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DIPLOMA THESIS

DIPLOMOVÁ PRÁCE

Characterization of barbiturates and benzodiazepines-human serum protein interactions by capillary electrophoresis

Charakterizace interakcí barbiturátů a benzodiazepinů s lidskými sérovými proteiny pomocí kapilární elektroforézy

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Hereby I affirm in lieu of an oath, that I made the present thesis autonomously and without other than the indicated auxiliary means. The data used indirectly or from other sources, and concepts are characterized with lists of sources.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

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ACKNOWLEDGEMENT

ABREVIATIONS

ACE	affinity capillary electrophoresis
AED	antiepileptic drugs
AGP	alpha-1-acid glycoprotein
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BMC	biopartitioning micellar chromatography
CE	capillary electrophoresis
CNS	central nervous system
CZE	capillary zonal electrophoresis
D	ligand such as drug
DAD	diode array detection
EOF	electroosmotic flow
FA	frontal analysis capillary electrophoresis
FACCE	frontal analysis electrophoresis capillary continual
GABA	gamma-amino butyric acid
HPLC	high-performance liquid chromatography
HSA	human serum albumin
HD	Hummel-Dreyer capillary electrophoresis
ID	internal diameter
P	receptor such as protein
UV	ultraviolet
VACE	vacancy affinity capillary electrophoresis
VP	vacancy peak capillary electrophoresis
WHO	World health organization

CONTENTS

1. INTRODUCTION

Drug action in living organisms is the result of a large number of pharmacological processes. In this sense, the interactions between drugs and biomembranes, plasma proteins, enzymes or receptors are decisive features of the final biological activity of drugs [1].

Plasma protein binding has a significant effect on the pharmacokinetic and pharmacodynamic properties of drugs. Pharmacokinetic studies are related to the drug absorption, distribution, metabolism and excretion, and plasma drug concentrations over time; pharmacodynamic studies are concerned with the relationships between drug effect and drug concentration at the site of action. At pharmacodynamic level, drug–plasmatic protein complexes serve as drug reservoirs to supply free drug, as the net drug concentration is diminished in the body by various elimination processes. So, drug–plasmatic protein binding can prolong the duration of drug action [2]. Thus, an important topic in the drug discovery and development of drugs is the role of drug binding to serum proteins [1].

THE AIM

The aim of the diploma thesis is to study the applicability of CE-FA procedure for the characterization of the interaction between barbiturates and benzodiazepines and HSA and AGP under physiological conditions.

Furthermore, to evaluate the binding of these drugs to all plasmatic proteins using ultrafiltration and capillary electrophoresis.

Finally, to outline the effect of some physicochemical properties of the compounds studied (acid–base ionization constants, octanol–water partition coefficients and retention data in biopartitioning micellar chromatography) and some structural properties (molar volume, molecular weight) on the affinity of drugs towards plasmatic proteins. The results obtained to compare and to discuss.

2. THEORETICAL PART

2.1 PLASMATIC PROTEINS

Human plasma contains over 60 proteins, human serum albumin, α_1-acid glycoprotein, lipoproteins and globulins being the most important drug binding proteins [3].

2.1.1 Human serum albumin

HSA is the most abundant plasma protein which accounts for approximately 60% of the total protein corresponding to a concentration of around 0.6mM [4]. HSA contains 585 amino acids and has a molecular mass of 66 500 Da [1].

2.1.2 Alpha-1-acid glycoprotein

Concentration of AGP can vary considerably in several physiological and pathological conditions; in healthy subjects it ranges from 10 to 30μM. Since AGP is an acute phase protein, several inflammatory states (infections, rheumatic disorders, and surgical injury) and pathological conditions (e.g. myocardial infarction, malignancies, and nephritis) elevate its serum concentration up to three- or four-fold [3].

2.1.3 Drug binding sites

While drug binding to plasma was first considered to represent a rather unspecific physicochemical phenomenon, similar to the adsorption of small molecules to charcoal or similar compounds, accumulated evidence indicates that drug binding to albumin and to AGP at low molar drug/protein ratios occurs at only a very few ligand binding sites of both proteins, respectively [5]. Therefore, a knowledge of the properties of these drug binding sites has become an important issue for understanding pharmacokinetically relevant binding phenomena such as displacement reactions between different drugs and the dramatically altered plasma protein binding of some drugs during several disease states as well [6].

2.1.3.1 Drug binding sites of human serum albumin

According to an X-ray crystallographic analysis of HSA and its recombinant version (rHSA), the polypeptide chain forms a heart-shaped conformation with the approximate dimension of 80× 80×80×30 Å and about 67 % of it consists of α-helices, but no β-sheet is evident (Fig. 1).
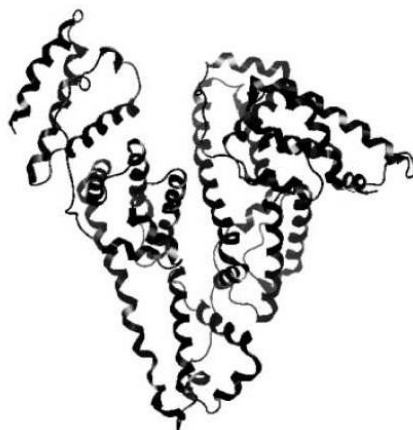
Figure 1: X –ray structure of human serum albumin [4].

X-ray diffraction has also shown that the protein has three homologous domains (I-III), each of which is comprised of two subdomains (A and B) [1].

HSA interacts reversibly with a broad spectrum of therapeutic agents. Typically, drugs bind to one or a very few high-affinity sites with typical association constants in the range of 10^4–10^6 M^{-1}. The pioneering work of Sudlow et al. [7] based on the displacement of fluorescent probes, revealed that most drugs bind with a high affinity to one of two sites, referred to as site I and site II.

Typical site I ligands appear to be dicarboxylic acids and/or bulky heterocyclic molecules with a negative charge localized in the middle of the molecule. Site I probes include warfarin, 5-dimethylaminonaphthalene-1-sulfonamide, dansylamide, dansyl-L-glutamine, dansyl-L-asparagine, dansyl-L-lysine and n-butyl-p-aminobenzoate.

Site I appears to be capacious and flexible and to contain a large number of individual ligand-binding sites that sometimes are independent of each other but in other cases, mutually influence one another [8].

The fact that ligands with very different chemical structures bind to the region with high affinity indicates that the site is adaptable. Mutual interactions between bound ligands have

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been observed and this can be attributed to partially overlapping binding sites, or conformational changes in the albumin molecule (allosteric effect or anti-cooperativity). Single residue mutations in this region of albumin have a significant effect on the conformational and thermal stability of the protein, much more than mutations in site II [9].

Site I is formed as a pocket in subdomain IIA and involves the lone tryptophan of the protein (Trp214) [10]. The inside wall of the pocket is formed by hydrophobic side chains, whereas the entrance to the pocket is surrounded by positively charged residues. Both residues contribute positively (e.g., Trp214 and Arg218) and residues that contribute negatively (e.g., Lys199 and His242) to the binding have been identified in the high affinity binding of ligands such as warfarin.

Ligands that bind to site II (also called the indolebenzodiazepine site) are often aromatic carboxylic acids with a negatively charged acidic group at one end of the molecule that is separate from a hydrophobic center. Dansylsarcosine, dansyl-L-proline, dansylglycine, and 7-alkylaminocoumarin-4-acetic acids all function as site II probes. Site II seems to be smaller, or narrower, than site I, because no large ligands apparently bind to it. It also appears to be less flexible, because binding often is strongly affected by stereo selectivity. Furthermore, the substitution of ligands with a relatively small group strongly influences the binding. Site II is a pocket that is formed in subdomain IIIA principally the same way as site I. Among the individual amino acid residue of this subdomain, Arg410 and Tyr411 are usually assumed to be important [11].

2.1.3.2 Drug binding sites of alpha-1-acid glycoprotein

The single polypeptide chain of serum AGP (183 residues, average Mw ≈ 41,000) is heavily glycosilated, the carbohydrate content is 41–45%. Acidity of the protein (isoelectric point = 2.7) is due to the presence of sialic acid residues.

Genetic polymorphism of AGP has been long known, it has three major variants (F1, S, and A) which differ from one another in their primary structure. The difference between F1 and S arises from a substitution of one amino acid, while the A variant differs from F1/S by 22 residues [12]. The relative abundance in commercial, pooled-source protein of the F1, S and A variants has been reported to be ≈40, ≈30, and ≈30%, respectively [13]. There

is a large difference in the binding of certain drugs to the F1/S and A variants of AGP [13-15].

Although AGP is among the best-studied plasma proteins, its three-dimensional structure and physiological function are still unresolved. AGP is an all-β protein with high β-sheet content (≈40%) and as the member of the lipocalin family its overall fold consists of eight antiparallel β-strands which form the so called β-barrel with a central hydrophobic pocket [16-18]. In relation to the large number of papers reporting AGP binding parameters of various drugs, much less attention has been paid on the identification of the ligand binding site(s) and binding mechanism though the detailed characterization of the binding sites of AGP is far from complete [4].

Some inconsistent findings indicate that AGP contains a wide drug-binding region for basic, acidic and neutral drugs (Fig. 2). Thus, more attention should be paid to the characterization of drug binding sites on AGP [19].
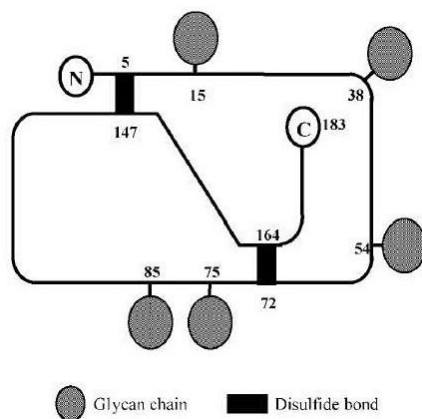
Figure 2: Structure of human alpha-1-acid glycoprotein [4].

2.2 OBSERVED DRUGS

2.2.1 Barbiturates

2.2.1.1 Mechanism of action

The principal mechanism of action of barbiturates is their affinity for the $GABA_A$ receptor. GABA is the principal inhibitory neurotransmitter in the central nervous system. Barbiturates bind to the $GABA_A$ receptor at the alpha subunit, which are binding sites distinct from GABA itself and also distinct from the benzodiazepine binding site. Like benzodiazepines, barbiturates potentiate the effect of GABA at this receptor. In addition to this GABA-ergic effect, barbiturates also block the AMPA receptor, a subtype of glutamate receptor. Glutamate is the principal excitatory neurotransmitter in the mammalian CNS. Taken together, the findings that barbiturates potentiate inhibitory $GABA_A$ receptors and inhibit excitatory AMPA receptors can explain the CNS-depressant effects of these agents. At higher concentration they inhibit the Ca^{2+} dependent release of neurotransmitters [20].

2.2.1.2 Therapeutic use

Barbiturates have been long used as anxiolytics and hypnotics. Today benzodiazepines have largely supplanted them for these purposes, because benzodiazepines have less potential for abuse and less danger of lethal overdose [21].

Phenobarbital is the most widely used anticonvulsant worldwide and the oldest still in use. It also has sedative and hypnotic properties but, as with other barbiturates, has been superseded by the benzodiazepines for these indications. The World Health Organization recommends its use as first-line for partial and generalized tonic-clonic seizures (those formerly known as Grand Mal) in developing countries. It is a core medicine in the WHO Model List of Essential Medicines, which is a list of minimum medical needs for a basic health care system [22]. In more affluent countries it is no longer recommended as a first-line medication, however it is relied on as an alternate when a patient fails to respond to treatment with more modern AED's [23]. It is still commonly used around the world to treat neonatal seizures [24].

Mephobarbital is used as a sedative, anxiolytic and anticonvulsant. It is the N-methylated analogue of phenobarbital, and has similar indications, therapeutic value, and tolerability [21].

