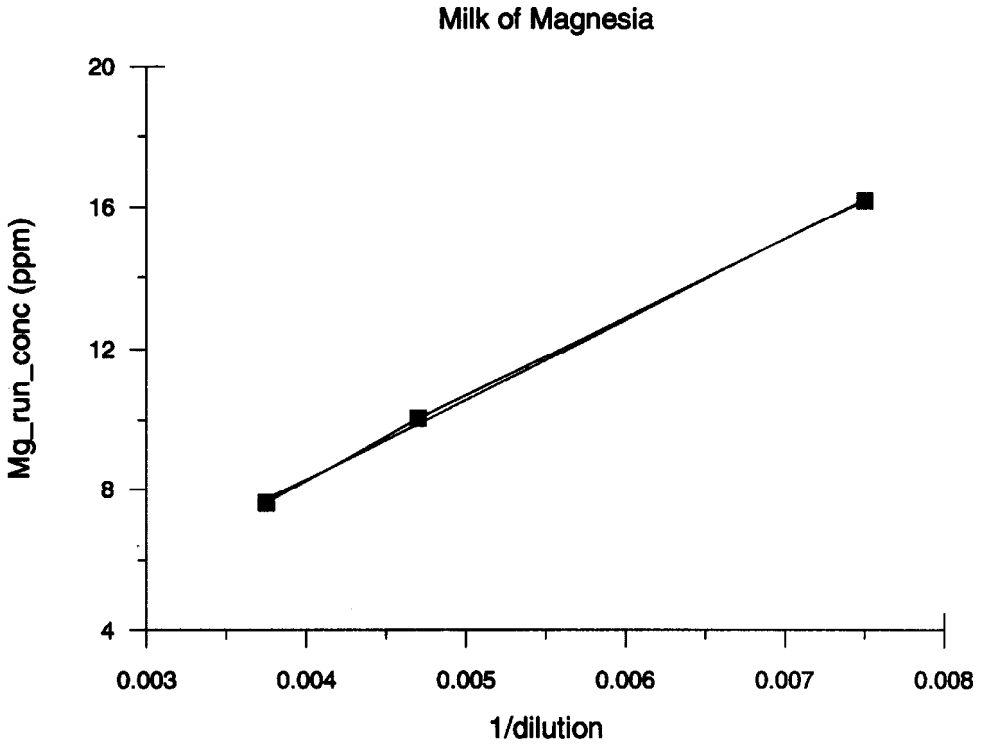

Figure 4.5 Dependence of run concentration on dilution factor; Milk of Magnesia®

Table 4.16 Dependence of formulation concentration on dilution factor; Milk of Magnesia®

	Parameter a	Std. Dev. a	**Parameter b**	**Std.Dev. b**	t_b^*
Magnesium	32648.53	1911.22	466846.96	344372.79	**1.356**

* For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95%.

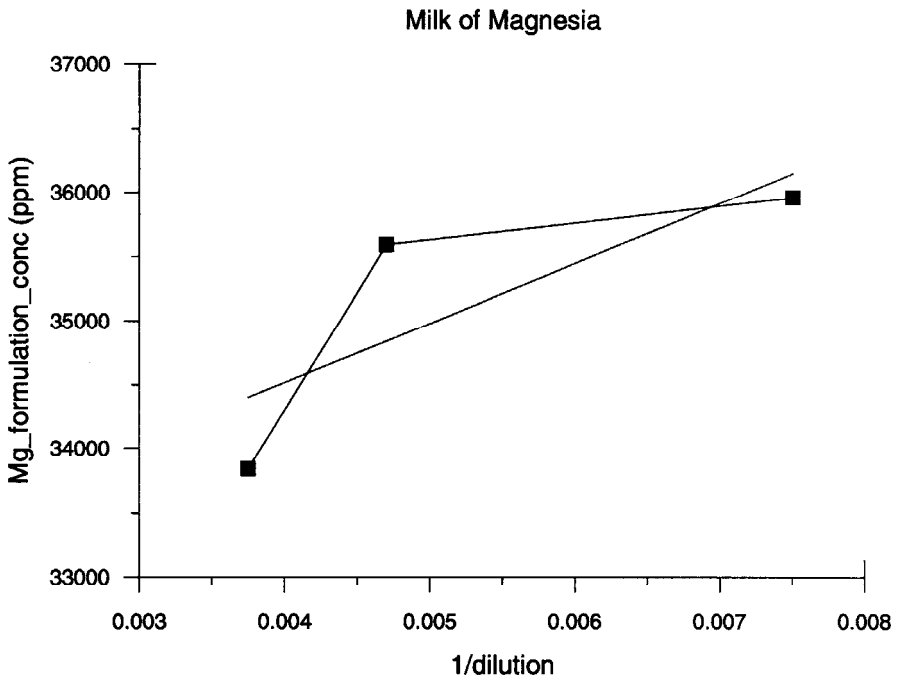

Figure 4.6 Dependence of formulation concentration on dilution factor; Milk of Magnesia®

