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Optimisation of a dialytic set-up for liquid chromatography: automated separation and preconcentration of ciprofloxacin

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Abstract

Continuous-flow and static dialysis coupled on-line to liquid chromatography was evaluated and an automated method for determination of ciprofloxacin in biological samples developed. A trace enrichment column packed with C_{18} material and coupled with a continuous dialysis and reversed-phase HPLC system with fluorescence detection enabled determination of ciprofloxacin in human blood serum at the 0.1-nmol/l level. The amount of analyte preconcentrated and loaded on the HPLC system was linearly proportional to the concentration in the dialysed sample over more than 4 orders of magnitude (up to $1 \cdot 10^{-6}$ *M*). Data for linearity, repeatability and detectability for each particular set-up are given. The trace enrichment step eliminates band broadening caused by solvents different from those of the eluent and affecting retention of ciprofloxacin on the analytical column (increase in k') due to the on-column change of eluent composition. In analysis of human serum samples phthalates leached from plastic materials may interfere due to coelution with the analyte.

Keywords: On-line dialysis; Trace enantiomer; Clean-up methods; Ciprofloxacin

1. Introduction

In the last decade column liquid chromatography (LC) and mass spectrometry have become the principal techniques for bioanalysis, mainly due to their high resolving power and sensitivity. Although both advanced techniques are used today in routine practice as well as in research, they cannot perform correctly without an appropriate sample preparation step. Removal of particulate matter and other matrix components from biosamples that will disturb the separation process and/or detection of the analyte is an unavoidable step prior to LC analysis of biological and other complex samples [1]. Several clean-up procedures such as precipitation, ultrafiltration and liquid–liquid extraction are currently used today in analytical practice; however none of them comply with the major requirements for chromatographic analysis [1,2]. As emphasized by Van de Merbel [3] only solid-phase extraction (SPE) fulfils all the major requirements for an ideal sample pretreatment technique and has become today one of the most frequently applied sample clean-up techniques for LC analysis. Sorption behaviour and on-line enrichment of polar compounds on SPE precolumns were studied in detail by Brinkman and colleagues [4]. It is known that the trace enrichment cartridge (TEC), besides clean-up, allows an efficient preconcentration of the analyte and thus extends the LC domain to ultratrace analysis. For bioanalysis the TEC should be coupled with an additional clean-up technique to prevent clogging of the cartridge.

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Membrane-based sample preparation coupled on-line with TEC and LC represents state-of-the-art methodology for the analysis of biological samples [2,3,5,6].

Dialysis, the most frequently used membrane-based sample pre-treatment step for chromatography, was described in detail in a review by Van de Merbel et al. [2], and from a recent review by the same author [3], the expanding growth of applications is evident. A significant contribution to the on-line coupling of dialysis with TEC and LC was made by Cooper and Turnell [7], by developing an automated sequential trace enrichment of dialysates (ASTED) system for the totally automated preparation of biological samples. After development of a commercially available ASTED system [8], this experimentally rather complicated and susceptible approach became reliable and robust, and today represents a very simple and effective automated sample pre-treatment technique in bioanalysis [6,9–14].

In a previous paper [15], a different mode of dialysis and the parameters influencing the dialytic separation and efficiency of ciprofloxacin (CF) fluoroquinolone were studied. Ciprofloxacin is an antibiotic usually used to treat bacterial infections and has recently been approved by the US Food and Drug Administration to reduce the incidence or progression of anthrax by inhalation. Numerous techniques and methods have been developed for the determination of fluoroquinolones in biomedical samples. Official, compendial and other methods of analysis of 4-quinolone antibacterials were reviewed briefly by Belal et al. [16], while HPLC analysis of fluoroquinolones in biological fluids was reviewed recently by Carlucci [17]. Although some very sensitive voltammetric methods appeared in the literature [18], HPLC became the method of choice in routine practice and in clinical and pharmacological studies of CF in body fluids [16,17,19–24]. Most of the recently published methods utilised UV [20,23–25] or fluorescence detection [19,21,22], since CF is one of the relatively few drugs that exhibits a high native fluorescence.

In papers reporting the determination of CF in biological samples by HPLC, samples are usually pre-treated by protein precipitation or by liquid–liquid extraction and no reports on dialysis on-line coupled to SPE were found. Since dialysis of CF was investigated in our previous paper [15] in detail, in the present study several aspects of a dialytic set-up regarding trace enrichment were evaluated. Both static and continuous modes of dialysis were used and some parameters influencing the chromatographic behaviour of CF caused by the incorporation a TEC in the system were studied. The analytical system consisting of a dialytic clean-up, trace enrichment and LC separation with fluorescence detection was optimised and automated using a few simple components commonly found in every analytical laboratory.