Hexobarbital is a barbiturate derivative having hypnotic and sedative effects. It was also marketed as a rapid-acting sleeping medication with short duration and is still used in some scientific research [21].

Butobarbital is a drug which is a barbiturate derivative. It is prescribed for severe insomnia [21].

2.2.1.3 Dependence, tolerance and overdose

Barbiturate use can lead to both psychological and physical dependence. Psychological addiction can occur quickly. Signs of drug dependence include relying on a drug regularly for a desired effect. The addicted abuser believes he or she must take a barbiturate to sleep, relax, or just get through the day. Continued use of barbiturates leads to physical dependence.

As people develop a tolerance for barbiturates, they may need more of the drug or a higher dosage to get the desired effect. This can lead to an overdose, which results when a person takes a larger-than-prescribed dose of a drug.

The amount of a fatal dosage of barbiturate varies from one individual to another. However, the lethal dose is usually ten to fifteen times as large as a usual dose. An overdose affects the heart and the respiratory system. Symptoms of overdose of barbiturates include confusion, decrease in or loss of reflexes, somnolence, pyrexia, irritability, hypothermia, poor judgment, shortness of breath or slow/troubled breathing, slow heartbeat, slurred speech, staggering, trouble in sleeping, unusual movements of the eyes, weakness. The user then falls into a coma and dies [21].

2.2.2 Benzodiazepines

Benzodiazepines gained popularity among medical professionals as an improvement upon barbiturates, which have a comparatively narrow therapeutic index, and are far more sedating at therapeutic doses. The benzodiazepines are also far less dangerous; death rarely results from benzodiazepines overdose, except in cases where it is consumed with large amounts of other depressants (such as alcohol or other sedatives) [25].

2.2.2.1 Mechanism of action

Benzodiazepines bind to a specific subunit on the $GABA_A$ receptor at a site that is distinct from the binding site of the endogenous GABA molecule [26-30]. The $GABA_A$ receptor is an inhibitory channel which, when activated, decreases neurologic activity. Because of the role of benzodiazepines as a positive allosteric modulators of GABA, when it binds to benzodiazepine receptors it causes inhibitory effects. This arises from the hyperpolarization of the post-synaptic membrane owing to the control exerted over negative chloride ions by $GABA_A$ receptors [26, 31].

Benzodiazepines have also calcium antagonist activity which may explain reports of systemic and coronary vasodilatation by benzodiazepine drugs such as diazepam. Benzodiazepines act via micromolar benzodiazepine binding sites as Ca^{2+} channel blockers and significantly inhibit depolarization-sensitive calcium uptake [32].

Benzodiazepines may influence neurosteroid metabolism and progesterone levels which in turn may adversely influence the functions of the brain and reproductive system. The pharmacological actions of benzodiazepines at the $GABA_A$ receptor are similar to those of neurosteroids. Neuroactive steroids are positive allosteric modulators of the $GABA_A$ receptor, enhancing GABA function. Many benzodiazepines potently inhibit the enzymes involved in the metabolism of neurosteroids. Long-term administration of benzodiazepines may influence the concentrations of endogenous neurosteroids, and thereby would modulate the emotional state. Factors which affect benzodiazepines ability to alter neurosteroid levels depend on the molecular make up of the individual benzodiazepine molecule. Presence of a substituent at N1 position of the diazepine ring and/or the chloro or nitro group at position 7 of the benzene ring contribute to potent inhibition of the isoenzymes, and in turn a bromo group at position 7 (for bromazepam) and additional

substituents (3-hydroxy group for oxazepam and tetrahydroxazole ring for cloxazolam and oxazolam) decrease the inhibitory potency of benzodiazepines on neurosteroids [33].

Diazepam an chlordiazepoxide inhibit acetylcholine release and sodium-dependent high affinity choline uptake which may play a role in diazepam's anticonvulsant properties [34].

Diazepam is metabolized via oxidative pathways in the liver via the cytochrome P450 enzyme system. It has a biphasic half-life of 1–2 and 2–5 days, and has several pharmacologically active metabolites [35].

Oxazepam is an active metabolite formed during the breakdown of diazepam [36]. Oxazepam may be safer than many other benzodiazepines in patients with impaired liver function because it does not require hepatic oxidation, but rather it is simply metabolized via glucuronidation. This means that oxazepam is less likely to accumulate and cause adverse reactions in the elderly or people with liver disease. The half life of oxazepam is 4-15 hours [29].

2.2.2.2 Therapeutic use

Diazepam, chlordiazepoxide and oxazepam is mainly used to treat anxiety, insomnia, and symptoms of acute alcohol or opiate withdrawal [37, 38]. Diazepam has a broad spectrum of indications including: treatment of anxiety, panic attacks, and states of agitation, as a premedication for inducing sedation, anxiolysis or amnesia before certain medical procedures (e.g. endoscopy), treatment of status epilepticus, adjunctive treatment of other forms of epilepsy, treatment of the symptoms of alcohol and opiate withdrawal, short-term treatment of insomnia [38], adjunctive treatment of painful muscle conditions [39], adjunctive treatment of spastic muscular paresis (para-/tetraplegia) caused by cerebral or spinal cord conditions such as stroke, multiple sclerosis, spinal cord injury (long-term treatment is coupled with other rehabilitative measures) [39].

Oxazepam is an intermediate acting benzodiazepine with a slow onset of action, so it is usually prescribed to individuals who have trouble staying asleep, rather than falling asleep. It is commonly prescribed for anxiety disorders with associated tension, irritability, and agitation. It is also prescribed for drug and alcohol withdrawal, and for anxiety associated with depression. Also prescribed for sleepwalking before a neurologist is involved when the sleepwalker may be a problem or danger to themselves [40].

2.2.2.3 Dependence, tolerance and overdose

Benzodiazepines can cause physical dependence, addiction and what is known as the benzodiazepine withdrawal syndrome. Withdrawal from chlordiazepoxide or other benzodiazepines often leads to withdrawal symptoms which are similar to those seen with alcohol and barbiturates. The higher the dose and the longer the drug is taken the greater the risk of experiencing unpleasant withdrawal symptoms. Withdrawal symptoms can however occur at standard dosages and also after short term use. Benzodiazepine treatment should be discontinued as soon as possible via a slow and gradual dose reduction regime [41].

Chronic use of benzodiazepines lead to the development of tolerance with a decrease in number of benzodiazepine binding sites [40, 42].

Benzodiazepines are drugs which are very frequently involved in drug intoxication, including overdose [43]. Benzodiazepines overdoses are considered a medical emergency and generally require the immediate attention of medical personnel. The antidote for an overdose of benzodiazepines are flumazenil [21]. An individual who has consumed excess benzodiazepines may display some of the following symptoms: Somnolence (difficulty staying awake), mental confusion, hypotension, hypoventilation, impaired motor functions - impaired reflexes, impaired coordination, impaired balance, dizziness, muscle weakness, coma [40].

2.3 ELECTROPHORESIS

2.3.1 General aspects of electrophoresis

The process of electrophoresis is defined as 'the differential movement or migration of ions by attraction or repulsion in an electric field'. In practical terms, a positive (anode) and negative (cathode) electrode are placed in a solution containing ions. Then, when a voltage is applied across the electrodes, solute ions of different charge, *i.e*., anions (negative) and cations (positive), will move through the solution towards the electrode of opposite charge. Capillary electrophoresis, then, is the technique of performing electrophoresis in buffer-filled, narrow-bore capillaries, normally from 25 to 100 µm in internal diameter.

2.3.2 Instrumentation

The instrumentation required for CE is remarkably simple in design, as fig. 3 illustrates.
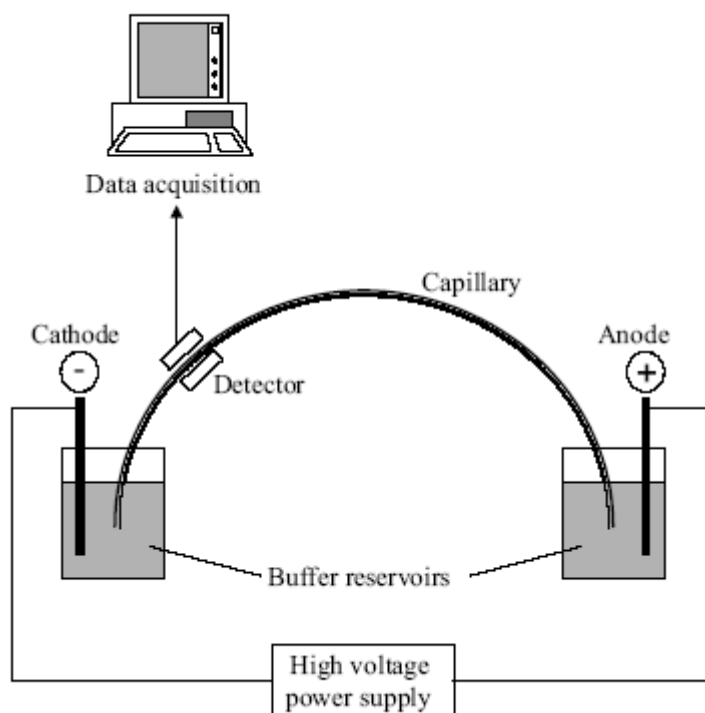

Figure 3: A schematic representation of the arrangement of the main components of a typical CE instrument [44].

The ends of a capillary are placed in separate buffer reservoirs, each containing an electrode connected to a high-voltage power supply capable of delivering up to 30 kV. The sample is injected onto the capillary by temporarily replacing one of the buffer reservoirs (normally at the anode) with a sample reservoir and applying either an electric potential or external pressure for a few seconds. After replacing the buffer reservoir, an electric potential is applied across the capillary and the separation is performed. Detection of separated analytes is achieved directly through the capillary wall near the opposite end (normally near the cathode).

Basic features of a CE instrument include an autosampler, a detection module, a high-voltage power supply, the capillary and, of course, a computer to control everything [44].

Sample injection

One of the main advantages of CE is its ability to inject extremely small volumes of sample. Typical injection volumes range from picoliters to nanoliters. There are two commonly used injection methods for CE: hydrodynamic and electrokinetic. Hydrodynamic injection is accomplished by the application of a pressure difference between the two ends of a capillary. Electrokinetic injection is performed by simply turning on the voltage for a certain period of time.

Capillary column

The capillary column is a key element of the CE separation. Fused silica is by far the most frequently used material, although columns have been made of teflon and borosilicate glass. The widespread use of fused silica is due to its intrinsic properties, which include transparency over a wide range of the electromagnetic spectrum and a high thermal conductance. Fused silica is also easy to manufacture into capillaries with diameters of a few micrometers. Many reports describe the covalent attachment of silanes with neutral or hydrophilic substituents to the inner wall of the capillary in order to reduce electroosmotic flow and prevent adsorption of the analyte. Coatings tend to stabilize the pH.

An uncoated fused silica capillary is prepared for its first use in electrophoresis by rinsing it with 10 to 15 column volumes of 0.1M NaOH followed by 10 to 15 column volumes of water and 5 to 10 column volumes of the separation buffer. For a coated capillary, the preparation procedure is the same except that 0.1M NaOH is replaced with methanol. In commercial instruments, the carrier fluid is forced through the capillary by either applying pressure to the inlet reservoir or reducing pressure at the outlet reservoir.

Detection modes

With some modifications, most HPLC detection modes can be applied to CE.

Table contains a list of commonly used CE detectors and their representative limits of detection (LODs) [45].

Table 1: CE detection modes and representative limits of detection.