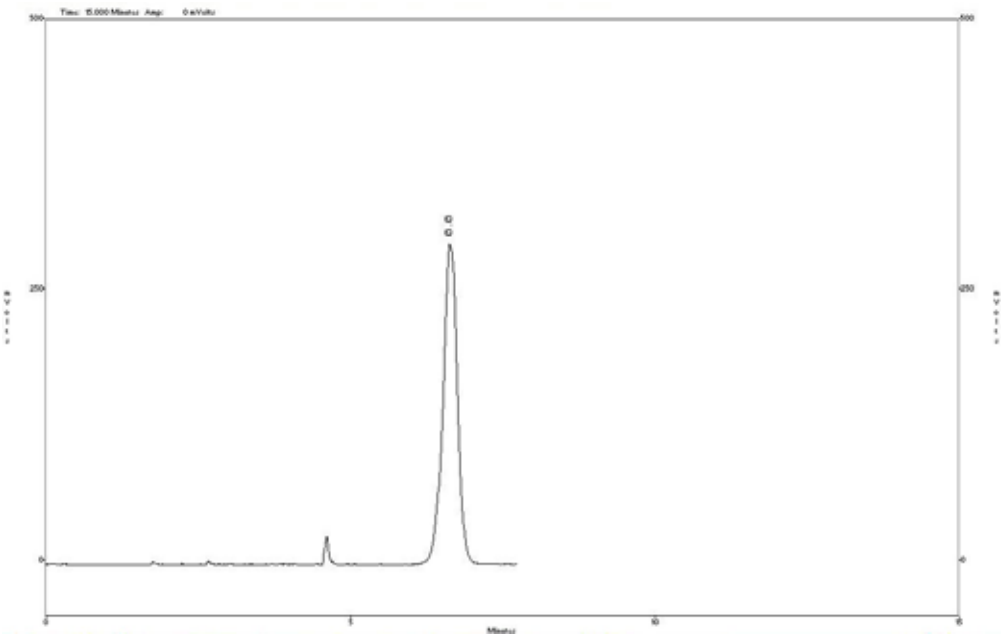

Figure 4.7 Typical chromatogram of Milk of Magnesia® trial using DIONEX® IONPAC® CS – 14 analytical column and isocratic elution 0.085% (v/v) TFA aqueous mobile phase. Magnesium (Rt=6.6 min) and not analyzed noise peak ahead of magnesium peak.

4.3.2. Tums ®

The formulation was analysed according to the new HPLC-ELSD method. The results obtained from the assay of calcium and magnesium revealed conformance to the European Pharmacopoeia [3]. The general chapter of pharmacopoeia is requiring the active substance content in one tablet within the range of 95.0 – 105.0 % of the labelled content, for tables its weight is greater than 250 mg. Individual values are presented in Table 4.17. A typical chromatogram of the analysis is depicted on Figure 4.10.

Table 4.17 Comparison of the HPLC-ELSD assay results to the label content; Tums®

Analyte Content in Formulation	Sample Run Concentration ($\mu g\ ml^{-1}$)	Content in One tablet (mg)	Percentage of Label Concentration (%)	n
Calcium	16.0	240.7	**100.16**	3
(240.3 mg in 1 tablet)	40.1	251.9	**104.83**	3
	64.2	242.3	**100.86**	2
Magnesium	9.6	36.3	**100.67**	4
(36.0 mg in 1 tablet)	15.7	37.2	**103.22**	2
	21.7	36.1	**100.23**	2

The accuracy of the new HPLC-ELSD method was evaluated by recovery experiments. Samples were fortified by adding known standard amounts of calcium and magnesium. For each component, five spiked samples were prepared. An excellent accuracy of the proposed method was confirmed since the individual recovery values are within the range of 97 – 103 % (Table 4.18).

Table 4.18 Spiked samples recovery; Tums®

	Formulation Concentration ($\mu g\ ml^{-1}$)	Standard Concentration ($\mu g\ ml^{-1}$)	Recovery (%)	Mean Recovery (%)	n
Calcium	16.0	14.4	**102.67**		3
	16.0	28.8	**101.89**		2
	16.0	43.2	**99.43**	**101.25**	2
	32.1	14.4	**100.38**		3
	48.1	14.4	**101.86**		2
Magnesium	4.0	4.0	**102.28**		2
	10.0	4.0	**101.94**		2
	18.0	4.0	**97.63**	**100.31**	2
	4.0	10.0	**98.09**		2
	4.0	18.0	**101.59**		2

Further study of the matrix effect on the determination was carried out by dilution experiments (determination of calcium and magnesium content using a varying dilution factor D ($V_{initial}/V_{final}$) at three different levels). The correlation curve of the concentration found (in the diluted solution) versus D was linear ($r > 0.95$) with a slope equal to the content of the formulation and a statistically (proven by t-test) zero intercept. Similarly, the correlation curve of formulation content found versus D was very linear with statistically (proven by t-test) zero slopes. These results confirmed the absence of any constant or proportional determinate error due to matrix (excipients) effect (Table 4.19; Table 4.20 and Table 4.21).

