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Review

Role of biological matrices during the analysis of chiral drugs by liquid chromatography

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Abstract

The review article covers advances of chiral drugs analysis by high-performance liquid chromatography (HPLC) methods achieved during last 10 years. Emphasis is given to various aspects of influence of biological matrix in pharmacodynamics, pharmacokinetics, HPLC analysis. Discussed is composition of main biological matrices from the point of view of potential interferences to above-mentioned fields of study. Beside typical analytical approaches to chiral recognition in HPLC, sample pretreatment and/or clean-up by conventional extraction procedures, column switching (CSW) techniques using restricted access materials (RAMs), microdialysis (MCD) is discussed. Measurement of unbound drug concentration and discussion of column maintenance and remedy is an additional source of information and field where knowledge on complex properties and interactions of biological matrix is usefully applicable.

Keywords: Reviews; Biological matrices; Pharmacodynamics; Pharmacokinetics; Chiral drugs

Contents

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1. Introduction

More than one-half of marketed drugs are chiral: that is their molecules contain one or more chiral centers. Many drugs developed from organic synthesis are still administered as a racemic mixtures which may even disturb other biological processes and may cause catastrophic side effects [1]. Chirality is common to reality, ranging from molecules to human beings and from objects to processes. Enantiomers behave differently in a chiral environment, such as biological systems or a chiral synthetic medium. Enantiomers exhibit diferent biological physiological and biochemical behaviour and may exert different pharmacodynamic, pharmacokinetic and toxicologic activities in a biological environment, although they have identical chemical and physical properties, except those bound to their space structure, e.g. the property of rotation of polarized light.

In the nature, via biological systems, asymmetry and optical activity are widely distributed as is illustrated, e.g. by sugars, proteins, enzymes and receptors. Thus, it is to be expected that chiral drugs and metabolites having stereogenic centres interact stereospecifically with biological systems. In fact, enantiomers are often behaving differently undergoing the processes of transport, absorption, distribution, metabolisation and elimination from the body [2,3].

The enantiomeric separation and analysis of chiral drugs have become essential for many reasons. Enantiomers cannot be separated using conventional analytical methods. In order to discriminate between enantiomers it is necessary to introduce, similarly to biological systems, an asymmetric or chiral environment and this can be achieved in a number of ways.

The most widely used approach in quality control of pharmaceuticals and pharmacokinetic studies of drugs is high-performance liquid chromatography (HPLC) separation, which has become a powerful and important technology also in various other fields.

HPLC methods devised for chiral separation can be classified into three categories:

(i) direct method using a chiral stationary phase (CSP);
(ii) separation on an achiral stationary phase by adding a chiral agent to the mobile phase which then forms adducts with the enantiomeric analytes (chiral mobile phase additive method (CMPA)); and
(iii) separation of the diastereomers formed by precolumn derivatization with a chiral derivatization reagent (indirect method) [4].

At first sight is apparent, that biological matrices and enantioselective or general interactions of drugs with their components can adversly influence the result of analysis. However, knowledge obtained from investigation of these interactions can be and actually is useful for the separation system design.

From the point of view of simplicity and clarity, the interactions among atoms and molecules are classified [5] into strong and weak. The classification is made on the basis of the energy liberated during the formation of an associate. Decisive interaction energy is about 40 kJ/mol.

The distance between interacting subsystems is shorter for strong interactions (0.08–0.2 nm, ΔH −40 to −400 kJ/mol, ΔS contribution to the Gibbs energy inversely depends on ΔH) and longer for weak interactions (for weak around 0.2–0.5 nm, ΔH −0.01 to −40 kJ/mol, ΔS is always significant), for which also geometric and electronic structure of interacting units change very little during associate formation. Product formed by weak interactions is called van der Waals molecule, or now supramolecule.

Intermolecular interactions play a key role also in liquid chromatography, influencing final resolution of the system via influence to efficiency, selectivity and retention. They influence either isolated molecules of analytes, or matrix component or functional groups of stationary phase by conformation changes and steric effects. Observable is presence of these interactions in liquids (mobile phases) where they influence bulk associations (e.g. solvophobic effects, micelle formation, etc.). These interactions are expressed also by interaction of a molecule with a set of different molecules, known as solvation phenomena, partitioning of molecules between two immiscible liquids (or liquid and chemically-bonded phase), physical sorption and solubilization of an inert gas in a liquid. Association of a few molecules creating true van der Waals molecules, or hydrogen-bonded systems, or electron donor–acceptor complexes performes at even more complex levels of interactions.

In separation systems used for analysis of biologically active substances in a biological matrices, all this complicated interactivity is present at once and considerably influences our chance to forecast the result of their influence to analytical signal, or chance to take consequences via correct interpretation of observed phenomena.

The determination of drugs, metabolites and endogenous compounds in protein-containing biological fluids (e.g. whole blood, plasma, cerebrospinal fluid, etc.) by direct injection of the sample into the LC system is very complicated owing to the high concentration of proteins and the large number of endogenous compounds present in this kind of samples. The major challenge is the removal of macromolecules (e.g. proteins) to avoid damage of expensive and smart chromatographic columns. Proteins in the biological fluids can precipitate or denature and adsorb onto the packing material, leading to backpressure build-up, changes in retention time and decreased column efficiency and capacity. The extent of sample pretreatment depends on the complexity of the sample, and is especially important when analysing drugs and endogenous compounds in biological matrices, such as plasma, urine or tissue homogenates [6,7].

With development of new analytical methods, there are growing new alternatives for direct injections of biological

samples into the chromatographic columns. A microdialysis (MCD) as a sampling methodology seems to be very perspective method which enables continuous monitoring of free drug concentrations in different body compartments [8].

The more manipulations the samples undergo prior to the quantitation step, the lower is the accuracy and precision obtained. It is advantageous to inject the sample directly into the liquid chromatographic system without off-line isolation procedures. It is still challenge for the bioanalytical chemist to develop methods where the sample preparation step can be automated and integrated with the LC procedure.

On-line column switching (CSW) devices combined with advanced separation media technology (restricted access media (RAM)) represent a powerful and reliable solution to the automated clean-up and trace enrichment of drugs in complex biological samples with a consequent improvement in the analytical process [9].

In the most of cases, the authors present only the best chromatograms from the analysis, but there is not enough information about the column behaviour after injection of a large volume of biological sample. In due to the complexity of handled problems not only a number, but also a variability of partial problems, which are needed to solve, is growing. The problem solving is depending on different conditions. Therefore, particular solutions are always unique and it is needed to take into account different approaches. This review considers an influence of biological matrices in the analysis of chiral drugs from different angles of view. It includes a specific discussion for the drug interactions in organism, the mutual interactions between drug and analytical system as well as different approaches for removal of interfering macromolecular compounds of biological samples.

2. Drug stereochemistry: general considerations

The new trend toward development of enantiospecific drugs has increased the interest in enantiospecific pharmacokinetic studies of chiral drugs, mainly in the case where only one of the two enantiomers is responsible for the pharmacologic activity. Enantiospecific bioassays are also useful in investigation of pharmacokinetic behaviour of two enantiomers when a given drug is marketed as a racemate [10].

Many biologically active substances exist as single enantiomers in organisms, and the stereochemistry plays an important role. The enantiomers administered as drugs have often different activities, toxicities and pharmacokinetic properties, because macromolecular substances such as enzymes and receptors have inherent chiral selectivity in biological systems [11].

The enantiomers share essentially the same physicochemical properties, including refractive index, melting point, boiling point and solubility. Because enantiomers exist as mirror images, they interact with plane-polarized light to an equal extent but in oposite direction. However, three-dimensional structure differences between the enantiomers can lead to significant biochemical differences [12].

A molecule containing a single chiral center can exist in two different forms which are mirror images of each other and are nonsuperimposable [13]. The mirror images may also be a result of a chiral axis or chiral plane. Enantiomers are stereoisomers that contain identical type and number of atoms, but they differ in a spatial arrangement. Enantiomers have identical physical and chemical properties, but they behave differently in a chiral environment involving also polarized light.

Enantiomers of chiral drugs may differ considerably in their pharmacological and toxicological effects because they interact with biological macromolecules, the majority of which are stereoselective. For chiral drugs, the most important addition to the normal requirements for drug approval is the need to justify on chemical, preclinical and clinical grounds the choices of the stereoisomeric forms for marketing [12].

Stereoisomers that are not mirror images of each other are called diastereomers. They can have similar chemical properties but different physical properties.

Because of the chiral environment of living systems, two enantiomers may exhibit the same pharmacological activity to different degrees, or may exhibit entirely different kinds of chemical or pharmacological activities. One good example is propranolol that has different pharmacodynamic and pharmacokinetic profiles for *R*- and *S*-enantiomers. *S*-propranolol is 100 times more potent than *R*-propranolol as a beta-blocker, because *S*-propranolol has a longer plasma half-life than *R*-propranolol.

The ability of our body to distinguish between two enantiomers of a compound was recognized very early and some well-known examples are found in the different smells and tastes of the optical antipodes of structurally rather simple organic compounds. A direct enantiomer-discriminating action by the receptor proteins means that the chiral binding site of the receptor is preferentially occupied by one of the enantiomers due to a stronger binding interaction. While direct effects are simple and easy to comprehend, the situation is more complex when it comes to the different actions of drug enantiomers.

3. Understanding to drug interactions with biological systems

Enantioselectivity plays an important role not only in pharmacodynamics involving the interaction of bioactive agents with macromolecules (enzymes and receptors) in the target organs, but also in pharmacokinetics, involving transport, absorption, distribution, metabolic conversion and excretion of the drug. The different pharmacodynamics and

pharmacokinetics of eutomers (isomers with higher affinity) and distomers (isomers with lower affinity) in racemates lead to variety of effects [14]. For instance, the distomer sometimes exhibits undesirable side effects, such as the hallucinogenic effect of (*R*)-ketamine, the mutagenicity of (*R*)-penicillamine and the teratogenicity of (*S*)-thalidomide [15]. Drug action is the result of pharmacological and pharmacokinetic processes by which it enters, interacts and leaves the body.

Stereoselectivity in biological activity may be related to the drug–receptor interaction or to the pharmacodynamics of the agent. It may also be due to differences in pharmacokinetics. Different enantiomers may have different plasma concentration–time profiles as a result of chiral discrimination in pharmacokinetic processes. These differences may be further influenced by other factors such as the route of administration, the age [16,17] and sex of the subject [18], disease states [19] and genetic polymorphism in drug metabolism [12].

Stereoselective disposition and/or dynamics were important in explaining anomalous concentration effect and drug–drug interaction findings with racemic drugs. The problems occur when the pharmacodynamic effect of the enantiomers is quantitatively and qualitatively significantly different, when enantiomers are metabolized by different enzymes and when these enzymes are either polymorphic in our population or can be induced or inhibited [11].

All the pharmacological activity may reside in one enantiomer, in which case the other enantiomer may be regarded to as an impurity [20]. The impurity may be inactive or have desirable or undesirable activity. Two isomers may have nearly identical qualitative and quantitative pharmacological activity. Enantiomers may have activity that is qualitatively similar but quantitatively different. Most drugs that exist as stereoisomers are in this category, e.g. warfarin, verapamil. Enantiomers may have qualitatively different pharmacological activity. Pairs of enantiomers may compete each with other for receptor binding. Pharmacokinetic differences between enantiomers are not as great as the pharmacodynamic differences but have to be considered in understanding drug disposition and the time course of drug action.

The rational design of novel drugs finds its basis in a thorough understanding of the interaction between a small ligand (hormone or drug) and a macromolecular target in the human body (e.g. a receptor or enzyme). Tools are available to study such interactions at a (sub)molecular level. Stereoselective is not only the interaction of enantiomers with biological macromolecules but also the enatiomers of a chiral drug can interact mutually in a different ways [1].