TABLE 1. CE detection modes and representative LODs.	
Detection principle	LOD (mol)
Spectrophotometric	
Absorption	$10^{-15} - 10^{-}$
Fluorescence	
Precolumn derivatization	$10^{-20} - 10^{-17}$
On-column derivatization	8×10^{-16}
Postcolumn derivatization	2×10^{-17}
Indirect	
UV	$10^{-13} - 10^{-12}$
Fluorescence	5×10^{-17}
Thermal lens	4×10^{-17}
Raman	2×10^{-15}
Mass spectrometric	1×10^{-17}
Electrochemical	
Conductivity	1×10^{-16}
Amperometric	7×10^{-19}

2.3.3 Electrophoresis theory

The theory that governs electrophoresis is directly applicable to CE and can be dealt with very briefly, with reference to a few equations. As mentioned earlier, electrophoresis is the movement or migration of ions or solutes under the influence of an electric field. Therefore, separation by electrophoresis relies on differences in the speed of migration (migration velocity) of ions or solutes. Now, ion migration velocity can be expressed as:

$$\upsilon = \mu_e \, E \qquad \text{(eq.1)}$$

where υ is ion migration velocity (m s^{-1}), μ_e is electrophoretic mobility (m^2 V^{-1} s^{-1}) and E is electric field strength (Vm^{-1}).

The electric field strength is a function of the applied voltage divided by the total capillary length.

Electrophoretic mobility is a factor that indicates how fast a given ion or solute may move through a given medium (such as a buffer solution). It is an expression of the balance of forces acting on each individual ion; the electrical force acts in favor of motion and the frictional force acts against motion. Since these forces are in a steady state during electrophoresis, electrophoretic mobility is a constant (for a given ion under a given set of conditions).

The equation describing electrophoretic mobility is:

$$\mu_e = \frac{q}{6\pi\eta r} \qquad \text{(eq.2)}$$

where q is the charge on the ion, η is the solution viscosity and r is the ion radius. The charge on the ion (q) is fixed for fully dissociated ions, such as strong acids or small ions, but can be affected by pH changes in the case of weak acids or bases. The ion radius (r) can be affected by the counter-ion present or by any complexing agents used. From (eq.2) we can see that differences in electrophoretic mobility will be caused by differences in the charge-to-size ratio of analyte ions. Higher charge and smaller size confer greater mobility, whereas lower charge and larger size confer lower mobility.

Electrophoretic mobility is probably the most important concept to understand in electrophoresis. This is because electrophoretic mobility is a characteristic property for any given ion or solute and will always be a constant. What is more, it is the defining factor that decides migration velocities. This is important, because different ions and solutes have different electrophoretic mobilities, so they also have different migration velocities at the same electric field strength. It follows that, because of differences in electrophoretic mobility, it is possible to separate mixtures of different ions and solutes by using electrophoresis.

2.3.4 Electroosmotic flow

A vitally important feature of CE is the bulk flow of liquid through the capillary. This is called the electroosmotic flow and is caused as follows. An uncoated fused-silica capillary tube is typically used for CE. The surface of the inside of the tube has ionisable silanol groups, which are in contact with the buffer during CE. These silanol groups readily dissociate, giving the capillary wall a negative charge. Therefore, when the capillary is filled with buffer, the negatively charged capillary wall attracts positively charged ions from the buffer solution, creating an electrical double layer and a potential difference (zeta potential) close to the capillary wall, as described according to Stern's model. Stern's model for an electrical double layer includes a rigid layer of adsorbed ions and a diffuse layer, in which ion diffusion may occur by thermal motion. The zeta potential is the potential at any given point in the double layer and decreases exponentially with increasing distance from the capillary wall surface. When a voltage is applied across the capillary, cations in the diffuse layer are free to migrate towards the cathode, carrying the bulk solution with them. The result is a net flow in the direction of the cathode, with a velocity described by:

$$\upsilon_{EOF} = \left(\frac{\varepsilon\varepsilon\xi}{4\pi\eta} \right) E \qquad\qquad (eq.3)$$

where ε is the dielectric constant of a vacuum, ε is the dielectric constant of the buffer, ζ is the zeta potential, η is the viscosity of the buffer and E is the applied electric field. The terms enclosed in brackets equate to the mobility of the EOF (μ_{EOF}). The relationship between EOF mobility and EOF velocity is analogous to that between electrophoretic

mobility and migration velocity. Indeed, the units for EOF mobility are the same as those for electrophoretic mobility.

2.3.4.1 Factors affecting EOF mobility

The main variables affecting EOF mobility are the dielectric constant and viscosity of the buffer and the size of the zeta potential.

The use of buffer additives and/or other modifications of the buffer composition may influence the dielectric constant and viscosity of the buffer. Buffer viscosity will also depend on the temperature at which the CE separation is performed.

Zeta Potential

The zeta potential is proportional to the charge density on the capillary wall, which itself is pH dependent. Therefore, EOF mobility will vary according to the buffer pH, such that at high pH the EOF mobility will be significantly greater than at low pH. Figure depicts the variation of EOF mobility with pH for a typical fused-silica capillary.
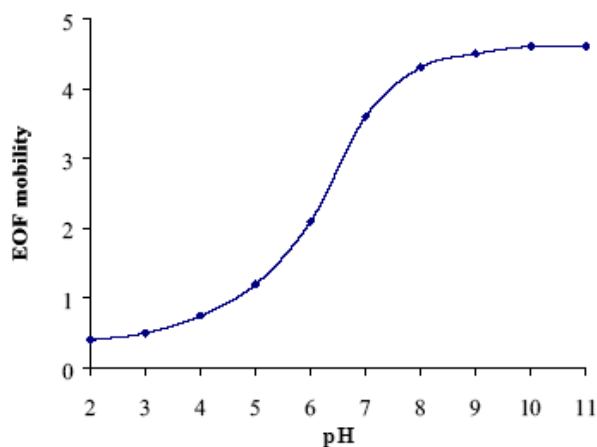
Figure 4: The variation of EOF mobility with changing pH for a typical uncoated fused-silica capillary (simulated data)[44].

Above pH 9, silanols are completely ionized and the EOF mobility is at its greatest. Below pH 4, the ionization of silanols is low and the EOF mobility is insignificant. The zeta potential will also depend upon the ionic strength of the buffer, because as ionic strength increases, the double layer will become compressed, which results in a decreased zeta potential and reduced EOF mobility. At pH ~7, the EOF mobility is sufficient to ensure the

net migration of most ions towards the cathode, regardless of their charge. Therefore, the observed migration velocity of a solute may not be directly related to its electrophoretic mobility. Instead, it is related to a combination of both its electrophoretic mobility and the EOF mobility. Therefore, a solute's apparent electrophoretic mobility (μ_a), which is calculated from its observed migration velocity, is the vector sum of its real (or effective) electrophoretic mobility (μ_e) and the EOF mobility (μ_{EOF}).

$$\mu_a = \mu_e + \mu_{EOF} \qquad\qquad (eq.4)$$

Since samples are normally introduced at the anode and EOF moves from the anode to the cathode, cations have positive μ_e, neutrals have zero μ_e and anions have negative μ_e. In other words, cations migrate faster than the EOF and anions migrate more slowly than the EOF. Neutrals migrate with the same velocity as the EOF.

2.3.4.2 Flow Profile in CE

A further key feature of EOF is that it has flat flow profile, which is shown in figure, alongside the parabolic flow profile generated by an external pump, as used for HPLC. EOF has a flat profile because its driving force (*i.e.* charge on the capillary wall) is uniformly distributed along the capillary, which means that no pressure drops are encountered and the flow velocity is uniform across the capillary. This contrasts with pressure-driven flow, yielding a parabolic or laminar flow profile. The flat profile of EOF is important because it minimizes zone broadening, leading to high separation efficiencies that allow separations on the basis of mobility differences as small as 0.05% [44].
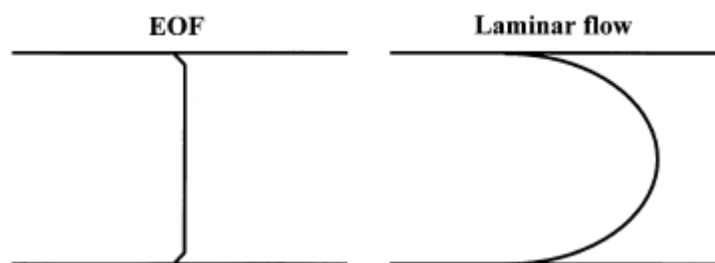

Figure 5: Flow profiles of EOF and laminar flow [44].

2.3.5 Capillary electrophoresis in the study of molecular interaction

CE is a well-developed technique for the study of molecular interactions. The method can be applied over a very wide pH range (pH 1–14), and be operated with aqueous media. Therefore, CE is suited to study almost any biomolecular interaction system.

The major drawback is still the low capacity of standard CE systems, equipped with UV detectors to yield specific information about sample components. CE coupled with other detectors, such as CE-mass spectrometry (CE-MS) [46], CE-inductively coupled plasma (CE-ICP-MS) [47], CE-nuclear magnetic resonation (CE-NMR) [48], CE-Fourier transform infrared spectroscopy (CE-FTIR) [49], CE-Raman spectroscopy (CE-Raman) [49], and CE-X-ray [50] is capable to provide more structural elucidation. At present, however, only the online MS technique has sufficient detection sensitivity for structural identification.

An attractive direction is to apply CE to high-throughput drug screening, for products of combinatorial chemistry as well as components of traditional Chinese medicine. With the aid of CE, binding constants and stoichiometry data can be obtained. An important practical aspect is that the sample may not be purified prior to CE separation. Therefore, a large number of samples can be screened for biological activity by a single CE run. With the rapid development of microfabricated devices, super high-throughput drug screening can become feasible on a chip-based array CE platform [51].

The method is a complementary and attractive alternative to other separation-based methods for evaluating binding parameters [52]. The application of CE to study receptor-ligand interactions was first reported in 1992 [53]. From then on, CE has been widely used to study molecular interactions and has become a powerful tool for the determination of the binding parameters of various bioaffinity interactions [51]. Provided that at least one of the interacting compounds is soluble, can be quantitatively recovered, and when complex formation results in changes in electrophoretic mobility, CE is well suited for binding studies [52].

The advantages often associated with CE for binding studies as compared with other methods are relatively short analysis time, low sample and reagent consumption, ease of automation, the versatility of compound that can be studied, and high separation efficiency [54, 55]. The approach is much less labor intensive than equilibrium dialysis and

ultrafiltration where a separate analytical step, usually HPLC, is required after the initial separation of species.

Errors associated with the presence of a membrane are absent and changes in affinity due to derivatization or immobilization and interactions with a chromatographic support are eliminated.

Its primary disadvantage is that it cannot be used for the preparation of the analytes [56] .

The general limitations of CE, however, also applies to affinity studies utilizing CE: requirements of mobility differences of charged species, lack of explicit temperature control, capillary wall adsorption [55].

Different modes or formats of CE have been developed and are compared in several studies [52, 57] and reviews [54, 55, 58-61] [54].

2.3.6 Capillary electrophoresis modes

Methods, which are most in use to study drug-protein interactions are: affinity capillary electrophoresis [61], the Hummel-Dreyer, the vacancy peak, the vacancy affinity capillary electrophoresis [57], the zonal capillary electrophoresis [51], Frontal analysis [62] and frontal analysis electrophoresis capillary continual [61]. The methods can be considered as complementary rather then competitive techniques, because each method exhibits specific ranges of applicability, advantages and disadvantages [57]. Methods are applicable for studying interactions between drugs with HSA and AGP, lipoproteins and globulins, but it could not be used directly with plasma. It is required to eliminate some particles in suspension, which causes problems in separation system, for instance ultrafiltration [61].

2.3.6.1 Affinity capillary electrophoresis

A capillary is filled with buffer containing P in varying concentrations and amount of D is injected. Since the equilibrium between D and P is established in the capillary, the apparent mobility of D depends on the binding constant and the difference between mobility of drug end the complex. The binding constant is calculated from the change in electrophoretic mobility of D. On the contrary, D can be also added on the buffer instead of P. In this case a small amount of P is injected as the sample. ACE has several potential advantages as follows: only a small amount of P and D is required, the injection sample need not be highly purified, and binding constants of several samples can be simultaneously determined [63].

