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Review

Direct injection of physiological fluids in micellar liquid chromatography

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Abstract

Micellar liquid chromatography (MLC), which uses mobile phases of surfactants above the critical micellar concentration, provides a solution to the direct injection of physiological samples by solubilizing the protein components, and coating the analytical column with surfactant monomers to avoid clogging. A review showing the advantages and limitations of this technique over other chromatographic techniques used in drug analysis, working protocols, and examples of application is presented. The possibility of direct sample introduction simplifies and greatly expedites the treatments with reduced cost, improving the accuracy of the procedures. Surfactant monomers and micelles appear to displace drugs bound to proteins, releasing them for partitioning to the stationary phase. The versatility of MLC encompasses the wide range of drug classes normally monitored, such as analgesics, anticancer drugs, antidepressants, bacteriostats, β-blockers, bronchodilators, catecholamines, diuretics and steroids, among others. Analytical procedures have been developed in urine, plasma, serum and cow milk samples. Most of them utilize sodium dodecyl sulphate as surfactant and a C_{18} column. UV detection is usual, but enhanced detection has been reported by measuring the absorbance in the visible region of drug derivatives formed precolumn, and with a variety of other techniques, such as fluorimetry, amperometry, inductively coupled plasma–mass spectrometry and immunoassay. Column-switching with on-line surfactant-mediated sample clean-up is shown as an attractive enrichment technique, which expands the practical use of MLC beyond the singular dimensional chromatographic process.

Keywords: Reviews; Micellar liquid chromatography; Physiological samples; Direct injection

Contents

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1. Introduction

The control of drugs and their metabolites in physiological fluids is of great interest in clinical chemistry, doping, toxicology, and pharmaceutical research. The therapeutic efficacy of many drugs is closely related to their concentration in blood and tissues, which depends on their dosage, route and frequency of administration. Drug monitoring may be necessary to adjust the dose of a drug to the patient's needs. Therefore, appropriate analytical assays that are simple and reliable are desired.

Drug analysis has been greatly enhanced through the development of high-performance liquid chromatography (HPLC) technology. The assay of drugs in physiological fluids presents, however, many problems. The drugs are frequently in very low concentration, strongly bound to proteins, and in a complex matrix where interference from numerous endogeneous compounds is expected. The high-molecular-mass proteins in these samples are particularly troublesome, since they tend to denature and precipitate in the injection valve or at the column head. This produces clogging of the system, and leads to a rapid degradation of chromatographic performance and an increase in back-pressure when traditional reversed-phase liquid chromatography (RPLC) silica supports are used. The most difficult fluids are those that contain a large fraction of proteins, mainly blood, plasma and serum. Urine, cerebrospinal and intersticial fluids are in general more compatible with liquid chromatographic systems, due to their lower protein content.

In an RPLC system, the harmful proteinaceous material must be removed from the sample prior to injection, to prevent irreversible adsorption to the stationary phase. Several approaches have been used to facilitate sample preparation for physiological fluids. One simple approach is to precipitate proteins by organics or sodium hydroxide, or remove them by ultrafiltration. Very often, other separation steps are required such as liquid–liquid or solid-phase extraction from the matrix, re-extraction and evaporation.

All these procedures are time-consuming, require heavy repetitive work, and introduce additional sources of error, because of incomplete recovery of the drugs. A suitable internal standard should be used to correct possible errors in the overall procedure (even a simple deproteinization step may cause analyte losses due to drug protein binding). The use of robotics can allow complex sample preparation to be carried out with high precision and minimal work, the equipment cost and development time of such methods are however only justifiable for cases where high sample throughput over an extended time period is expected. An additional problem of the extraction processes which should be considered is the use and disposal of toxic solvents and chemicals, which are dangerous not only to the analyst but also to the environment. The complexity of these labor-intensive methods have prevented their application for routine clinical use.

Previous sample preparation steps constitute the main share of bioanalytical methods. This is largely the reason why much effort has been dedicated to the

development of liquid chromatographic systems that can tolerate the direct injection of physiological fluids. Any of the existing HPLC methods performing a deproteinization step and/or a sample enrichment is slower than one-step direct injection methods.

2. Direct injection techniques

The past decade has witnessed a proliferation of methods and media for direct injection of physiological fluids into HPLC systems [1–4]. Conventional RPLC media are not adequate to tolerate direct injection of drugs in protein-containing matrices. Special restricted-access materials (internal-surface reversed-phases, shielded hydrophobic phases, semipermeable surface phases, dual-zone phases and mixed-functional phases) have been designed for column packings to minimize the deleterious effects of protein adsorption [5,6]. Most systems utilize a precolumn (changed regularly to keep the system back-pressure at normal levels) for the reception of the physiological fluids, trace enrichment and preliminary clean-up, before the analyte fraction is transferred to the analytical column. The precolumn serves the dual function of acting as a guard column, as well as effecting a preseparation of the analytes from the physiological fluid matrix. Column-switching procedures have been used in the foreflush and backflush mode [7].

The most common solid-phase in precolumns is silanized silica, but many other types have been used. One interesting approach is the pretreatment of small-pore silica with plasma [8] and/or solutions containing bovine serum albumin (BSA) [9]. Proteins are only adsorbed on the external surface of the support particles. Another concept is the use of a solid-phase where a hydrophobic tripeptide is attached to glycerylpropyl-derivatized small pore silica [10]. This support, named internal surface reversed-phase (ISRP), exposes to the mobile phase an internal surface that permits hydrophobic partitioning and an external surface with a hydrophilic phase, which is non-adsorptive towards proteins.

Another alternative of direct injection consists in the use of conventional RPLC columns and eluents capable of solubilizing the proteins in the physiological matrix, such as solutions of surfactants at a concentration above the critical micellar concentration (cmc). In these conditions, in addition to the formation of micelles, the column packing is covered with a constant layer of monomers of surfactant, which protects and modifies the underlying alkyl-bonded silica phase. This chromatographic mode has been called micellar liquid chromatography (MLC). In 1981, Armstrong and Nome [11] first suggested the possibility of making analytical use of these media and explained the chromatographic behaviour of solutes through a three-phase model where solutes partition among bulk water, micelles and stationary phase modified by the adsorption of surfactant. These equilibria are mainly governed by hydrophobic forces, although electrostatic attraction and repulsion with ionic groups may be important.

Organic modifiers, such as alcohols, added to the micellar eluents enhance the efficiencies of chromatographic peaks (which are rather low when only the surfactant is present), and permit the correct control of the retention and selectivity [12]. The elution strength of alcohols depend on their chain-length. Methanol is rarely used because of its low elution strength; in contrast, strongly retained compounds require a micellar eluent with a small amount of pentanol. The eluent strength of acetonitrile is similar to that of propanol, but efficiency enhancements are greater with the former eluent [13].

An interesting feature of MLC is the accurate prediction of retention factors with mobile phase composition, which facilitates the optimization of chromatographic procedures [14,15]. The variety of interactions inside the chromatographic column give a large versatility to this technique and makes it appropriate for analyzing a wide range of solutes. Mixtures of hydrophilic and hydrophobic compounds can be separated in one run, without the need of gradient elution.

The first application of MLC to the assay of drugs in physiological fluids was reported in 1985 [16], but most analytical procedures have appeared since 1989. In that time, two specific review articles on direct injection procedures with micellar eluents were published (1989 and 1990) [17,18]. A kit for drug monitoring utilizing MLC was patented in 1989 [19]. Later, other reviews on the bioanalytical use of MLC have dedicated some short section to the direct

injection capability [20–24]. The scope of this paper is to give a specific and complete view of the published procedures on this field up to date.

