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Strategy for the development of automated methods involving dialysis and trace enrichment as on-line sample preparation for the determination of basic drugs in plasma by liquid chromatography

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Abstract

Among the sample preparation techniques, dialysis followed by clean-up and enrichment of the dialysate on a pre-column has proved to be a useful approach for the LC determination of drugs in plasma. By use of sample processors, like the ASTED system, such bioanalytical methods can be fully automated, the dialysis and trace enrichment steps being directly coupled to LC. In order to facilitate the development of such automated methods, a strategy based on a decision tree has been elaborated. After the selection of appropriate conditions for the LC analysis, the decision tree provides information about suggested starting conditions and guidelines for the optimisation of the most important parameters likely to influence analyte recovery and method selectivity. The plasma samples are dialysed on a cellulose acetate membrane in the static-pulsed mode and the dialysate is enriched on a trace enrichment pre-column packed with octadecyl silica or with a strong cation-exchange material. This decision tree is until now restricted to the analysis of basic drugs in plasma. In order to demonstrate the applicability of this method development strategy, an automated procedure based on the coupling of dialysis with trace enrichment has been developed for the LC determination of antifungal agents (clotrimazole, econazole and miconazole) in plasma.

Keywords: Dialysis; Sample preparation; Automation; Method development; Clotrimazole; Econazole; Miconazole; Basic drugs

1. Introduction

Methods for the quantitative analysis of drugs in biological fluids most often involve nowadays the use of reversed-phase liquid chromatography (LC). However, the direct injection of a biological sample into an LC system is generally not possible. Therefore, the majority of bioanalytical methods include one or more sample preparation steps. Many strategies have been developed to eliminate proteins and other matrix macromolecules, isolate the analyte(s) from potentially low molecular mass sample components and increase the analyte concentration.

Over the past few years, fully automated bioanalytical systems have been used increasingly for the determination of drugs and metabolites in biofluids, especially when the number of samples to be analysed is rather large, such as bioavailability, bioequivalence and clinical studies. Automated sample pretreatment cannot only increase productivity, but also improve significantly the precision and the trueness of the whole procedure.

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Among the different sample preparation strategies combined with LC to develop automated methods in bioanalysis, dialysis followed by clean-up and enrichment of the dialysate on a short pre-column has proved to be an attractive approach for the LC determination of drugs in biofluids and especially in plasma [1–16]. The use of a semi-permeable dialysis membrane allows the removal of matrix macromolecules and in particular proteins as well as the diffusion through the pores of this membrane of low molecular mass compounds, such as the analyte(s) of interest, into a dialysis liquid. Moreover, the sample dilution caused by the dialysis step can be overcome by the enrichment of the analyte(s) on a pre-column (TEC or trace enrichment column). By use of a sample processor, like the ASTED (Automated Sequential Trace Enrichment of Dialysate) XL system, dialysis and trace enrichment are directly coupled with LC.

In spite of the interesting features of this sample handling technique [17,18], its use is still rather limited, probably because method development is not straightforward and can be relatively time consuming. For automated systems, the lack of information about method development probably represents the main limitation.

In order to facilitate the development of such automated methods, a strategy based on a decision tree has been elaborated on the basis of our expertise in the optimisation of procedures with the ASTED XL system [5,6,13–16], in the same way as that used earlier for developing methods in which automated solid-phase extraction is coupled to LC for the determination of drugs in plasma [19]. This decision tree, which is until now restricted to the analysis of basic drugs in plasma, provides information about suggested starting conditions and guidelines for the optimisation of the most important parameters likely to influence analyte recovery and method selectivity. The plasma samples are dialysed on a cellulose acetate membrane (cut-off: M_r 15,000) and the dialysate is flushed onto a TEC packed with octadecyl silica or with a strong cation-exchange material on which the analyte(s) of interest is concentrated. By means of a switching valve, the target analyte(s) is desorbed from the TEC in the back-flush mode by the LC mobile phase and transferred to the analytical column.

The main purpose of this paper is to present the method development strategy and to describe the decision tree on which this strategy is based. The optimisation scheme of the dialysis and trace enrichment steps is divided in two parts: firstly, the procedure is developed by use of aqueous solutions and secondly, the application of plasma samples is considered. Then, the applicability of this strategy will be tested for the development of automated procedures based on the combination of dialysis and trace enrichment using three antifungal agents (clotrimazole, econazole and miconazole) (Fig. 1) as model compounds. The operating conditions obtained for the LC determination of these drugs in plasma will be presented.

A few procedures have been reported for the analysis of these compounds in plasma using either liquid chromatography [20–22] or gas chromatography [23]. In these bioanalytical methods, sample preparation prior to chromatographic analysis consisted of deproteinization [20] or liquid–liquid extraction after alkalinization followed by double extraction, the first into an acidic aqueous solution and the second into a basic medium [23]. Solid-phase extraction on disposable cartridges was also proposed [21,22]. To our knowledge, no automated procedure has been reported for the determination of these antifungal agents in biofluids.