2.3.6.2 Vacancy affinity capillary electrophoresis

VACE is a relatively new method in CE for the estimation of binding constants [57, 64]. A capillary is filled with a running buffer containing both D and P. The concentration of either D or P is fixed and that of the other component is varied. The binding constants are estimated from the migration behavior of D as well as ACE. An advantage of the VACE is the fact that the absolute number of the binding sites (n) can be calculated [57]. In addition, VACE is particularly convenient for the estimation of binding constant of weakly soluble D in water because the solubility should be increased in the running buffer containing P [57].

2.3.6.3 Frontal analysis electrophoresis capillary continual

Recently, FACCE has been developed by Gao et al. [65] as a novel CE method. The capillary is filled and equilibrated with buffer containing P prior the run, and the inlet of the capillary is immersed in the sample solution containing D and P. In this method, a voltage is applied for the analysis keeping the sample solution as the inlet vial. The sample is continuously introduced into the capillary during the analysis and the separation process progresses at the same time. When the mobility of D is higher than of P, the first plateau in the electropherogram is due to elution of free D and the second plateau is due to free D and drug-protein complex. A disadvantage of this method is greater sample consumption than the other CE methods. However, FACCE offers lower detection limits and is free from the reproducibility problems arising from slow binding kinetics as compared to the other CE methods, such as HD and the conventional FA [65].

2.3.6.4 Hummel-Dreyer

In this type of experiment the capillary is filled with buffer containing the drug D to be studied at varying concentrations. When a small amount of protein P is injected a typical elution profile appears. The positive peak corresponds to the drug-protein complex and the free protein, and the negative peak emerges at the migration time of the drug. The area of the negative peak is directly related to the amount of drug bound to the protein. The number of bound drug molecules per protein molecule is fixed by the drug concentration in the buffer and therefore can be considered constant during elution [52].

2.3.6.5 Vacancy peak

In a vacancy peak experiment, the capillary is filled with buffer containing both the protein and the drug [52]. The concentration of one compound, for instance the protein, is fixed and the concentration of the other component is varied. Then a small amount of plain buffer is injected and typical elution profiles appear. The first negative peak arises due to a vacancy in the drug-protein complex and the free protein, and the second negative peak is due to a vacancy in the free drug concentration indicated by. The area of the second negative peak depends directly on the amount of free drug in the buffer. The amount of free drug in each buffer may be quantified from internal calibration [52, 58]. A series of samples (neat buffer) with increasing drug concentrations are injected. For large drug concentrations in the sample, the area of the trough peak changes from negative to positive. A simple interpolation allows the determination of the drug concentration for which the negative peak vanishes. From this value the amount of free drug in the buffer can be found. It is assumed that the protein and the complex have approximately the same mobility [58]. The VP method offers the possibility to check the stoichiometric model. Using the measured value for free drug, the known total drug concentration in the buffer, and the known protein concentration, the amount of drug bound by the protein can be calculated [57].

2.3.6.6 Capillary zonal electrophoresis

In CZE the capillary is filled with buffer and sample, which contains drug and protein in concentrations variables in equilibrium, is injected. The separation is produced and there are fractions of free drug and drug-protein complex. This methodology can be applied to the systems, where affinity constants are elevated and kinetics is slow [51].

2.3.6.7 Capillary electrophoresis – frontal analysis

In frontal analysis, the equilibrium between D and P is obtained in the sample vial. After injection of the equilibrated sample into the capillary filled with buffer electrolytes, the free and bound components are separated in the capillary.

2.3.6.8. Summary

In CZE, the receptor, ligand, and complex should differ in mobility.

In ACE, the free receptor must show a different mobility to the complex.

In FA, there are two requirements: (i) the receptor and the complex have approximately the same mobility, and (ii) the mobility of the ligand differs sufficiently from the mobility of the complex.

Also, in VP the receptor has an approximate mobility with the complex and HD requires that the ligand has a different mobility with respect to the receptor and the complex [57].

For instance with the HD, the VP, the FA and the VACE method, both association constants and the number of the different binding sites can be determined, while with the ACE method only the binding constant can be determined and no information about the number of binding sites can be obtained [57].

Among these five CE modes, HD and VP are analyte consuming compared with the other modes but they are well suited for calculating the binding stoichiometry [66].

Table 2: Summary of CE methods and their experimental details for the estimation of binding constants [51, 61].

Method	Sample	Running solution	Applicability	Estimation of K
ACE	D P	Buffer + P Buffer + D	Fast on/off kinetics	Change in mobility of D Change in mobility of P
VACE	Buffer	Buffer + D + P		Change in mobility of D or P (vacancy peak)
HD	Buffer + D + P	Buffer + P (or D)	Fast on/off kinetics	Peak area of vacancy peak corresponding to $[D]_f$
VP	Buffer	Buffer + D + P	Fast on/off kinetics	Peak area of vacancy peak corresponding to $[D]_f$
FA	Buffer + D + P	Buffer	Fast on/off kinetics	Plateau height corresponding to $[D]_f$
FACCE	Buffer + D + P	Buffer		Plateau height corresponding to $[D]_f$
CZE	Buffer + D + P	Buffer	Fast or slow on/off kinetics	Peak area of vacancy peak or plateau height corresponding to $[D]_f$

In the case of strong binding, the interacting compounds and the complex have their unique migration mobilities, respectively. In weak binding systems, the complex dissociates progressively during the run, interacting compounds but not the final complex can be detected [67]. The distinction between strong and weak affinity interactions in CE studies does rest not only on a fixed value of the binding constant but also on the experimental setup, primarily on the separation time and buffer conditions [67].

Generally, CZE is appropriate for the assay of strong binding systems in which the complex will not dissociate during the required separation time period. When a strong binding assay is performed, either the area or height of the peak of the interacting components can be used in the quantitative determination [66].

Weak binding is typically analyzed by ACE or FA [68]. According to recent observations [69, 70], CZE can be also applied to study weak binding systems, in contrast to some former reports [54, 59]. It was found in CZE that the peak height can also be used to evaluate the concentration of free ligand or receptor as in FA. When only small amounts of samples fact, CZE and FA are identical except for a difference in the length of the analyte zone, which means that in CZE, the receptor and ligand can be injected immediately after mixing, or after certain time of incubation.

In the case of weak binding, the use of peak height is better than the peak area for the quantification of analytes.

The profiles of the interacting components depend on the degree of association of the receptor- ligand interaction. Besides, Joule heat, high electric field, wall effect, and properties of the running buffer may also influence the migration of analytes to some extent [66]. In studies of molecular interactions, different CE modes, equipments, or even differences in ligand or receptor concentrations may cause quantitative discrepancies [52, 71, 72]

2.3.7 Advantages and limitations of CE-FA at drug-plasma protein binding studies

In general, CE-FA is subject to the same advantages and limitations as other CE methods, but CE-FA is the method of choice because of its robustness, ease of implementation, capability to study multiple equilibriums, and smaller material consumption. Advantages include also the well-known characteristics of CE such as speed, ease of automation, separation power. Simplicity may be one of the main advantages of CE-FA. (Following equilibration of the interacting species the sample is introduced into the capillary containing neat buffer and voltage applied.) Separation of free ligand and analysis occur in one integrated step [14] and the free concentration of ligand in a particular sample can be determined directly [73].

Certainly, the fact that very little effort has to be put into method development and optimization is a major strength of the CE-FA method [57].

Furthermore, accordance with literature data was better than for the Hummel-Dreyer and vacancy peak methods [13].

Also, CE-FA is not subject to constraints in the binding stochiometries that can be analyzed [57].

CE-FA uses sample volumes 10–50 times higher than those normally used in CE. However, none of the interacting species has to be added to the electrophoresis buffer. This also avoids a large background detector response from analyte and/or ligand added to the electrophoresis buffer [52] which may be present in the ACE, vacancy peak, and HD methods as is the limitation associated with the use of indirect UV detection in terms of a small dynamic range [73].

Lack of sensitivity may be the main limitation to the use of CE-FA and have been pointed out in several studies. Therefore, capillaries with expanded light pathways have found some use [74-77].

To the best of our knowledge, other more sensitive detection methods than UV have not been applied in CE-FA studies so far.

In addition, limitations may apply to the mobilities of the free and complexed species subject to investigation.

Adsorption to the capillary may be a problem especially for proteins. Different types of coated capillaries have been applied in drug-plasma protein binding studies performed [78-83].

It is not possible to obtain uniform thermostating conditions across the length of a capillary [84], therefore lack of precise temperature control in CE-FA and other CE methods may be a drawback compared to conventional methods [62].

Finally, in contrast to ACE, CE-FA may be limited to the study of pure sample and protein preparations [73].

The results obtained with the FA, the VP, the HD, the VACE and the ACE method clearly show that for drug-protein binding studies the FA method seems to be the most favorable one [57].

2.3.8 Principles of capillary electrophoresis – frontal analysis

Similar to most other CE affinity methods CE-FA has been adapted from chromatography [52, 57, 85]. The use of frontal analysis in chromatographic settings has been reviewed [85-90]. FA was first applied in a gel filtration setup by Nichol and Winzor [91] in 1964 studying protein-protein interactions [73]. The main area of application has been drug-plasma protein binding studies. Although CE-FA appears to have evolved from FA chromatography [85], it should be recognized that the theory on which FA chromatography is based was originally developed for electrophoresis and ultracentrifugation [91, 92]. Thus, there is a link from CE-FA back to early affinity studies performed using moving boundary electrophoresis, see [93] for a historical presentation [73].

The principle of FA by CE is the same as for the chromatographic approach. However, separation occurs due to differences in electrophoretic mobilities of free and bound ligand rather than due to a size-exclusion mechanism affected by a chromatographic support.

CE-FA is based on the separation of ligand from protein and ligand-protein complex after the introduction of a relatively large volume (60–200 nL) of equilibrated sample mixture into the buffer-filled capillary. It is assumed that the protein and the complex have approximately the same mobility [54, 59] and the mobility of the drug differs sufficiently from the mobility of the complex. Differences in mobility cause the free drug to leave the protein zone. Bound drug remains within the protein zone. Provided the on- and off-kinetics is rapid, steady-state conditions and, thus, equilibrium is maintained where the drug and protein zones are overlapping.

Injection of the large sample plugs give rise to the appearance of two plateau peaks provided the mobility of the free drug is sufficiently different from that of the protein. The height of the free drug plateau peak is proportional to the free drug concentration $[D_f]$ in the original sample and the degree of binding can be determined by aid of a calibration curve. Plots of the number of bound ligand molecules per molecule of protein as a function of the free ligand concentration yields the binding curve. Direct fitting to the experimental data using nonlinear regression analysis for obtaining the binding parameters is recommended [73].

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2.3.9 Requirements for using CE-FA

The primary prerequisite in CE-FA is the requirement of sufficiently different migration times between the free and bound form of one of the species studied [52]. Otherwise determination of the free ligand concentrations is not feasible.

It is suggested that CE-FA is also feasible for studying equilibrium processes irrespective of the magnitudes of the association and dissociation rate constants.

Based on computer simulations Busch *et al*. [94] found that CE-FA is limited to systems where the mobility of the complex is equal to interacting receptor. Otherwise the free concentrations determined would be associated with an error. Experiments strongly indicate that low-molecular-weight drug-HSA [62], AGP [74, 75, 80, 95], and drug-lipoprotein [96] binding fulfill these requirements. The order of magnitude of the errors arising from not complying with the mobility restrictions has not been assessed. It seems unlikely that similar size/charge ratios of complex and one of the free species can be found in all cases. Thus, studies addressing the influence of the mobilities of the interacting species and the effect of fast or slow kinetics are warranted to validate results obtained by CE-FA and could significantly expand the application of the method.

Recently, it was found that plateau peaks may not be an absolute requirement in order to estimate binding correctly; however, the presence of plateau peaks may lead to a more robust method of analysis.