3. Attractive advantages of micellar eluents

MLC appears as a promising and useful technique for the direct injection of physiological samples. The compatibility with conventional RPLC column packings is particularly attractive. Surfactant monomers and micelles tend to bind proteins competitively [25], thereby releasing protein-bound drugs, which are free to partition into the stationary phase. Meanwhile, the proteins rather than precipitating on the column, are solubilized and swept harmlessly away, eluting with or shortly after the solvent front. Moreover, surfactants are non-toxic, non-flammable, biodegradable with low pollution impact, and inexpensive in comparison to aqueous-organic solvents. The use of surfactants in direct injection is also much less complex than column-switching procedures which require additional instrumentation (precolumns, switching valves and HPLC pumps), and accurate and precise timing of valve switching for a successful separation.

In MLC, untreated physiological fluids directly injected into the HPLC system have been reported to be simple, allowing repetitive serial injections with no increase in system pressure, no noticeable clogging of the injection valve or analytical column, no changes in retention factors, or system contamination evident. However, the analytical column should be protected with a guard precolumn to saturate the micellar mobile phase with silica. Control of the precipitation of proteinaceous material into the column can be made by monitoring the pressure of the system, and by the daily injection of a probe solute to check for possible changes in retention.

4. Useful surfactants

The anionic surfactant sodium dodecyl sulphate (SDS) is the most common in MLC, but the non-ionic polyoxyethylene(23)dodecyl (Brij-35) has also been employed successfully. Cationic surfactants cause proteins to precipitate and cannot be usually used with physiological samples. An example has been reported on the simultaneous analysis of nitrophenol and its glucuronide where cetyltrimethylammonium bromide (CTAB) was used [26]. Attempts have been made to use other surfactants without success, such as sodium decyl sulphate and sodium pentadecyl sulphate [27]. One non-denaturing anionic surfactant, sodium desoxycholate, was found to elute BSA from serum injections. A zwitterionic surfactant, 3-(dimethyldodecylammonium propanesulfonate (C12 DAPS), was used for the determination of theophylline [28], but the performance of the procedure was similar to that found using SDS as surfactant [29]. Certainly, the good characteristics and availability of SDS will make it difficult for it to be replaced in the future by other surfactants in MLC drug analysis.

5. Surfactant concentration

Conditions unfavorable for micelle formation or for protein solubilization should be avoided. It has been suggested that the eluents typically used in MLC may only represent a subset of the surfactant-containing eluents, that will permit direct injection of physiological samples onto C_{18} chromatographic columns. In a study carried out with a model serum of 0.05 *M* BSA injected into a mobile phase of 0.001 *M* SDS–15% methanol (where the cmc of SDS was not reached), it was shown that good results and no column clogging occurred after a hundred injections [27]. However, SDS interactions with both protein and C_{18}-bonded phase appear to be too limited to allow direct injection, in the presence of relatively high amounts of methanol. The use of other alcohol modifiers is even more problematic.

Indeed, several authors have reported problems of pressure increases and irreproducible retention times, when low concentrations of surfactants are employed (below 0.05 *M*) in mobile phases with alcohols. Thus, after serial injections of spiked serum or plasma containing bumetanide into a mobile phase of 0.02 *M* SDS–10% 1-propanol at pH 3.5, the operating pressure increased noticeably and the retention time of the drug decreased [30]. Shortening retention times of the analyte were also observed with a similar mobile phase with plasma samples [31].

Direct injection of plasma fortified with chlorthalidone and furosemide using a mobile phase of 0.05 *M* SDS–5% 1-propanol also gave a rapid increase in column back pressure, as well as loss of analyte sensitivity [32]. These effects were explained as changes in the stationary phase characteristics produced by the complex matrix. The determinations were appreciably improved by increasing the concentration of SDS to 0.1 *M*, probably due to better solubilization of proteins.

Protein material can only be effectively removed from samples and column packing material, if sufficiently surfactant is used in the mobile phase. An adequate coating of surfactant should be maintained on the reversed-phase packing to prevent protein adsorption. For this reason, the concentration of surfactant should be well above the cmc, and the organic solvent content relatively low.

6. Displacement of drug bound to proteins

The drug bound to proteins is mostly displaced by the surfactant monomers and/or micelles in the mobile phase. Drug recovery in MLC with direct injection can however be incomplete. It depends on the nature of the drug, protein binding and mobile phase composition. Doubled peaks ascribed to the protein-bound and unbound drug were observed for cephalosporins in serum samples, injected directly into a C_{18} column and eluted with an SDS eluent. The single or double peaks appeared depending on the pH of the eluent [33,34].

Fig. 1 shows the chromatograms of a mixture of cefmenoxime hemihydrochloride (CMX) and cefotiam dihydrochloride (CTM), eluted with 0.08 *M* SDS–8% 2-propanol at pH 3.3. CMX showed two peaks at retention times of 3.8 and 5.2 min, while CTM gave a single peak. Acidification of the serum sample to pH 1.2 also changed the two peaks of CMX into a single peak. The two peaks of CMX were demonstrated to be produced by drug bound (the peak at shorter retention time) and unbound (the peak at longer retention time) to proteins, since the protein binding estimated as the peak area ratio of the bound to total CMX, was 79%, which coincided with a previously determined value (80%). Also, after ultrafiltration of the serum samples, only the peak at longer retention time was observed. The results suggested that the protein binding of a drug might be evaluated, and that for a strongly bound drug, the alteration of drug-protein binding by changing the conditions, such as pH, is required for the recovery and quantitation of the total drug.

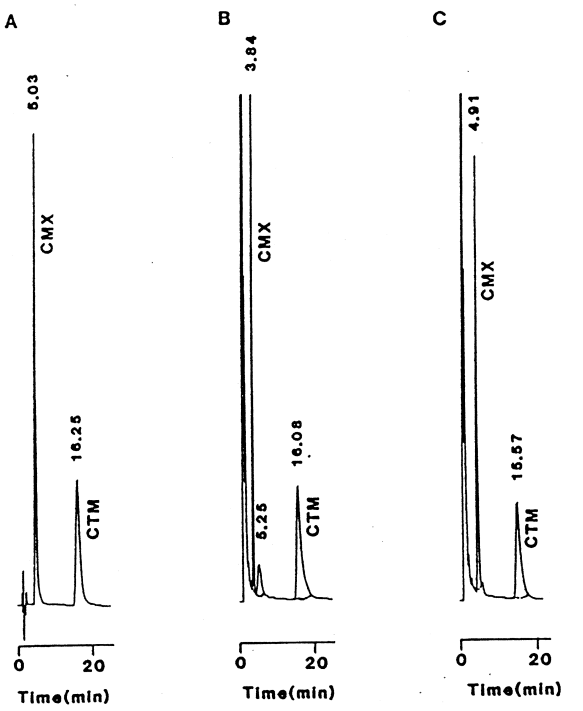

Fig. 1. Chromatograms of cefmenoxime hemihydrochloride (CMX) and cefotiam dihydrochloride (CTM) in: (A) distilled water, (B) serum sample, and (C) acidified serum sample. Mobile phase: 0.08 *M* SDS–8% 2-propanol in 0.05 *M* phosphate buffer at pH 3.3. The figures over the peaks represent the retention times. Reprinted from Ref. [33] with the permission of the American Chemical Society.

7. Background signal of the matrix fluid

7.1. Nature of the background

The high background signal of the matrix at the beginning of the chromatogram, observed with direct injection, is one of the limitations of the procedure. This signal is produced by the presence of proteins and endogeneous compounds and may overlap the peaks of the analytes, resulting in a useless zone

(Fig. 2A). The protein–surfactant complexes, excluded from the pores of the stationary phase support, show a broad band at the solvent front. Also, an unidentified endogeneous compound produces a peak that stands out among other smaller. A similar but smaller background signal is observed in conventional RPLC with physiological matrices, even when previous separation steps are utilized.