Table 4.19 Dependence of run concentration and formulation concentration on dilution factor; Tums®

	1/Dilution Factor	**Run Concentration ($\mu g\ ml^{-1}$)**	**Formulation Concentration ($\mu g\ ml^{-1}$)**
Calcium	0.010	16.1	160713.0
	0.025	42.0	168208.7
	0.040	64.7	161836.6
Magnesium	0.040	9.7	24229.0
	0.065	16.2	24842.1
	0.090	21.7	24123.6

Table 4.20 Dependence of run concentration on dilution factor; Tums®

	Parameter a	**Std. Dev. a**	**t_a^***	Parameter b	Std. Dev. b	**Corelation Coefficient**
Calcium	0.40	1.77	**0.2227**	1621.67	63.70	**0.9992**
Magnesium	0.23	0.69	**0.3332**	240.40	10.16	**0.9991**

* For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95%.

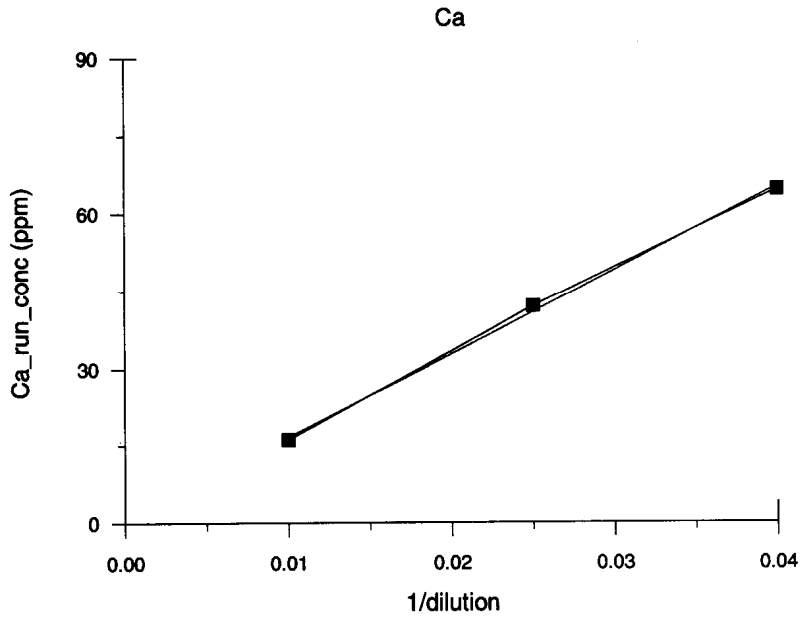

Figure 4.8 Dependence of run concentration on dilution factor; Tums®

Table 4.21 Dependence of formulation concentration on dilution factor; Tums®

	Parameter a	Std. Dev. a	**Parameter b**	**Std. Dev. B**	t_b^*
Calcium	162649.67	7429.90	37466.67	266889.78	**0.1404**
Magnesium	24534.83	1047.09	2100.00	15369.06	**0.1366**

* For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95%.