For the drug-HSA interactions investigated, the extent of binding tended to be underestimated when small sample volumes were introduced into the capillary and plateau peak conditions were absent. FA is more robust because the height and thus concentration is not affected by changes in migration times, EOF, length of the capillary, and applied voltage to the same extent in a plateau peak as in a zonal peak because of the nondispersed zone constituting the plateau region [73].

Practical considerations in CE-FA include solubility in the electrophoresis buffer of species to be investigated, matching of the ionic strength in samples and electrophoresis buffer applied in order to avoid stacking phenomena, linearity of detector responses, and the absence of adsorption phenomena causing distortion of plateau peak shape [73].

2.3.10 Application of CE-FA for the study of drug-plasma protein binding

Knowledge of the extent of binding to plasma proteins is reflected in the large number of papers dealing with the determination of plasma protein binding. Also, studies using CE-FA have focused on the most important drug-binding plasma proteins; HSA, AGP, and lipoproteins. CE-FA studies of drug-plasma protein binding, a few other studies have been performed using CE-FA only [52, 76, 97, 98]. FA in the CE format is relatively new; the first publication appearing in 1992 [52], thus, in many of the studies development, evaluation, and validation of the method have been the major theme [73].

2.3.10.1 Human serum albumin binding

Kraak et al. [52] studied binding of the site I ligand warfarin to BSA. CE-FA was found to be the most favorable CE format when compared to Hummel-Dreyer and vacancy peak.

Busch et al. [57] confirmed these findings in a comprehensive study including ACE and VACE in addition to CE-FA, Hummel-Dreyer, and vacancy peak methods.

Østergaard et al. [62] studied the HSA binding of 12 low-molecular- weight ligands with widely different physicochemical properties using identical experimental conditions. Only two compounds failed to be analyzed with the original experimental setting. One of the compounds (phenylbutazone) had too small a difference in mobility from the protein and a distinct plateau due to the free drug was not obtained. The other compound (phenol) had a very weak affinity and thus the HSA concentration had to be increased to obtain reliable measurements of the free drug concentration [62].

McDonnell et al. [99] studied the binding of 8 β-adrenoceptor blocking drugs.

Jia et al. the binding of 17 cationic and neutral drugs to HSA and AGP with only minimal alterations of the experimental conditions. Jia et al. used pressure-assisted CE-FA to shorten analysis times and improve sample throughput. In addition, application of air pressure was found to prevent loss of protein from sample and provide superior drug plateau peaks by minimizing capillary wall adsorption [100].

The utility and validity of binding studies performed with CE-FA have been demonstrated in several studies using HSA and various cationic ligands such as propranolol and verapamil [78, 81, 82, 99-101]. CE-FA is ideally suited for the measurement of these interactions because the electrophoretic mobilities of the cationic ligands and HSA

(negatively charged at physiological pH) are very different; moreover, the interactions between HSA and cationic drugs in general are relatively weak [1].

2.3.10.2. Acid glycoprotein binding

AGP has also been subject to study by CE-FA. Using verapamil as a ligand Shibukawa *et al*. [78] obtained good accordance between results generated using CE-FA and ultrafiltration. CE-FA studies of AGP binding include work focused on estimation of the degree of binding of cationic drugs [78, 99, 100] and studies directed at elucidating the effect of AGP structure and glycation on binding [77, 80, 83].

2.3.11. The application of the short-end capillary injection

This is a new, fast and simple CE-FA procedure for the study of drug-protein interactions at near-physiological conditions. This methodology involves the use of short-end capillary injection in order to reduce the analysis time. The results obtained are similar to conventional capillary electrophoresis and also agree with the reported bibliographic values. In addition, the protein binding percentages obtained with the long- and short-end injection schemes are in good agreement with the values found in literature. All these results confirm the applicability of the short-end capillary injection for the FA studies of drug- protein interactions with the advantage of increasing speed of analysis [102].

2.3.12 Biopartitioning micellar chromatography

It was demonstrated that the retention data obtained in a chromatographic system constituted by a C18 reversed stationary phase and a polyoxyethylene lauryl ether micellar mobile phase in adequate experimental conditions are helpful in describing the biological behaviour of different kinds of drugs [103-114]. The usefulness of BMC in describing the biological behaviour of drugs could be attributed to the following features:
The retention of a drug in this chromatographic system is mainly governed by its hydrophobic, electronic properties and, to a less extent, by its steric properties. These features of compounds also determine their passive permeability across cell membranes. In order to study the similarity between BMC and other well-recognised natural systems that mimics biomembranes, it is correlated [115] the retention data on BMC - log k_{BMC}.

2. EXPERIMENTAL PART

2.1 INSTRUMENTATION

A Hewlett-Packard HP 3DCE capillary electrophoresis system (Agilent, AZ, USA) equipped with a diode array detection (DAD) system and HP 3DCE Chemstation software was used.

Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. and 363 μm O.D. with total and effective length of 48.5 and 40 cm, respectively, were used. The capillary cassette temperature was set at 36.5°C and UV detection was performed at 200, 220 and 240 nm.

For ultrafiltration, Microcon YM-10 filters of cellulose of a molecular weight of 10000 VCO (Millipore, Bedford, MA, USA) and a centrifuge Heraeus Biofuge Strate (Heraeus, Madrid, Spain) were used.

All solutions were degassed in an ultrasonic bath (JP Selecta, Barcelona, Spain) prior to use. A Crison Micro pH 2000 pH meter from Crison Instruments (Barcelona, Spain) was employed to adjust the pH of the electrophoretic buffer.

2.2 CHEMICALS AND SOLUTIONS

All reagents were of analytical grade except of oxazepam. HSA and AGP were purchased from Sigma-Aldrich (St. Louis, U.S.A); sodium dihydrogenphosphate dihydrate was from Fluka Chemie GmbH (Steinheim, Germany);

Hexobarbital, mephobarbital, butabarbital were from Sigma Chemical CO (St. Louis, MO, USA); phenobarbital were from Bayer (cod. 12154); Chlordiazepoxide and diazepam were from Guinama (Valencia, Spain); oxazepam (Adumbran[®]) was from Boeringer Ingelheim, S.A., (Barcelona, Spain); Barnstead E-pure deionized water (Sybron, Boston, MA, USA) was used to prepare solutions.

The running buffer in all CE experiments was 67mM sodium phosphate pH 7.4 and was prepared by dissolving the appropriate amount of sodium dihydrogenphosphate dihydrate in water and adjusting the pH with NaOH 5 or 1.5M.

500μM human serum albumin stock solutions and 40μM AGP stock solutions were daily prepared by dissolving the corresponding amount of protein powder with the phosphate buffer. Working protein solutions were obtained by dilution of HSA and AGP stock solutions with phosphate buffer to a final concentration of HSA 0, 25, 75, 150, 250, 350, 475μM and of AGP 0, 5, 10, 15, 20μM.

Stock standard solutions of the drugs (2mM) were prepared in methanol. Working solutions were obtained by dilution with phosphate buffer from the corresponding stock solution to a final concentration of 100µM. All solutions were filtered prior to use through 0.45 µm pore size nylon membranes (Micron Separation, Westborough, MA, USA).

2.3 PROCEDURES

2.3.1 Capillary conditioning

New capillary was activated for 10 min flush with 1M NaOH at 60°C. Then, it was rinsed for 5 min with water and 15 min with phosphate buffer at 36.5°C. In order to obtain good peak shapes and reproducible migration times, the capillary was conditioned at the beginning of the day and between runs with the following sequence: (i) 2 min rinse with deionised water, (ii) 2 min rinse with 1M NaOH, (iii) 2 min rinse with deionised water, and (iv) 2 min rinse with running phosphate buffer at 1000 mbar.

2.3.2 Procedure for the study of drug interactions with HSA and AGP by CE-FA

Series of mixtures with increasing HSA concentration (from 0 to 475µM) and a fixed total concentration of drug (100µM) were prepared in duplicate by dilution of the stock solutions of drugs and proteins with the electrophoretic buffer.

Mixtures containing fixed total concentration (100µM) drug and increasing AGP concentration (from 0 to 20µM) in phosphate buffer were also prepared in duplicate.

The concentration protein range studied was selected according to their physiological concentration (HSA, 550µM; AGP, 20µM). However, the maximum HSA concentration studied was slightly lower than the physiological concentration in order to avoid problems connected with the viscosity of the solution and the Joule effect. All these solutions were allowed to reach equilibrium for at least 20 min before injection into the capillary system. Samples were injected hydrodynamically into the capillary at 50 mbar for 30 s and a running voltage of 15 kV was applied.

2.3.3 Procedure for the study of drug interactions with total plasmatic proteins by ultrafiltration and CE

Samples were prepared in duplicate by adding 10 µl of 2mM drug solution to 200 µl of tested human plasma and pre-equilibrated for 30 min. The mixture was filtered through cellulose filters in order to prevent problems related to viscosity and capillary plugging by centrifugating at 12000 rpm for 40 min after having reached equilibrium. Finally, the ultrafiltrated fraction was injected hydrodynamically into the capillary at 50 mbar for 4 s. The running voltage applied was 15 kV.

2.3.4 Data sources, software and data processing

Binding parameters of drug to HSA were determined by using equation 5 that relates the free drug concentration $[D]_f$ to the total protein concentration C_P and total drug concentration C_D [102].

$$[D]_f = \frac{-(1 - K_1 C_D + n_1 K_1 C_P) + \sqrt{(1 - K_1 C_D + n_1 K_1 C_P)^2 + 4 K_1 C_D}}{2 K_1}$$

(eq.5)

where n_1 is the number of primary binding sites and K_1 its corresponding affinity constant. This equation assumes only one type of independent binding site in the protein molecule as responsible for drug binding under physiological conditions and at therapeutic drug concentration (10^{-7} to 10^{-4}M) [102]. The parameters n_1 and K_1 were determined by adjusting the experimentally measured $[D]_f$ obtained for each C_P value to eq.5 by nonlinear regression using MATLAB 5.3 (Matlab® Ver. 5.3.0.10183 (R11), ©The Mathworks Inc., Natick, MA). The determination of the protein binding percentages of drugs PB can be performed calculating the $[D]_f$ value obtained for a total protein concentration of 475µM (eq.6).

$$PB\,(\%) = 100 \times \frac{C_D - [D]_f}{C_D}$$

(eq.6)

3.1 THEORETICAL BACKGROUND

When a certain drug solution is equilibrated with protein, the concentration of bound drug $[D]_b$ can be expressed as the difference between the total drug concentration C_D and the concentration of free drug $[D]_f$.

$$[D]_b = C_D - [D]_f \qquad \text{(eq.7)}$$

Let to consider m types of independent binding sites per HSA molecule and drugs binding to any of these sites with 1:1 stoichiometry. Considering the law of mass action, the concentration of drug bound $[D]_b$ can be expressed as:

$$[D_b] = \sum_{i=1}^{m} [D]_{b,i} = C_P \sum_{i=1}^{m} \frac{n_i K_i [D]_f}{1 + K_i [D]_f} \qquad \text{(eq.8)}$$

where $[D]_{b,i}$ is the concentration of drug-bound at the i-site while n_i and K_i are the number of binding sites and the affinity constant for each site, respectively. The product n_{iCP} represents the total concentration of binding sites of i-class. Eq. 8 is the basis of the traditional data treatment for determining the binding parameters of drugs to proteins from the nonlinear fitting of eq. 9:

$$r = \frac{[D]_b}{C_P} = \sum_{i=1}^{m} \frac{n_i K_i [D]_f}{1 + K_i [D]_f} \qquad \text{(eq.9)}$$

where r is the concentration of bound drug molecules per HSA molecule. Substituting eq.7 into eq. 9 and rearranging the terms it is possible to obtain the following expression that allows the estimation of the binding parameters by nonlinear regression assuming that one or two types of independent binding sites ($m = 1$ or $m = 2$) in the HSA molecule are the main responsible of drug binding [62].