2. Experimental

2.1. Chemicals and reagents

Econazole nitrate, clotrimazole and miconazole nitrate were obtained from Sigma (St. Louis, MO, USA). They were used without further purification. Potassium dihydrogenphosphate, potassium hydroxide, phosphoric acid (85%, w/w), hydrochloric acid (37%, w/v) and sodium azide were purchased from Merck (Darmstadt, Germany) and were of analytical grade. Monochloroacetic and trichloroacetic acids were supplied by Acros Chimica (Geel, Belgium). Methanol and far-UV acetonitrile, both of LC grade, were purchased from Fisher Scientific (Leicestershire, UK). The water was purified by means of a Milli-Q system (Millipore, Bedford, MA, USA).

The analytical column was preceded by a guard column, both pre-packed with LiChrospher 60 RP Select B (particle size: 5 μm) (Merck). The trace

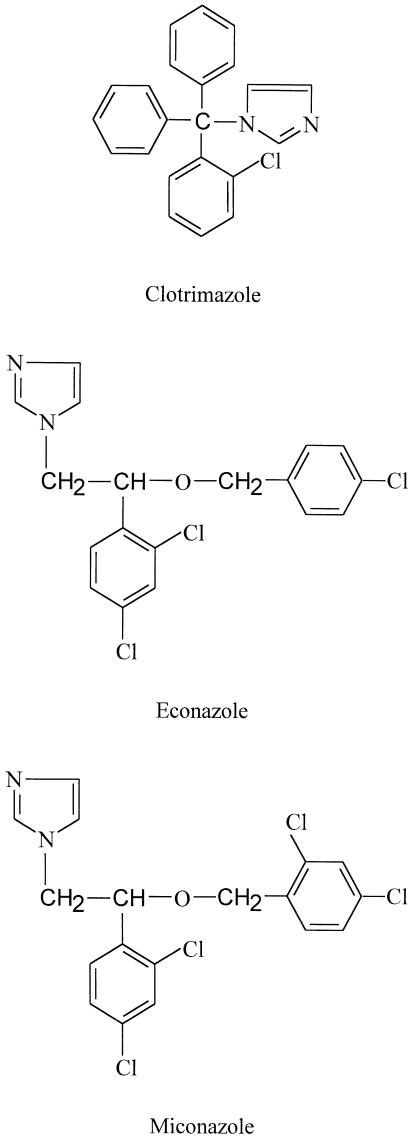

Fig. 1. Structure of the antifungal agents used as model compounds.

enrichment column (TEC) contained 70 mg of octadecyl silica (Hypersil, particle size: 10 μm) and was purchased from Gilson (Villiers-le-Bel, France).

2.2. Apparatus

The dialysis and trace enrichment steps were performed on a Gilson ASTED XL module connected on-line with the LC system. A schematic representation of the ASTED XL system is depicted in Fig. 2. It consisted of an auto-sampler equipped with two model 401C dilutors fitted with 1-ml syringes, a flat-bed dialyser with donor and acceptor channel volumes of 370 and 650 μl, respectively, and two Rheodyne model 7010 six-port switching valves (Berkeley, CA, USA), one of which being connected with the TEC. The donor and acceptor channels were separated by a cellulose acetate membrane (Cuprophan) with a molecular mass cut-off of 15,000. The TEC consisted of a titanium tube (5.8×4.6 mm I.D.) contained in a stainless steel holder (Gilson).

The LC system was composed of a model 305 pump coupled with a model 805 manometric module (Gilson) and of a model Dynamax UV-1 variable-wavelength UV–visible absorbance detector (Rainin, Woburn, MA, USA).

A Manu-CART system contained a LiChroCART analytical column (125×4 mm I.D.) and a short LiChroCART guard column (4×4 mm I.D.) (Merck) and was thermostated at 35±0.1°C in a model 20 B/VC Julabo waterbath (Seelbach, Germany).

The ''715 HPLC System Controller'' software loaded on an IBM compatible computer (PC-AT; CPU 80486) and the ''722 Keypad'' software were used to control the LC and the ASTED systems, respectively.

2.3. Chromatographic conditions

The LC mobile phase consisted of a mixture of methanol–0.02 *M* phosphate buffer (pH 3.5) (63:37, v/v) and was degassed for 15 min in an ultrasonic bath. The flow-rate was 1.0 ml/min. Prior to use, the phosphate buffer was passed through a 0.45 μm membrane filter from Schleicher and Schuell (Dassel, Germany). The antifungal agents were monitored photometrically at 230 nm.