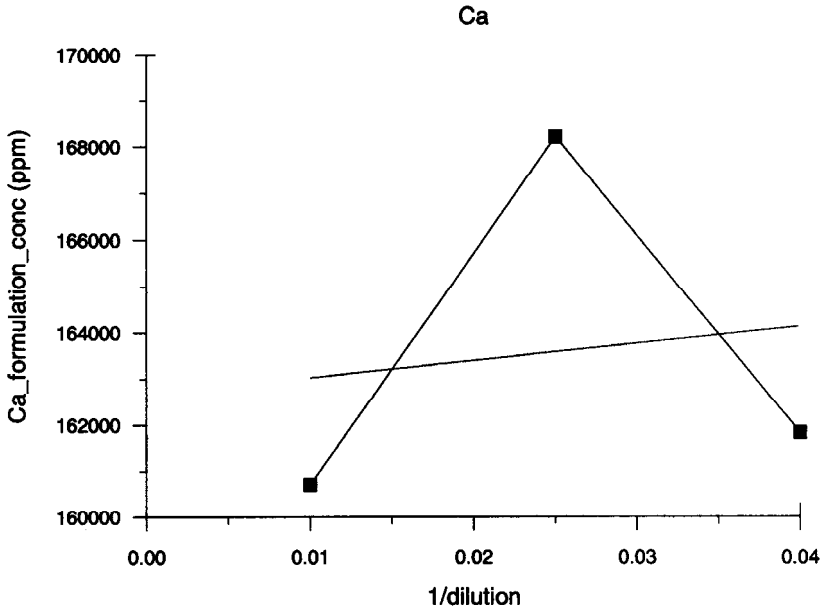

Figure 4.9 Dependence of formulation concentration on dilution factor; Tums®

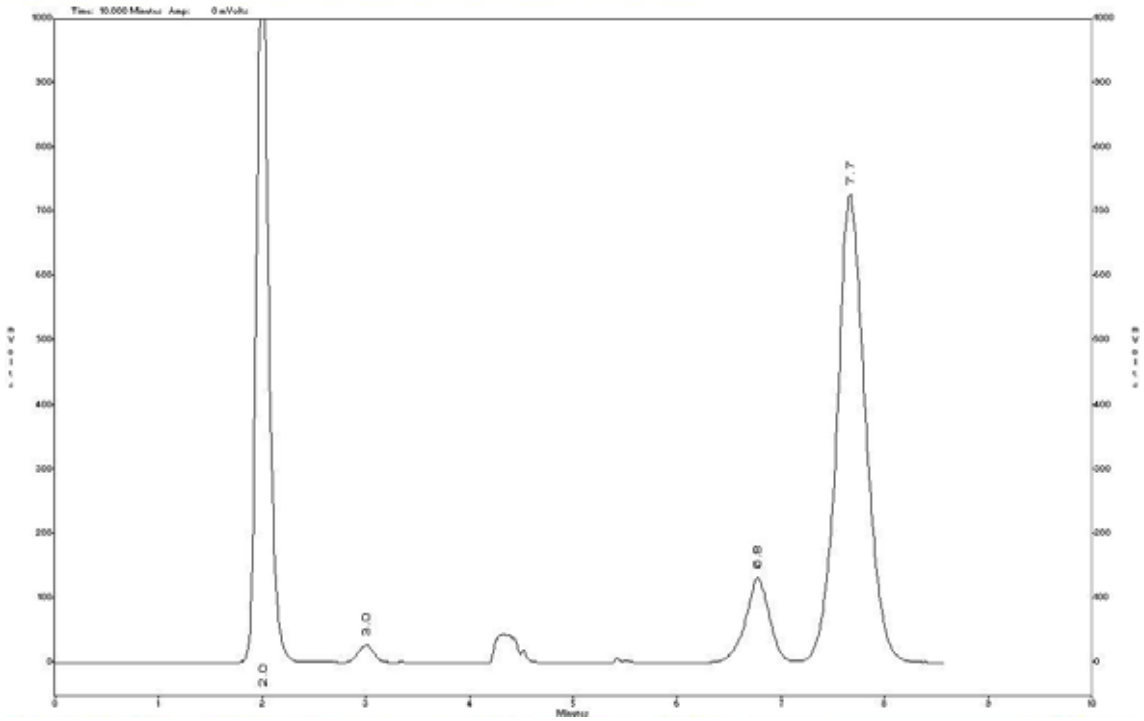

Figure 4.10 Typical chromatogram of Tums® trial using DIONEX® IONPAC® CS – 14 analytical column and isocratic elution 0.085% (v/v) TFA aqueous mobile phase. Magnesium (Rt=6.8 min); calcium (Rt=7.7 min); peaks of excipients (Rt=2.0 min and Rt=3.0 min) and not analyzed noise peak between peaks of second excipient and magnesium.

4.3.3. Aludrox®

The formulation was analysed according to the new HPLC-ELSD method. The results obtained from the assay of aluminium revealed conformance to the European Pharmacopoeia [3]. The general chapter of pharmacopoeia is requiring the active substance content in one tablet within the range of 95.0 – 105.0 % of the labelled content, for tables its weight is greater than 250 mg.

Detected quantity of magnesium was lower than was declared. Despite of maximal effort to find the reason for low gain of magnesium from the formulation, it was not successful. The supposition that magnesium complexes with TFA may be retented in lipophilic excipients and removed by centrifuging was not confirmed. Calcium inner standard technique was used, but calcium standard recovery from sample was 100%. The most probable explication is the lower magnesium content or magnesium occurrence in different form, than is declared on the formulation label. It's not possible to discover the truth reason without the detailed date about formulation composition from the producer or confrontation

with another analytical method. Individual values are presented in Table 4.22. A typical chromatogram of the analysis is depicted on Figure 4.11.