The interaction of a chiral drug molecule with asymmetric macromolecules, such as hepatic metabolizing enzymes and plasma proteins, results in the formation of transient diastereomeric complexes which might lead to differences in drug disposition profiles. Differences in the disposition of drug enantiomers usually result from differences in hepatic metabolism and plasma protein binding, since the processes of absorption, distribution and excretion would normally involve passive diffusion which would not be expected to be affected by drug chirality [2,21–23].

A number of mechanisms in the body can be stereoselective, among them first-pass metabolism, metabolic clearance, renal clearance, protein and tissue binding [24]. Differences in first-pass metabolism may cause differences in the ratio of plasma concentrations of enantiomers when a drug is given by the intravenous route compared with the oral route [21].

Perhaps the most interesting and certainly the most important of stereoselective processes in drug disposition is stereoselective metabolism. It is generally accepted that it is exactly drug metabolism that introduces the greatest degree of stereoselectivity into drug disposition. Any alteration of the activity of the enzymes involved in metabolism of clinical compounds can effect stereoselective disposition. Different scenarios have to be taken into account:

(1) both enantiomers of a chiral drug can be metabolized by the same enzymes at different rates (verapamil). Induction or inhibition of these enzymes will affect metabolism of both enantiomers, but possibly to a different extent;
(2) enantiomers of some chiral drugs (such as warfarin) are metabolized by different enzymes [11].

The pharmacokinetics and metabolism of an increasing number of drug enantiomers following administration of the racemate have been documented and there are now many examples of stereoselectivity in the processes of drug absorption, distribution, metabolism and excretion.

The potential advantages of using pure enantiomeric drugs are:

- less complex and more selective pharmacological profile;
- greater therapeutic index;
- less complex pharmacokinetics;
- less complex drug interactions; and
- less complex plasma concentration–response relationships [21].

Stereoisomers of a drug may be metabolized by two different enzyme systems, resulting in different rates of metabolic clearance; age and sex may also affect the rates of enzymatic metabolism of stereoisomers [24]. Drug binding to specific plasma transport proteins (human serum albumin (HSA), α_1-acid glycoprotein (AGP), lipoproteins) is an integral part of many other types of intermolecular interactions in a cellular or organ environment. Different aspects of drug–protein interactions have been reviewed recently including their molecular nature, biological functions, pharmacological significance as well as methodological approaches applied and their potential shortcomings.

Substantial differences in the protein binding of two enantiomers may result in a difference in their glomerular filtration rates, when one enantiomer is more highly bound to plasma proteins, it will have a lower free fraction and

free drug concentration in the plasma than the other enantiomer, resulting in decreased filtration of the more highly protein-bound enantiomer. It combines both passive and active processes, including glomerular filtration, passive reabsorption, active secretion and reabsorption and renal metabolism, but unlike absorption, these may be bidirectional and dependent on protein binding.

Clinically important differences occur in the binding of various drug enantiomers to plasma proteins. The extent of stereoselective plasma binding of drugs ranges up to a factor of about 1.5, reflecting a diastereomeric association with both albumin, the major binding protein for most acids (warfarin, ketoprofen [25], zopiclone [26]) and with AGP, which binds predominantly basic compounds (verapamil [27], propranolol [28]). Propranolol illustrates a case where the stereoselectivity of binding occurs in opposite directions for the different proteins. Plasma binding can independently affect both distribution and elimination of the drug. The plasma protein binding is stereoselective for many chiral drugs [29].

Pharmacokinetics describes the time course of total plasma or serum concentrations. Modern pharmacokinetic theory underlines the importance of knowledge about the unbound fraction of drug for better understanding concepts of clearance and distribution.

Protein binding is important in many processes that determine the eventual activity and fate of a drug once it has entered the body [30]. Some examples of such binding include the interactions of a drug with a target enzyme or receptor. Another type of interaction is the binding of pharmaceutical agents with blood proteins. These interactions can be important in determining the overall distribution, excretion, activity and toxicity of a drug. In some instances, this protein binding occurs with general ligands, such as the interaction of many drugs with HSA or AGP. This binding is highly specific in nature.

4. Analytical approaches

Conventional analytical techniques for drug analyses often do not differentiate enantiomers, however one enantiomer of drug may be absorbed very well and/or metabolized and/or excreted at different rates than the other enantiomeric forms. The importance of stereoselective assays in pharmacokinetic and pharmacodynamic drug interaction studies is now widely recognized [31–34].

In a chromatographic assay, separation enantioselectivity can be achieved by:

- use of a proper CSP capable of separating enantiomers (direct method);
- use of CMPA (direct method);
- formation of a diastereomeric derivative through a reaction with another chemical substance possesing a stereogenic centre (indirect method).

Detection enantioselectivity via chiroptical detectors (e.g. circular dichroism, polarimetry) can aid the analysis in all cases where we are in doubt about changes in enantiomeric ratio after administration of pure enantiomer, or when chromatographic resolution of enantiomers falls below 0.6, or in many other cases (e.g. coelution of matrix component).

Potential source of useful ideas and concepts for HPLC can be found in (now complementar) methods creating background of chiral immuno- and radioimmuno assays.

Accepted explanation of fundamental mechanism for chiral recognition is based on mechanistic three-point fit model of Dalgliesh [35]. At least three interactions are required between the chiral selector and one of the enantiomers in the racemate to be separated [11,19,36,37]. At least one of these interactions must depend on the stereochemistry at the chiral center of the chiral selector and the enantiomer. Examples of the required interactions are hydrogen bonds, π–π interactions and dipole–dipole interactions.

4.1. Direct method

It relies on the formation of temporary diastereomeric molecular complexes between the drug enantiomers and a chiral discriminant (CSP or CMPA) or chiral environment within the chromatographic system [4]. Chiral discriminant must have an enantioselective affinity for the individual enantiomers. Discrimination between the enantiomers by the chromatographic system requires that one of the enantiomers undergoes a three-point interaction with the CSP or the CMPA. When this occurs, the other enantiomer is therefore able to undergo prevalently a two-point interaction.

4.1.1. Chiral stationary phases

Most of enantioseparations have been achieved with the direct methods that employ CSPs. During last two decades, the number of CSPs available for the separation of enantiomers has grown rapidly and made it possible to develop an analytical separation of nearly any racemic mixture [9,38]. Different types of chiral selectors have been used for CSPs, such as: proteins and enzymes [39–41]; polysaccharides and their derivates [42,43]; cyclodextrins or derivatized CD [12,44]; macrocyclic antibiotics [45–47]; molecularly imprinted polymers [48,49]; crown-ether [50,51].

Polysaccharide derivatives (cellulose esters, cellulose carbamates, amylose carbamates) belong to the most widely used CSPs for HPLC [52–56]. They can be successfully used not only in the common size HPLC columns but also in a miniaturized techniques. Although reverse and normal phase modes cover almost completely the potential applications of CSPs in HPLC, the polar organic mode becomes increasingly popular for the various CSPs during last few years. Popular pure organic eluents may offer the advantages of alternative chiral recognition mechanisms, higher solubility of some analytes, can be cheaper and easier to be removed from the analytes, however their compatibility with biological matrices can be questionable.

Protein-based CSPs (i.e. ovomucoid (OVM)[57–60], α_1-acid glycoprotein [61–63], ovoglycoprotein (OVG) [58,64–66], avidin (AVD) [57], flavoprotein (FLA) [57]) are of special interest because of their unique enantioselective properties and because they are suitable for separation of a wide range of enantiomeric forms. Protein CSPs (e.g. chymotrypsin CSP) [40,41] are compatible with aqueous mobile phases. Cellobiohydrolase I (CBH I) [56,60,67] immobilized on porous silica is supposed to be now the best CSP suited for resolving chiral drugs containing in their structure one or more basic nitrogen atoms and one or more hydrogen-acceptor or hydrogen-donor groups.

Although, the protein-based CSPs provide the possibility to use aqueous eluents (e.g. buffers with organic modifiers), disadvantageous features of these phases may be their tendency to loose enantioselectivity during the use for bioanalytical applications due to remnant contaminating matrix components as well as low separation capacity, lack of column ruggedness and limited understanding of the chiral recognition mechanism [39]. Insufficient column efficiency may also be caused by higher LODs [59].

In CSPs enantiomeric separation arises from the formation of transient diastereomeric complexes involving the chiral selector and enantiomers. The retention times of enantiomers depend on the differences in stabilities of these complexes. The drawbacks associated with this approach include the high price of most chiral columns and the fact that some chiral columns exhibit higher batch-to-batch variability in comparison to achiral columns, what probably relates to more complex chemical nature of CSPs. Column efficiencies generally tend to be lower than those for conventional HPLC columns, what corresponds to lower probability of optimal arrangement of functional groups of CSP and analyte during the enantioselective interaction needed for chiral resolution. The prediction of suitable phases is difficult and often based on an empirical experience. One of the most important criteria for the separation of enantiomers is the chiral selectivity expressed by a particular CSP, which is determined by the difference in the stability of the diastereomeric complexes formed between the individual enantiomers and the chiral selector.

Since there is no derivatization step in direct resolution, no racemization occurs during the reaction with a chiral tagging reagent. The direct method, based upon CSP columns seems to be preferable for the analysis of traces of antipode enantiomers in main components.

The choice of the best column for the separation of each racemate is difficult, because the separation higly depends on the interaction between CSP and enantiomer. Furthemore, the elution order of enantiomers is also dependent upon the CSP column used, and cannot be changed easily.

Surprisingly, there is almost no evidence in literature about investigation of achiral selectivity and the other chromatographic figures-of-merit related to possible interactions of potential achiral sample components with complex functional groups of CSPs. The reason is obvious, we are too focused to chiral analyte separation using CSPs and moreover why to waste expensive CSP, time and money for achiral separation? The authors opinion is that this approach could help to understand such phenomena as is CSP column poisoning by sample components, lifetime shortage, its remediation and maybe elucidation of its chiral selectivity, too.

4.1.2. Chiral mobile phase additive

The transient diastereomeric complexes formed between the drug enantiomers and the CMPA may be resolved on a non-chiral HPLC column. Various chiral mobile additives may be employed, including proteins (albumin, AGP), cyclodextrins, L-proline chiral counter ions [11,37,68].

The applicability of the method in the biomedical and pharmaceutical areas is limited because of purity of chiral selectors and potential problem with detection. CMPAs offer the advantage of using less expensive conventional columns and higher flexibility in proper additive selection step of optimization of chiral separation. In comparison to chiral columns, conventional columns are more rugged, more efficient and have higher sorption capacities. The most significant disadvantage is that large amounts of expensive CMPA or counter ion may be needed, what however can be solved by use of microcolumn HPLC.

4.2. Indirect method

This approach is based on a diastereomers formation by reaction of a pair of enantiomers with a pure chiral compound. These diastereomers are further separated using an achiral stationary phase or a CSP. Although the derivatization methods have been widely practiced, they suffer from potential racemization during the derivatization steps and can be used only when the enantiomeric molecule possesses an easily derivatized functional group. With respect to pharmaceutical applications, the indirect techniques have been used extensively for the enantioselective analysis of drugs in biological fluids. Review about precolumn derivatization and diastereomer formation has been published [13,69,70].

However, there is a trend to use the CSP in preference to either the derivatization CMPA approaches.

4.3. Chiroptical detection

Laser-based polarimetric detectors may open the field for the determination of enantiomer ratios using UV and polarimetric detection in series. The enantiomeric purity can be calculated even in a case of a significant degree of peak overlap.