2.4. Standard solutions used for method development

Stock solutions of each analyte were prepared in methanol at a concentration of 1.0 mg/ml. Each standard solution was stored in a refrigerator at 4°C when not in use. During method development, a mixed solution was then prepared by diluting 1.0 ml of each stock solution with a mixture of water–

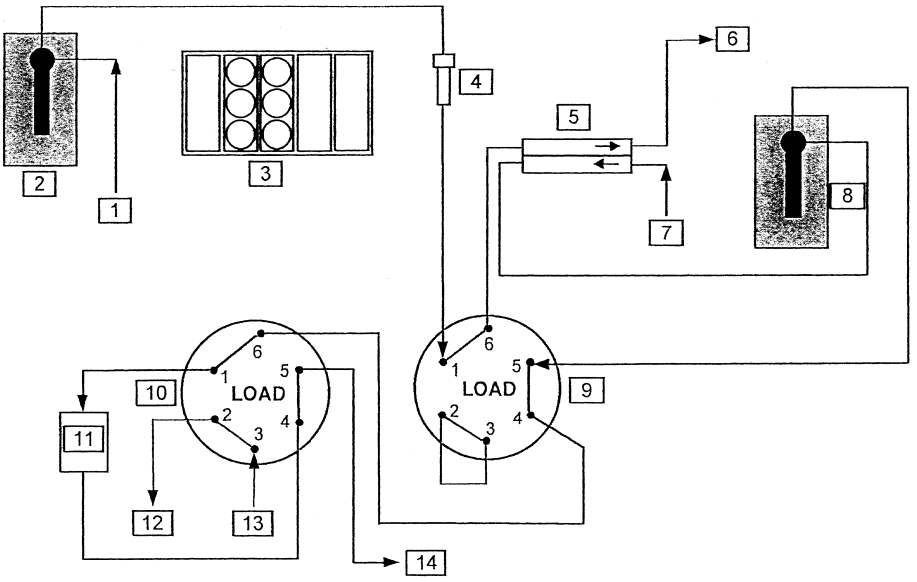

Fig. 2. Schematic representation of the ASTED XL system. 1, Priming solution; 2, dilutor no. 1; 3, racks; 4, injection port; 5, flat-bed dialyser; 6, waste; 7, dialysis liquid; 8, dilutor no. 2; 9, switching valve no. 1; 10, switching valve no. 2; 11, trace enrichment column (TEC); 12, LC column; 13, LC pump; 14, waste. Both switching valves are in LOAD position during dialysis and trace enrichment steps.

methanol (80:20, v/v) in order to obtain a concentration of 10 μg/ml for each compound. This intermediate solution was stored in a refrigerator at 4°C and was found to remain stable for at least 1 week.

This solution was then diluted with a mixture of 0.02 *M* phosphate buffer (pH 2.5)–methanol (90:10, v/v) or plasma to reach a final concentration of about 1.5 μg/ml for each analyte. These solutions were prepared daily.

2.5. Other solutions

The solution used as protein releasing reagent was prepared in a 50-ml volumetric flask by mixing 4.3 ml of hydrochloric acid with 25 ml of a mixture of acetonitrile–water (80:20, v/v) and by adding the same solvent mixture to the mark. The final concentration of hydrochloric acid was 1 *M*.

2.6. Automated sample preparation

After thawing of the plasma sample at ambient temperature, the only manual operations were its centrifugation at 3900 *g* for 10 min and the introduction of a low volume (0.6 ml) of the sample into a polypropylene vial (0.85 ml) located in the appropriate rack of the sample processor.

All other sample handling operations were then executed automatically by the ASTED XL system. Between each step, the needle was rinsed with 1.0 ml of a mixture of 0.02 *M* phosphate buffer (pH 2.5)–methanol (80:20, v/v) (flow-rate: 30 ml/min) and an air-gap volume of 5 μl was generated inside the transfer tubing before pipetting the next liquid in order to avoid cross-contamination.

Unless stated otherwise, the automatic sequence of operations was performed in the following way.

TEC conditioning (flow-rate: 2.0 ml/min): the TEC was conditioned with 1.0 ml of 0.02 *M* phosphate buffer (pH 3.5) containing 0.005% (w/v) of sodium azide.

Addition of the protein releasing reagent to the plasma sample: 400 μl of plasma were introduced into a vial placed on the collector rack at a flow-rate of 1.0 ml/min. A 100-μl volume of 1 *M* hydrochloric acid in hydro-organic solution was aspirated by the needle of the first dilutor at a flow-rate of 0.2 ml/min and then dispensed at the same flow-rate in the collector vial. Afterwards,

the sample was homogenised by air bubbling (air volume: 0.3 ml; flow-rate: 0.5 ml/min).

Dialysis: the donor channel of the dialyser was filled with 0.37 ml of the sample at a flow-rate of 1.0 ml/min. During the dialysis process, the sample was kept static while 8.1 ml of the dialysis liquid consisted of a mixture of 0.02 *M* phosphate buffer (pH 2.0)–methanol (80:20, v/v) were passed through the acceptor channel at a flow-rate of 1.5 ml/min in 25 successive 0.325-ml pulses. After dialysis, each pulse was dispensed onto the TEC at a flow-rate of 1.0 ml/min.

TEC washing (flow-rate: 1.0 ml/min): when dialysis in the static-pulsed mode was discontinued, the TEC was washed with 1.0 ml of 0.02 *M* phosphate buffer (pH 3.5).

Elution (flow-rate: 1.0 ml/min): by rotation of a switching valve, the analytes were desorbed from the TEC and transferred to the analytical column in the back-flush mode with the LC mobile phase.