Table 4.22 Comparison of the HPLC-ELSD assay results to the label content; Aludrox®

Analyte Content in Formulation	Sample Run Concentration ($\mu g\ ml^{-1}$)	Content in One Tablet (mg)	Percentage of Label Concentration (%)	n
Aluminium	29.7	77.4	**96.03**	1
(80.6 mg in 1 tablet)	30.3	79.4	**98.54**	1
Magnesium	11.9	31.1	**63.50**	1
(49.0 mg in 1 tablet)	12.3	32.3	**65.93**	1

The accuracy of the new HPLC-ELSD method was evaluated by recovery experiments. Samples were fortified by adding known standard amounts of aluminium and magnesium. For each component, four spiked samples were prepared. A good accuracy of the proposed method was confirmed since the individual recovery values for aluminium are within the range of 95 – 105 % (except of one measurement) Recovery values of magnesium were out of the range (Table 4.18).

Table 4.23 Spiked samples recovery; Aludrox®

	Formulation Concentration ($\mu g\ ml^{-1}$)	Standard Concentration ($\mu g\ ml^{-1}$)	Recovery (%)	Mean Recovery (%)	n
Aluminium	32.0	4.9	**101.02**		1
	18.5	7.5	**98.82**	**98.38**	1
	12.3	7.5	**94.69**		1
	12.3	15.0	**98.99**		1
Magnesium	19.5	3.2	**71.48**		1
	11.2	5.0	**72.88**	**71.08**	1
	7.5	5.0	**69.03**		1
	7.5	5.0	**70.92**		1

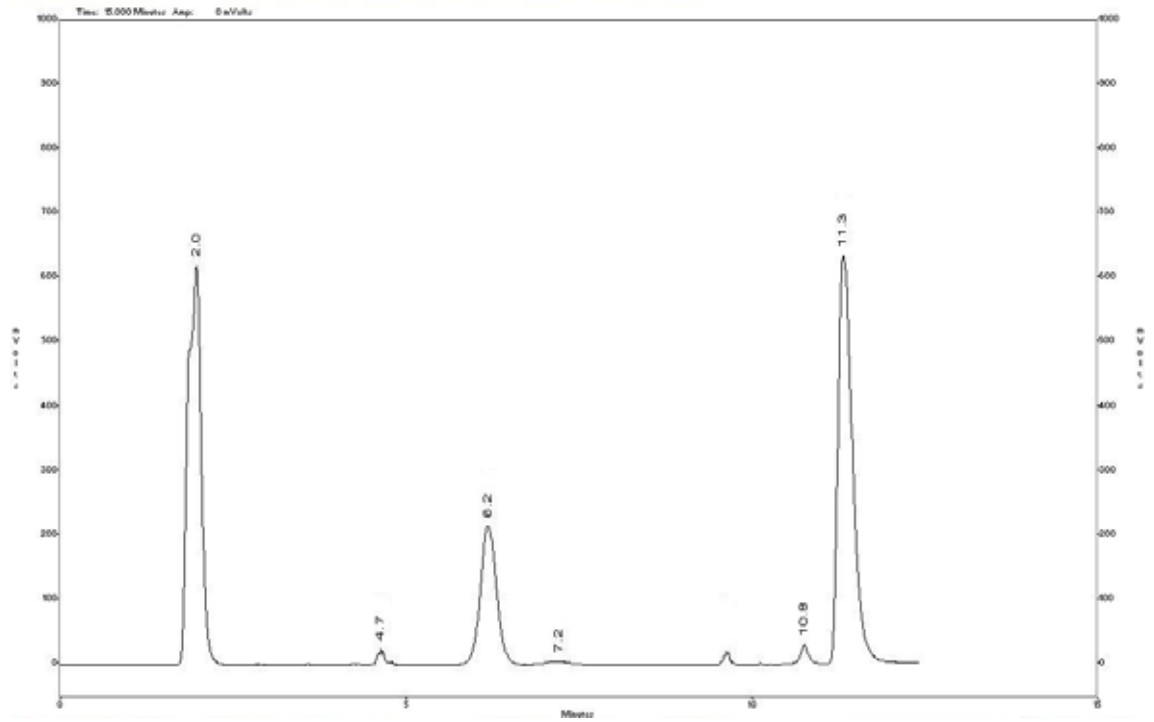

Figure 4.11 Typical chromatogram of Aludrox® trial using DIONEX® IONPAC® CS – 14 analytical column and gradient elution program: from 0 min to 6 min 0.096% (v/v) TFA aqueous mobile phase and from 6 min to 7 min linear gradient to aqueous 0.640% (v/v) TFA aqueous mobile phase. Magnesium (Rt=6.2 min); aluminium (Rt=11.3 min); peaks of excipients (Rt=2.0 min; Rt=7.2 min and Rt=4.7 min); peak of TFA (Rt=10.8 min) and not analyzed noise around 9. min.