Therefore, eq. 9 can be expressed as follows:

$$r = \frac{C_D - [D]_f}{C_D} = C_P \left(\frac{n_1 K_1 [D]_f}{1 + K_1 [D]_f} + \frac{n_2 K_2 [D]_f}{1 + K_2 [D]_f} \right) \qquad \text{(eq.10)}$$

In the most general case of one type of binding site, different linearized forms of eq. 10, such as the Scatchard and the Klotz plots, have been developed and reviewed recently [51]. However, one disadvantage of eq. 10 and its linearized forms is that experimentally measured concentration of free drug and its derived corresponding r-values are present in both dependent and independent variable. Therefore, small experimental errors are included in both axes leading sometimes to difficulties in the interpretation of results and to wrong estimations of the binding parameters. One possible alternative to overcome this problem was proposed by McDonnell et al. [99]. They proposed an equation relating the experimentally measured percentage of drug bound to the protein with the total drug concentration. Therefore, this approach enables the determination of the binding parameters isolating experimental errors in the y-axis. However, these authors limited the application of this equation to a single class of interaction site ($m = 1$) assuming one binding site ($n = 1$). A more general treatment can be applied from eq. 10, taking into account that the usual K_1 and K_2 values for HSA are in the range between 10^5–10^4 and 10^3–10^2 M, respectively. This fact makes that the concentration of drugs bound at the secondary binding site ($m = 2$) under physiological conditions would only be appreciable at free drug concentrations over 10^{-3} M, well above the usual therapeutic levels (10^{-7}–10^{-4} M) [116]. Therefore, the second addend in eq. 10 can be neglected in most of the cases. This leads to a simplification of eq. 10 giving the following expression:

$$[D]_f = C_D - C_P \left(\frac{n_1 K_1 [D]_f}{1 + K_1 [D]_f} \right)$$

(eq.11)

Rearranging the terms of this last equation it is possible to obtain a second degree polynomial whose resolution provides eq. 5 that relates the free drug concentration $[D]_f$ with the total protein C_P and drug C_D concentrations.

$$[D]_f = \frac{-(1 - K_1 C_D + n_1 K_1 C_P) + \sqrt{(1 - K_1 C_D + n_1 K_1 C_P)^2 + 4 K_1 C_D}}{2 K_1}$$

(eq.5)

Then, a nonlinear plot of the experimentally measured $[D]_f$ vs. C_P or C_D can be applied to the data analysis being the number of primary binding sites (n_1) and its corresponding

constant (K_1) the fitting parameters. In order to estimate drug-protein interactions at nearphysiological conditions, it is possible to work with two experimental setups: (A) series with increasing total concentration of drug (C_D) and a fixed total concentration of protein (C_P, approximately 500mM); or (B) series keeping constant C_D (close to the therapeutical level) and increasing C_P (from 0 up to 500mM). The experimental approach (A) would result in a great consumption of protein and thus higher costs per analysis. The experimental setup (B) allows reaching the physiological HSA concentration with a much lower cost and therefore this last setup would be preferable for high throughput applications and was selected to perform these studies. It is worth mentioning that reaching physiological protein concentration is a requirement if good estimations of drug-protein interactions are to be obtained since it has been reported that displacement of bound drug may happen due to albumin aggregation at high protein concentration [57, 117, 118]. On the other hand, due to the lack of sensitivity inherent to CE, total drug concentration below 10^{-4}M, leading to free drug concentrations between 10^{-4} and 10^{-6} M, could not be used in this kind of studies, and therefore this total concentration value was selected for all experiments. Once the n_1 and K_1 values have been obtained using eq. 5, it is possible to use this equation to estimate the free drug concentration, $[D]_f$, for a physiological HSA concentration (550mM). Then, the determination of the physiological drug protein binding percentages can be performed substituting the $[D]_f$ value obtained into eq. 6, considering a 10^{-4} M total drug concentration.

$$PB\,(\%) = 100 \times \frac{C_D - [D]_f}{C_D}$$

(eq.6)

3. RESULTS AND DISCUSSION

3.1 EVALUATION OF PLASMATIC PROTEIN BINDING OF BENZODIAZEPINES AND BARBITURATES

The interactions of 4 barbiturates and 3 benzodiazepines with the main plasmatic proteins at near physiological conditions were studied.

For this purpose, mixtures containing a fixed drug concentration (100µM) and increasing concentration of HSA (from 0 to 475µM) or AGP (from 0 to 20µM) were prepared. Samples of benzodiazepines were injected in the short-end capillary in order to reduce analysis time according to the methodology described in [102], and samples containing barbiturates that were injected in the long-end capillary to improve the separation between the zones of free drug and proteins.

The free drug concentration at each total protein concentration was determined by the plateau height ratio between each sample and a sample containing only drug measured in the electrophoreograms.

For example, fig. 6 shows the electrophoreograms corresponding to the study of interaction of oxazepam with HSA and fig. 7 shows oxazepam with AGP. As it can be observed, a plateau corresponding to the free drug was obtained followed by the band of the protein. The height of the plateau decreased with increasing protein concentration reflecting the evolution of the complexation process.
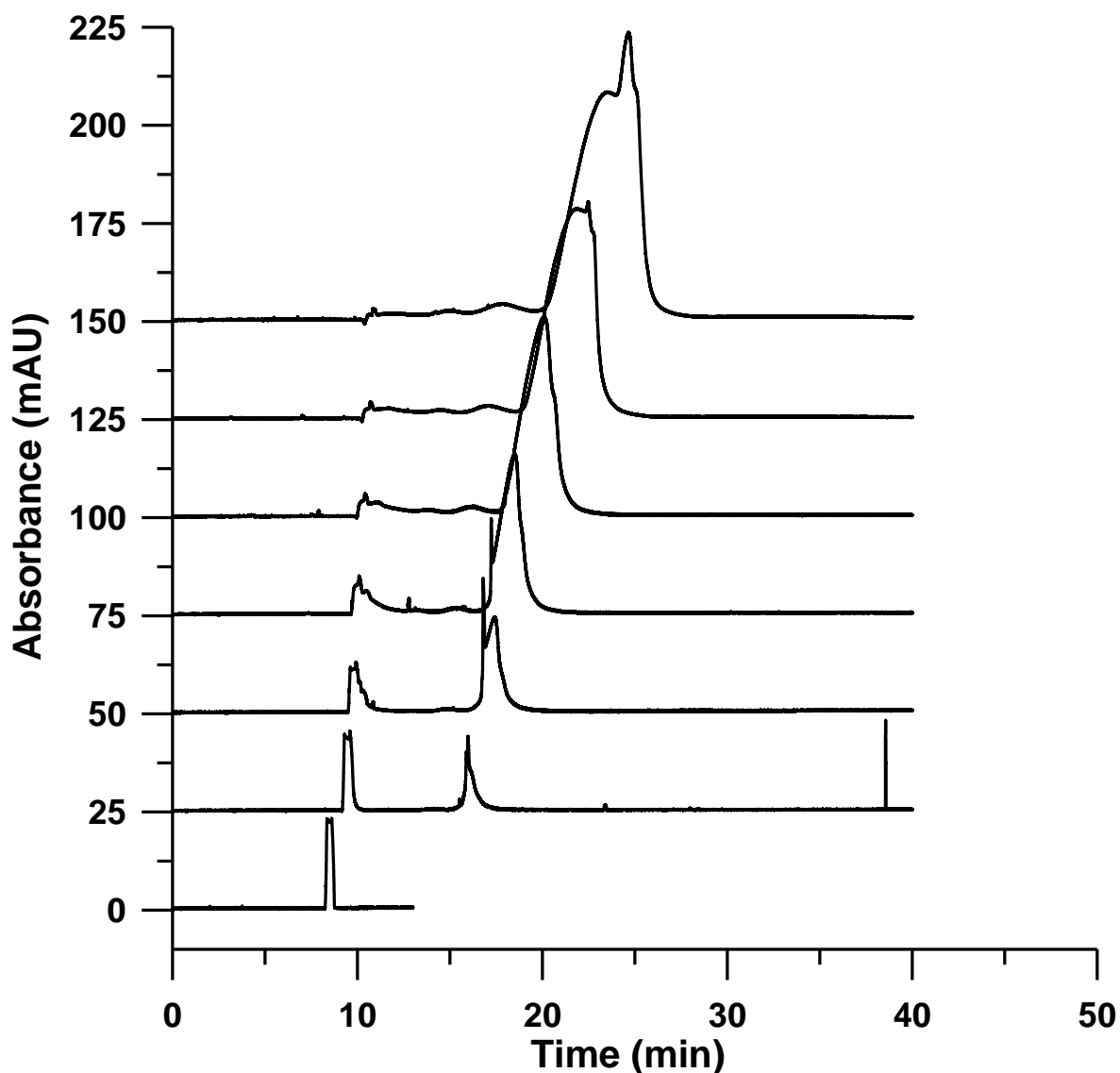
Figure 6: Set of representative electrophoreograms of the binding of oxazepam to HSA. Injected solutions contained 20 µl with increasing protein concentration (HSA: 0, 25, 75, 150, 250, 350 and 475µM from bottom to top). The electrophoretic buffer was 67mM phosphate solution at pH 7.4; temperature, 36.5°C. UV detection was performed at 240nm. An uncoated fused silica capillary with 48.5cm total length was used throughout. The injections were performed in the short-end capillary by 50mbar vacuum for 15 s and a running voltage -15 kV.
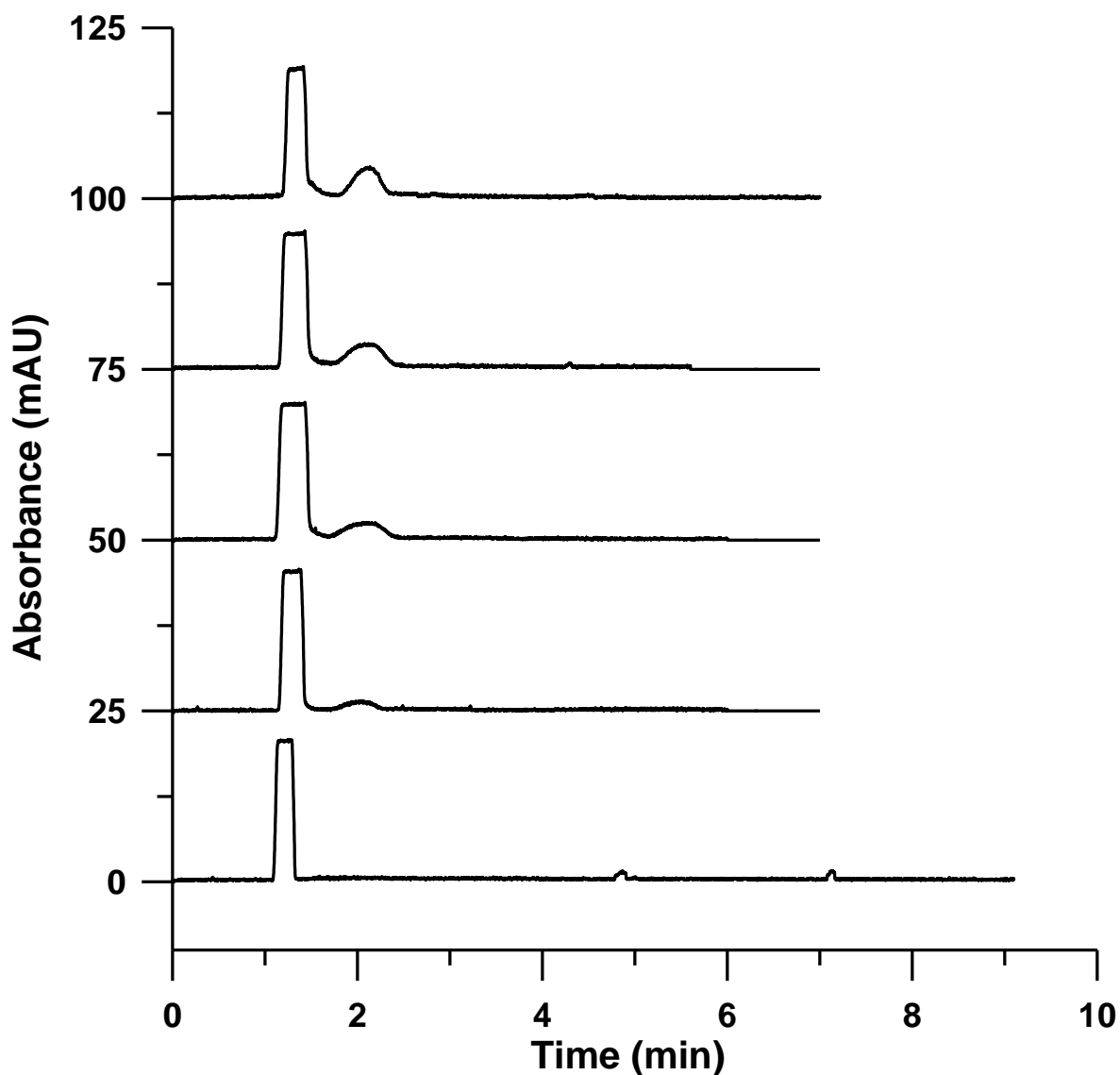
Figure 7: Set of representative electrophoreograms of the binding of oxazepam to AGP. Injected solutions contained 20 μl with increasing protein concentration (AGP: 0, 5, 10, 15 and 20μM from bottom to top). The electrophoretic buffer was 67mM phosphate solution at pH 7.4; temperature, 36.5°C. UV detection was performed at 240 nm. An uncoated fused silica capillary with 4.5cm total length was used throughout. The injections were performed in the short-end capillary by 50 mbar vacuum for 15 s and a running voltage -15 kV.