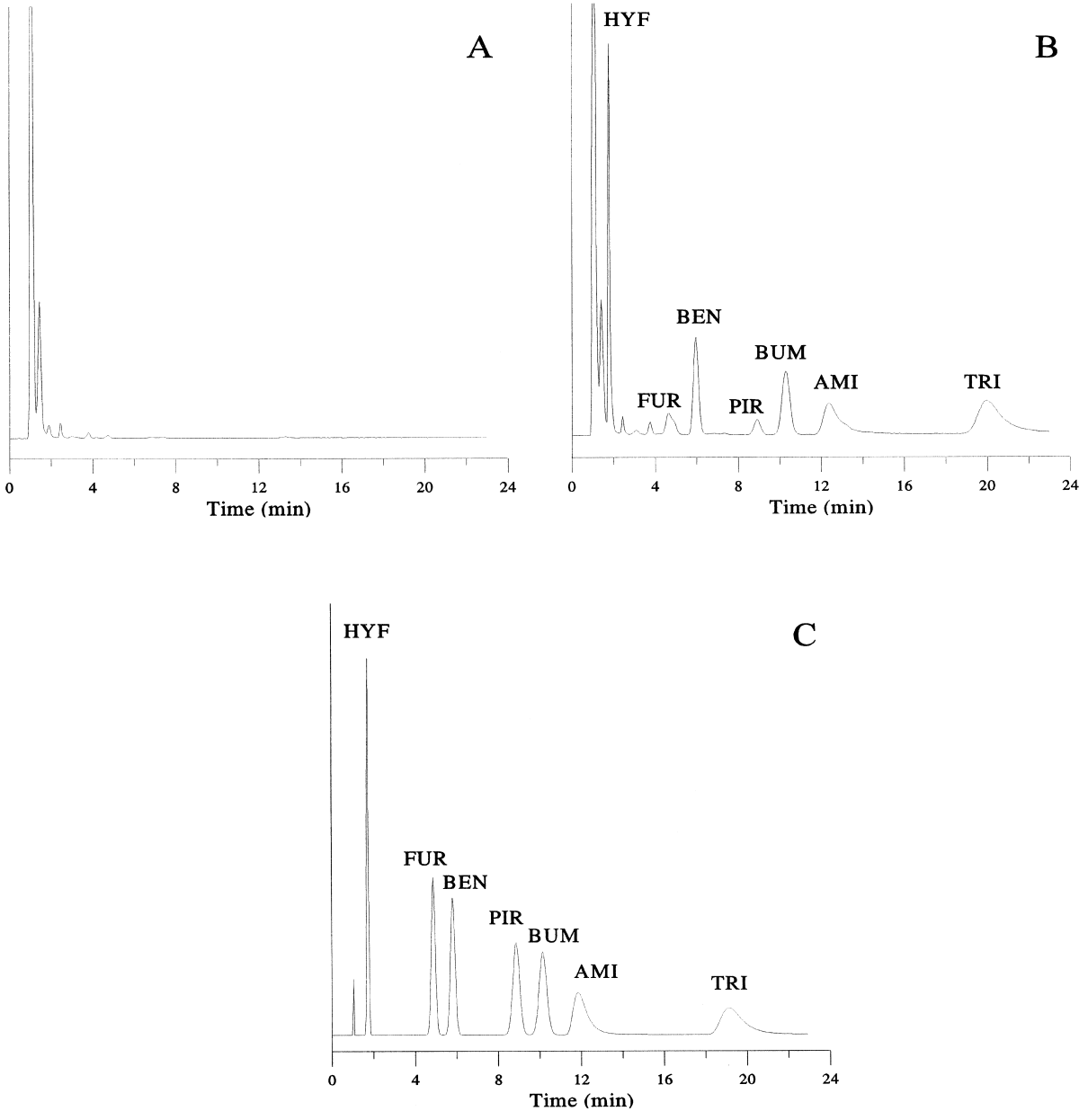

Fig. 2. Chromatograms of a mixture of diuretics: (A) urine matrix, (B) urine matrix spiked with 1 μg/ml of each diuretic, and (C) predicted chromatogram. Mobile phase: 0.055 *M* SDS–8% 1-propanol in phosphate buffer at pH 3. Drugs: hydroflumethiazide (HYF), furosemide (FUR), bendroflumethiazide (BEN), piretanide (PIR), bumetanide (BUM), amiloride (AMI), and triamterene (TRI).

7.2. Modification of the background with mobile phase composition

The profile of the background depends on the composition of the mobile phase [35], and can be reduced at an increased detection wavelength. The retention of the endogeneous compound giving the largest peak in the chromatogram of urine matrix was observed to be minimized and remained constant in the pH-range 5.5–7.5, for varying surfactant (SDS and Brij-35) concentration, particularly with SDS mobile phases [36]. At pH $\leq$5.5, an important increase in retention was observed for both surfactants. The shape of the k vs. pH curve for the endogeneous compound was sigmoidal, which indicated that it was protonated in acidic medium, with a protonation constant $\log K_H = 4.5–5.0$ [35]. The retention was also higher at decreasing concentrations of surfactant. It was concluded that for the analysis of physiological fluids with pure micellar mobile phases (without modifier), the pH should be in the range of 6–7 in order to minimize the retention of the matrix, or at a lower pH, the concentration of SDS should be increased. However, the elution of the drugs should also be considered. The retention of many acidic drugs decreases at larger pH and/or SDS concentration. The efficiency of the chromatographic peaks also deteriorates appreciably with increasing SDS.

In most analytical procedures for the determination of drugs in physiological fluids by MLC, an alcohol is added to the micellar eluent. The presence of an alcohol not only reduces the retention of the analytes, it also affects the matrix [35]. The retention times of the endogenous compounds decrease, a smaller effect is observed for the protein band.

The retention of the endogeneous compounds leaves, however, a detection window for drug analysis and is reproducible. Thus, the mean retention time for the endogenous compound giving the largest peak, with a mobile phase of 0.042 *M* SDS–4% 1-propanol at pH 4.5, was 8.3±0.3 min (relative standard deviation, RSD=4%), for urine samples taken from eleven healthy males and females. Otherwise, the RSD for 12–16 urine samples of two volunteers, taken over 36 h, was 3–4% [35].

For the determination of many compounds, the retention of the protein band and the peak of the cited endogeneous compound should be decreased, as much as possible. It should be noted that only if the elution of the drug occurs after the protein band, will the determination be possible. Drugs eluting at shorter times will need extraction procedures.

8. Experimental protocol

8.1. Column conditioning

In MLC, the chromatographic column needs special care. It is usual to keep the column with methanol–water. Changing to the micellar eluent should be made by washing with some volumes of water to decrease the concentration of the organic solvent and to avoid crystallization of the surfactant inside the system. Next, the micellar eluent should be pumped for 1 h at least (at 1 ml/min flow-rate) to assure equilibration of the stationary phase. With the micellar eluent inside the column, the flow should not be stopped, but when the column is not used, the flow-rate can be decreased to a minimum (i.e., 0.1 ml/min). Before the pump is stopped, the micellar eluent should be replaced by methanol. After one or two weeks of continuous experimental work, the elimination of strongly retained compounds may be convenient by substitution with methanol. This is made following the same procedure as above, in reverse, changing first the micellar solution to water, which should be pumped for at least 2 h to assure that no surfactant remains in the system, and afterwards, methanol at a low flow-rate.