2. Experimental

2.1. Chemicals and reagents

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecar-boxylic acid, MW 331.4) was of synthesis grade and supplied as CF-hydrochloride salt by Krka (Novo mesto, Slovenia). Analytical grade phosphoric acid (85%) was from Merck (Darmstadt, Germany) and HPLC-grade acetonitrile was purchased from Scharlau (Barcelona, Spain). Human blood serum was supplied by the Institute for Blood Transfusion (Ljubljana, Slovenia) and stored in a deep freezer. Double deionised water was additionally purified by a Milli-Q system (Millipore, Bedford, MA, USA).

A stock solution of CF ($1 \cdot 10^{-4}$ *M*) was prepared once per week in the mobile phase and stored in a refrigerator at 6 °C. Working solutions of lower concentrations were prepared daily by dilution with the mobile phase or deionised water. The mobile phase (MP) consisted of 0.01 *M* phosphoric acid–acetonitrile (70:30, v/v) of operational pH 2.64, determined in the aquo-organic mixture according to the procedure recommended by Barbosa and Sanz-Nebot [26], if not stated otherwise.

2.2. Equipment

The liquid chromatographic system consisted of a ConstaMetric IIIG pump (LDC Milton Roy, Riviera Beach, FL, USA), a Shimadzu fluorescence detector (D) RF-535 (Kyoto, Japan; excitation wavelength

287 nm and emission wavelength 455 nm) and a Hewlett-Packard HP 3395 integrator (Waldbronn, Germany). In all LC experiments the analytical columns (AC) were equipped with a 10×4 mm guard cartridge, packed with 3-μm Hypersil BDS C_{18} material in a stand-alone holder (ThermoQuest, Hypersil Division, Runcorn, UK). A Thermo Hypersil 150×4.6 mm column (column 1) packed with 3-μm Hypersil C_{18} stationary phase (ThermoQuest, Hypersil Division, Runcorn, UK) and a Luna Phenyl-Hexyl 150×4.6 mm column (column 2), packed with 5-μm particles (Phenomenex, Torrance, CA, USA) was used for LC separations. For preconcentration a 10×2 mm I.D. Spark (Aj Emmen, The Netherlands) trace enrichment cartridge (TEC), packed with 40-μm C_{18} highly hydrophobic material (A05) and installed in a Spark cartridge handclamp, was used. The TEC was mounted on a Knauer (Berlin, Germany) six-port Model A0377 switching valve and actuated manually or automatically by a home-made interface via an autosampler or computer. Samples were introduced into the on-line LC-dialytic system via an ISMATEC, MS-4 Reglo/8-100 peristaltic pump (Glattbrugg-Zuerich, Switzerland) and/or with a Spark (Aj Emmen, The Netherlands) Basic Marathon 816 autosampler (AS). CF standards were injected by a Model 7125 Rheodyne valve (Cotati, CA, USA) or via the autosampler, using a 20-μl sample loop. LC measurements were performed at a flow-rate of 0.5 ml/min at ambient (22±2 °C) temperature, if not stated otherwise.

The dialytic system consisted of a B 54535 Gilson (Villiers-le Bel, France) dialysis cell with donor and acceptor channel volumes of 100 and 175 μl, respectively. A Gilson B 54561 cellulose acetate membrane with 15-kDa molecular mass cut-off was used throughout the work. All connections on the low pressure side of the dialysis system were made by 0.5-mm I.D. Teflon tubing with the exception of the peristaltic pump tubing, which was made of tygon.

2.3. Dialysis set-up and procedure

The on-line dialysis–LC system consisted of the following main components: a sample introducing unit (autosampler and/or peristaltic pump), a dialysis cell, a trace enrichment column and liquid chromatograph (HPLC pump, guard and analytical columns and fluorescence detector with integrator). Two types of dialysis were used: static dialysis and continuous-flow dialysis [5]. In the static mode the donor phase was stagnant, while in continuous dialysis both phases were continuously pumped by the peristaltic pump at a flow-rate of 0.5 ml/min, if not stated otherwise. According to the dialysis mode different set-ups were used as described below.

The experiments were performed in triplicate using 0.01 *M* phosphoric acid (PA) of pH 2.24 both as the acceptor solution for the dialysis process, as well as for the transfer of the dialysate to the TEC. In the static mode sample was introduced by the digital dispensing unit (DDS) of the autosampler. After a definite collecting period, i.e. dialysis time, the analyte accumulated on the TEC was backflushed (injected) by the mobile phase to the analytical column by switching the valve, and the chromatogram was recorded. Between each series of experiments the sample line and the donor channel were flushed with deionised water, and the acceptor line including TEC with phosphoric acid, for 3–5 min. Determination of CF in serum samples was performed by the standard addition technique, i.e. after dialysis of the original sample an appropriate amount of CF was added to a sample aliquot and dialysed at the same conditions. The concentration of CF in the original sample was calculated from the increase in peak area.