Washing of the dialyser (flow-rate: 3.0 ml/min): the donor channel was rinsed successively with 1.0 ml of a mixture of 0.02 *M* phosphate buffer (pH 2.5)–methanol (80:20, v/v) containing 0.005% (w/v) of sodium azide, 2.0 ml of a solution consisted of the same mixture in the proportions 50:50 (v/v) and 1.0 ml of the mixture used initially. Meanwhile, the acceptor channel was washed with 4.0 ml of the solution used as dialysis liquid.

The liquid chromatographic analysis of the prepared sample was then performed during the handling of the next sample (concurrent mode).

3. Results and discussion

3.1. Strategy for the development of bioanalytical LC procedures using dialysis and trace enrichment as on-line sample preparation techniques

The strategy proposed for the optimisation of procedures using dialysis and trace enrichment in combination with LC for the determination of drugs in plasma involves four steps. The first step is the selection of a suitable detection mode according to the analyte(s) of interest as well as the sensitivity and selectivity required for the determination method. Although the ASTED system can be combined to liquid chromatography–mass spectrometry (LC–MS), dialysis followed by trace enrichment is not quite suitable as sample preparation techniques for the analysis of drugs in biofluids by LC–MS. Indeed, in the field of the quantitative bioanalysis by LC–MS, sample clean-up appears to be the rate-limiting step. In order to reduce the time for sample pretreatment and to achieve high throughput, it would be better to apply high speed sample preparation techniques, such as on-line solid-phase extraction (SPE) methods or off-line SPE methods using cartridges mounted in a 96-well plate format.

The second step consists of the selection of appropriate chromatographic conditions. In order to avoid interference with the front peak, which is relatively large in the high-sensitivity range when using UV detection, the capacity factor of the analyte(s) should be higher than 3–4.

Then, the development of the sample preparation technique based on dialysis coupled to trace enrichment was considered in detail. Firstly, under suggested starting conditions, the direct injection of an aqueous standard solution on the TEC as well as the dialysis of the same solution coupled to trace enrichment were carried out. At this stage of development, the presence of possible memory effects was investigated. Memory effects give rise to a poor repeatability of the results and residual analyte peaks on the blank chromatogram obtained after the analysis of a spiked sample. Such effects are due in most cases to the limited solubility of the analytes in the dissolution medium or to their adsorption on the dialysis membrane or on tubes of the ASTED XL system [7,13]. The next step consisted of the elaboration of the sample preparation procedure by means of a decision tree. The latter provides guidelines for the optimisation of the most important parameters likely to influence analyte recovery and method selectivity. The analyte recovery is usually expressed in terms of relative recovery (%), calculated by comparing the peak area obtained after dialysis and trace enrichment with that found by direct injection of aqueous standard solutions of the analyte at the same concentration on the TEC.

3.1.1. Suggested starting conditions

Table 1 shows the suggested starting conditions by

Table 1
Suggested starting conditions

Step	Operating conditions
TEC conditioning (C_{18} support)	
Liquid	LC mobile phase buffer+azide (0.005%, w/v)
Volume (ml)	1.0
Dispensing flow-rate (ml/min)	2.0
Dialysis	
Mode	Static-pulsed
Dialyser (volumes of donor and acceptor channels (μl))	370–650
Sample	Aqueous solution
Sample volume (μl)	370
Dialysis liquid	LC mobile phase buffer
Volume of dialysis liquid (ml)	6.5
Volume of pulses (μl)	325
Number of pulses	20
Aspirating flow-rate of the dialysis liquid (ml/min)	1.5
Dispensing flow-rate of the dialysate (ml/min)	1.0
TEC washing	
Liquid	LC mobile phase buffer
Volume (ml)/flow-rate (ml/min)	1.0/1.0
Washing of the donor channel of the dialyser	
Liquid	LC mobile phase buffer+azide (0.005%, w/v)
Volume (ml)/flow-rate (ml/min)	2.0/3.0
Washing of the acceptor channel of the dialyser	
Liquid	Dialysis liquid
Volume (ml)/flow-rate (ml/min)	2.0/3.0

applying aqueous standard solutions of the analyte(s) for the direct injection on the TEC packed with octadecyl silica as well as the coupling of dialysis to trace enrichment. These operating conditions have been selected on the basis of our expertise in the optimisation of the bioanalytical procedures with the ASTED XL system [13–16].

Dialysis can be performed by means of different modes. The simplest and the least efficient mode of dialysis is equilibrium dialysis, where both the sample contained in the donor channel of the dialyser and the dialysis liquid are stagnant. The drawback of equilibrium dialysis is that the concentration gradient across the membrane and the diffusion rate decrease as time increases. Consequently, analyte recovery is low. This dialysis mode can be used when the analyte concentration is high and/or the sensitivity is not a problem. To overcome these disadvantages, continuous dialysis can be applied. In this case, the sample remains stagnant while the dialysis liquid is passed through the acceptor channel in successive pulses (static/pulsed mode) or continuously at a constant flow-rate (static/continuous mode). The comparison between these two dialysis modes has demonstrated that the recoveries obtained with the static/continuous mode were lower than those observed with the static/pulsed mode [15].