After a series of injections of physiological samples, the back pressure of the system may increase or the retention of the analytes change. In this case, the stationary phase should be regenerated by flushing water, methanol and chloroform, one after the other. In an experiment done with relatively large volumes of urine sample (20 μl, 40–50 injections), the mobile phase (a volume of approximately 500 ml) was recycled for 4–5 days through the chromatographic system [37]. Under these conditions, changes in the retention times were observed for the analytes (β-blockers). Normal values were, however, again obtained after cleaning the system and changing the solution being used as mobile phase. With low limits of detection (LODs), dilution of the urine samples

has been suggested to increase the life of the column [37,38].

For micellar solutions of ionic surfactants, the temperature of the column should be always above the Krafft point (15°C for SDS), to avoid clogging and possible destruction of the column and chromatographic system.

8.2. Optimization of mobile phase composition

The type of surfactant and modifier, their concentrations, and the pH of the mobile phase have been identified as the key parameters that can be varied to obtain the required resolution in drug analysis. Mobile phase parameters should be properly chosen to improve the selectivity of the separation between drugs and endogeneous compounds, in the physiological fluids. Optimal control of mobile phase composition permits the development of MLC procedures for the determination of virtually any drug in these samples.

The pH of the micellar mobile phase is an important factor for the analysis of ionizable drugs, using non-polar column stationary phases. Micellar solutions of SDS increase the apparent protonation constants due to the stabilization of acidic species by the anionic micelles. This benefits the observation of the maximal limiting retention of acidic species within the operable limits of silica-based columns [39]. One example of the importance of pH is given by the assay of acetylsalicylic acid ($\log K_H = 3.5$) in a serum sample, eluted at pH 3.0 and 6.5 with 0.08 *M* SDS from a C_{18} column [16]. At pH 6.5, the anionic species of the drug was not evident, it probably eluted with the serum proteins. However, at pH 3.5, the neutral species eluted at approximately 3.5 min, appreciably distinct from the serum components.

The determination of anticancer 6-thiopurine drugs and their metabolites in untreated serum is another useful example (Fig. 3) [40]. With a mobile phase of 0.04 *M* SDS at pH 2.2, the blank serum produced a background response that had completely eluted after 6 min, with the exception of a peak at 8 min. The peaks of 6-thioguanidine riboside and 6-thioguanine were well resolved, but unfortunately, the three earlier-eluted compounds (6-mercaptopurine riboside, 6-thioxanthine and 6-mercaptopurine) were overlapped by the matrix peaks. A lower pH (2.0) permitted complete separation of 6-mercaptopurine from the serum background signal. Under these conditions, 6-thioguanine eluted too late (at around 40 min).

In MLC, the optimization of mobile phase composition (surfactant and modifier) can be made through the use of an interpretive method based on a checked mechanistic model, which takes into account the different interactions that occur inside the micellar chromatographic system [41]. Reliable optima are obtained using, as global resolution criterion, the product of overlapped fractions for each individual peak [42]. The methodology is computer-assisted [43]. Fig. 2 shows the predicted chromatogram together with an experimental chromatogram obtained for the optimum mobile phase for the separation of a mixture of diuretics in a spiked urine sample. As observed, the agreement between experimental and predicted chromatograms is excellent.

8.3. Use of eluents containing two surfactants

Mixed surfactant mobile phases were used to improve the selectivity in the separation of hydrochlorothiazide and its hydrolysis product 5-chloro-2,4-disulfamoylaniline, which is formed in aqueous solution upon standing at room temperature [36]. Although the diuretic drug was well separated from the urine matrix with a Brij-35 mobile phase, both related compounds could not be resolved. In contrast, base-line separation was obtained between the two compounds with SDS mobile phases, but the separation of the diuretic from the peak of an endogeneous compound in urine was unsatisfactory. A mobile phase of 0.004 *M* SDS (below its cmc) and 0.02 *M* Brij-35 provided instead good resolution between the diuretic and its hydrolysis product, without compromising the separation of the drug from the urine background.

8.4. Gradient elution

Micellar mobile phases containing modifiers permit the adequate elution of mixtures of solutes with a wide range of hydrophobicity, under isocratic conditions. A good example of the capability of MLC is given by the isocratic screening of twelve sulphonamides in human urine and cow milk, using a

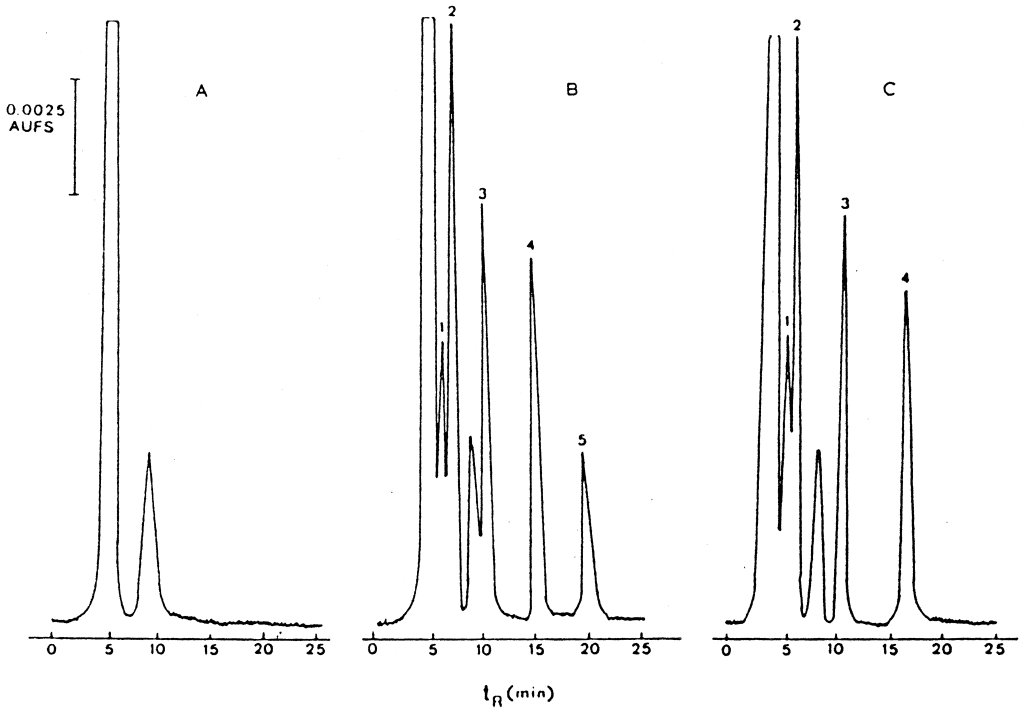

Fig. 3. (A) Chromatograms of serum blank, and (B) and (C) serum spiked with 1 μg/ml of 6-thiopurine compounds. Mobile phase: 0.04 *M* SDS in 0.01 *M* phosphate buffer at pH 2.3 for (A) and (B), and pH 2.0 for (C). Drugs: (1) 6-mercaptopurine riboside, (2) 6-thioxanthine, (3) 6-mercaptopurine, (4) 6-thioguanidine riboside, and (5) 6-thioguanine. Reprinted from Ref. [40] with the permission of Elsevier.

0.07 *M* SDS–6% 1-propanol eluent at pH 3.0 and a hydrophilic endcapped C_{18} column [44]. The drugs eluted within 15 min.