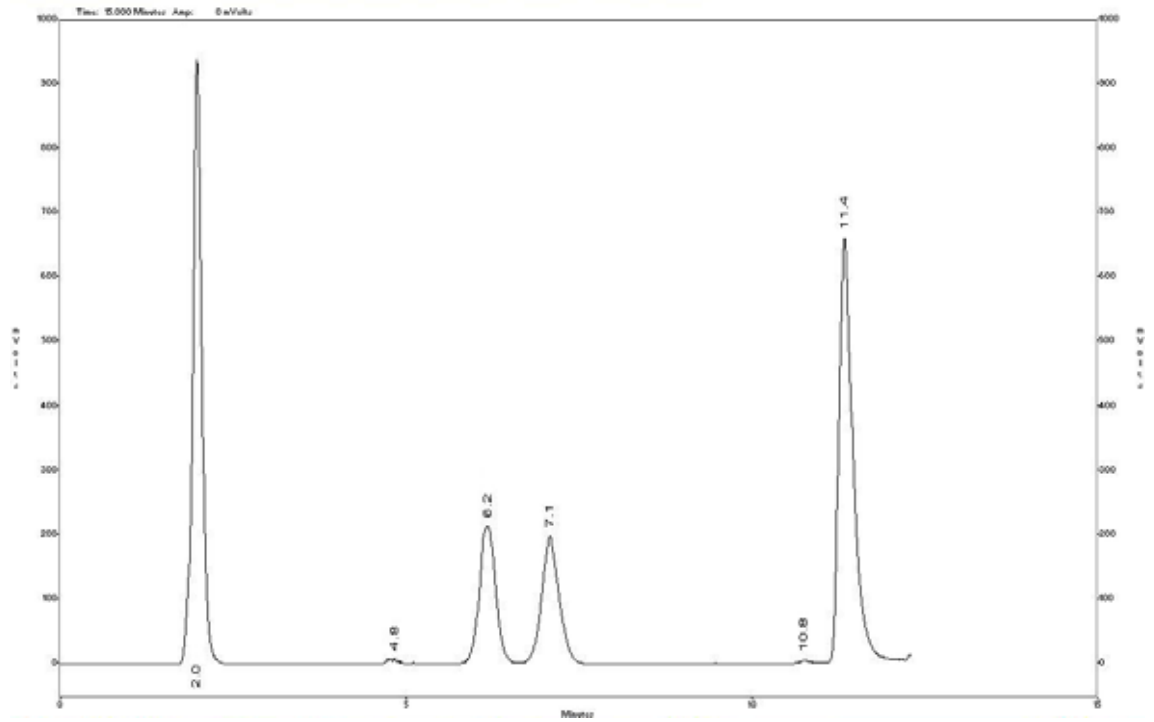

Figure 4.12 Typical chromatogram of Aludrox® with addition of calcium inner standard using DIONEX® IONPAC® CS – 14 analytical column and gradient elution program: from 0 min to 6

min 0.096% (v/v) TFA aqueous mobile phase and from 6 min to 7 min linear gradient to aqueous 0.640% (v/v) TFA aqueous mobile phase. Magnesium (Rt=6.2 min); calcium (Rt=7.1 min); aluminium (Rt=11.4 min); peaks of excipients (Rt=2.0 min and Rt=4.8 min); peak of TFA (Rt=10.8 min).

5. CONCLUSIONS

According to pharmacopoeia two or more individual methods are required for simultaneous determination of metal cations. Cation exchange HPLC-ELSD isocratic and linear gradient methods developed and validated in this paper shows, that simultaneous determination of magnesium and calcium (isocratic method) or magnesium, calcium and aluminium (linear gradient) can be reliably achieved. Also standards of organic acids were examined in order to observe separation abilities of cation exchange analytical column in this application, because metal cations in form of organic salts or inorganic salt with certain organic component are commonly contained in pharmaceutical formulations. Nevertheless no method simultaneously determining both inorganic cation and organic anion had been published. This paper reveals strong advantage of this method-potential efficiency to determine metal cations and organic anions in one injection. However this utilization of HPLC-ELSD methods must be further investigated and validated before its application on pharmaceutical formulations. HPLC-ELSD methods are able to determine several analytes in one step, simply, rapidly and for low cost. They have very easy sample pretreatment, requiring no derivatization of analyte as it is common for spectrometric methods. Direct determination saves the time and lowers the expenses for analyses. HPLC-ELSD method validation data are fully acceptable for active substance analysis of pharmaceutical formulations.