3.1.2. Optimisation scheme of dialysis and trace enrichment steps

The optimisation scheme of dialysis and trace enrichment steps has been elaborated on the basis of a decision tree, which can be applied to any basic drug irrespective of its polarity. As can be seen in Figs. 3 and 4, the decision tree has been divided in two parts: the first one represents the optimisation scheme for an aqueous standard solution (Fig. 3), whilst the second one depicts the optimisation of

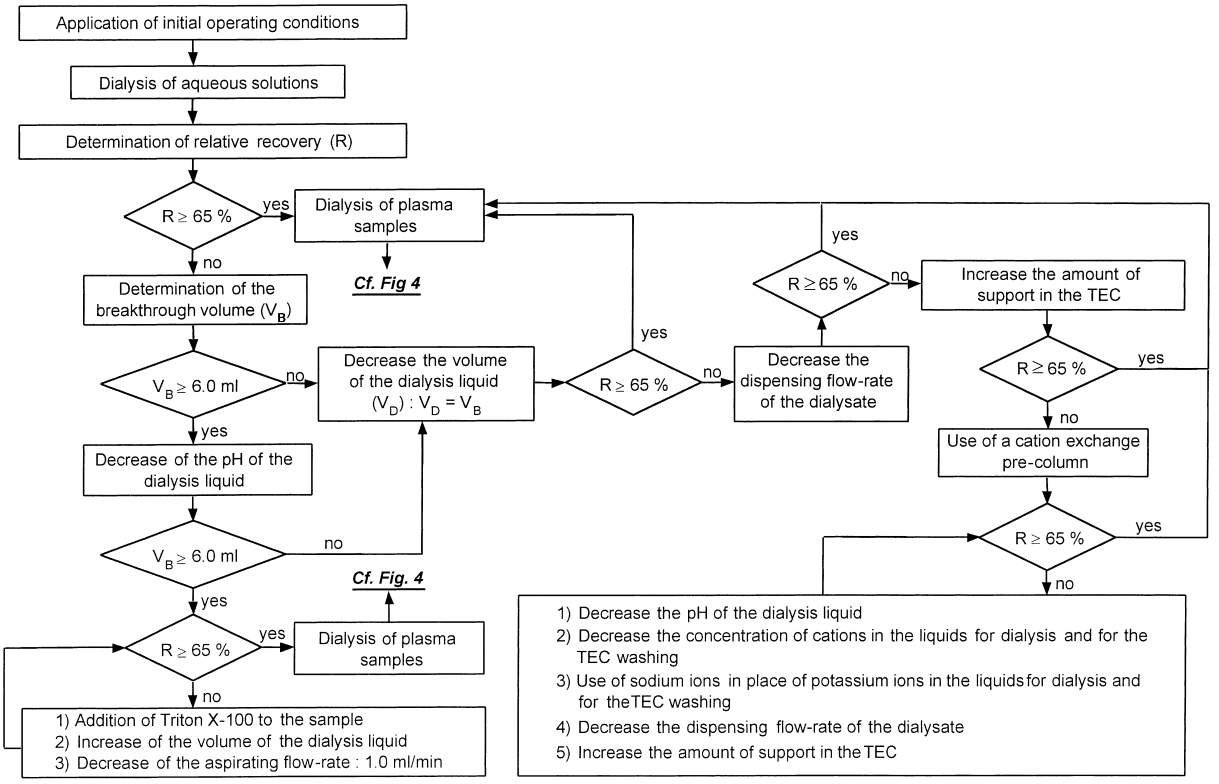

Fig. 3. Optimisation scheme of the dialysis process (part A; aqueous samples).

dialysis and trace enrichment by applying plasma samples (Fig. 4). The objective is to obtain operating conditions that guarantee a selective method towards endogenous components from plasma as well as a high analyte recovery. The latter should be equal or superior to 65%. This target value was selected according to the recoveries of the different compounds for which we have developed automated LC procedures based on the combination of dialysis to trace enrichment [13–16]. The observed recoveries were close or superior to 65%.

When the recovery of the analyte(s) of interest was not satisfactory from an aqueous standard solution, the breakthrough volume was first determined. The breakthrough volume of a compound is defined here as the volume of liquid needed for its 50% elution from the TEC [5,15] and it can be determined using the method described previously [5]. If the breakthrough volume was higher than 6 ml, the pH of the dialysis was decreased. Indeed, a decrease of dialysis pH could have a significant influence on analyte recovery. At high pH, a strong hydrophobic interaction between the basic compounds and the dialysis membrane could take place, which would lead to a decrease in analyte recovery. The analyte recovery was evaluated unless the breakthrough volume was lower than 6 ml. Then, if needed, several options, such as the addition of Triton X-100 to the sample, the increase of the dialysis volume or the decrease of the flow-rate for the dispensing of the dialysate onto the TEC, could be tested in order to increase the analyte recovery.