3. Results and discussion

Dialysis through porous membranes is based on diffusion, which is a rather slow process. In liquids, its rate is ~0.5 mm/min and can be accelerated substantially by forced convection. Although the mass transfer through membranes is affected by diverse phenomena [27], dialysis efficiency (DE) can be generally optimised by a proper choice of the membrane (material, porosity, thickness), dialysis cell (exposed membrane area per channel volume), chemical composition of the donor and acceptor phases, and flow regime and rate through the channels. When optimising the dialysis set-up, understanding of basic principles is necessary and all the experimental details of importance should be careful-

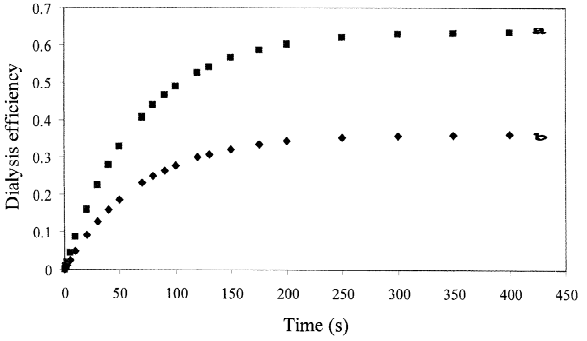

Fig. 1. Dependence of DE for equilibrium dialysis [2] on time at two channel volumes: (a) $V_a = 175$ μl, $V_d = 100$ μl; (b) $V_a = 100$ μl, $V_d = 175$ μl. Other parameters: $D = 1 \cdot 10^{-5}$ cm^2/s, $l = 0.02$ mm, $\tau = 3$, $A = 3.7$ cm^2.

ly considered. In Fig. 1, the effect of the channel volumes on the time dependence of DE for the dialysis cell used in this work is simulated for stationary equilibrium dialysis according to the theoretical equation [2]. It is evident that in equilibrium dialysis using a channel with a smaller volume for the acceptor phase (curve b) results in a substantially lower DE (more than 50%) than when a channel with a larger volume was used for collection of the analyte (curve a). Therefore, for ''do it yourself'' analysts a careful study of the theory and relevant literature in this field [2,3,8,27] is highly recommended.

3.1. Influence of the TEC on LC performances

As described previously [15], CF is an amphiprotic compound and can be enriched by dialysis alone if appropriate pH values of donor and acceptor are used. To ensure a high flux through the membrane CF should be in its zwitterionic $CF^{\pm}$ or neutral CF^0 form in the donor phase (pH 7–8) and in its protonated form HCF^+ in the acceptor phase (pH$<$ 4). Therefore, we applied an aqueous solution of CF standards or samples to the donor channel and 0.01 *M* phosphoric acid was used as the acceptor solution. Instead of a static acceptor, where the collected analyte was transferred to the LC via a sample loop [15], we incorporated a trace-enrichment step and continuously flowing acceptor to further improve clean-up and to achieve additional enrichment [2–6].

On the basis of chromatographic experiments with an RP column and mixtures of 0.01 *M* H_3PO_4/KH_2PO_4–acetonitrile as mobile phase, it was found that the retention factor, k', of CF increases with increasing pH (between pH 2 and 6) and decreases with increasing amount of acetonitrile in the mixture. The retention time, t_R, of CF equals ~2.6 min in 70:30 mobile phase and column 2 (described in the Experimental section), and increases from 4.5 to 19.5 min in 80:20 and 90:10 (v/v) mixtures of phosphoric acid–acetonitrile, respectively. CF is retained completely in pure 0.01 *M* phosphoric acid. Since we intended to determine CF at trace level and in a small volume of samples (less than 1 ml), a relatively coarse (40 μm) highly hydrophobic preconcentration column and 0.01 *M* phosphoric acid solution as acceptor were chosen to adsorb the analyte in a small segment of the sorbent bed. To achieve desorption of the analyte from the TEC in a narrow zone profile, backflushing was carried out with a mixture of 0.01 *M* phosphoric acid–acetonitrile with the volume fraction of the organic component between 0.15 and 0.35, i.e. the same eluent as used for LC.