6. SOUHRN

Magnesium a kalcium hrají esenciální roli v existenci živých organismů. Magnesium, se účastní kolem 300 základních enzymatických reakcí, je důležité pro energetický metabolismus, zastává klíčovou roli v neurotransmisi, při imunitních funkcích a regulaci neuromuskulární aktivity srdce. Kalcium kromě strukturální, elektrofyziologické a intracelulárně regulační funkce je také kofaktorem extracelulárních enzymů a regulačních proteinů. Rovnováha těchto minerálů v těle naplňuje důležitou podmínku potřebnou k udržení zdraví. Jejich nedostatek nebo naopak nadbytek v organismu je spojen s řadou vážných syndromů a onemocnění. Aluminium neplní žádnou fyziologickou funkci, důležité je z hlediska jeho toxikologie. Magnesium, kalcium a aluminium jsou využívány samostatně nebo v kombinaci ve formě různých solí, oxidů, hydroxidů nebo komplexů při výrobě mnoha farmaceutických přípravků nebo potravinových doplňků.

Český lékopis 2005 používá ke stanovení magnesia, kalcia a aluminia chelatometrickou titraci edetanem disodným (přímou nebo zpětnou). Další metody užívané k detekci magnesia, kalcia nebo aluminia jsou například ASS, ET-AAS, SIA s UV-VIS detekcí, multikomponentní FIA s detekcí diodovým polem, FIA za iontově selektivní elektrodové detekce, iontově selektivní elektrody, iontová chromatografie s piezoelektrickou detekcí, ICP-AES nebo MS, kapilární isotachoforéza a jiné elektrogravimetrické, coulometrické, polarografické, voltmetrické nebo thermogravimetrické metody. Používané metody bývají často spojeny s řadou nevýhod. Vzorky kovových kationů je například nutné před stanovením náročně a zdlouhavě upravovat nebo metoda vyžaduje drahé vybavení, proto bylo jedním z cílů této práce vyvinout a validovat jednoduchou a rychlou přímou metodu bez vysokých nároků na vybavení.

Metoda je založena na spojení iontově výměnné HPLC s ELSD (Evaporative Light Scattering Detector). ELS detektor je quasi-univerzální detektor, který v současné době nachází stále širší uplatnění, a to především u analytů, které nemají ve své molekule chromoforové skupiny a nemohou být tudíž bez derivatizace detekovány spektrofotometricky. Oproti ostatním univerzálním detektorům, jako např. refraktometrický (RID) nebo MS, vykazuje určité přednosti: a) kompatibilita s gradientovou elucí (na rozdíl od

RID); b) podstatně lepší detektabilitu ve srovnání s RID (běžný limit detekce se pohybuje v nanogramových množstvích, v závislosti na těkavosti a molekulové hmotnosti); c) nízké náklady a snadná obsluha (na rozdíl od hmotnostního spektrometru). Nicméně ELSD vykazuje také určité nevýhody. Tou hlavní je požadavek na těkavost mobilní fáze. Nesmí být použity netěkavé reagenty, pufry ani jiné složky mobilní fáze. Výběr vhodných kyselin a bazí se tím značně omezuje; mezi často používané patří kyselina octová, mravenčí, triflouroctová, pentaflouropropionová a heptaflouromáselná a jejich amonné soli v nízkých koncentracích (<0,1 M). ELSD je destruktivní detektor, proto musí být poslední v řadě, pokud je použit v sérii s jinými detektory. Vykazuje také nedostatečnou detektabilitu pro analýzu např. nečistot a reziduí, které se vyskytují v množstvích ng ml^{-1} (LOQ je obvykle vyšší než 0,1 $\mu g\ ml^{-1}$). Tyto vzorky je obvykle třeba upravit prekoncentrací, což bývá obtížné, neboť není možné použít netěkavé reagencie. Základní princip detektoru sestává ze tří následných kroků: a) nebulizace chomatografického eluentu; b) vypaření mobilní fáze a c) detekce netěkavých částic na základě rozptylu světla.

Pro separaci byla použita kationtově výměnná analytická kolona IONPAC® CS – 14 od firmy DIONEX® s karboxylovými funkčními skupinami.

Dobré rozlišení píků hořčíku a vápníku bylo kritickou podmínkou při hledání optimálního složení mobilní fáze. Nejlépe se osvědčila vodná mobilní fáze s TFA v koncentraci 0,085 % (v/v). Vyzkoušena byla také kombinace TFA/NFPA, ale rozdíly v retenčních časech a rozlišení byly minimální (Table 4.1). Předmětem výzkumu byly i organické aniony, proto proběhlo testování separační schopnosti metody v případě organických kyselin (Table 4.2, Table 4.3). Používaná analytická kolona je primárně určena k analýze jednomocných a dvojmocných kationů, přesto se podařilo užitím lineárního gradientu stanovit hliník. Program lineárního gradientu začínal s koncentrací 0,096% (v/v) TFA ve vodné mobilní fázi a mezi 6. – 7. minutou od nástřiku se koncentrace plynule změnila na 0,640% (v/v) TFA. V jednom měření je takto možné stanovit hořčík, vápník i hliník (Table 4.4, Table 4.5).