Detection by polarimetry at one wavelenght is helpful to LC on optically active or inactive sorbents because of the (+) and (−) signs provided. The combination with simultaneous photometric detection can be used for the determination of optical purity, especially in case of overlapping peaks. The acquisition of circular dichroism spectra during LC means

that racemates can be applied instead of the antipodes, provided an analytical LC separation is accomplished. Advantage of this technique is its ability to determine relative configurations of interconvertible enantiomers [71–73].

4.4. Chiral immuno- and radioimmuno assays

The use of immunoassay techniques represents an alternative approach to the more common chromatographic methods for the determination of the concentrations of chiral drugs. The inherent stereoselectivity of the antibodies used appears to be an important parameter to be studied. The structural features of the drug, including the environment around the asymmetric center, the flexibility of the molecule and the ability of the molecule to undergo racemization, contribute to the molecule's ability to be recognized by stereoselective antibodies. These parameters are intrinsic and cannot be influenced by the investigator. On the other hand, other parameters can be modified to favor the raising of highly stereoselective antibodies [74].

5. Sample pretreatment

The determination of drugs, metabolites and endogenous compounds in protein-containing biological fluids by direct injection of the sample into the LC system is very complicated owing to the high concentration of proteins and the large number of endogenous compounds present in this kind of sample [5,6,75]. Proteins in the biological fluids can precipitate or denature and adsorb onto the packing material, leading to backpressure build-up, changes in retention time and decreased column efficiency and capacity. Sample preparation separates an analyte of interest from proteinaceous material, allowing the total amount of the analyte (protein-bound and free) to be determined and preventing or reducing the adsorption of protein and other interferences onto the analytical column. At the same time, the analyte is concentrated to improve sensitivity and detection capabilities. The major challenge is the removal of macromolecules, proteins, to avoid damage to chromatographic columns. During a deproteinization, there is necessary to take into account the fact that some fractions of drug can be free and the others can be bind to proteins. Except of this, solvents of deproteinization can interfere during the analytical determination. They must completely remove proteins and at the same time a precipitate must not bind a drug (absorption, precipitation, occlusion). A traditional sample pretreatment focused to removal of proteins is based on:

- precipitation with solvents (to prevent of protein precipitation on the chromatographic column during a contact with a mobile phase, which includes organic solvents or buffers);
- enzymatic deproteinization (employing proteolytic enzymes);
- ultrafiltration within membranes (separation of molecules according to their molecular weight on the membrane);
- precipitation by metal complex formation;
- salting-out; and
- thermal denaturation.

Sample pretreatment is very often the most time-consuming step of an analytical process. The extent of sample pretreatment usually depends on the complexity of the sample.

Plasma and serum samples are known to be the most complex biological matrices because they contain a very large number of endogenous compounds of different kinds, both low-molecular mass compounds of diferent hydrophobicity and macromolecules [75]. Proteins (such as albumin, AGP, lipoproteins) and other potential interferences (immunoglobulins) possess different physical and chemical characteristics and are present in matrices at high levels. The prevalent cation in blood plasma is sodium cation and the most important anions are chloride and hydrogencarbonate anions. Other cations Ca^{2+}, K^{+}, Mg^{2+}, as well as anions HPO_4^{2-}, $H_2PO_4^{-}$ and SO_4^{2-} are present at minor concentrations [76]. Contents of mineral compounds in a normal plasma is shown in Table 1. Functional compounds of blood plasma, which define its most important characteristics, are electrolytes and proteins (60–80 g/l). Electrolytes (Na^{+}, Cl^{-}, HCO_3^{-}) are responsible for its physical and chemical properties, such as osmotic pressure, volume and pH. Other plasma components are small organic molecules, which have only incosiderable influence on plasma properties, however as matrix constituents can cause in some instances serious problems. Table 2 offers brief summary of main plasmatic proteins.

Separation of analytes from matrices and detection at low concentration levels are generally required for an analytical method applied in pharmacokinetic and biopharmaceutical studies. The endogenous compounds, drugs and metabolites are retained on the extraction column and the amount extracted is affected by the affinity to the plasma proteins and the affinity to the internal surface of the extraction column. Some endogenous compounds in plasma are highly retained on the extraction column even when the column is eluted with a mobile phase containing a high concentration of organic modifier. The mobile phase composition and the flow-rate as well as wavelength of the detector play a very important role during the determination of the elution profile of sample matrix [75].

Urine of a healthy person contains small concentration of protein but it does contain major amounts of various salts, uric acid, creatinine and urea (for details see Table 3). When kidneys cannot operate properly, toxic materials accumulate in blood and other physiological fluids, leading to death within 10–12 days. Molar mass cut-off value of healthy men kidney (glomerular filtration) [77] is approximately 6000 (g/mol). However, at trace levels (ppm) urine contains hundreds of substances and at even lower levels (ppb) thousands of substances, which can interfere by coelution or cause in-

Table 1
Main inorganic compounds of normal blood plasma [76]

Substance	Concentration (mmol/l)	Concentration (mg/l)	Molar mass (g/mol)
Sodium [Na^+]	137.0–147.0	3.2×10^3–3.4×10^3	22.9
Potassium [K^+]	3.8–5.1	148.6–199.4	39.1
Calcium [Ca^{2+}]	2.3–2.8	92.2–112.2	40.1
Magnesium [Mg^{2+}]	0.8–1.3	19.5–31.6	24.3
Chloride [Cl^-]	98.0–106.0	3.5×10^3–3.8×10^3	35.5
Hydrogencarbonate [HCO_3^-]	24.0–35.0	1.5×10^3–2.1×10^3	61.0
Inorganic phosporus P (total)	0.7–1.6	21.7–49.6	30.9
Iron (Fe) in men	18.1×10^{-3} to 25.4×10^{-3}	1.0–1.4	55.9
Iron (Fe) in women	16.3×10^{-3} to 21.7×10^{-3}	0.9–1.2	
Iodine I (total)	275.0×10^{-6} to 630.0×10^{-6}	34.9×10^{-3} to 79.9×10^{-3}	126.9
Copper (Cu) in men	11.0×10^{-3} to 22.0×10^{-3}	0.7–1.4	63.6
Copper (Cu) in women	13.3×10^{-3} to 24.3×10^{-3}	0.9–1.5	

terferences to detection. In some cases (e.g. alkaptonuria, metabolic disorders) [77] concentration of certain substances can rise up to gram per liter levels (e.g. homogentisic acid), what should be also taken into consideration.

Saliva is an easily accessible and sometimes useful body fluid for therapeutic drug monitoring and for pharmacokinetic and pharmacodynamic studies. The total protein content of saliva is approximately one-tenth that of plasma [78]. Drug concentration in saliva correlates well with unbound drug concentration in plasma. Since saliva is one of biological fluids which can be collected by using a non-invasive method, the collection of saliva from patients is non-invasive, easy to realise and does not give any pain to patients. Pharmacokinetic parameters calculated from the disopyramide concentration–time data in saliva were also in good agreement with those from the unbound concentraton-time data. Drug concentrations in saliva may be regarded as equivalent to the plasma unbound concentrations on the elimination phase [79]. The usefulness of saliva for drug monitoring is limited to a selected array of drugs owing to intersubject and intrasubject variations of saliva–plasma concentration ratios. A drug should exhibit a constant saliva/plasma ratio that is independent of drug concentration, resistant to effects of salivary flow and consistent among individuals. The degree of drug protein binding is an important determinant of the availability of drug for diffusion [80].

Table 3
Main components of normal human urine [76]

Substance	Concentration (mg/l)
Sodium [Na^+]	3500
Hydrogen [H^+]	1500
Calcium [Ca^{2+}]	150
Magnesium [Mg^{2+}]	60.0
Chloride [Cl^-]	6000
Phosphates	1500
Sulphates	1800
Urea	20000
Uric acid	500
Creatinin	750
Ammonium [NH_4^+]	400

Table 2
Main macromolecular compounds of normal blood plasma [76]

Protein	Average concentration in plasma (g/l)	Average concentration in plasma (mol/l)	Molar mass (g/mol)
Prealbumin	0.1–0.4	1.6×10^{-6} to 6.5×10^{-6}	61000
Albumin	42.0	6.1×10^{-6}	69000
Acid α_1-glycoprotein	0.2–0.4	5.0×10^{-6} to 1.0×10^{-5}	40000
Apolipoproteins (globulins)	4.0– 9.0	2.0×10^{-5} to 3.0×10^{-6}	2×10^6–3×10^6
Haptoglobin (α_2-globulin)	1.0	1.2×10^{-5}	85000
Hemopexin (β_1-globulin)	0.7	1.2×10^{-5}	57000
Transferin (β_1-globulin)	2.9	3.8×10^{-5}	77000
Ceruloplasmin (α_2-globulin)	0.4	2.5×10^{-6}	160000
Transcortin (α_1-globulin)	0.04	8.1×10^{-7}	49500
Transcobalamin	94.0×10^{-8}	1.6×10^{-11}	60000
α_2-Macroglobulin	2.5	3.1×10^{-6}	820000
α1-Antitrypsin	2.5	5.0×10^{-5}	50000
Protein binding metal (α_1-globulin)	0.06	1.9×10^{-7}	308000
Antitrombin III (α_2-globulin)	0.2	3.1×10^{-6}	65000
Fibrinogen	4.0	1.2×10^{-5}	340000
Imunoglobulines (γ-globulins)	15.0–16.0	1.0×10^{-4} to 1.7×10^{-5}	150000–960000

In all the cases, microbiological contamination of the samples can cause either change of important characteristics of the samples or introduction of new substances–metabolites. Suppression of the microorganisms can be done by deep freezing, membrane filtration, chemical conservation or physical treatment (thermal, photochemical, gamma and X-ray irradiation sterilization). However, we should always consider that every one of these approaches can potentially influence also substances of interest.

5.1. Conventional extraction procedures

The most commonly used sample preparation techniques include liquid–liquid extraction (LLE), protein precipitation and solid-phase extraction (SPE). These methods are labor intensive, increase the total analysis time and reduce the total recovery of the analyte of interests [6,7,81,82].

The extraction techniques like LLE are laborious, time-consuming and difficult to automate. They require relatively large sample volumes ($\geq$1 ml) and there is need for an internal standard due to a loss of analyte during a multi-step sample manipulations. Moreover, the next disadvantage can be a production of emulsions with organic solvents; large quantities of organic solvents which are often expensive, toxic, carcinogenic and hazardous to the environment; a relatively low extraction efficiency and in some cases; insufficient reproducibility and a possibility of contamination.

Although there is a lot of disadvantages of LLE, it is still employed for sample pretreatment of biological samples concerning their simplicity of performing (appropriate choose of organic solvents, pH, etc.) and it is still using together with SPE for enantioselective as well as achiral determination of chiral drugs (see Table 4). An ideal sample preparation technique should be solvent-free, simple, inexpensive, efficient, selective and compatible with a wide range of separation methods.

SPE became a widely used laboratory technique used for sample preparation instead of deproteinization or LLE [82]. SPE on disposable extraction cartridges can be directly coupled to HPLC. SPE columns provide higher chemical selectivity than conventional LLE based only on different solubility or distribution rates. An attractive feature of SPE is the availability of various extraction materials (including numerous siloxane-bonded materials with different functional groups, porous synthetic polymers, immunosorbents and RAM), which favour and incorporate different types of interactions.

In typical SPE, the sample is passed through a minicolumn filled with an appropriate extraction material. In the most frequent case of drug trace analysis, compounds of interest are retained on column while interferences are washed away. The analytes are recovered by eluting the column with a proper solvent. SPE can also be automated and coupled on-line to LC [83]. SPE has also some important limitations: plugging of the cartridge or blocking of the pores by matrix components, high elution volumes and batch-to-batch variations. Moreover, it is a multi-step process and is therefore suspected to analyte loss. Finally SPE often involves a concentration step through solvent evaporation and in this way it is not applicable to the extraction of volatile or thermolabile compounds.