To study the sorption/desorption process and loading capacity of the TEC, the simple set-up shown in Fig. 2 was arranged. According to the

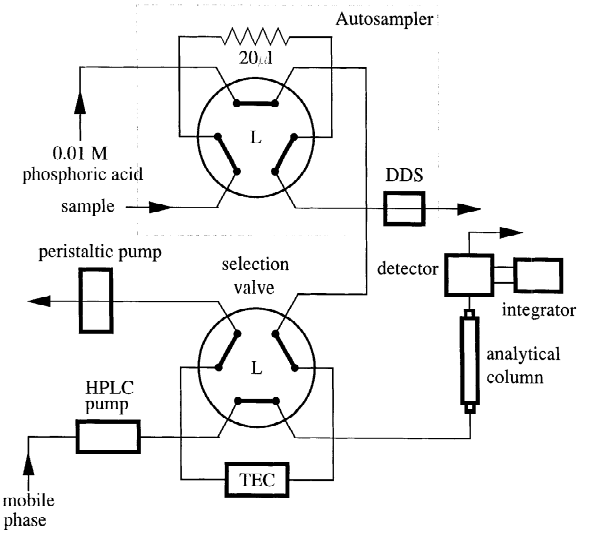

Fig. 2. Schematic representation of the set-up for study of TEC capacity and its influence on LC performance. Both valves shown in the load positions (L); flow-rates of HPLC pump and peristaltic pump are 0.5 ml/min.

scheme, standard solutions of CF were introduced via autosampler into a stream of phosphoric acid aspirated by peristaltic pump (0.5 ml/min) through the TEC (30 s) and then injected to the LC system. The procedure begins with flushing of the sample needle, aspiration of sample through the sample loop, injection of sample into a stream of phosphoric acid and proceeds automatically by entering an appropriate time sequence through the autosampler keyboard. The following steps, i.e. sample transfer to the TEC, sample injection to the LC and start of the chromatogram recording, were triggered via an interface, optically coupled to the auxiliary output of the autosampler. In this way all steps were exactly reproducible and controlled simply by the software of autosampler.

Recovery of the sorbed analyte was investigated by varying the concentration of CF between $1\cdot10^{-8}$ and $1\cdot10^{-6}$ *M* and the number of successive injections (one to five) of the same analyte concentration. In the observed range its value was between 93 and 102%. Repeatability of the recovery at lower concentrations was 7.5% (RSD), while at higher concentrations the uncertainty decreased to 3.2%. Successive injections of a particular CF standard solution and/or injection of different concentrations between 0.2 pmol and 0.1 nmol resulted in an almost linear ($r=0.9985$) relationship between the amount of CF and peak area. The minimal concentration of CF which could be detected ($S/N=3$) by a single injection via the 20-μl loop was $5\cdot10^{-9}$ *M*, which corresponds to 0.1 pmol of CF and a limit of quantitation ($S/N=10$), LOQ, equal to 0.2 pmol. By varying the flow-rate of the acceptor solution between 0.1 and 1.5 ml/min with an injection of 20 μl of $5\cdot10^{-8}$ *M* CF, a practically constant peak area was obtained (RSD 4.8–7.8%) thus indicating that sorption of CF on the TEC is fast and not influenced by the transport rate in the flow range studied.

A series of chromatograms obtained by direct injection of CF in different solvents into the LC (autosampler–column–detector), and after loading on the TEC (autosampler–TEC–column–detector), were compared to investigate the influence of the TEC on the chromatographic behaviour of CF. In Fig. 3, chromatograms obtained by direct injections of an equal volume (20 μl) and concentration ($1\cdot10^{-7}$ *M*) of CF standard solutions prepared in mobile phase,

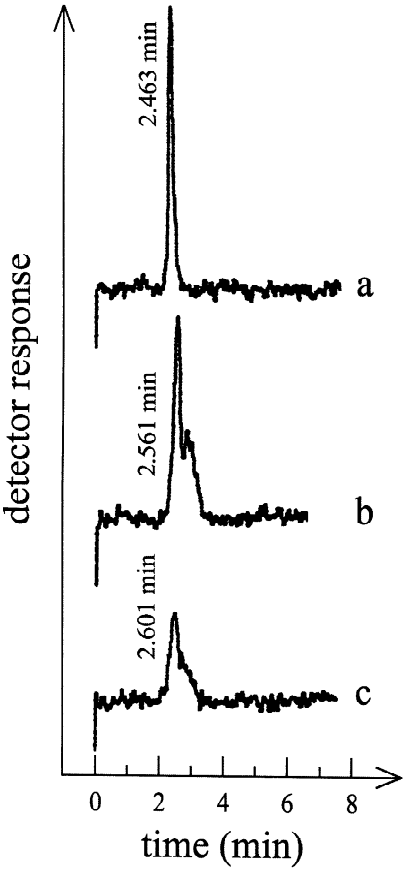

Fig. 3. Chromatograms obtained after direct injection of 20 μl $1\cdot10^{-7}$ *M* CF prepared in (a) mobile phase, (b) water and (c) 0.01 *M* phosphoric acid. Eluent, 0.01 *M* phosphoric acid–acetonitrile (70:30) at 0.5 ml/min; column 2, fluorescence detection.