In some cases, gradient elution should be used to expedite the elution of strongly retained compounds. In fact, the capability of performing rapid gradient of surfactant concentration is another advantage of MLC, since the stationary phase must not be re-equilibrated with the original mobile phase composition after each run [45]. A gradient of modifier seems to be also possible [46]. The reported MLC procedures using gradient elution with direct injection are however few. One example is the determination of the diuretic bumetanide in serum, using a mobile phase of SDS–3% 1-propanol at pH 3.5 [30], where the initial concentration of SDS was held at 0.1 *M* until the peak of bumetanide was completely eluted (7 min), and then increased to 0.2 *M* over 5 min to ensure that any adsorbed serum components were washed from the column. Another application is the determination of thiazide diuretics and furosemide, where an SDS gradient was used to accelerate the elution of furosemide [47].

9. Precolumn derivatization

Precolumn derivatization is applied in liquid chromatography to improve the sensitivity and selectivity of analytical procedures. Amino acids are a classical example. Accordingly, a procedure was developed for the MLC determination of a mixture of proline, glutamine, threonine and tyrosine in urine, by precolumn formation of the cupper(II) complexes and detection at 235 nm, using a 0.03 *M* SDS–8% 1-propanol mobile phase at pH 5.5 [48].

The high background observed at the beginning of the chromatogram of a physiological sample, when the detection is performed at 230–280 nm, is eliminated in the visible region. In order to exploit this fact, several sulphonamides were coupled with *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) in

micellar solution, to form azo dyes before injection in an MLC system [49]. The derivatization reaction was enhanced in the micellar medium and yielded high molar absorptivities at long wavelengths (488 nm). The chromatogram of urine matrix treated with the derivatization reagents gave only one peak at 13.5 min, which corresponded to an unknown endogeneous compound that was also derivatized. This peak did not interfere however with the quantitation of sulphonamide azo dyes, which eluted at shorter retention times. The procedure combined the advantages of precolumn derivatization and chromatography with micellar mobile phases. The derivatization of sulphonamides increased their retention by reducing the polarity of the drugs, and improved peak resolution; the longer detection wavelengths also improved the signal-to-noise ratios.

Another example of an MLC procedure with precolumn derivatization is the determination of thiazide diuretics and furosemide after hydrolysis and formation of NED azo dyes [47]. Thiazide diuretics yielded only two different arylamines by hydrolysis, depending on the existence of Cl or CF_3 substituents in the thiazide nucleus. Therefore, only two peaks were observed in the chromatograms for mixtures of these compounds. Detection of underivatized thiazides was difficult, especially for those with a Cl substituent, due to overlapping with the band of proteins and the peak of an endogeneous compound in the physiological sample.

10. Detection techniques

The experimental characteristics of the published MLC procedures for the analysis of physiological samples, using direct injection, are given in Table 1. Most procedures utilized UV detection. This is not optimal for many drugs and samples. Although opportunities to further reduce matrix interferences and confirm peak identity through chemometric techniques and diode array detection [50] present challenges for future studies, the sensitivity of UV detection is insufficient for some trace amount determinations. Fluorescence detection of compounds that yield measurable fluorescence emission may be preferable, owing to the higher sensitivity and selectivity when compared to absorption methods. In certain cases, LODs will be significantly lower, more than adequate for drug monitoring of concentration ranges normally encountered in serum and urine.

The fluorescence background signal of the physiological matrix at the solvent front, due to unretained proteins, is again the limiting factor in the LODs, although the background signal seems to be substantially reduced with respect to UV detection. The response varies by changing the excitation wavelength and pH. It has been found that serum background is completely eliminated by using a 470-nm cut-off filter [58].

MLC with sensitized terbium fluorescence detection has been presented as a viable alternative for steroid analysis in physiological fluids. A mobile phase of 0.1 *M* SDS–20% acetonitrile–0.01 *M* $Tb(NO_3)_3$ simplified the analysis, by permitting the direct injection of urine into the column, with adequate LODs (in the ng/ml range) [60].

Elemental speciation is becoming more and more important, since the environmental toxicity and biological importance of many elements depend on their oxidation states and different chemical forms. The low level detection capability of inductively coupled plasma–mass spectrometry (ICP–MS) is especially attractive as an element-specific chromatographic detector in chromatography. In this field, the applicability of MLC direct injection with ICP–MS detection of 'dirty samples', in 'real-life' situations, was demonstrated to analyze arsenic compounds [As(III), As(V), monomethylarsonic acid and dimethylarsenic acid], in urine [68].

There is no reference of the use of mass spectrometry (MS) as a detection system for MLC. Actually, direct on-line coupling of MLC with MS is hampered by the presence of high concentrations of surfactant in the mobile phase. A similar problem is found in other surfactant-mediated separation techniques. Thus, an on-line microcolumn switching method was developed for the removal of SDS with a specific ionic detergent trapping column, from tryptic digest samples, before separation by capillary liquid chromatography with electrospray mass spectrometry (ESI–MS) detection [70]. Otherwise, partial-filling micellar electrokinetic chromatography (PF-MEKC) provides a possible way to overcome the problematic on-line coupling between MEKC

Table 1
Characteristics of published direct injection MLC procedures for the analysis of physiological fluids

Compounds	Sample; column; mobile phase; detection method; limit of detection	Reference
Therapeutically distinct drugs: acetylsalicylic acid (I), chloramphenicol (II), theophylline (III), acetaminophen (IV), carbamazepine (V), phenobarbitone (VI), phenytoin (VII), procainamide (VIII), and quinidine (IX)	Serum; Supelcosil LC-18 (I, II), μ-Bondapak C_{18} (III) and Supelcosil LC-CN (IV, V, VI, VII, VIII, IX); 0.02 to 0.10 *M* SDS or Brij-35, pH 3; UV 254 nm (I–VIII) and fluorescence λ_{exc}=336 nm, λ_{em}=370 nm (IX); 0.2–3 (I–VIII), and 0.3 (IX) μg/ml	[16,51]
Therapeutically distinct drugs: carbamazepine (I) and theophylline (II)	Serum; Supelcosil LC-CN (I) and Supelcosil LC-18 (II); 0.02 *M* SDS (I) and 0.05 *M* SDS (II); UV 254 nm	[52]
Bronchodilator: theophylline	Serum; μ-Bondapak phenyl; 0.001 *M* C12 DAPS–3% 1-propanol; UV 273 nm; 0.5 μg/ml	[28]
Stimulant: caffeine (I) and their metabolites, theophylline (II) and theobromine (III)	Urine; Spherisorb ODS-2; 0.075 *M* SDS–1.5% 1-propanol; UV 273 nm; 1.2 (I) and 0.4 (II, III) μg/ml	[29]
Analgesic: acetaminophen	Urine; Nucleosil C_{18}; 0.05 *M* SDS–3% 1-propanol; amperometry with a wall-jet cell and carbon fibre microelectrode; 0.02 μg/ml	[53]
Cephalosporins: cefmenoxime hemihydrochloride and cefotiam dihydrochloride	Serum; Nucleosil C_{18}; 0.08 *M* SDS–8% 2-propanol in 0.05 *M* phosphate buffer at pH 3; UV 260 nm; 2 μg/ml	[33]
Cephalosporins: cephalexin (I), cefradoine (II), cefotaxime (III) and cefmenoxime (IV)	Serum; Develosil ODS; 0.02 *M* SDS in 0.05 *M* phosphate buffer at pH 6.1 (I, II), and 0.15 *M* in 0.05 *M* phosphate buffer at pH 3.1 (III, IV), at 40°C; UV 254 nm	[34]
Anti-herpes: acyclovir	Serum and plasma; Separon SGX C_{18}; 0.05 *M* SDS in 0.05 *M* phosphate buffer at pH 2.0; fluorescence λ_{exc}=285 nm and λ_{em}=370 nm; 0.08 μg/ml	[54]
Sulphonamides: sulphacetamide, sulphadiazine, sulphamerazine, sulphathiazole, sulphamethazine, sulphamethoxypyridazine, sulphachloropyridazine, sulphamonomethoxine, sulphabenzamide, sulphadimethoxine, sulphaquinoxaline and sulphisomidine	Human urine and cow milk; hydrophilic endcapped ODS at 40°C; 0.07 *M* SDS–6% 1-propanol; UV 254 nm	[44]
Sulphonamides: sulphadiazine (I), sulphaguanidine (II), sulphamethizole (III), sulphamethoxazole (IV) and sulphathiazole (V)	Urine; Spherisorb ODS-2; 0.05 *M* SDS–2.4% 1-pentanol, azo dye precolumn derivatization with nitrite and NED; visible 550 nm; 0.1 (I, IV), 0.2 (II, V) and 0.3 (III) μg/ml	[49]
Diuretic: bumetanide	Serum and urine; Nucleosil RP-18; 0.10 *M* SDS–3% 1-propanol in 0.05 *M* phosphate buffer at pH 3.5; UV 305 nm	[30]
Diuretic: hydrochlorothiazide	Urine; Hypersil C_{18}; 0.02 *M* Brij 35–0.004 *M* SDS in 0.01 *M* phosphate buffer at pH 6.5; UV 271 nm	[36]
Diuretics: bendroflumethiazide and chlorthalidone	Urine; Spherisorb ODS-2; 0.05 *M* SDS–5% methanol at 50°C; UV 224 nm; 0.1–0.5 μg/ml	[55]
Diuretics: amiloride (I), bendroflumethiazide (II), bumetanide (III), chlorthalidone (IV), ethacrynic acid (V), furosemide (VI), spironolactone (VII), triamterene (VIII) and xipamide (IX); also probenecid (X)	Urine; Spherisorb ODS–2; 0.042 *M* SDS–4% 1-propanol in 0.01 *M* phosphate buffer at pH 4.5; UV 254 nm; 0.8 (I, V), 0.4 (III, VI, VII), 0.3 (IV), 0.2 (VIII, X) and 0.08 (IX) μg/ml	[56]