Compared with the solid-phase cartridge technique, the disc solid-phase technique is relatively new with advantage of reduced bed mass (less capacity of retaining impurities), no channeling, large flow area and rigid structure [84]. Mixed-mode discs (combination of hydrophobic and cation-exchange extraction mechanisms) achieved a higher reproducibility SPE, reduction of volume of organic solvents and higher selectivity [85].

In spite of the low capacity of disc, it has been applied for determination of methadone enantiomers in serum [118,119]. The particle-loaded membranes and particle-embedded glass fiber disks referred to generically as disk technologies, are examples of alternative formats to the cartridge design for SPE [82].

Davankov and co-workers [120,121] described a microporous hypercrosslinked polystyrene sorbent Styrosorb for extraction of non-polar compounds with extremely high adsorption capacity, which readily adsorbs both nonpolar and polar compounds. It has been used, for example, in sample clean-up of propranolol from serum and for stereoselective determination of (*R*, *S*)-propranolol [122] as well as analysis of propranolol and its metabolites in blood plasma [121]. Great hydrophobic surface area and the small pore sizes makes the material suitable for use as a restricted access packing.

An alternative is the so-called micro-SPE (SPME). The dimensions of the sorbent beds are minimised in order to carry out the extraction in a disc or a packed pipette tip. In exhaustive extractions with a solvent or on a solid-phase the equilibrium between matrix compounds and drug is disturbed, leading to a shift towards the freely dissolved fraction. An overview of the SPME applications for the analyses of biological samples is described by Theodoridis et al. [81].

The application of molecularly imprinted polymer (MIP) as chromatographic material in SPE [123] provides a combination of polymer mechanical and chemical robustness with highly selective molecular recognition comparable to biological systems. Problems with peak broadening and tailing peaks play in this case only a minor role.

Boos et al. [124] described a multi-dimensional SPE sample-processing platform (so-called Six-S ProcEdure (Six-SPE)), which involves a size-selective sample preparation step followed by a solvent-switch. As a first application, they combine LiChrospher ADS RP-18 and Tramadol imprinted polymer for on-line analysis of the analgesic drug tramadol in human plasma.

5.2. Column switching techniques

A sample clean-up and an enrichment of traces of the investigated compounds must be usually carried out prior to

Table 4
HPLC analysis of chiral drugs in biological matrices using LLE sample pretreatment

Drug	Matrix	Sample clean-up	Analytical column	LOQ	Detection	Influence of matrix	Reference
Citalopram	Human plasma	LLE	Chirobiotic V	5 (ng/ml)	FSC	–	[86]
Propranolol	Human urine	LLE	Chiralcel OD	0.1 (μg/ml)	FSC	–	[87]
Trimipramine	Human serum	SPE	Chiralcel OD-R	10 (ng/ml)	UV	–	[85]
Ondansetron	Human serum	SPE	Chiralcel OD-R	7 (ng/ml)	UV	–	[88]
Halofantrine	Erythrocyte pellets	LLE	Chiralpak AD	25 (ng/ml)	FSC	–	[89]
Celiprolol	Human plasma	LLE	Puresil C18	5 (ng/ml)	FSC	Non-directly	[90]
Salbutamol	Human urine	LLE, SPE	Chirex™ 3022	10.4–10.8 (ng/ml)	FSC	Non-directly	[91]
Bisoprolol	Human plasma,urine	LLE, SPE	Chiralcel OD	2 (ng/ml)	FSC	Non-directly	[92]
Oxprenolol	Rat plasma	LLE	Spherisorb C18 ODS-2[a]	2.5 (ng/ml)	FSC	–	[93]
Citalopram	Human plasma	LLE	Chiral-AGP	1 (ng/ml)	UV	Yes	[94]
(±)-Ethopropazine	Rat plasma	LLE	(α-*R*-naphtyl)ethylurea	25 (ng/ml)	UV	–	[95]
Praziquantel	Human serum	SPE	Chiralcel OJ-R	5 (ng/ml)	UV	–	[84]
	Human plasma	LLE	Chiralcel OD-H	5.10 (ng/ml)	UV	–	[96]
Bupivacaine	Human plasma, ultrafiltrate	SPE	Chiral-AGP	8.10 (ng/ml)	UV	–	[97]
Thalidomide	Human plasma	SPE	Bioptic AV-1	50 (ng/ml)	UV	–	[98]
Propafenone	Human plasma	SPE	Chiralcel OD-R	25 (ng/ml)	UV	–	[99]
Labetalol	Human plasma, urine	SPE	Chirex™ 3022	1.5–1.8 (ng/ml)		–	[100]
Losigamone	Human plasma	LLE	Chiradex LiChroCart	0.0078 (μg/ml)	UV	–	[101]
Diltiazem	Human plasma	LLE	Ultron-OVM	1.8, 3.2 (ng/ml)	UV	–	[102]
Ketoprofen	Human plasma	LLE	Chiralpak AD	0.025 (μg/ml)	UV	–	[103]
Verapamil + metabolites	Human serum	SPE	Chiral-AGP	1.0–5 (ng/ml)	FSC	–	[104]
Tramadol	Human urine	LLE	Chiralpak AD	80 (ng/ml)	UV	–	[105]
	Human plasma	LLE	Chiralcel OD-R	0.5 (ng/ml)	FSC	–	[106]
Verapamil	Human plasma	LLE	Ultron OVM	–	UV	–	[107]
Clenbuterol	Human plasma	LLE	Chirobiotic T	0.25 (μM)	UV	–	[108]
Metoprolol	Human urine	SPE, LLE	Hypersil5 C18	5 (ng/ml)	FSC[b]	Non-directly	[109]
Mefloquine	Human plasma	LLE	ODS[b]	250 (nmol/l)	FSC, UV[b]	–	[110]
	Capillary blood	FSC		10 (nmol/l)			
Ketoprofen	Human plasma, urine	LLE	Chiralpak AD	–	UV	–	[111]
Metoprolol	Human plasma	LLE	Chiralcel OD	4 (ng/ml)	FSC	–	[112]
Clenbuterol	Bovine retinal tissue	LLE	LiChrospher 100 RP-18	5 (ng/ml)	EC	–	[113]
Propafenon	Human plasma	LLE	Chiralpak AD	25 (ng/ml)	UV	–	[114]
Metoprolol	Human plasma	LLE	Chiralpack AD	1 (ng/ml)	FSC	–	[115]
		SPE	Chiralcel OD-H	5 (ng/ml)			
Pantoprazol	Human serum	SPE	Chiralcel OJ-R	0.10 (μg/ml)	UV	–	[116]
Amlodipine	Human plasma	SPE	Chiral AGP	0.5 (ng/ml)	EC	Non-directly	[117]

FSC: fluorescence detection; EC: electrochemical detection; (–) influence of biological matrix not mentioned within the article.
[a] Derivatization with S-NEIC:S(–)-1-(1-napthyl)-ethylisocyanate.
[b] Derivatization procedure with FLEC:-(–)-1-(9-fluorenyl)ethyl chloroformate.

analysis. CSW is a powerful technique for the clean-up and separation of multi-component mixtures [6,7,125,126]. The CSW includes all techniques by which the direction of the mobile phase flow is changed by valves, so that effluent from a primary column is directed to secondary column for a defined period of time. The transferred fraction must be reconcentrated to reduce the dispersion of the analyte within the chromatographic system and the dead volumes in the connections between columns and in switching valves must be minimized to achieve maximum separation efficiency. Desorption of the analytes from the precolumn should occur in as small volume of mobile phase as is possible to maintain. If the analytes on the precolumn are strongly retained, the elution may require a high proportion of organic modifier. This type of solvent system can cause a problem in the analysis step. One limitation with conventional types of precolumns is that the content of organic solvent that can be used in the mobile phase is limited. High organic solvent concentration can cause buffer precipitation, which can clog the precolumn and/or connecting capillaries [126]. Moreover, concentration of organic modifier (methanol, acetonitril, isopropanol, etc.) higher than 15–20% precipitate plasma proteins, especially albumin, which can be present in blood or plasma sample at high concentration levels.

The simplest configuration consists of two columns connected by a switching valve and this has been succesfully applied in many sample clean-up procedures [125,126]. Two main problems are associated with direct injection of proteineous samples: strong and irreversible adsorption of some proteins combined with protein denaturation resulting in a rapid pressure build-up at the head of the column. Resolution decreases and the column lifetime is substantialy shortened. The principle of CSW for sample clean-up is to trap, in a primary column or precolumn, the fraction of the sample that contains the analytes. In most of cases, biological samples are injected onto the precolumn, which retaines only small molecules. Interfering macromolecular compounds are eluted to the waste whereas the cut-off effluent containing the analytes is diverted to the secondary column or analytical column, where they are separated for identification and/or quantification. The fraction of effluent to be further transferred may elute at the front (front-cut technique), in the middle (heart-cut technique) or at the end (end-cut technique) of the poorly resolved zones from primary precolumn [125,127]. Zone cutting probably is one of the most useful and versatile of all CSW techniques. The different CSW sets-up were described in several reviews [7,125,126,128].

The simplest configuration described for processing of biological samples by CSW is so-called straight-flush mode. Samples are initially chromatographed by precolumn, the unwanted components being directly vented to waste. By switching usually a six-port valve, the cut fraction of eluate containing the analytes is directed to the analytical column for further separation. A more powerful system can be achieved by operating in back-flush mode. This technique reverses the mobile phase flow through the precolumn, so the analytes retained at the head of the precolumn are directly transferred to the analytical column. The precolumn can also be back-flushed to remove the components of the matrix that are strongly retained, after the fraction of interest has been eluted; thereby the analytical column is protected from contamination by late-eluting matrix components. The back-flush configuration also minimizes peak broadening.

CSW is used for

- trace enrichment of selected analytes;
- improvement of resolution of part of a complex sample (optimal resolution can be achieved by using different separation modes, stationary phases and mobile phases);
- increase of sample throughput by using heart cutting, back-flushing, front- or end-cutting and recycle chromatography

The potential of the on-line CSW technique lies not only in the selection of different modes for the primary and secondary steps but also in the ability to use different stationary and mobile phases to provide separation selectivity and adjustment of retention factors of analytes. An important consideration in CSW is the requirement that the mobile phases must be compatible [125,126].

With a single mobile phase, columns may be of the same type but with different phase ratios or of different types. The advantages of an isocratic mobile phase in CSW include

- ability to use one pump;
- elimination of column regeneration time required in a gradient elution; and
- capability to use gradient-incompatible detectors.

The major disadvantage is that only a few components can be analyzed because of the limited peak capacity provided by isocratic HPLC analysis.

The optimization of the chromatographic conditions should include careful choice of the type of precolumn (dimensions and packing), eluent conditions and system design, to obtain good recovery and minimum peak broadening of the compounds of interest. The best enrichment factors can be achieved by back-flushing the analytes from the precolumn to the analytical column [7,125,126]. Various precolumns with different hydrophobicities are available that have different extraction properties with respect to apolar sample components.

The goal of integrated sample preparation and analysis is the complete elimination of matrix components that would otherwise interfere with the separation and quantification of the analytes. Optimization the fractionation step consists of achieving as large a time interval as possible between t_M (elution of the sample matrix) and t_A (start of the elution of the analyte) on the precolumn [8,129].