phosphoric acid and water are shown. It can be seen that the peak was narrow and symmetric when CF standard dissolved in the mobile phase was injected (chromatogram a) and that a significant band broadening appeared when CF dissolved in phosphoric acid or water was injected (chromatograms b and c). Although the effect of solvent on chromatographic behaviour is commonly known, it is interesting that the TEC completely eliminated band broadening caused by the solvent in which CF was prepared. This is evident from Table 1, where data for a series of chromatograms for 2 pmol of CF injected directly (experiments a–c) and after loading of equal amounts of CF on the TEC (experiments d–f) according to the set-up shown in Fig. 2, are

Table 1
Effect of configuration on chromatographic parameters for the injection of ciprofloxacin in different solvents directly to LC and via TEC[a]

Experiment	Solvent	Configuration	Area	t_R (min)	Peak height (mm)	w_h (min)
a	MP	AS–AC–D	2.087	2.46	46	0.21
b	H_2O	AS–AC–D	2.106	2.56	32	0.29
c	PA	AS–AC–D	1.517	2.60	13	0.51
d	MP	AS–TEC–AC–D	1.696	4.30	63	0.12
e	H_2O	AS–TEC–AC–D	1.35	4.35	55	0.11
f	PA	AS–TEC–AC–D	1.788	4.54	70	0.12

AS, autosampler; AC, analytical column; D, detector.
[a] Average values of three measurements, $V=20$ μl, $1\cdot10^{-7}$ M CF.

compared. All chromatographic peaks obtained by the sorption/desorption of CF on TEC are well shaped, i.e. narrow and practically of the same area and/or height. Another favourable property of the sample clean-up by TEC is, therefore, efficient removal of the matrix effect on the band broadening of the analyte, and should be applied whenever the microenvironments of the sample and the standard solution of analyte are different.

From Table 1 it can be seen that introduction of the TEC into the LC system results in a significantly increased retention factor, k'. Namely, CF injected directly into the column is eluted at a retention time of ~2.6 min (experiments a–c) but when the trace enrichment step is involved (experiments d–f), the peak of CF appeared ~2 min later. Barbosa et al. [28] investigated the influence of eluent pH on k'. As described above, an increase in pH results in an increased retention of CF and this finding agrees with that of Barbosa et al. [28]. Therefore, it can be supposed that the shift in retention time of CF is caused by the difference in pH of the inflowing mobile phase and that of the sample plug within which the analyte is moved through the column. We have found similar effects previously in ion chromatographic processes [29]. However, the pH difference between the eluent (2.64) and that of 0.01 M phosphoric acid (2.24) used for the enrichment step is very small and according to the above statements should act in the opposite direction, i.e. k' should be decreased.

To obtain a rational explanation for the change in retention of CF due to inclusion of the TEC into the LC system, a series of experiments with different configurations was performed. Fig. 4 shows a comparison of detector response/chromatograms obtained by successive injection of 2 pmol of CF dissolved in mobile phase on applying different instrumental configurations. As can be seen, direct injection of CF onto the detector without the column (curve a) results in a very fast response ($t_R=0.14$ min) and a narrow and high peak characteristic of the flow-injection mode (FIA) with low physical disper-

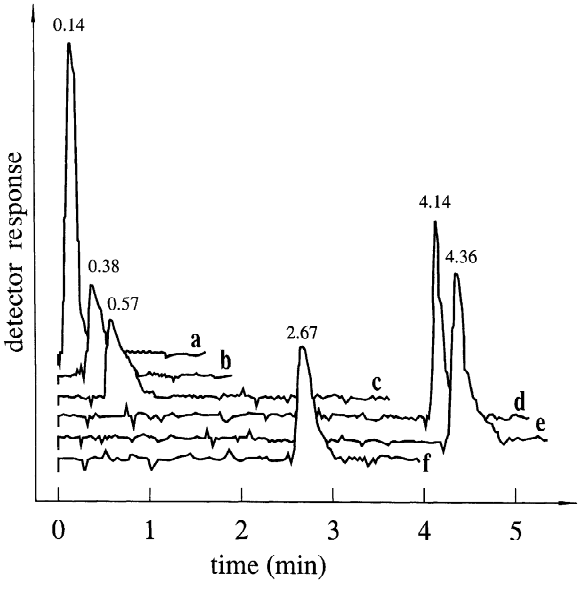

Fig. 4. Comparison of fluorescence detector response after injection of 2 pmol of CF dissolved in mobile phase for different configurations: (a) autosampler–detector, (b) autosampler–TEC–detector, (c) autosampler–TEC–guard cartridge–detector, (d) autosampler–TEC–analytical column–detector, (e) autosampler–TEC–guard cartridge–analytical column–detector and (f) autosampler–guard cartridge–analytical column–detector. Other conditions are the same as in Fig. 3.