(*Continued on next page*)

Table 1 (*continued*)

Compounds	Sample; column; mobile phase; detection method; limit of detection	Reference
Thiazide diuretics: althiazide (I), bendroflumethiazide (II), chlorothiazide (III), hydrochlorothiazide (IV), hydroflumethiazide (V) and trichlorothiazide (VI); also furosemide (VII) and chloraminophenamide	Urine; Spherisorb ODS-2; 0.05 *M* SDS–8% 1-propanol with precolumn azo dye derivatization of the hydrolized diuretics with nitrite and NED; visible 525–550 nm; 2.9 (I), 2.5 (II), 2.2 (IV), 2.0 (VI) and 1.7 (VII) μg/ml	[47]
Diuretics: amiloride (I), bendroflumethiazide (II), bumetanide (III), hydroflumethiazide (IV), piretanide (V) and triamterene (VI)	Urine; Spherisorb ODS-2; 0.055 *M* SDS–8% 1-propanol; fluorescence λ_{exc}= 270 nm and λ_{em}=430 nm; 9.7 (I), 10 (II), 1.4 (III), 7.1 (IV), 54 (V) and 1.7 (VI) ng/ml	[38]
β-Blockers: acebutolol (I), atenolol (II), celiprolol (III), labetalol (IV), metoprolol (V), nadolol (VI) and propranolol (VII)	Urine; Spherisorb ODS-2; 0.1 *M* SDS–15% 1-propanol-1% triethylamine in 0.02 *M* phosphate buffer at pH 3; fluorescence λ_{exc}=230 nm and λ_{em}= 440 nm (I, III, IV), 300 nm (II, V, VI) and 340 nm (VII); 30 (I), 19 (II), 200 (III), 20 (IV), 16 (V), 8 (VI) and 3 (VII) ng/ml	[37]
Mixtures of diuretics and β-blockers: amiloride (I) bendroflumethiazide (II), piretanide (III), triamterene (IV), acebutolol (V), atenolol (VI), labetalol (VII), metoprolol (VIII), nadolol (IX) and propranolol (X)	Urine; Spherisorb ODS-2; 0.11 *M* SDS–8% propanol, except for mixtures of I/VIII, I/VII and IV/X; fluorescence λ_{exc}=230 nm and λ_{em}=300 nm (I, II, III, IV, V, VII), and 440 nm (VI, VIII, IX, X); 10.6 (I), 17.5 (II), 12.3 (III), 2.8 (IV), 27.6 (V), 3.8 (VI), 28.3 (VII), 19.2 (VIII), 12.3 (IX) and 11.8 (X) ng/ml	[57]
Distinct drugs: codeine (I), morphine (II), propranolol (III), quinidine (IV) and quinine (V)	Serum and urine; μBondapak C_{18} and Supelcosil LC-CN; 0.02–0.05 *M* SDS–10% 1-propanol; fluorescence λ_{exc}=215 nm and λ_{em}=300 nm; 0.3 (I, II), 0.01 (III) and 0.03 (IV, V) μg/ml	[58]
Illegal drugs in sport: amiphenazole (I), amiloride (II), amphetamine (III), clostebol (IV), ephedrine (V), phenylpropanolamine (VI), methandienone (VII), methoxyphenamine (VIII), nandrolone (IX) and spironolactone (X)	Urine; Spheri-5 RP-18; 0.1 *M* SDS–3% 1-pentanol; UV 260 nm; 2.6 (I), 11 (II), 4.1 (III), 1.2 (IV), 4.2 (V), 2.2 (VI), 0.4 (VII), 8.7 (VIII), 0.07 (IX) and 1.6 (X) μg/ml	[59]
Steroids: bolasterone (I), cortisone (II), methyltestosterone (III), progesterone (IV), testosterone (V) and testosterone acetate (VI)	Urine; C_{18}; 0.10 *M* SDS–20% acetonitrile–0.01 $Tb(NO_3)_3$; sensitized terbium fluorescence λ_{exc}=245 nm and λ_{em}=547 nm; 10 (I, IV, VI) and 50 (III, V) ng/ml	[60]
Steroids: hydroxycorticosterone (I), corticosterone (II), norhisterone (III), testosterone (IV), medroxyprogesterone acetate (V) and progesterone (VI)	Urine; Spheri-5 RP-18; 0.05 *M* SDS–9% 1-butanol; UV 245 nm; 50 (I, II) and 100 (III, IV, V, VI) ng/ml	[61]
Alkaloid: nicotine (I) and its metabolite cotinine (II)	Urine; Econosphere CN-bonded silica; 0.2 *M* SDS–3% 2-propanol at pH 4.6 and 40°C; UV 260 nm; 0.2 (I) and 0.1 (II) μg/ml.	[62]
Antiinflammatory drug: 5-lipoxygenase inhibitor Zileuton and its *N*-dehydroxylated metabolite	Urine; CN-bonded silica; 0.025 *M* SDS–3% 1-propanol in 0.01 *M* phosphate buffer at pH 3; UV 262 nm; 0.1 μg/ml	[63]
Anticancer drugs: 6-mercaptopurine (I), 6-thioguanine (II) and their metabolites 6-mercaptopurine riboside (III), 6-thioguanine riboside (IV) and 6-thioxanthine (V)	Serum; LiChrosorb RP-18; 0.08 *M* SDS in 0.01 *M* phosphate buffer at pH 3, and 30°C; UV 320 nm; 0.56 (I), 0.21 (II) and 0.10 (IV) μg/ml	[40]
Anticancer drug: methotrexate	Serum and urine; LiChrospher 100 RP-18; 0.1 *M* SDS in 0.05 *M* phosphate buffer at pH 5.7 for serum, pH 5.2 for urine; UV 305 nm; 90 n*M*.	[50]
Antineoplastic drug: teniposide	Plasma; Chromspher C_{18}; 0.04 *M* SDS–0.5 *M* 1-propanol; amperometry with glassy carbon electrode; 500 ng/ml	[64]