Vyvinuté metody se podařilo úspěšně validovat pro užití v analýze léčivých přípravků. Přípravky obsahující hořčík a/nebo vápník (Milk of Magnesia®, Tums®) byly analyzovány metodou isokratickou, k analýze léčivých přípravku obsahujícího hliník

(Aludrox®) byla použita lineárně gradientová metoda. Pro obě metody byly určeny validační parametry rozlišení a asymetrie (Table 4.6, Table 4.7), linearita a rozsah (Table 4.8), přesnost z hlediska opakovatelnosti (Table 4.9) a reprodukovatelnosti (Table 4.10), limity detekce a kvantifikace (Table 4.11).

Validované metody byly použity k analýze tří léčivých přípravků registrovaných v Řecku. Jednalo se o suspenzi Milk of Magnesia®, účinná látka: hydroxid hořečnatý 425 mg v 5 ml suspenze; žvýkací tablety Tums®, účinné látky: uhličitan vápenatý 600 mg a uhličitan hořečnatý 125 mg v 1 tabletě; žvýkací tablety Aludrox®, účinné látky: hydroxid hlinitý 233 mg a hydroxid horečnatý 83 mg v 1 tabletě (viz 3.3.4 Pharmaceutical Formulations). Při analýze se porovnávaly naměřené koncentrace iontů v přípravku s údajem od výrobce. Podle požadavků řeckých norem pro registrované léčivé přípravky musí být údaje ve shodě v rozmezí 95 - 105% obsahu. Tři vzorky z každého léčivých přípravků ve třech různých koncentracích byly změřeny většinou ve třech replikacích. Obsahovému kritériu odpovídaly všechny kovové kationy (Table 4.12 a Table 4.17) s výjimkou hořčíku u přípravku Aludrox® (Table 4.22). Důvod nižšího naměřeného obsahu v léčivém přípravku se nepodařilo zjistit. Poté co byla vyloučena adsorpce komplexů hořečnatých kationů s TFA na lipofilní excipienty léčivého přípravku změnou úpravy vzorku a použitím vnitřního standardu vápníku, je možné uvažovat o nepřesnosti v udání složení ze strany výrobce. Případně by bylo nutné pokusit se stanovit hořečnaté kationy jinou metodou a porovnat zjištěné výsledky. Dalším předmětem analýzy bylo měření pěti vzorků s přídavkem standardu příslušného kationu. Stanovení proběhlo většinou ve třech replikacích a opět vyhovovaly všechny vzorky kromě hořčíku u přípravku Aludrox® (Table 4.13, Table 4.18 a Table 4.23). Z naměřených údajů (Table 4.14, Table 4.19) byla pomocí t-testu hodnocena významnost odchylky u parametru a od nulové hodnoty pro závislost koncentrace vzorku na obrácené hodnotě jeho zředění (Table 4.15, Table 4.20) a významnost odchylky u parametru b od nulové hodnoty pro závislost koncentrace formulace na obrácené hodnotě jeho zředění (Table 4.16, Table 4.21). Všechny hodnoty byly menší než 4,303; což je maximální hodnota předepsaná pro tři měření zajišťující konfidenční interval 95%.

Přestože hořčík a vápník se v léčivých přípravcích často vyskytují ve formě organických solí nebo přípravek s anorganicky vázaným kovem obsahuje navíc organický anion, nebyla dosud publikovaná žádná metoda stanovující anorganický kation i

organický anion současně. Každý z iontů bylo potřeba kvantifikovat odděleně. Tato práce poskytuje údaje pro další výzkum v oblasti, tak aby bylo možno s nenáročným vybavením stanovit v jednom nástřiku kationický i anionický komponent, metodu validovat a v další fázi ji aplikovat na farmaceutické přípravky. Prezentovaná metoda dokazuje, že hořčík, vápník a hliník lze, volbou správných podmínek, spolehlivě separovat a kvantifikovat využitím iontově výměnné chromatografie, za současného výskytu anorganického anionu. Měření standardů organických kyselin (aspartát, askorbát, citrát) teoreticky dokazuje, že by ani výskyt organického anionu v přípravku neměl bránit současnému stanovení kationické i anionické složky účinné látky léčivého přípravku. Hlavní výhodou metody je rychlost, snadná úprava vzorků, značná přesnost, dostatečná citlivost a nízké náklady na přístrojové vybavení.

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