To estimate the drug–protein affinity constant, K_1 and the number of primary binding sites, n_1, the free drug concentration $[D]_f$ obtained for each protein concentration Cp and drug concentration C_D in duplicate were adjusted to eq. 5 and 6 respectively.

Table 3: Estimated K_1 and n_1 values.

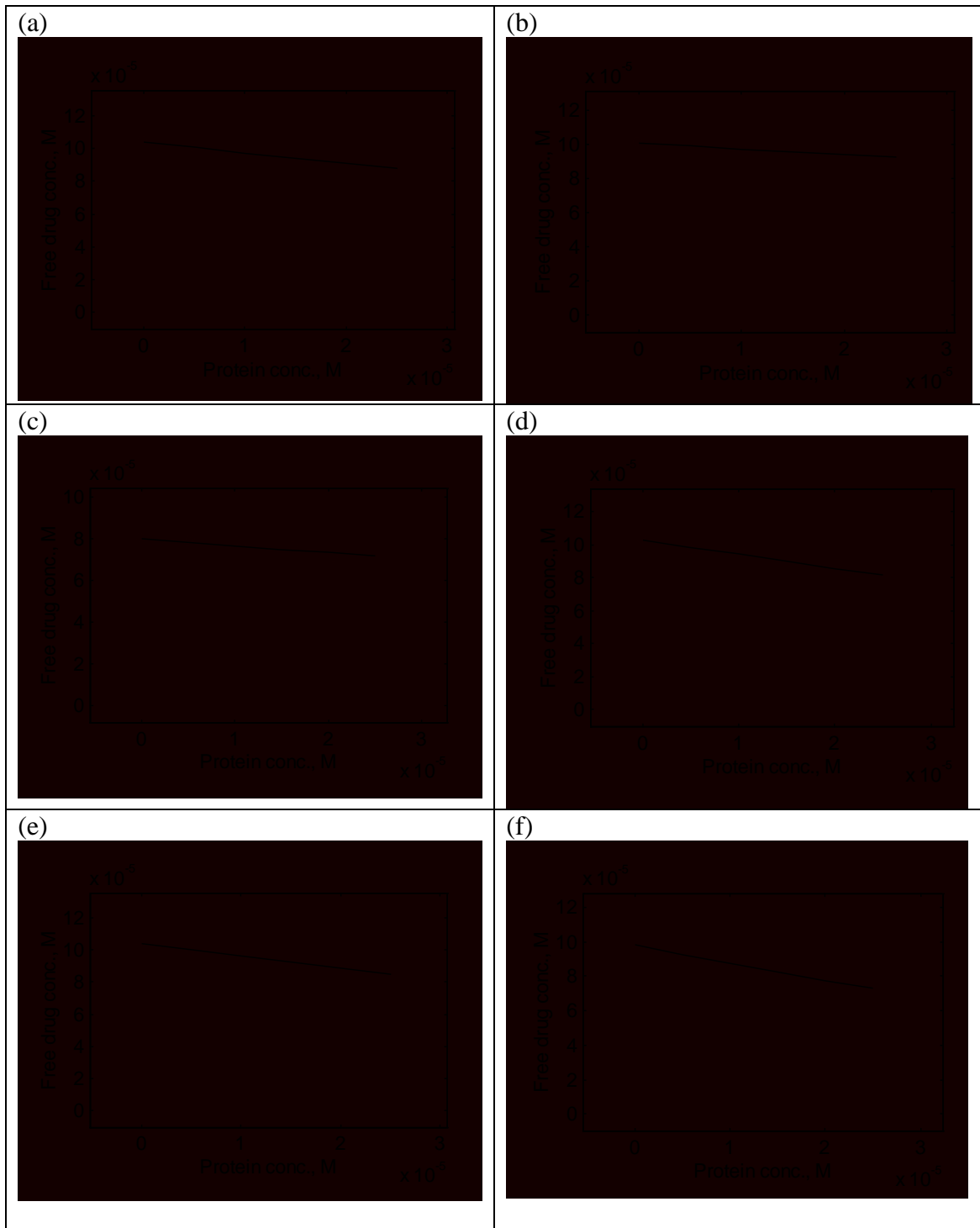| DRUG	K_{HSA} (M^{-1})	n_{HSA}	PB(%)	R^2	K_{AGP} (M^{-1})	n_{AGP}	PB(%)	R^2
Hexobarbital	$1.1\ 10^3$	1	34.7	0.93	$2.1\ 10^4$	1	14.1	0.94
Butabarbital	$1.2\ 10^3$	1	38.6	0.96	$5.3\ 10^3$	1	6.6	0.86
Mephobarbital	$1.1\ 10^4$	1	78.9	0.99	$3.7\ 10^4$	1	16.1	0.85
Oxazepam	$2.3\ 10^4$	1	95.1	0.99	$7.0\ 10^3$	1	11.5	0.87
Chlordiazepoxide	$6.3\ 10^4$	1	99.8	0.99	$7.0\ 10^4$	1	20.5	0.93
Diazepam	$7.1\ 10^4$	1	89.9	0.91	$1.4\ 10^4$	2	22.7	0.98

Figure 8: Binding curves. Drug-AGP relationships. (a) Hexobarbital, (b) Butabarbital, (c) Oxazepam, (d) Chlordiazepoxide, (e) Mephobarbital, (f) Diazepam.
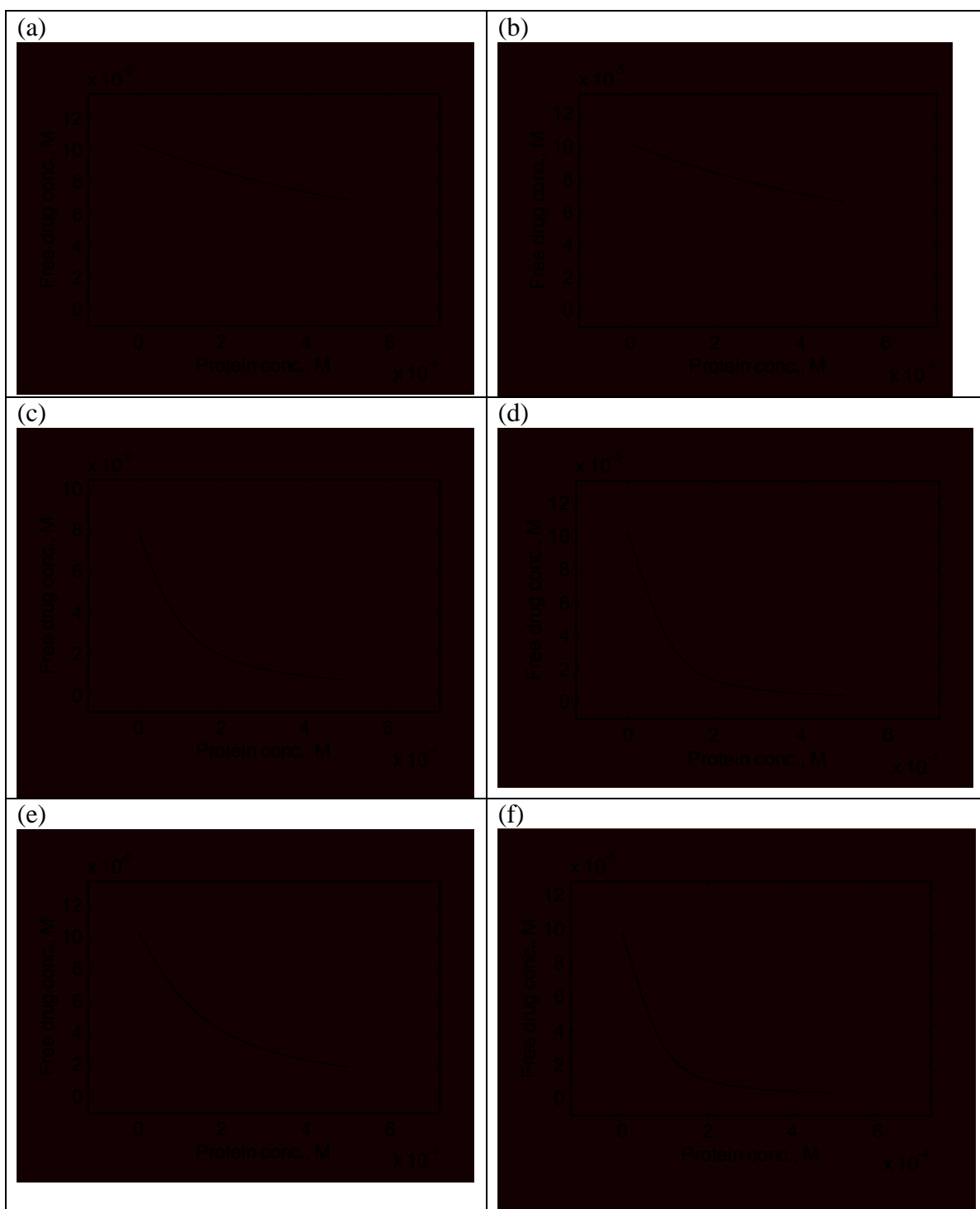
Figure 9: Binding curves. Drug- HSA relationships. (a) Hexobarbital, (b) Butabarbital, (c) Oxazepam, (d) Chlordiazepoxide, (e) Mephobarbital, (f) Diazepam.

To characterize the interactions of drugs with plasmatic proteins, the drug–protein binding percentage values to HSA (PB_{HSA}) and to AGP (PB_{AGP}) in physiological conditions were determined.

The free drug concentration was determined by the plateau height ratio between sample containing proteins and the sample containing only drug. To evaluate the interaction of drugs to the total plasma proteins, samples containing drug (100mM) and 200 µl of tested human plasma were prepared. The mixture was injected in the CE system. The free drug concentration was determined by the plateau height ratio between the ultrafiltrated mixture and the drug solutions.

Table 4: Protein-binding values of barbiturates and benzodiazepines to HSA, AGP and whole plasma proteins.