The CSW can tolerate many injections if the special precolumn packings are applied and ensure that the valve-switching times are set properly to flush the proteins from the precolumn completely. In a typical application of CSW, you can select the precolumn to either retain the compound of interest or to allow it to pass unretained. Moreover, CSW system definitely provides advantages over single-column systems in terms of their selectivity (ability to couple precolumns and analytical columns of different selectivities), detection sensitivity (analyte enrichment due to larger sample volumes and reduced number of interfering peaks) and higher variability of mobile phases and detection modes. Employing reversed-phase packing in precolumn proteins, salts an other highly polar compounds are eluted to the waste, while from the other hand, endogenous compounds are retained by precolumn before their injection to the analytical column [6,7,125,129].

The on-line achiral–chiral systems were developed for the determinations of metoprolol in human urine [130]; verapamil and its metabolites in serum [104]; tramadol enantiomers in human urine [105]; verapamil in human plasma [131]; eliprodil in human plasma and urine [132]. Two different types of CSW set-up (single-pump system with ovomucoid CSP as well as two-pumps set-up with teicoplanin CSP) were compared for determination of propranolol enantiomers in rat blood microdialysate [59].

5.3. Restricted access media

Conventional reversed-phase columns cannot tolerate larger volumes of biological fluids than several μl and chromatographic performance of such columns deteriorates very rapidly. The backpressure also increases after injection of small volumes of biological fluids on such columns [129,133–135].

A special type of packing material, so-called RAM were developed. Desilets et al. [136] introduced the term restricted access media, which expresses the limited accessibility of macromolecular sample compounds to the adsorption sites of porous supports surface due to their molecular size and are eluted in the column void volume. RAM are tailor-made porous packing materials that prevent macromolecules such as proteins from penetrating the pores but allow free access for low molecular weight compounds. The different types of RAM have been classified and reviewed in some publications [137–140]. Beside size exclusion, which plays the dominant role in the restricted retention of proteins, irreversible protein binding and accumulation is prevented by the generation of a protective, non-adsorptive, hydrophilic outer packing surface by a protein coating [141–143], polymer coating [138,144,145], or enzyme-catalyzed hydrolysis [146].

New kind of silica-based RAM has been tested by Chiap et al. [147]. The outer surface of RAM contains hydrophilic diol groups, while sulphonic acid groups are bound to the internal surface, which gives the sorbent the properties of a strong cation-exchange towards low molecular mass compounds (atropine, fenoterol, ipratropium, procaine, sotalol, terbutaline). The internal surface reversed-phase (ISRP) packing has a hydrophobic or ion-exchange partitioning phase on the internal surface and a hydrophilic diol phase on the external surface. Gisch [144], and Feinbush and Santasania [148] introduced shielded hydrophobic phase (SHP) which has a hydrophilic polymer through the bonding of polyethylene oxide to porous silica with hydrophobic groups embedded in the polymer. Polyoxyethylene (or polyethylene glycol (PEG)) network with a phenyl phase enclosed within the polymer mesh. Williams and Kabra [149] reported novel dual-zone materials (DZM) having perfluorobutylethylene dimethylsilyl (PFB) and octadecylsilyl groups on external and internal zones, respectively. A hydrophilic perfluorobutyethylene dimethylsilyl phase is bound to the outer surfaces of the silica and alkyl-bonded phase on the inside of the packing.

The semi-permeable surface phase (SPS) is based on the non-ionic surfactants coated on reversed phase silica. Polyoxyethylene was both adsorbed hydrophobically (through the use of non-ionic surfactants) and covalently bonded to reversed-phase HPLC packings, thereby establishing a semi-permeable hydrophilic layer over the alkylsilane [138,150].

Mixed-functional phases (MFP) combine different chromatographic modes or different functionalities on a uniform support surface. It is based on hydrophilic materials that are nonadsorptive for proteins, that is, porous glass materials modified by glyceryl (diol) ligands or porous polymeric supports (copolymers of oligoethylenglycol and methacrylates) [151].

Rudolphi and Boos [133] compared several RAM packings used in a single- and coupled-column mode and discussed the advantages and disadvantages of the two approaches for the determination of epirubicin and its metabolites in human plasma.

Alkyl-diol silica (ADS) sorbents, the ISRP sorbent, introduced by Boos et al. [146] which has been developed for use as precolumn packing and for on-line liquid–solid extraction (LSE) and enrichment of hydrophobic compounds in proteinaceous fluids by classical reversed-phase or ion-pair chromatography.

The on-line LSE with the RAM is based on the complete non-adsorptive size-exclusion of macromolecules (such as proteins) and on the simultaneous extraction and enrichment of low-molecular weight target compounds (such as drugs). LC-integrated sample clean-up of fluids using such materials like LiChrospher RP-ADS and coupled CSW has some advantages: repeated direct injection of untreated fluids, quantitative removal of the matrix, on-column enrichment of analytes, quantitative, matrix-independent analyte recovery, no requirement for addition of an internal standard, total automation, safe handling of hazardous or infectious samples, high number of analysis cycles and low costs per analysis [152].

LiChrospher RP-ADS has a pore diameter of approximately 6 nm (physical diffusion barrier) and excludes spherical macromolecules (e.g. proteins) having a relative molar mass larger than 15,000 in the void volume of a packed column. It possesses two chemically different surfaces. The outer surface (glycerylpropyl, i.e. diol moieties) of the support is hydrophilic, electroneutral and non-adsorptive towards proteins. This means that the protein matrix of a biological sample can be directly flushed into the waste as the applied precolumn excludes the macromolecules in the void volume. Meanwhile, the analyte fraction having free access to the binding centers at the hydrophobic internal surface of the porous particles is selectively extracted and enriched at the stationary phase of the precolumn. The bonded phase which exclusively covers the internal pore surface of a glyceryl-modified silica is a butyryl-(C-4), capryloyl-(C-8) or stearoyl-(C-18) moiety. The broad hydrophobic retentive capability of these packings permits chromatographic determinations of a wide variety of compounds in biological matrices (Table 5). Out of the three types of LiChrospher RP-ADS phases covering the whole range of hydrophobic retention factors, the most suitable precolumn for a given analyte has to be determined in each specific case [135].

As compared to Pinkerton sorbents GFFII (ISRP type) [127], ADS sorbents proved the higher adsorption capacity. For example, the lifetime of ADS exceeds more than 200 injections of 500 μl of plasma) [161]. Centrifugation of sam-

Table 5
HPLC analysis of chiral drugs in biological matrices by sample pretreatment using LiChrospher ADS precolumn in column switching technique

Drug	Matrix	LiChrospher ADS precolumn	Analytical column	Reference
Methylphenobarbital	Human plasma	RP-18	Chiralcel OJ-R	[153]
Atenolol	Human urine	RP-18	Chirobiotic T	[154]
Ketoprofen	Plasma	RP-18	Chiral-HSA	[155]
8-Metoxypsoralen	Plasma	RP-8	LiChrospher RP-18	[156]
Drugs and metabolites[a]	Plasma, serum	RP-18	LiChrocart RP-18	[152]
Propafenon	Serum	RP-18	Gromsil ODS AB	[157]
Pirlindole	Human plasma	RP-4	Chiralcel OD-R	[158]
Propranolol	Blood	RP-8	Ultron-OVM	[59]
	Microdialysate	RP-18	Chirobiotic T	
Bupivacaine	Plasma	RP-18	Kromsil C18	[159]
Arachidonic acid	Urine	RP-18	Hypersil ODS	[160]
Cortisol	Plasma		LiChrospher 100 RP18	

[a] Talinolol, celiprolol, metoprolol, trimterene, trimethoprim, tiracizine, articaine, detajmium, ajmaline, lamotrigine.

ples, use of in-line filters and addition of organic modifiers to the mobile phase can prolong the life of precolumns and analytical columns.

Although the aforesaid recommended pretreatmets and procedures for an optimal column lifetime were maintained, some problems with a change of retention times or increase of the precolumn backpressure occured during the analyses of selected analytes with different hydrophobicity and lipophilicity (atenolol, pindolol, propranolol) arosen. Detailed investigation of sample behaviour on ADS precolumn has been discussed by Mišlanová and Hutta [162]. Similar effects have been described by Lamprecht et al. [154] for enantioselective HPLC analysis of atenolol in human urine and plasma samples.

Yu and Westerlund [163] have studied the chromatographic behaviour of two types (ADS and SPS supports) of RAM in CSW system with regard to peak performance, retention and column backpressure. There were observed no differences between the individual columns. The results indicate that the surfaces of packings have been altered by proteins after loading a large quantity of plasma. Being adsorbed on the sealings and/or the external and internal surfaces through hydrogen bonding, proteins may act as new surfaces.

The increase of column dead volume may mainly be attributed to these effects and broadening or deformation of inert peaks observed on all columns after plasma injection supports the hypothesis of the change of surface characteristics. It seems that majority of pores available to the analyte, i.e. the hydrophobic ligand, is not affected by such adsorption.

Unlike ADS sorbents, BioTrap 500 is a new generation column, which is based on the same basic principles, but the surface chemistry is different. The particles have a biocompatible outer surface and C18 groups on the surface within the pores. The biocompatibility has been obtained by attaching the plasma AGP to the outer surface of the silica particles [141,142,164–166].

This type of sorbent has been applied for determination of ibuprofene, naproxen, propranolol, carbamazepine and phenytoin in human plasma [167]. Two different extraction columns have been tested, BioTrap Amine C18 (for extraction of basic drugs) and BioTrap Acid C18 (for extraction of acidic drugs). The external surfaces are identical, but the internal surface of amine precolumn is end-capped for minimization of peak broadening on the extraction column.

By combining the Chiral-AGP chromatography of amlodipine with the achiral HPLC on a narrow bore column Zorbax SB-Ph in a coupled-column system a sensitive on-line assay for determination of amlodipine enantiomers was obtained. Since the peaks from the Chiral-AGP were broad, they were trapped on short hydrophobic reversed-phase columns CT-sil Phenyl before final chromatography with high sensitivity electrochemical detection [117].

6. Measurement of unbound drug concentration

Measurement of the total drug concentration does not provide the needed information concerning the unbound fraction of drug in plasma, which is available for distribution, elimination and pharmacodynamic action [8,168,169]. Accurate determination of unbound plasma drug concentrations is essential in the therapeutic drug monitoring of drugs. Protein binding has a great influence on drug distribution, elimination and pharmacodynamic action. Highly plasma protein-bound drugs typically have a relatively low volume of distribution since their strong association with plasma proteins confines them to vascular spaces. On the other hand, drugs that remain largely unbound in plasma are generally available for distribution out of the vascular system. The volume of distribution is also affected by the magnitude of drug binding by tissue proteins. Unbound drug concentration is also important in consideration of drug clearance. Plasma protein binding is dependent on drug concentrations over the

therapeutic range. A drug–drug protein binding interaction is known to alter drug binding to plasma proteins. Changes in the fraction of unbound drug can result in changes in drug concentrations available at the biological target site (receptors/channels/membranes).

Many methodologies are available for determination of the extent of plasma protein binding of drugs. In the clinical evaluation of drug therapy, equilibrium dialysis, ultrafiltration and ultracentrifugation are the most routinely utilised methods [8,170]. The characterisation of the interactions between drug and protein molecules is essential for the assessment of the pharmacokinetic implications of drug–protein binding.

Equilibrium dialysis employs a dialysis cell that contains two reservoirs (with plasma and buffer) separated by a semi-permeable dialysis membrane. This membrane with various molecular weight cut-offs (MWCO) is available and should be selected on the basis of the molecular weight of the compounds and proteins of interest. After reaching equilibrium, the fluid from each reservoir is removed and analysed for drug concentration. Since the post-dialysis drug concentration in the buffer reservoir will be in equilibrium with unbound concentration, in the plasma reservoir, the drug concentration in the buffer is unbound concentration. Equilibrium dialysis is an in vitro technique and so used buffer solution is usually not physiologically identical to serum. A major disadvantage is that the drug concentration in plasma changes from its initial value. This is important for drugs exhibiting concentration-dependent binding. Equilibrium dialysis is also somewhat time-consuming.