If, in spite of the decrease of the pH of the dialysis liquid, the breakthrough volume remained inferior to 6 ml, this volume was taken as the maximal volume of the dialysis liquid. Under such conditions, since the affinity of the analyte is low for the sorbent (octadecyl silica) of the TEC, it is advisable first to decrease the dispensing flow-rate of the dialysate, second to increase the amount of support in the TEC

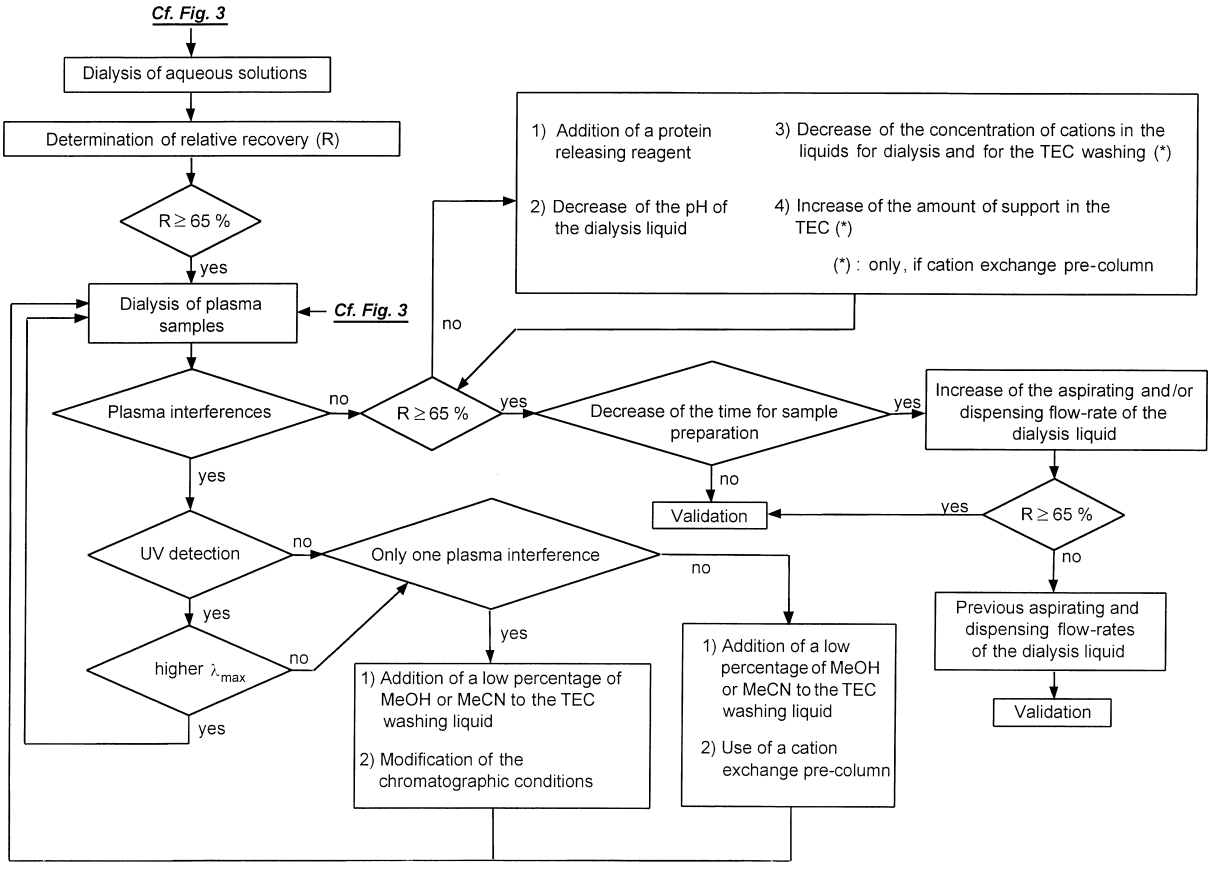

Fig. 4. Optimisation scheme of the dialysis process (part B; plasma samples).

and third to use a cation exchange pre-column for trace enrichment, like the HEMA-SB (S-hydroxyethylmethacrylate-BIO 1000 sulphobutyl) phase [14–16]. At each proposed option, the analyte recovery had to be evaluated before considering the next step.

If the analyte recovery was lower than the target value when using a TEC packed with a cation exchange material, it was then suggested to decrease the pH of the dialysis liquid, to decrease the concentration of cations in the liquids used for dialysis and for the TEC washing, and/or to use sodium ions instead of potassium ions in these liquids. Indeed, it has been demonstrated that an increase of the ionic strength of the dialysis liquid gave rise to a decrease in the breakthrough volumes, due to competition effects from the co-ions [15,16]. Moreover, lower breakthrough volumes were obtained in the presence of potassium ions, due to a stronger competition effect from these ions, compared to that of sodium ions [15]. Other options were also proposed, such as decreasing the dispensing flow-rate of the dialysate or increasing the amount of cation exchange material in the TEC. Between each modification in the operating conditions, it is necessary to check whether the analyte recovery has been influenced significantly.