	PB_{HSA} (%)		PB_{AGP} (%)		$PB_{Whole\ plasma}$ (%)	
	Observed	Bibliographic	Observed	Bibliographic	Observed	Bibliographic
Hexobarbital	34.7		14.1		50,2±0,1	42-52 [116]
Butabarbital	38.6		6.6		24,5±0,5	<26[119]
Mephobarbital	78.9		16.1		60,2±0,1	59-67[120]
Diazepam	89.9		22.7		100	98,7±0,2[121]
Chlordiazepoxide	99.8		20.5		100	94-97[121]
Oxazepam	95.1		11.5		100	98,8±1,8[121]

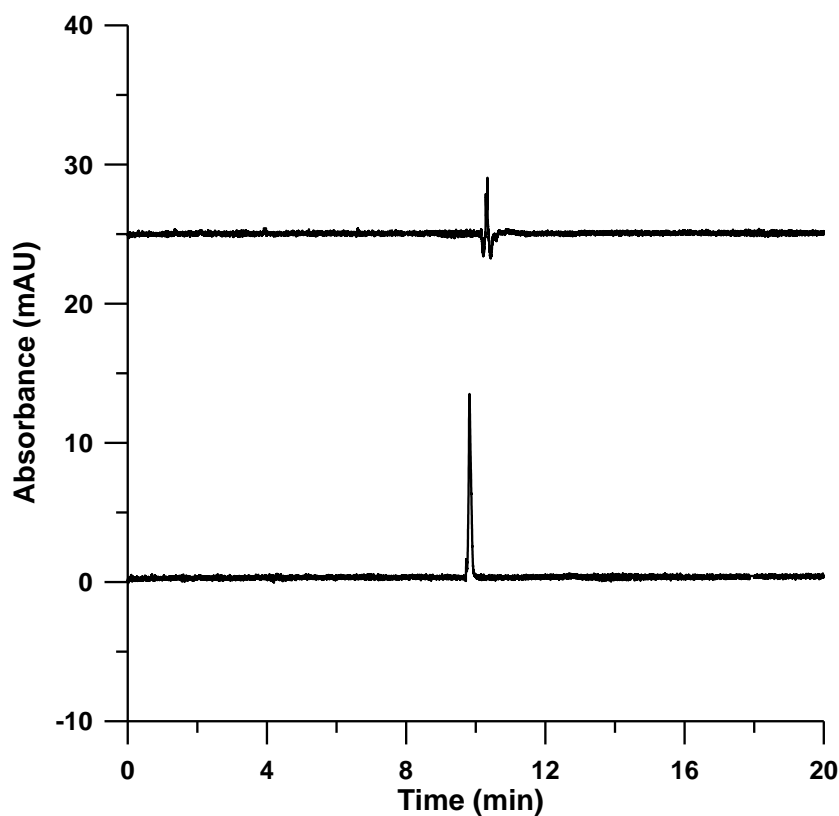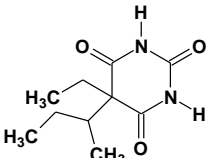As an example fig. 10 shows the electrophoreograms obtained for oxazepam.
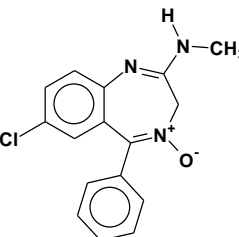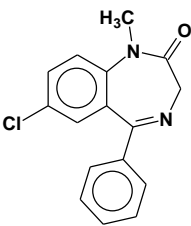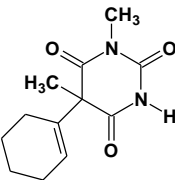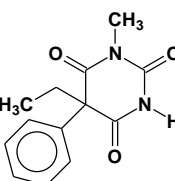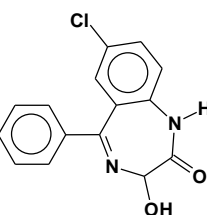
Figure 10: Representative electrophoreogram of the binding of oxazepam to plasmatic proteins . The sample solutions contained 100μM oxazepam in phosphate buffer; 100μM oxazepam in phosphate buffer and plasma from bottom to top. The injection was performed in the long-end capillary by 50 mbar vacuum for 4 s and a running voltage of 15 kV. UV detection was performed at 240 nm. The other experimental conditions are the same as before.

Table 5: Structure, logarithm of octanol-water partition coefficients (log$P$), protonation constants (pK_a), retention data in biopartitioning micellar chromatography (log k_{BMC}), molar volume (V_M) and molecular weight (M_W) of the compounds studied

Compound	CAS Number	Structure	log$P$	pK_a	log k_{BMC}	V_M (cm^3)	M_W (g/mol)
Butabarbital	125-40-6		1.65	7.9 (A)	1.36	193	212,2
Chlordiazepoxide	58-25-3		2.44	4.76 (B)	1.59	231	336,2
Diazepam	439-14-5		2.80	3.3 (B)	1.65	226	284.74
Hexobarbital	56-29-1		1.49	8.2 (A)	1.37	193	236.3
Mephobarbital	115-38-8		1.84	7.8 (A)	1.48	203	246.3
Oxazepam	604-75-1		2.24	11.6 (A) 1.7 (B)	1.47	202	286.7

Protonation constant for and acidic (A) and for a basic (B) group.

Table 3 shows, that all observed drugs – diazepam, oxazepam, chlordiazepoxide, hexobarbital, mephobarbital and butabarbital present interaction with AGP and HSA. The number of primary binding sites to HSA and AGP for the majority of drugs was close to one. In relation to affinity constants, benzodiazepines showed similar affinity to HSA and AGP while in case of barbiturates the affinity constants towards AGP were higher than towards HSA.

Table 4 shows the protein-binding values of benzodiazepines and barbiturates to HSA and AGP. As it can be observed, the PB values of benzodiazepines to HSA were close to 100 % while for barbiturates these values ranged between 35 and 79%. The differences observed between benzodiazepines and barbiturates are not so high to AGP, the values ranged between 7-23 %.

The PB whole plasma protein values obtained for benzodiazepines and barbiturates and bibliographic data available have been included. As it can be observed, the result obtained agreed with the bibliographic values taken from different sources. On the other hand, the protein- binding values of benzodiazepines to the whole plasma proteins were 100 % and mainly, they bound to HSA, to a lesser extent to AGP. For barbiturates PB values to whole plasma proteins was 24-60 %, and also bind mainly to HSA and lesser to AGP.

The following step was to evaluate the importance of some of the physicochemical parameters of drugs in the drug–protein interaction. Table 5 shows the structure and physicochemical characteristics of compounds studied. Benzodiazepines are basic drugs with similar hydrophobic character, the logarithm of octanol–water partition coefficient ranged between 2.24 and 2.80. Barbiturates are weak acids also with similar hydrophobic character – log P ranged between 1.49-1.84. In this table the retention data of compounds in biopartitioning micellar chromatography (BMC), are included. These values could be considered as a measure of the hydrophobicity of compounds in near physiological conditions [104, 122]. The molar volume and molecular weight values have been included in order to consider steric factors.

4. CONCLUSIONS

All observed drugs – diazepam, oxazepam, chlordiazepoxide, hexobarbital, mephobarbital and butabarbital present interaction with AGP and HSA. The number of primary binding sites to HSA and AGP for the majority of drugs was close to one. In relation to affinity constants, benzodiazepines showed similar affinity to HSA and AGP while in case of barbiturates the affinity constants towards AGP were higher than towards HSA.

The analysis of the results obtained for PB_{HSA}, PB_{AGP}, $PB_{whole\ plasma}$ revealed that the hydrophobic character of compounds expressed as $logP$ and/or $\log k_{BMC}$ is the most important factor to describe drug–protein interactions. That is why benzodiazepines bind to HSA with larger extent than barbiturates. The rest of variables were less important or unreliable with the present data. Fig. 11 shows the variation of PB_{HSA}, PB_{AGP}, $PB_{whole\ plasma}$ protein with $\log k_{BMC}$ of compounds. As it can be observed, the PB of basic drugs to proteins increased with increasing $\log k_{BMC}$, which is the hydrophobicity of compounds.
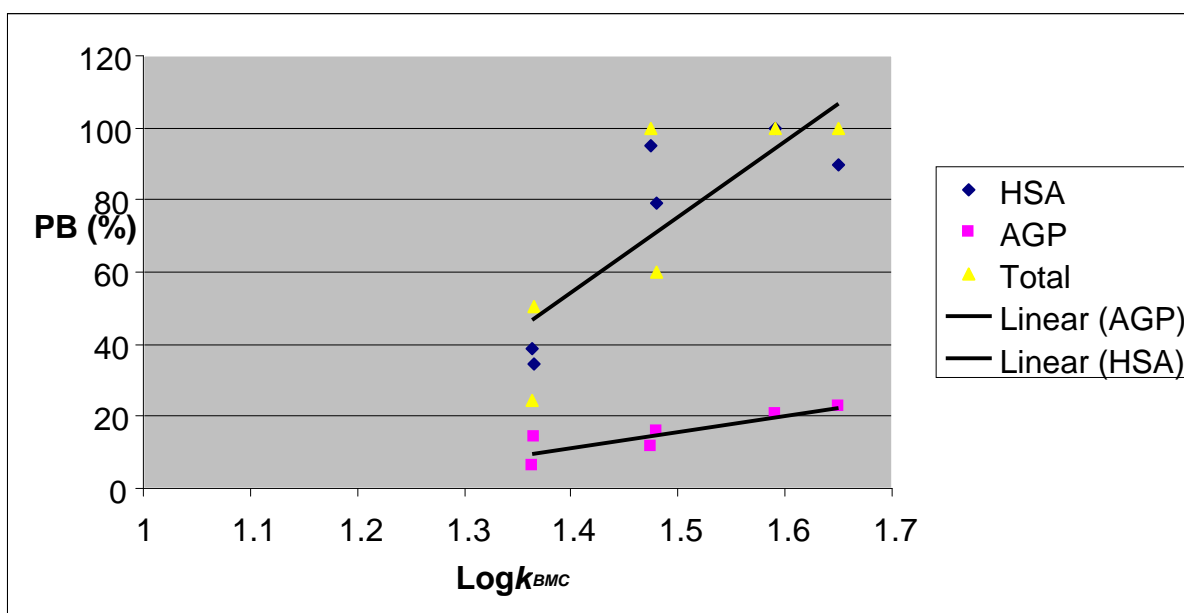

Figure 11: Variation of PB_{HSA}, PB_{AGP}, $PB_{whole\ plasma}$ protein with log k_{BMC} of compounds.

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ABSTRACT

An important topic in the drug discovery and development process is the role of drug binding to plasma proteins. In this diploma thesis the characterization of the interaction between benzodiazepines and barbiturates towards human serum albumin and α-1-acid glycoprotein under physiological conditions by capillary electrophoresis-frontal analysis is presented. Furthermore, the binding of these drugs to all plasma proteins is evaluated by using ultrafiltration and capillary electrophoresis. The results indicate that the hydrophobic character of compounds seems to be the key factor on the interaction between these drugs towards proteins. In fact, hydrophobic basic drugs (benzodiazepines) bind in great extension to HSA, while less hydrophobic acid drugs (barbiturates) present lower interactions with proteins and bind especially to AGP.

ABSTRAKT

Důležitým směrem výzkumu při vývoji nových léčiv je jejich vazba na plasmatické proteiny. Tato diplomová práce charakterizuje interakce vybraných barbiturátů a benzodiazepinů s lidským sérovým albuminem a alfa glykoproteinem za fyziologických podmínek metodou kapilární elektroforézou – frontální analýzou. Navíc je sledována vazba vybraných barbiturátů a benzodiazepinů celkově ke všem proteinům plasmy kapilární elektroforézou po provedení ultrafiltrace. Výsledky ukazují, že rozhodující faktor pro vazbu na sérové proteiny je hydrofóbní charakter sloučenin.