The drug-containing plasma is placed in an ultrafiltration unit (two reservoirs separated by a filter) [171]. The filter allows low molecular mass substances of plasma pass through, while larger molecules, such as plasma proteins, are retained. As the plasma sample is filtered, the ultrafiltrate is considered to contain unbound fraction. A disadvantage of ultrafiltration is that the protein concentration in the plasma sample is concentrated as plasma water is filtered (concentration polarization of the filter). Only a small volume of ultrafiltrate should be collected to maintain an appropriate protein concentration in the reservoir. Small ultrafiltrate volume of drugs with extensive protein binding may produce an ultrafiltrate drug concentration that is below the limit of measurement of the assay method [8].

The use of microdialysis perfusion is proposed as an alternative to these techniques (e.g. dialysis, ultrafiltration) to study drug–protein interactions.

6.1. Microdialysis

This sampling methodology is undoubtedly an in vivo experimental alternative to the commonly used indirect in vitro methods, since it enables one to visualise free (i.e. protein-unbound) concentration, c_f, at the site of drug action, or eventually in tissues acting as drug depot compartments and/or to monitor biotransformation. Its potential is valuable also for chiral drugs, where different enantiomeric ratios in plasma and tissues may occur as a result of complex distribution phenomena or different metabolic pathways and reaction routes [172–174].

Microdialysis is based on the principle that solute diffusion between two compartments separated by a semipermeable membrane results from the concentration gradient of particular low molecular substance across the membrane. These two compartments are represented by the tissue extracellular fluid and the artificial physiological perfusion fluid inside the microdialysis probe. A MCD probe containing a dialysis membrane is surgically implanted in a blood vessel, fluid containing space or tissue space. The perfusion physiological fluid (the ionic strenght and pH will match those of the extracellular space) is continuously pumped at low flow rates through the probe and unbound drug in the blood or tissue fluid diffuses across the membrane into the probe via a concentration gradient. Endogenous (such as hormones) and exogenous (such as drug and its metabolites) compounds can cross the dialysis probes and can be collected over time and transported for the next analysis. On the other hand, large molecules (such as proteins and complexes drug protein) are excluded by the dialysis membrane.

MCD probes generally consist of an inlet tube, a semipermeable membrane and an outlet tube, and are commercially available in various sizes, design and materials [175]. Basically, there are four probe geometries: linear, loop, side-by-side and concentric. The choice of probe geometry depends on the site of placement. Linear and loop styles are typically suitable for sampling soft peripheral tissue such as liver, muscle, skin and tumor. Both side-by-side and concentric types of probe have the inlet and outlet tubes positioned in a parallel fashion, thus requiring only one-point of entry into the tissue. Dialysis membrane performance is characterized by the molecular weight at which 90% of the solute will be retained (preventing from permeating) by the membrane. This value is called the molecular weight cut-off. It is controlled by the size and shape of the pores in the membrane. Separation efficiency is influenced by pore size distribution and the presence of a substantial number of pores much larger than the average will allow leakage of high molecular weight solutes [169,174].

All dialysis membranes are made of highly biocompatible and inert materials, such as cellulose acetate, regenerated cellulose, polycarbonateether copolymer, polyacrylonitrile and polyther sulfonate. The MWCO expressed as a relative molecular mass is from 5000 to 100,000 thus allowing a variety of chemicals to be sampled by microdialysis [175].

One of the critical and most difficult questions in microdialysis is how to estimate the true concentration of an analyte in the interstitial fluid of the tissue from the measured concentration in the dialysate. The dynamic nature of microdialysis due to the continuous perfusion of dialysate fluid and removal of the analyte, normally results in analyte concentration in the dialysate being less than that in the extra-

cellular fluid. The ratio between these two concentrations is commonly expressed as a percentage and defined to be the relative recovery. Absolute recovery is defined as the mass of an analyte collected in the dialysate during a defined time period. The term recovery will be used hereafter to refer to relative recovery because it is most often the parameter of interest. The recovery expresses the degree of equilibrium reached [176].

Since equilibrium is not achieved, it is essential that drug recovery across MCD membrane is quantitated. An in vitro method accomplishes this by placing the MCD probe in a buffer containing a known concentration of drug. The drug concentration of the dialysate after perfusing the solution can be determined and used to calculate recovery. Concerning the relative recovery of intravenous (i.v.) microdialysis sampling, it has been shown previously by Lunte and co-workers that probe recoveries determined in vitro correspond well to probe recovery placed intravenously [172,173,177,178].

It is important to recognize that MCD sampling is a continuous process that results in drug concentration versus time data that are a reflection of the mean analyte concentration (concentration averaging) at the sampling site over a discrete time interval. This contrasts with traditional pharmacokinetic sampling in blood or tissue which yields instant drug concentrations at discrete time points.

There are many factors that can affect MCD probe recovery. Characteristics of analyte (molecular weight and charge), probe membrane (surface area, pore size, interaction between the analyte and the membrane) and perfusion medium (composition, flow-rate, temperature) will all affect the rate of diffusion [175].

The greatest advantage of using MCD is that free analyte (drug) concentration can be determined i.v. With MCD, c_f can be measured directly from the plasma or tissue fluid of interest over a desired time period.

In spite of volume limitations, MCD sampling provides several advantages over conventional techniques including a means of continuous sampling with no fluid loss. No internal standard is needed and drugs can be separated from enzymes that might catalyze their degradation. Moreover, the volume of the sample is constant and the concentrations of the components of the sample do not change during the experiment. The fact that no fluid is removed from or introduced into the system during the process is especially advantageous [174]. Microdialysis results in obtaining of minute quantities of relatively simple, basically protein-free matrix (according to the cut-off and chemical properties of the membrane used) [172,179–181] which in principle, could be injected directly into HPLC system via a sophisticated on-line interface [179,180] or simply via an off-line transfer [181–183]. On-line systems, reduce the problems associated with sample transfer and evaporation of the small volume microdialysates. On-line coupling of the analytical instrumentation to the sample flow allows for the analysis of samples in near real-time, providing rapid feedback of data.

However, from an analytical point of view, three inherent or potential problems could be identified by:

- usually relatively small recovery of the microdialysis probe used;
- low amount (fraction) of free drug to be analysed in case of highly protein-bound drugs; and
- low perfusion rates resulting in small sample volumes.

This means that relatively sensitive analytical techniques are required to measure drug concentrations, particularly for highly bound drugs.

The temporal resolution of these sampling intervals depends on the sensitivity of the analytical method employed and the time needed to perform the analysis [169]. Several analytical methods have been employed to measure compounds of interest in microdialysates. However, while MCD sampling is a continuous sampling method, LC requires discrete samples. The dialysate is collected over some fixed time interval to provide the required sample volume. The high temporal resolution of microdialysis is lost and becomes dependent upon the sample requirements of the chromatographic system. To minimize the sample volume required and therefore increase the temporal resolution of the experiment, microbore chromatographic systems are often employed with MCD sampling [179–181,184]. The on-line system provides a near real-time response to multiple chemical species in an awake, freely moving experimental animal [180].

A methodological risk may reside in contamination of an incorporated CSP by remnant low-molecular mass endogenous components of a microdialysate matrix which may lead to fouling of the CSP resulting in diminishing enantioselectivity. A general problem with chiral separations, especially in the case of protein-based columns (CBH-I, OVM, AGP) often resides in relatively poor efficiency, which causes a decrease of the peak height and hence a lower detection sensitivity. On the other hand, the teicoplanin CSP provided a good stability and efficiency for determination of propranolol enantiomers in rat blood microdialysate as compared to OVM-CSP [59]. Some authors [182,183] proved and validated already a rather complicated three-step procedure for stereoselective determination of β-blockers (propranolol, alprenolol, metoprolol) in microdialysates: initial enantioseparation was followed by trapping and on-column sharpening of the individual enantiomers on two separate achiral precolumns which were sequentially switched in-line to an achiral analytical column, increasing finally the separation efficiency and detection sensitivity (employing CBH-I CSP).

In studies of the distribution and metabolism of exogenous compounds (i.e. pharmacokinetic studies), there is no baseline concentration of the exogenous substance (i.e. drug and/or its metabolites) to which subsequent measurement can be related. The interpretation of data derived from pharmacokinetic studies utilizing MCD therefore requires an understanding of the qualitative and quantitative process involved.

7. Column maintenance and remedy

Despite analysts' best attempts to protect HPLC columns from foreign substances, certain analyte-matrix combination can effect stationary phases detrimentally. Contaminated columns can generate poor peak shapes, nonreproducible retention, high backpressures and baseline artifacts. Majors [185] discussed about cleaning procedures for bonded-silica as well as other types of reversed-phase columns in details. When contamination results from the accumulation of strongly retained substances from repeated injections, a simple washing process to strip these contaminants often can restore column performance. A recommended column washing system with organic solvents and reagents (such as 100% methanol, 100% acetonitrile, 100% isopropanol, etc.) that can break the interactions with the bonded phase or underlying silica surface can clean these columns. In case of protein residues from biological matrices (plasma, serum, urine), neat organic solvents such as acetonitrile or methanol do not dissolve peptides and proteins and are ineffective for cleaning reversed-phase columns. However, mixtures of organic solvents with buffer, acids (acetic acid, trifluoroacetic acid, etc.), bases (triethanolamine, etc.) and sometimes, ion-pairing reagent can be effective.

If metallic ions are sorbed to the silica or bonded phase, washing with organic solvents can fail to remove the column contaminants. A chelating reagent such as 0.05 mol/l ethylenediaminetetraacetic acid (EDTA), citric acid or the other chelating agents can be flushed through the column. The EDTA creates soluble complexes with many metallic species and dissolves them. If the sample matrix contains ionizable compounds, the change of pH could put them into an nonionized form and they can be flushed from the column with water–organic solvent mixtures.

We suggested the process of the precolumn treatment employing the cleavage effect of proteolytic enzyme pepsin (0.1% solution in 10 mM HCl, pH 2) for removal of potentially adsorbed protein molecules in combination with release of metals by EDTA complexation (1 mM, pH 6) for removal of metals (mainly Fe) [162]. Due to the fact that bioassays are run frequently using conventional stainless steel instrumentation (that gradually dissolves at trace levels) and also biological sample usually contains constitutional metals released during sample collection and/or treatment—formation of mixed complexes is probably the main source of troubles.

8. Conclusion

Role of biological matrices in the analysis of (not only) chiral drugs is ambiguous. Biological matrices are unavoidable items in very important fields of clinical (pharmacological, partly environmental and the other) analyses. They, their properties and intrinsic behavior, as well as their interactions with the analytes can serve as a potential source of topics for creative application in separation science, including HPLC. Knowledge of relations of drugs and biological matrices stereochemistry, composition and interactions as studied by various branches of science, e.g. pharmacodynamics and pharmacokinetics is also potentially transferable HPLC analysis of chiral drugs. However, more often we consider biological matrix as an enemy hindering our effort to separate and analyze similar substances—enantiomers, and not only them.

In the review article basic properties and composition of most common biological matrices of clinical and pharmacological interest are discussed. Analytical approaches to chiral HPLC of drugs are discussed and attempt to reveal matrix effect is presented. Surprisingly, in reviewed articles information on negative consequences of biological matrix is mentioned only seldom. The reason can be either perfect sample pretreatment and/or clean-up by conventional extraction procedures, column switching techniques using restricted access materials, microdialysis, etc. or good habit to publish only positive and acceptable results. Measurement of unbound drug concentration and discussion of column maintenance and remedy is an example of areas where knowledge on complex properties and interactions is usefully applicable.