Table 1 (*continued*)

Compounds	Sample; column; mobile phase; detection method; limit of detection	Reference
Antibiotic: tinidazole	Serum; Bondapak CN; 0.05 *M* SDS–6% 1-propanol; UV 320 nm; 0.1 μg/ml	[65]
Amino acid: proline	Urine; C_{18}; 0.03 *M* SDS–8% 1-propanol in 0.01 *M* acetate buffer at pH 5.3 and 40°C, precolumn derivatization with 0.001 *M* Cu^{2+}; UV 235 nm; 10 μg/ml	[48]
Catecholamine: dopamine	Urine; Micropak ODS; 0.01 *M* SDS–3% 1-propanol in 0.02 *M* citrate buffer at pH 4.15 and 0.001 *M* EDTA; amperometry with glassy carbon electrode; 4 pg	[66]
Peptide 520	Plasma; Supelco LC-18; 0.232 g sodium octyl sulphate–22% methanol–78% phosphate buffer at pH 6.5; photolytic amperometry with glassy carbon electrode	[67]
Arsenic compounds: dimethylarsenic acid (I), monomethylarsonic acid (II), As(III) (III), and As(V) (IV)	Urine; Hamilton PRP-1; 0.05 *M* CTAB–10% 1-propanol in 0.02 *M* borate buffer at pH 10.2; ICP–MS; 90 (I) and 300 (II, III, IV) pg	[68]
Aluminium	Serum; Capcell Pak MF pH-1; 0.010 *M* SDS–20% acetonitrile (precolumn formation of the 8-quinolinol chelate); fluorescence λ_{exc}=370 nm and λ_{em} =504 nm; 1 ng/ml	[69]

and MS [71,72]. In PF-MEKC, only a part of the capillary is filled with an electrolyte solution containing micelles, thus allowing a separation without the surfactant entering the mass spectrometer. Micelles gradually break down under PF-MEKC conditions and migrate as surfactant monomers at a concentration at/or below the cmc [72].

Electron transfer processes also offer highly sensitive and selective methods for detection of solutes in flowing streams. Various techniques have been devised for these measurements, with the most popular being based on the application of a fixed potential to a glassy carbon solid electrode. Mobile phases containing a surfactant have been reported to prevent the effects of adsorptive fouling of these electrodes [73]. Amperometric detection was successful in detecting endogeneous dopamine in urine samples [66].

RPLC is often used in combination with immunoassay to increase the selectivity. After separation, the fraction containing the analyte is collected and the eluent is usually evaporated to remove organic modifiers, which may have a negative effect on immunochemical and enzymatical reactions. The sample is then dissolved in a buffer compatible with the immunoassay. This time-consuming procedure is hard to automate and may lead to erroneous results due to a decrease in recovery and a potential risk for contamination. For this reason, it is desirable to simplify the procedure through direct transfer of the analyte for immunochemical detection. Laborious sample handling procedures can be obviated when using MLC with a mobile phase compatible with the immunoassay. An example was given for the detection of the corticosteroids budesonide and cortisol eluted with micellar mobile phases of Tween 20 [74].

11. Determination of illegal drugs in sport

The use of performance-enhancing drugs by sportsmen and sportswomen is today recognized as one of the key problems in sport practice. More than 200 drugs used as doping agents or their metabolites must be tested for a variety of sport events. Laboratories are receiving more and more samples, and are expected to keep turn-around times to a minimum. The tedious sample preparation and lengthy analysis times preclude the general use of the existing conventional HPLC procedures for rapid screening. Methods needing little or no sample preparation, resulting in a reduction of analysis time, are preferred. The capability of direct injection MLC in the screening of illegal drugs in sport (stimulants, narcotic analgesics, anabolic steroids, β-blockers and

diuretics) has been studied, mostly in urine [59,75,76]. This is the physiological fluid of choice in doping control because of convenience in taking the samples, relative simplicity and accumulated experience. Particularly, MLC was considered as a worthy technique for the analysis of β-blockers in urine samples [37].

12. Previous extraction of the drugs

Quantitation at low concentration levels of some drugs by MLC with direct injection may not be feasible for several reasons: (i) the drug peak is overlapped by the protein band and the peaks of endogeneous compounds in the physiological matrix, (ii) the drug peak is overlapped by the peaks of other drugs consumed by the individual, or (iii) the LOD is insufficient. Effectively, as with any singular dimension approach with a small injection volume, and the lack of an enrichment step, the sensitivity of the determination is inadequate for many applications.

Some authors have considered the MLC approach still interesting for the control of drugs in physiological fluids, when a previous separation step is required. It is expected that the combined selectivity of extraction procedures and MLC will provide wide resolution power to avoid presumable restrictions. Thus, the antipyrine metabolites 4-aminoantipyrine,-4-methylaminoantipyrine and 4-formylaminoantipyrine were well separated in plasma samples through liquid–liquid extraction with methylene chloride [77], or disposable C_{18}-bonded porous silica cartridges using methanol as eluent [78]. The organic extraction solvent was evaporated to dryness and reconstituted with mobile phase before injection into the chromatographic system.

Also, previous to the MLC separation, chlorthalidone was extracted from plasma into diethyl ether-2-propanol [32], catecholamines were extracted from urine through alumina columns with 0.5 *M* perchloric acid [79,80], and clenbuterol from urine through a cation-exchange sorbent and elution with the same solvent used as mobile phase (0.1 *M* SDS–12% 1-butanol) [81].

13. Column-switching with micellar clean-up

The lack of an enrichment step precludes simple one-dimensional chromatographic separations with direct injection from providing the required LODs for many determinations. In contrast, a multidimensional approach can improve the sensitivity through trace enrichment, use of large injection volumes (e.g., 100 μl), and peak compression due to solvent focusing.

Some reported multidimensional procedures take advantage of conventional RPLC (high column efficiency), and MLC (extended column life with direct injection of physiological fluids, and extraction of protein-bound drugs). This approach demonstrates a practical use of MLC expanded beyond the singular dimensional chromatographic process. The first chromatographic dimension provides the sample extraction and clean-up with a micellar mobile phase. The second dimension, coupled on-line to the first, utilizes conventional RPLC media for the analytical separation. With the proteinaceous material removed in the extraction step, any reversed-phase packing material is compatible with the system. The clean-up and analytical column chromatography should be developed independently. The multidimensional system will be, afterwards, integrated with a column-switching valve.

The excluded components from the clean-up column should occupy the first minutes of the chromatogram with the micellar eluents. Also, the analytical phase should be more retentive for the drug component than the extraction phase with the eluent used during the purge phase. Because of trace enrichment, the purity of all reagents, primarily the surfactant, is especially critical. Otherwise, high protein binding still prevent a satisfactory recovery of drugs from plasma samples. It was demonstrated that if the surfactant (SDS) is added directly to the plasma sample instead of merely being used as a micellar carrier phase, the recoveries are greatly improved [82].