The objective was reached when the analyte recovery was equal or higher than 65%. Once an acceptable method had been developed for aqueous standard solutions, method selectivity and analyte recovery were evaluated with spiked plasma samples. If needed, the optimisation process was continued, as shown in Fig. 4.

If the determination of the analyte was disturbed

by interfering peaks, some options were given, such as the use of a more selective detection system or, with UV detection, the use of a higher measuring wavelength. The analyst could also add a low percentage of methanol or acetonitrile to the TEC washing liquid, adapt the chromatographic conditions in order to separate the interfering peaks from that of the analyte or use a cation exchange material in the TEC, which usually improves method selectivity towards endogenous components of plasma.

Once the method selectivity had been demonstrated, the analyte recovery was determined. A problem commonly encountered using dialysis for the treatment of plasma samples is a substantial decrease in recovery, often caused by the binding of the analytes to plasma proteins [1,3,7,13,16]. Indeed, only the unbound drug fraction can actually diffuse through the membrane. In order to determine the total drug fraction and to increase analyte recovery, a protein releasing reagent, such as monochloracetic acid, trichloroacetic acid or hydrochloric acid, could be added to the plasma sample prior to the dialysis process. With these reagents, the structure of proteins is modified by a change in the sample pH, which increases the concentration of the free analyte. Another approach to increase the free concentration of a drug before the application of dialysis was the use of a compound, such as *n*-octanoic acid, which could displace the compound of interest from its binding sites. The volume of the protein releasing reagent to be added to the sample should be minimal in order to avoid a precipitation of proteins and a too important dilution of the sample. An additional possibility was to decrease the pH of the dialysis liquid. Moreover, if a cation exchange pre-column was used in the enrichment step, decreasing the concentration of cations in the liquids used for dialysis and for the TEC washing or increasing the amount of support in the TEC could lead to an increase in analyte recovery.

Once the recovery had reached the target value, the time for sample preparation could be reduced before validation by increasing the aspirating flow-rate of the dialysis liquid and/or the dispensing flow-rate of the dialysate. If the analyte recovery becomes lower than 65%, it is advisable to keep the previous aspirating and dispensing flow-rates. The last step is the validation of the optimised procedure.

3.2. Applicability of the method development strategy

The usefulness of the proposed method development strategy was then tested for the development of automated procedures using dialysis and trace enrichment as on-line sample preparation techniques for the LC determination of some antifungal drugs (clotrimazole, econazole and miconazole) used as model compounds. The full results obtained for method optimisation as well as for validation will be presented elsewhere [24]. Only a summary of the method development is given hereafter.

Under the starting conditions presented in Table 1, the LC mobile phase buffer used as liquid for the TEC conditioning, the dialysis step, the TEC washing and the washing of the dialyser was 0.02 *M* phosphate buffer (pH 3.5). Relatively important memory effects were observed not only for the direct injection of a standard aqueous solution of the analytes onto the TEC, but also for the combination of dialysis with trace enrichment. Under the starting conditions for dialysis, the memory effects for miconazole, econazole and clotrimazole were 10%, 4% and 3%, respectively. However, these memory effects were eliminated by decreasing the buffer pH (from 3.5 to 2.5) used as liquid for dialysis and the washing of the dialyser as well as by adding a small percentage of methanol (20%, v/v) to the dialysis liquid and to the washing liquid of the dialyser. Under such conditions, the relative recoveries of the three model compounds were close to 60%.

Then, in order to increase the analyte recovery and reach the target value of 65%, the optimisation scheme of the dialysis process shown in Fig. 3 was applied. Since the breakthrough volume for each compound of interest was higher than 6 ml and the pH of the dialysis liquid had been already decreased to eliminate memory effects, the volume of the dialysis liquid was increased from 6.5 to 8.1 ml, which resulted in recoveries higher than 65%. The dialysis liquid was passed through the acceptor channel of the dialyser in 25 successive pulses.

Once the procedure had been developed for aqueous samples, method selectivity towards endogenous components of plasma and analyte recovery were evaluated with spiked plasma samples.