9. Nomenclature

HPLC	high-performance liquid chromatography
LC	liquid chromatography
CSW	column switching
RAM	restricted access media
CSP	chiral stationary phase
CMPA	chiral mobile phase additive
HSA	human serum albumin
AGP	α_1-acid glycoprotein
CD	cyclodextrin
OVM	ovomucoid
OVG	ovoglycoprotein
AVD	avidin
FLA	flavoprotein
CBH I	cellobiohydrolase I
LOD	limit of detection
LOQ	limit of quantitation
LLE	liquid–liquid extraction
SPE	solid-phase extraction
LSE	liquid–solid extraction
SPME	micro solid-phase extraction
MIP	molecularly imprinted polymer
ISRP	internal surface reversed-phase
SHP	shielded hydrophobic phase
PEG	polyethylene glycol
DZM	dual-zone material
PFB	perfluorobutyethylene
SPS	semi-permeable surface
MFP	mixed-functional phase

ADS	alkyl-diol silica
MCD	microdialysis
MWC	molecular weight cut-off
EDTA	ethylenediaminetetraacetic acid
FSC	fluorescence
UV	ultraviolet
EC	electrochemical
i.v.	intravenous
c_f	free drug concentration
ΔH	enthalpy change
ΔS	entropy change
ΔG	change of Gibbs free energy

References

[1] Z.J. Li, D.J.W. Grant, J. Pharm. Sci. 86 (1997) 1073.
[2] A.J. Hutt, S.C. Tan, Drugs 52 (1996) 1.
[3] E.J.D. Lee, K.M. Williams, Clin. Pharmacokinet. 15 (1990) 109.
[4] J. Haginaka, J. Pharm. Biomed. Anal. 27 (2002) 357.
[5] P. Hobza, R. Zahradník, Weak Intermolecular Interactions in Chemistry and Biology, Academia, Prague, 1980.
[6] K.-S. Boos, A. Rudolphi, LC–GC 15 (1997) 602.
[7] D.J. Anderson, Anal. Chem. 65 (1993) 434R.
[8] J.D. Wright, F.D. Boudinot, M.R. Ujhelyi, Clin. Pharmacokin. 30 (1996) 445.
[9] K. Fried, I.W. Wainer, J. Chromatogr. B 689 (1997) 91.
[10] S. Andersson, S.G. Allenmark, J. Biochem. Biophys. Methods 54 (2002) 11.
[11] K.M. Rentsch, J. Biochem. Biophys. Methods 54 (2002) 1.
[12] S.M. Han, Biomed. Chromatogr. 11 (1997) 259.
[13] D.B. Campbell, Eur. J. Drug Metab. Pharamcokinet. 15 (1990) 109.
[14] T. Toyo'oka, J. Bioechem. Biophys. Methods 54 (2002) 25.
[15] T. Eriksson, S. Björkman, B. Roth, A. Fyge, P. Höglund, Chirality 7 (1995) 44.
[16] A.M. Vermeulen, F.M. Belpaire, E. Moerman, F. de Smet, M.G. Bogaert, Chirality 4 (1992) 73.
[17] J.B. Schwartz, H. Capili, J. Daugherty, Clin. Pharmacol. Ther. 55 (1994) 509.
[18] U.K. Walle, T.C. Fagan, M.J. Topmiller, E.C. Conrad, T. Walle, Br. J. Clin. Pharmacol. 37 (1994) 21.
[19] K.J. Skeith, A.S. Russell, F. Jamali, J. Clin. Pharmacol. 33 (1993) 1052.
[20] E.J. Ariens, Eur. J. Clin. Pharmacol. 26 (1984) 663.
[21] G.T. Tucker, M.S. Lennard, Pharmacol. Ther. 45 (1990) 309.
[22] J.H. Lin, Drug Metab. Dispos. 23 (1995) 1008.
[23] F. Jamali, J. Clin. Pharmacol. 32 (1992) 930.
[24] W.R. Crom, Am. J. Hosp. Pharm. 49 (1992) S9.
[25] N. Dubois, F. Lapicque, M. Abiteboul, P. Netter, Chirality 5 (1993) 126.
[26] C. Fernandez, F. Gimenez, A. Thuillier, R. Farinotti, Chirality 11 (1999) 1.
[27] S.A. Bai, S.M. Lankford, L.M. Johnson, Chirality 5 (1993) 436.
[28] H. Takahashi, H. Ogata, S. Kanno, H. Takeuchi, J. Pharmacol. Exp. Ther. 252 (1990) 272.
[29] T. Itoh, J. Maruyama, Y. Tsuda, H. Yamada, Chirality 9 (1997) 354.
[30] I. Fitos, J. Visy, M. Simonyi, J. Biochem. Biophys. Methods 54 (2002) 71.
[31] S. Allenmark, Acta Pharm. Nord. 2 (1990) 161.
[32] E.R. Francotte, J. Chromatogr. A 906 (2001) 379.
[33] N.M. Maier, P. Franco, W. Lindner, J. Chromatogr. A 906 (2001) 3.
[34] J. Ward, Anal. Chem. 72 (2000) 4521.
[35] C.E. Dalgliesh, J. Chem. Soc. 137 (1952) 3940.
[36] T.D. Booth, D. Wahnon, I.W. Wainer, Chirality 9 (1997) 96.
[37] C. Pettersson, Eur. Chromatogr. News 2 (1988) 16.
[38] B.-A. Persson, A. Andersson, J. Chromatogr. A 906 (2001) 195.
[39] J. Haginaka, J. Chromatogr. A 906 (2001) 253.
[40] G. Félix, V. Descorps, Chromatographia 49 (1999) 606.
[41] G. Félix, V. Descorps, Chromatographia 49 (1999) 595.
[42] E. Francotte, D. Huynh, J. Pharm. Biomed. Anal. 27 (2002) 421.
[43] H.Y. Aboul-Enein, Biomed. Chromatogr. 12 (1998) 116.
[44] S. Piperaki, A. Tsantili-Kakoulidou, M. Parissi-Poulou, Chirality 7 (1995) 257.
[45] T.J. Ward, A.B. Farris III, J. Chromatogr. A 906 (2001) 73.
[46] D.W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, J.-R. Chen, Anal. Chem. 66 (1994) 1473.
[47] D.W. Armstrong, Y. Liu, K.H. Ekborgott, Chirality 7 (1995) 474.
[48] L. Schweitz, L.I. Andersson, S. Nilsson, Chromatographia 49 (1999) S93.
[49] B. Sellergren, J. Chromatogr. A 906 (2001) 227.
[50] Y. Machida, H. Nishi, K. Nakamura, Chromatographia 49 (1999) 621.
[51] H.Y. Aboul-Enein, V. Serignese, Biomed. Chromatogr. 11 (1997) 7.
[52] K. Tachibana, A. Ohnishi, J. Chromatogr. A 906 (2001) 127.
[53] H.Y. Aboul-Enein, J. Chromatogr. A 906 (2001) 185.
[54] Y. Tang, Chirality 8 (1996) 136.
[55] B. Chankvetadze, E. Yashima, Y. Okamoto, J. Chromatogr. A 670 (1994) 39.
[56] E. Yashima, J. Chromatogr. A 906 (2001) 105.
[57] N. Mano, Y. Oda, N. Asakawa, Y. Yoshida, T. Sato, T. Miwa, J. Chromatogr. A 687 (1994) 223.
[58] J. Haginaka, H. Takehira, J. Chromatogr. A 777 (1997) 241.
[59] C. Mišl'anová, A. Štefancová, J. Oravcová, J. Horecký, T. Trnovec, W. Lindner, J. Chromatogr. B 739 (1999) 151.
[60] C. Vandenbosch, D.L. Massart, Anal. Chim. Acta 270 (1992) 1.
[61] M. Enquist, J. Hermansson, J. Chromatogr. 519 (1990) 285.
[62] D. Haupt, C. Pettersson, D. Westerlund, Fresenius J. Anal. Chem. 352 (1995) 705.
[63] A. Nystron, A. Karlsson, J. Chromatogr. A 763 (1997) 105.
[64] J. Haginaka, Y. Okazaki, H. Matsunaga, J. Chromatogr. A 840 (1999) 171.
[65] J. Haginaka, H. Takehira, J. Chromatogr. A 773 (1997) 85.
[66] J. Haginaka, H. Matsunaga, T. Tsukamoto, J. Chromatogr. A 830 (1999) 81.
[67] H.Y. Aboul-Enein, V. Serignese, Biomed. Chromatogr. 11 (1997) 47.
[68] G. Egginger, W. Lindner, C. Vandenbosch, D.L. Massart, Biomed. Chromatogr. 7 (1993) 277.
[69] T. Toyo'oka, Biomed. Chromatogr. 10 (1996) 265.
[70] X.X. Sun, L.Z. Sun, H.Y. Aboul-Enein, Biomed. Chromatogr. 15 (2001) 116.
[71] L. Thunberg, S. Andersson, S. Allenmark, J. Vessman, J. Pharm. Biomed. Anal. 27 (2002) 431.
[72] F. Brandl, N. Pustet, A. Mannschreck, J. Chromatogr. A 909 (2001) 147.
[73] M. Meyring, J. Muhlbacher, K. Messer, N. Kastner-Pustet, G. Bringmann, A. Mannschreck, G. Blaschke, Anal. Chem. 74 (2002) 3726.
[74] P.A. Got, J.M. Scherrmann, Pharm. Res. 14 (1997) 1516.
[75] Z. Yu, D. Westerlund, Chromatographia 44 (1997) 589.
[76] S. Trojan, et al., Lékařská Fyziologie, Grada Avicenum, Prague, 1994 (in Czech).
[77] P. Karlson, W. Gerok, W. Gross, Pathobiochemie, Academia, Prague 1987 (in Czech).
[78] R.K. Drobitch, C.K. Swensson, Clin. Pharmacokinet. 23 (1992) 365.
[79] K. Sagawa, S. Shimada, M. Shimizu, J. Muramatsu, Eur. J. Clin. Pharmacol. 52 (1997) 65.
[80] W.J. Jusko, R.L. Milsap, Ann. N. Y. Acad. Sci. 694 (1993) 36.
[81] G. Theodoridis, E.H.M. Koster, G.J. de Jong, J. Chromatogr. B 745 (2001) 49.