sion. Incorporation of the TEC (curve b) and of the guard column (curve c), increases retention and band broadening only slightly in accordance with the theory, and slow desorption of CF from the TEC could therefore be excluded as the reason for increased retention in the presence of the TEC. The addition of the analytical column (chromatogram d) and guard column (chromatogram e) to the TEC, further increases retention time to 4.14 and 4.36 min, respectively, in accordance with expectations. As seen, the chromatographic column decreases band broadening caused by the TEC and improves peak shape, i.e. increases peak height and decreases peak width (w_h). From chromatogram (f), obtained by direct injection of CF onto the analytical column without a preconcentration step, a significantly lower retention time of 2.67 min is evident. The difference in retention between chromatograms (e) and (f) of 1.7 min cannot therefore be ascribed to slow sorption processes or to the increased void volume, V_0, of the system when the TEC is incorporated, where a delay of only 0.4 min (curve b) could be expected.

A reasonable explanation for these phenomena should be sought in the changes of eluent composition when the trace enrichment step was applied. Namely, during the enrichment step the analyte is adsorbed on the top of the TEC and the precolumn is continuously flushed with the acceptor solution, i.e. 0.01 *M* phosphoric acid. When the analyte is injected onto the column, instantaneous desorption of the analyte from the TEC proceeds in 70:30 phosphoric acid–acetonitrile eluent. However, the plug of phosphoric acid released from the TEC during the injection also moves with the same speed as the analyte, especially at the beginning of the chromatographic column and disturbs the eluent microcomposition around the analyte in such a way that it contain less of the organic component than the inflowing eluent. The higher local fraction of phosphoric acid in the eluent results in stronger retention (as mentioned above) and elution of the analyte is shifted to a longer retention time. The increased retention of analyte caused by the incorporation of the TEC coupled on-line to the LC, appeared therefore to be due exclusively to the on-column changes of eluent microcomposition and not to the change in pH, slow desorption from the TEC or the increased void volume of the system.

3.2. Optimisation of a set-up for continuous dialysis

Continuous dialysis can be performed according to the transport of the donor and the acceptor solutions in static, pulsed or continuous-flow modes [5]. The static or pulsed mode of operation is usually applied when samples are available in small quantities, e.g. <0.2 ml. Fig. 5 shows a set-up for continuous-flow dialysis where both sample and acceptor solutions (0.01 *M* PA) are aspirated through the dialysis cell continuously. This type of dialysis consumes more sample (>0.5 ml) and according to the time available t_s, the sample volume to be processed V_s, the volume of the donor channel V_d and the volume of the sample line V_0, the optimum donor flow-rate, F_d, is expressed by [30]:

$$F_d = \frac{V_s + V_d + V_0}{t_s}$$

We applied this type of dialysis for determination of CF in blood serum samples where, due to the presence of proteins, the sample should be appropriately diluted (dilution with water in the ratio of 1:5 to 1:10 is recommended) to prevent clogging of the sample line and the donor channel of the DC. The advantage of continuous-flow dialysis lies in the fact that the pH of the donor phase does not change

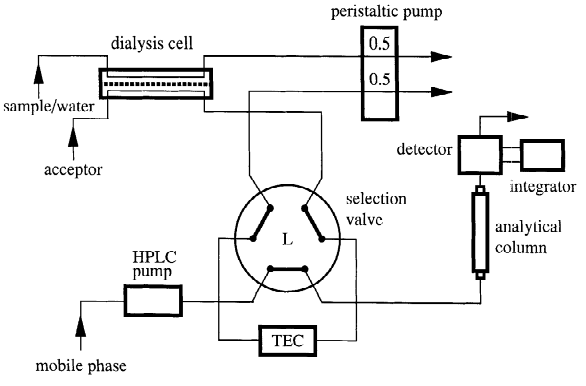

Fig. 5. Schematic representation of a set-up for continuous-flow dialysis coupled on-line to an LC system. The selection valve is shown in the load position for preconcentration of dialysate on the TEC; flow-rates of sample, acceptor (0.01 *M* phosphoric acid) and mobile phase are 0.5 ml/min.

during dialysis since the phosphoric acid penetrating through the membrane is continuously removed from the donor channel by the inflowing sample. In this way, during the entire dialysis time a convenient and constant pH gradient through the membrane is assured and DE does not decrease with increasing dialysis time, as is observed in equilibrium dialysis of CF [15] or the static dialysis described below.