In some reported procedures (see Table 2), the surfactant used in the clean-up column was added to the analytical mobile phase to minimize artifacts from the switching process, or to act as an ion-pair reagent for the analyte. At the high levels of organic

Table 2
Characteristics of published column-switching procedures including an MLC clean-up for the analysis of physiological fluids

Compounds	Sample; clean-up and analytical columns and mobile phases; detection method; limit of detection	Reference
Therapeutically distinct drugs: carbamazepine (I), chloramphenicol (II) and procainamide (III)	Serum; clean-up column: C_8 (I, II), CN (III), 0.04 *M* SDS–14% acetonitrile (I), and 0.02 *M* SDS–4% acetonitrile (II, III); analytical column: Spheri-5 RP-18 and 0.04 *M* SDS–methanol (45:55) in 0.04 *M* phosphate buffer at pH 3 (I), 0.02 *M* SDS–methanol (72:28) in 0.02 *M* phosphate buffer at pH 4.6 (II), and 0.04 *M* SDS–acetonitrile 30:70 in 0.04 *M* phosphate buffer at pH 3 (III); UV 287 nm (I), 278 nm (II) and 280 nm (III); 100 (I), 50 (II) and 70 (III) ng/ml	[7]
Corticosteroid: cortisol	Urine; clean-up column: C_{18} and 0.02 *M* SDS–18% methanol–25% 1-propanol at pH 6, analytical column: C_{18} and 0.02 *M* SDS–38% methanol–2% 1-propanol at pH 6; UV 254 nm; 1.2 ng/ml	[83]
Corticosteroids: budesonide and cortisol	Plasma; for budesonide, clean-up column: Apex II aminopropyl and 0.1% Tween 20 in 0.01 *M* glycine buffer at pH 2.5; analytical column: Spherisorb C_1 and 0.1% Tween 20–3% 1-propanol in 0.01 *M* glycine buffer at pH 2.5; for cortisol, clean-up column: Spherisorb C_1 and 0.1% Tween 20 in 0.01 *M* glycine buffer at pH 2.5; analytical column: Spherisorb C_1 and 0.1% Tween 20–3% 1-propanol in 0.005 *M* Tris–HNO_3 at pH 7.3; immunoassay	[74]
Benzodiazepine: diazepam	Serum; clean-up column: ODS and 0.01 *M* SDS, analytical column: Adsorbosphere ODS and methanol–water 65:35; UV 242 nm; 30 ng/ml	[84]
Antineoplastic drug: teniposide	Plasma; clean-up column: Chromsep C_{18} and 0.02 *M* SDS, analytical column: Bondapak Phenyl and methanol–water 55:45 at pH 7; amperometry with glassy carbon electrode; 10 ng/ml	[64,82]
Anticancer drug: hexamethylene bisacetamide	Plasma and urine; clean-up column: C_{18} and 0.02 *M* SDS–10% methanol at pH 6, analytical column: C_{18} and methanol–water 30:70 at pH 6; UV 210 nm; 1 μg/ml	[85]
Anticancer drug: mitomycin-C	Plasma; clean-up column: C_{18} and 0.02 *M* SDS–10% methanol at pH 6.8, analytical column: C_{18} and methanol–water 30:70 at pH 6.8; UV 365 nm; 1 μg/ml	[86]
Phenols: nitrophenol and its glucuronide	Urine; clean-up column: C_4 and 0.03 *M* CTAB–7% acetonitrile-6-aminohexanoic acid at pH 5, analytical column:C_{18} and 0.03 *M* CTAB–20% acetonitrile-6-aminohexanoic acid at pH 5; UV 300 nm	[26]

modifiers present in these solutions, micelles were severely altered compared to more aqueous media. Also, a specified amount of organic modifier was usually added to the micellar clean-up mobile phase to increase the eluting power of the otherwise relatively weak micellar solution. Saturation of the stationary phase in the clean-up column was maintained by addition of surfactant to the wash solution.

A comparison study was made on the potential of MLC, on-line surfactant-mediated sample clean-up involving column-switching prior to RPLC, and labour-intensive off-line isolation step using liquid–liquid extraction prior to chromatography, for the determination of the anti-neoplastic drug teniposide in plasma, using electrochemical detection [64]. The linearity, reproducibility and recovery of the surfactant-mediated techniques were similar to those of the conventional procedure. With the column-switching procedure, the degree of sample clean-up or preconcentration was similar to that of the conventional procedure, whereas these steps did not exist in MLC resulting in a moderate LOD. In addition to the analytical performance, aspects such as cost, speed and sample throughput per column were considered. An important selection criterion is the manual sample handling that is involved in the procedure. A

skilled technician was occupied almost full-time with the sample preparation in the conventional procedure, much less time was required with the surfactant-mediated procedures. As for the costs involved (disposables, chemicals and labour), these procedures were much cheaper mainly because of the reduced labour cost. In contrast, instrumental complexity was increased in the column-switching technique. Finally, using the surfactant-mediated clean-up procedure, a maximum of 150 samples could be processed on a single precolumn, which was simple and inexpensive to clean, whereas with MLC the column should be replaced after less than 100 injections [64]. Careful care of the column should increase column life.

14. Conclusions

MLC is a useful technique to avoid the sample preparation step entirely, by making direct injection of the physiological sample in the chromatographic system. An important feature of direct injection is that the same column can be used for all assays in a series of specimens, the risk of non-reproducibility of off-line procedures previous to the chromatographic separation is then eliminated. Another advantage is the low sample demand of only a few microliters. For blood analysis, for instance, finger-puncture samples are adequate for analysis, an important consideration in pediatric applications. Great care should be taken however in order to keep the endogeneous proteins solubilized during chromatography. Certain eluent restrictions exist to ensure that the matrix components remain in solution (e.g., the concentration of surfactant should be well above the cmc, and the organic modifier content should be kept as low as possible, preferably below 10%).

There are, however, also occasions where direct injection principles are unsuitable. Since there is no elimination of interferences of both exogeneous and endogeneous compounds, nor pre-concentration of the drug, frequently large volumes of untreated physiological fluids need to be injected in order to determine drugs whose therapeutic ranges lie in the ng/ml range. Injection of large volumes will create however blockage of the analytical column and buildup of strongly retained endogeneous compounds. Also, because there is no sample clean-up, the physiological matrix will produce a large signal eclipsing the peaks of early eluting compounds. Optimization should be achieved by judicious selection of the detection scheme. Enhanced detection of the eluted compounds in the micellar mobile phases has been reported for various techniques, but more work is needed to investigate their real potentiality. Finally, column-switching with on-line surfactant-mediated sample clean-up is an attractive sample enrichment technique which merits future development.

Some of the reasons that explain the relatively few applications reported to date for MLC in clinical and toxicologic analysis may be that most of the present extraction/reconstitution methods are well established, or the method development in MLC is unfamiliar. The ability to inject physiological samples directly into a micellar system will no doubt in the future be exploited.

15. Abbreviations

Brij-35	polyoxyethylene(23)dodecyl
BSA	bovine serum albumin
C12 DAPS	3-(dimethyldodecylammonium propanesulphonate
Cmc	critical micellar concentration
CMX	cefmenoxime hemihydrochloride
CTM	cefotiam dihydrochloride
ESI–MS	electrospray mass spectrometry
HPLC	high-performance liquid chromatography
ICP–MS	inductively coupled plasma–mass spectrometry
ISRP	internal surface reversed-phase
LOD	limit of detection

Log K_H	protonation constant
MEKC	micellar electrokinetic chromatography
MLC	micellar liquid chromatography
MS	mass spectrometry
NED	*N*-(1-naphthyl)ethylenediamine dihydrochloride
PF-MEKC	partial-filling micellar electrokinetic chromatography
RPLC	reversed-phase liquid chromatography
RSD	relative standard deviation
SDS	sodium dodecyl sulphate

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