[82] C.F. Poole, S.K. Poole, D.S. Seibert, C.M. Chapman, J. Chromatogr. B 689 (1997) 245.
[83] J.D. MacFarlane, J. Autom. Chem. 19 (1997) 175.
[84] J. Liu, J.T. Stewart, J. Chromatogr. B 692 (1997) 141.
[85] J. Liu, J.T. Stewart, J. Chromatogr. B 700 (1997) 175.
[86] M. Kosel, C.B. Eap, M. Amey, P. Baumann, J. Chromatogr. B 719 (1998) 234.
[87] V.L. Herring, J.A. Johnson, J. Chromatogr. 612 (1993) 215.
[88] J. Liu, J.T. Stewart, J. Chromatogr. B 694 (1997) 179.
[89] E. Gorichon, C. Martin, K.N. Bangchang, J. Karwang, A. Thuillier, R. Farinotti, F. Gimenez, J. Chromatogr. B 712 (1998) 259.
[90] F.C.K. Chiu, K. Raymond, J. Chromatogr. B 687 (1996) 462.
[91] R. Bergés, J. Segura, X. de la Torre, R. Ventura, J. Chromatogr. B 723 (1999) 173.
[92] T. Suzuki, Y. Horikiri, M. Mizobe, K. Noda, J. Chromatogr. 619 (1993) 267.
[93] M.E. Laethem, M.T. Rosseel, P. Wijnant, F.M. Belpaire, J. Chromatogr. 621 (1993) 115.
[94] D. Haupt, J. Chromatogr. B 685 (1996) 299.
[95] M. Maboudian-Esfahani, D.R. Brocks, J. Chromatogr. B 715 (1998) 417.
[96] V.A.P. Jabor, G.M. Rocha, P.S. Bonato, J. Chromatogr. B 696 (1997) 307.
[97] K. Groen, P.W.M. Zeijlmans, A.G.L. Burm, J.W. van Kleef, J. Chromatogr. B 655 (1994) 163.
[98] A. Haque, J.T. Stewart, J. Liq. Chromatogr. Relat. Technol. 21 (1998) 2151.
[99] A.M. de Gaitani, V.L. Lanchote, P.S. Bonato, J. Chromatogr. B 708 (1998) 177.
[100] J.M. Dakers, D.W. Boulton, J.P. Fawcett, J. Chromatogr. B 704 (1997) 215.
[101] C.D. Torchin, W.D. Yonekawa, I.M. Kapetanovic, H.J. Kupferberg, J. Chromatogr. B 724 (1999) 101.
[102] G. Rosell, A. Camacho, P. Parra, J. Chromatogr. 619 (1993) 87.
[103] R. Lovlin, M. Vakily, F. Jamali, J. Chromatogr. B 679 (1996) 196.
[104] E. Brandšteterová, I.W. Wainer, J. Chromatogr. B 732 (1999) 395.
[105] B. Elsing, G. Blaschke, J. Chromatogr. 612 (1993) 223.
[106] M.A. Campanero, B. Calahorra, M. Valle, I.F. Troconiz, J. Honorato, Chirality 11 (1999) 272.
[107] Y. Oda, N. Asakawa, T. Kajima, Y. Yoshida, T. Sato, Pharm. Res. 8 (1991) 997.
[108] H.Y. Aboul-Enein, V. Serignese, Biomed. Chromatogr. 13 (1999) 520.
[109] F. Li, S.F. Cooper, M. Côté, J. Chromatogr. B 668 (1995) 67.
[110] Y. Bergqvist, M. Doverskog, J. Al Kabbani, J. Chromatogr. B 652 (1994) 73.
[111] R.A. Carr, G. Caillé, A.H. Ngoc, R.T. Foster, J. Chromatogr. B 668 (1995) 175.
[112] V.L. Herring, T.L. Bastian, R.L. Lalonde, J. Chromatogr. 567 (1991) 221.
[113] L.A. Lin, J.A. Tomlinson, R.D. Satzger, J. Chromatogr. B 762 (1997) 275.
[114] L.R.P. de Abreu, V.L. Lanchote, C. Bertucci, E.J. Cesarino, P.S. Bonato, J. Pharm. Biomed. Anal. 20 (1999) 209.
[115] V.L. Lanchote, P.S. Bonato, P.M. Cerqueira, V.A. Pereira, E.J. Cesarino, J. Chromatogr. B 738 (2000) 27.
[116] M. Tanaka, H. Yamazaki, Anal. Chem. 68 (1996) 1513.
[117] M. Josefson, B. Norlander, J. Pharm. Biomed. Anal. 15 (1996) 267.
[118] S. Rudaz, W. Haerdi, J.-L. Veuthey, Chromatographia 44 (1997) 283.
[119] S. Rudaz, J.-L. Veuthey, J. Pharm. Biomed. Anal. 14 (1996) 1271.
[120] V.A. Davankov, L.A. Pavlova, M.P. Tsyurupa, D.R. Tur, J. Chromatogr. B 689 (1997) 117.
[121] M. Beth, K.K. Unger, M. Tsyurupa, V.A. Davankov, Chromatographia 36 (1993) 351.
[122] D.O. Rumiantsev, T.V. Ivanova, J. Chromatogr. B 674 (1995) 301.
[123] L.I. Andersson, J. Chromatogr. B 739 (2000) 163.
[124] K.-S. Boos, C.T. Fleischer, Fresenius J. Anal. Chem. 371 (2001) 16.
[125] P. Campíns-Falcó, R. Herráez-Hernández, A. Sevillano-Cabeza, J. Chromatogr. 619 (1993) 177.
[126] R.E. Majors, K.-S. Boos, C.-H. Grimm, D. Lubda, G. Wieland, LC–GC 14 (1996) 554.
[127] F. Mangani, G. Luck, C. Fraudeau, E. Vérette, J. Chromatogr. A 762 (1997) 235.
[128] K.A. Ramsteiner, J. Chromatogr. 456 (1988) 3.
[129] Z. Yu, D. Westerlund, Chromatographia 44 (1997) 589.
[130] K.H. Kim, H.J. Kim, J.S. Kang, W. Mar, J. Pharm. Biomed. Anal. 22 (2000) 377.
[131] Y. Oda, N. Asakawa, T. Kajima, Y. Yoshida, T. Sato, Pharm. Res. 8 (1991) 997.
[132] B. Malavasi, M. Ripamonti, A. Rouchouse, V. Ascalone, J. Chromatogr. A 729 (1996) 323.
[133] A. Rudolphi, K.-S. Boos, LC–GC 15 (1997) 814.
[134] K.-S. Boos, C.-H. Grimm, Trends Anal. Chem. 18 (1999) 175.
[135] W.R.G. Baeyens, G. Van der Weken, J. Haustraete, H.Y. Aboul-Enein, S. Corveleyn, J.P. Remon, A.M. García-Campaña, P. Deprez, J. Chromatogr. A 871 (2000) 153.
[136] C.P. Desilets, M.A. Rounds, F.E. Regnier, J. Chromatogr. 544 (1991) 25.
[137] K.K. Unger, Chromatographia 31 (1991) 507.
[138] T.C. Pinkerton, J. Chromatogr. 544 (1991) 13.
[139] A. Haque, J.T. Stewart, Biomed. Chromatogr. 13 (1999) 51.
[140] K. Kimata, K. Hosoya, N. Tanaka, T. Araki, R. Tsuboi, J. Haginaka, J. Chromatogr. 558 (1991) 19.
[141] J. Hermansson, A. Grahn, I. Hermansson, Curr. Separations 16 (1997) 55.
[142] J. Hermansson, A. Grahn, I. Hermansson, J. Chromatogr. A 797 (1998) 251.
[143] Y. Oda, N. Mano, N. Asakawa, Y. Yoshida, T. Sako, T. Nakagawa, Anal. Sci. 9 (1993) 221.
[144] D.J. Gisch, B.T. Hunter, B. Feibush, J. Chromatogr. 433 (1988) 264.
[145] H. Wang, C. Desilets, F.E. Regnier, Anal. Chem. 64 (1992) 2821.
[146] K.-S. Boos, A. Rudolphi, S. Vielhauer, A. Walfort, D. Lubda, F. Eisenbeiβ, Fresenius J. Anal. Chem. 352 (1995) 684.
[147] P. Chiap, O. Rbeida, B. Christiaens, P. Hubert, D. Lubda, K.-S. Boos, J. Crommen, J. Chromatogr. A 975 (2002) 145.
[148] B. Feinbush, C.T. Santasania, J. Chromatogr. 544 (1991) 41.
[149] D.E. Williams, P.M. Kabra, Anal. Chem. 62 (1990) 807.
[150] H. Wang, C. Desilets, F.E. Ragnier, Anal. Chem. 64 (1992) 2821.
[151] T. Kanda, H. Kutsuna, Y. Ohtsu, M. Yamaguchi, J. Chromatogr. A 672 (1994) 51.
[152] R. Oertel, K. Richter, T. Gramatté, W. Kirch, J. Chromatogr. A 797 (1998) 203.
[153] A. Ceccato, B. Boulanger, P. Chiap, P. Hubert, J. Crommen, J. Chromatogr. A 819 (1998) 143.
[154] G. Lamprecht, T. Kraushofer, K. Stoschitzky, W. Lindner, J. Chromatogr. B 740 (2000) 219.
[155] W.R.G. Baeyens, G. Van der Weken, J. Haustraete, H.Y. Aboul-Enein, S. Corveleyn, J.P. Remon, A.M. García-Campaña, P. Deprez, Biomed. Chromatogr. 13 (1999) 450.
[156] S. Vielhauer, A. Rudolphi, K.-S. Boos, D. Seidel, J. Chromaogr. B 666 (1995) 315.
[157] P. Kubalec, E. Brandšteterová, J. Chromatogr. B 726 (1999) 211.
[158] P. Chiap, A. Ceccato, R. Gora, P. Hubert, J. Géczy, J. Crommen, J. Pharm. Biomed. Anal. 27 (2002) 447.
[159] Z. Yu, D. Westerlund, J. Chromatogr. A 725 (1996) 149.
[160] R.A.M. van der Hoeven, A.J.P. Hofte, M. Frenay, H. Irth, U.R. Tjaden, J. van der Greef, A. Rudolphi, K.-S. Boos, G. Marko-Varga, L.E. Edholm, J. Chromatogr. A 762 (1997) 193.
[161] LiChrospher RP ADS Operating Instructions, Merck KgaA, Darmstadt, Germany, 1996.
[162] C. Mišlanová, M. Hutta, J. Chromatogr. B 765 (2001) 167.
[163] Z. Yu, D. Westerlund, J. Chromatogr. A 725 (1996) 137.

[164] C. Gunaratna, Curr. Separations 17 (1998) 83.
[165] Z. Yu, D. Westerlund, K.-S. Boos, J. Chromatogr. B 704 (1997) 53.
[166] K. Benkestock, J. Chromatogr. B 700 (1997) 201.
[167] J. Hermansson, A. Grahn, J. Chromatogr. A 660 (1994) 119.
[168] A.M. Herrera, D.O. Scott, C.E. Lunte, Pharm. Res. 7 (1990) 1077.
[169] S. Sarre, K. van Belle, I. Smolders, G. Krieken, Y. Michotte, J. Pharm. Biomed. Anal. 10 (1992) 735.
[170] J. Oravcova, B. Böhs, W. Lindner, J. Chromatogr. B 677 (1996) 1.
[171] M.C. Linhares, P.T. Kissinger, Anal. Chem. 64 (1992) 2831.
[172] M. Telting-Diaz, D.O. Scott, C.E. Lunte, Anal. Chem. 64 (1992) 806.
[173] C.E. Lunte, D.O. Scott, Anal. Chem. 63 (1991) 773A.
[174] W.F. Elmquist, R.J. Sawchuk, Pharm. Res. 14 (1997) 267.
[175] J. Chu, J.M. Gallo, Adv. Drug Deliv. Rev. 45 (2000) 243.
[176] L. Stahle, Eur. J. Drug Metab. Pharmacokin. 18 (1993) 89.
[177] C.E. Lunte, D.O. Scott, P.T. Kissinger, Anal. Chem. 63 (1991) 773A.
[178] R.K. Palsmeier, C.E. Lunte, Life Sci. 55 (1994) 815.
[179] B.L. Hogan, S.M. Lunte, J.F. Stobaugh, C.E. Lunte, Anal. Chem. 66 (1994) 596.
[180] A. Chen, C.E. Lunte, J. Chromatogr. A 691 (1995) 29.
[181] K. van Belle, V. de Koster, S. Sarre, G. Ebinger, Y. Michotte, J. Chromatogr. B 657 (1994) 149.
[182] M. Johansson, P. Sjöberg, A.-M. Hesselgren, T. Salmonson, Chirality 7 (1995) 290.
[183] T. Fornstedt, A.-M. Hesselgren, M. Johansson, Chirality 9 (1997) 329.
[184] K.J. McLaughlin, A.A. Faibushevich, C.E. Lunte, Analyst 125 (2000) 105.
[185] R.E. Majors, LC–GC 21 (2003) 19.