As seen from Fig. 5, the set-up is very simple and a computer can easily control all the steps. The dialysis efficiency, DE, can be altered and/or optimised simply by variation of the flow-rates of the donor and acceptor solutions. It is known that increasing the flow-rate of the acceptor phase results in increased recovery [6], but as pointed out previously [15], other parameters such as the hydrostatic pressure in both channels and the pH gradient may affect DE of CF significantly. The dialysis rate is usually dependent on the diffusion rate of the analyte through the membrane pores and a lower F_d yields a higher DE [15]. As a compromise, we used a flow-rate of 0.5 ml/min in both channels and when the volume of the sample is small, i.e. less than 1 ml, the flow-rate of the sample was decreased to 0.1 ml/min. It is to be noted that peristaltic pump should be connected as shown in Fig. 6 and not positioned somewhere else, e.g. before the dialysis cell. There are several reasons to keep strictly to this configuration: to avoid contamination and carry-over between samples, the sample should be brought into contact with inert materials only (Teflon tubing), the line should be short (low dead volume) and the dialysis membrane should be exposed to a very similar hydrostatic pressure gradient over the whole channel length and to a low pressure difference between the channels.

To find the operational range of the continuous-flow dialysis set-up shown in Fig. 5, aqueous CF standards between $1\cdot10^{-9}$ and $1\cdot10^{-6}$ *M* were dialysed for different times. It was found that dialysis of $1\cdot10^{-9}$ *M* CF results in a linear increase in peak area with dialysis time up to more than 30 min. In dialysis of $1\cdot10^{-6}$ *M* CF, the peak area–time relationship is linear only up to 3 min and then levels off. CF peak area is also linearly dependent on the concentration of CF in the sample when it is dialysed for a constant time. Dialysis of CF standard solutions between $1\cdot10^{-9}$ and $3\cdot10^{-8}$ *M* ($n=6$) for 2 min resulted in a linear increase in peak area, *A*, described by the equation: $A=(5.5\pm1)\cdot10^5+(1.36\pm0.07)\cdot10^{14}\cdot C_{CF}$, ($r=0.996$). With repeated dialysis ($n=3$) of the same standard solution for 2 min, the RSD of the peak area was 13% for $1\cdot10^{-9}$ *M* CF and 4% for $3\cdot10^{-8}$ *M* CF. With an increased dialysis time of 5 or 10 min, sensitivity increases and a $1\cdot10^{-10}$ *M* concentration of CF was quantified ($S/N>10$) with an uncertainty below 30% (RSD).

It is interesting that dialysis of 1:10 diluted human blood serum spiked with CF under the same conditions of dialysis resulted in a very similar *A vs.* C_{CF} response. The dependence between peak area and concentration of CF in spiked serum (between $1\cdot10^{-9}$ and $5\cdot10^{-8}$ *M*), for 2 min of dialysis ($n=3$) gave a calibration line: $A=(1.8\pm0.4)\cdot10^6+(1.39\pm0.18).\ 10^{14}\cdot C_{CF}$, ($r=0.97$). Since the sensitivities of dialysis of aqueous and serum-containing solutions of CF are practically the same, it is evident that the presence of serum does not affect DE and that protein binding of CF at the given conditions is negligible. In the presence of serum the reproducibility also remains practically the same as when aqueous standards of CF were dialysed (18% at $1\cdot10^{-9}$ *M* CF and 6% at $1\cdot10^{-8}$ *M* CF).

A special problem arising during the continuous-flow dialysis of aqueous solutions and samples containing blood serum at low concentrations of CF was the rather high blank signal. This is also evident from the calibration lines given above, where a relatively high intercept at the origin is evident for both, especially in the presence of serum. Since we excluded contamination by careful cleaning, we presume that another coeluting and fluorescing compound must be present in deionised water and in serum samples. To confirm this statement we added UV detector to the LC system and observed its response at 254 and 274 nm. We found that our presumption was true and that CF is coeluted in 70:30 mobile phase within the zone of unknown substances which appeared in the chromatogram as a high and broad peak (~0.01 a.u. at 254 nm). When the fraction of acetonitrile in the mobile phase was decreased from 0.3 to 0.18 the interfering peak was completely resolved from that of CF, i.e. the CF peak was shifted to longer retention times (6.42 min). By extended experimentation we found that a

number of phthalate derivatives are responsible for interference with UV detection and some of them also exhibit weak fluorescence at the wavelength used for detection of CF. Since most plastic materials contain phthalates, it is practically impossible to avoid contamination with these substances in the laboratory. To diminish such interferences an additional cleaning of deionised water by filtration through a column filled with C_{18} material is recommended, and all reagents prepared with this clean water should be stored in glass vessels. However, if the sample is taken by plastic syringe or stored in plastic bags or vessels, as was the case with our serum sample, contamination is unavoidable and a serious analytical error may appear, although clean-up of the sample by dialysis and SPE was performed and an LC separation of the analyte followed by selective detection applied. It should be pointed out that dialysis does not remove the majority of low molecular mass organic compounds present in biosamples and that many of them can be adsorbed and/or accumulated on TECs and, as seen above, some of them can also coelute with the analyte and interfere in their detection. Careful work and a critical interpretation of the results, especially at the trace level, is therefore still needed despite the use of sophisticated instrumentation and/or highly automated and advanced techniques.