Fig. 5 presents typical chromatograms of blank

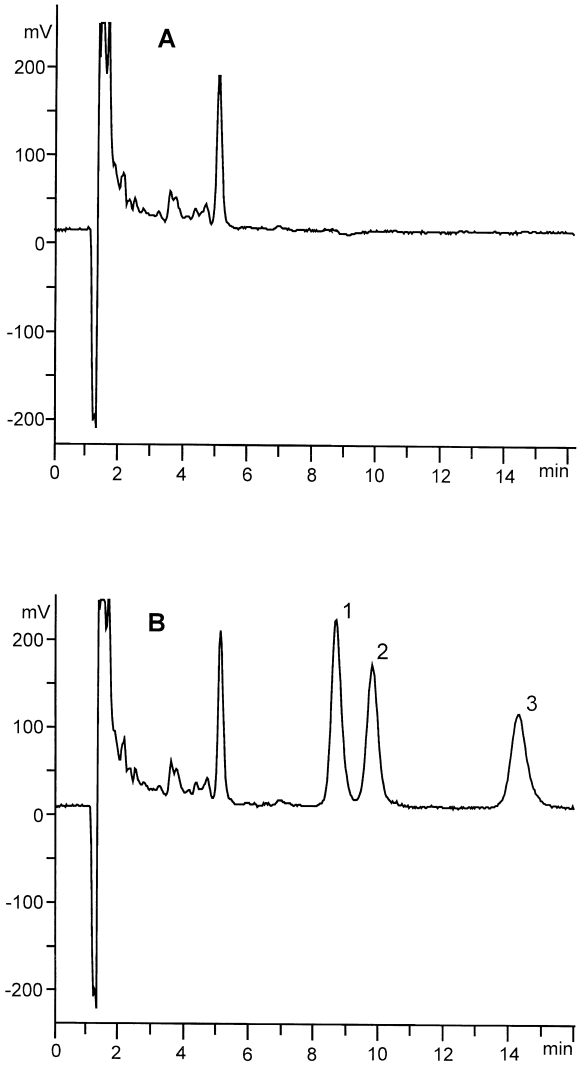

Fig. 5. Typical chromatograms obtained by on-line coupling of dialysis and trace enrichment to LC. (A) Blank plasma sample. (B) Spiked plasma sample (concentration: 1.5 μg/ml). Chromatographic and dialysis conditions as given in Section 2. Peaks: 1, econazole; 2, clotrimazole; 3, miconazole.

and spiked plasma samples obtained by on-line coupling of dialysis and trace enrichment to LC. As shown in this figure, no endogenous sources of interference were observed at the retention time of the different compounds.

However, the relative recoveries for the three compounds were lower than those observed with aqueous solutions of analytes. This decrease in recovery can be attributed to the binding of these drugs to proteins. As suggested in the optimisation scheme presented in Fig. 4, a protein releasing reagent was added to plasma samples prior to the dialysis process in order to displace the protein binding. Monochloroacetic acid (MCA), trichloroacetic acid (TCA) and hydrochloric acid in methanolic solutions were first selected as protein releasing reagents. As expected, the relative recoveries of the three compounds were increased by adding a protein releasing reagent. As can be seen in Table 2, the effect of trichloroacetic or monochloroacetic acids was found to be comparable with respect to the gain in analyte recoveries. Moreover, the most important increase in relative recoveries was observed by adding a relatively high volume (100 μl) of a hydroorganic solution of hydrochloric acid. However, since the target recovery of 65% was not reached, in particular for miconazole, it was decided, as indicated in Fig. 4, to decrease the pH of the dialysis liquid. The decrease of the pH from 2.5 to 2.0 gave rise to an increase in relative recoveries for the three compounds above the target value.

4. Conclusions

A fully automated procedure was developed for the LC determination of three antifungal agents (clotrimazole, econazole and miconazole) with dialysis coupled to trace enrichment as on-line sample handling. The final operating conditions were selected from the conditions settled a priori according to our expertise for the development of procedures with the ASTED XL system and by applying the proposed schemes for the optimisation of the dialysis and trace enrichment steps.

The developed method was selective towards endogenous components of plasma and the recoveries of the three model compounds were higher than 65%, the target value considered as satisfactory for a method based on the dialysis coupled to trace enrichment.

These results demonstrate the applicability of the proposed method development strategy. Although the latter is until now restricted to the analysis of basic drugs in plasma, it can be applied to a number of drugs in order to facilitate the development of

Table 2
Influence of the addition of a protein releasing reagent and effect of the pH of the dialysis liquid on analyte recovery

Protein releasing reagent Composition/volume (μl)	pH of the dialysis liquid	Relative recovery (%; $n=2$)		
		Econazole	Clotrimazole	Miconazole
–	2.5	43.8	44.6	43.8
0.5 *M* TCA[a]/40	2.5	48.4	49	42.3
0.5 *M* TCA[a]/40	2.5	50.3	49.1	44.6
0.5 *M* MCA[a]/80	2.5	55.7	54.3	50.7
1 *M* HCl[a]/40	2.5	50.2	53.4	49.1
1 *M* HCl[a]/80	2.5	56.1	58.5	54.8
1 *M* HCl[a]/100	2.5	59.7	59.7	57.4
1 *M* HCl[b]/100	2.5	64.5	64.8	62.2
1 *M* HCl[b]/100	2.0	67.7	68.8	65.4

MCA, monochloroacetic acid; TCA, trichloroacetic acid; HCl, hydrochloric acid; sample, spiked plasma sample (concentration: 1.5 μg/ml); dialysis liquid, 0.02 *M* phosphate buffer–methanol (80:20, v/v). Other conditions as given in Section 2.

[a] Methanolic solution.

[b] Mixture of acetonitrile and water (80:20, v/v).

automated bioanalytical procedures. Moreover, it should lead to the elaboration of a knowledge-based system.

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