Another problem which frequently appears in dialytic clean-up of biological samples is the influence of macromolecular constituents on membrane permeability and consequent changes of DE. To avoid needing a knowledge of DE and its concentration or the sample matrix dependence, the evaluation of the analyte concentration should be performed via the standard addition procedure and not by a calibration curve. Although the sample consumption and analysis time due to the need for at least two successive analyses of the original and the spiked sample is higher, the reliability and accuracy of the results thus obtained is substantially improved.

3.3. Continuous static dialysis of CF

Fig. 6 shows the set-up for static or pulsed mode continuous dialysis, constructed from the few instrumental components described above. Static dialysis coupled to a TEC is usually applied when high efficiency is required and the volume of sample is limited. It is performed by holding the donor phase stagnant and moving the acceptor phase continuously through the TEC. The set-up is similar to that used for characterisation of the TEC (Fig. 2), with the exception that a dialysis cell is incorporated into the system and the injection valve of the autosampler is accomodated in such a way that sample/water are aspirated by the dispensor through the donor channel of the dialysis cell instead of the sample loop. As described previously [15], membrane bending and/or compression may change the channel volume and affect the reproducibility of continuous dialysis. Therefore, in static dialysis an open-ended donor channel does not ensure a constant volume of the sample due to membrane compression caused by the continuous flow of the acceptor. From Fig. 6 it can be seen that by turning the injection valve from load to inject position, the sample contained in the donor channel becomes a part of a closed loop and membrane bending is thus avoided. Another feature of the arrangement shown in Fig. 6 is that all steps of the analysis can be very simply automated and

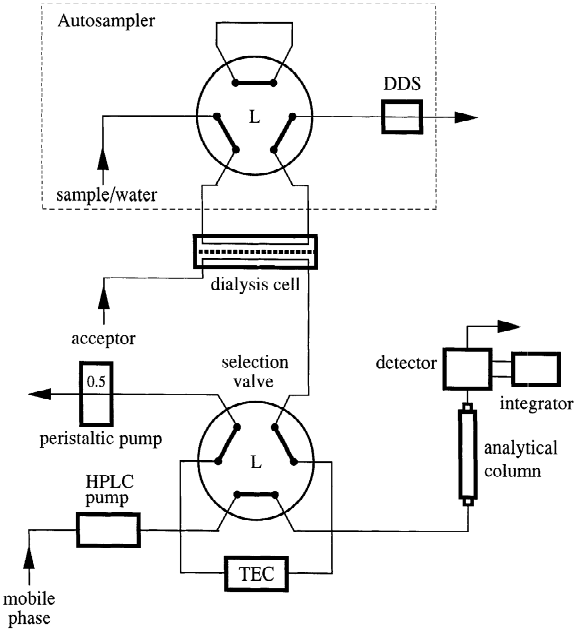

Fig. 6. Schematic diagram of a set-up for static dialysis coupled on-line to an LC system. Both valves shown in the load position; flow-rates of acceptor (0.01 *M* phosphoric acid) and mobile phase are 0.5 ml/min; volume of sample is 150 μl.

controlled by software incorporated in the autosampler, as described above (Section 3.1). This improves the repeatability of all particular phases and simplifies operation of the system.

In a series of experiments it was shown that, similarly to continuous-flow dialysis, the amount of dialysed CF retained on the TEC and loaded onto the LC is linearly proportional to its concentration in the dialysed sample from $1\cdot10^{-9}$ to $5\cdot10^{-6}$ mol/l. At low concentrations ($<1\cdot10^{-8}$ *M*) a dialysis time of 5–10 min should be applied and at CF concentrations above $1\cdot10^{-7}$ *M* 2 min of dialysis results in a satisfactory recovery of the analyte and a reproducibility below 7% (RSD). In investigations of static dialysis it was found that the recovery of the analyte decreases rapidly with increase in dialysis time. On the one hand this is normal due to the falling analyte concentration in the donor compartment and consequent decrease in concentration gradient. But on the other hand acidification of the sample in the donor channel occurs due to penetration of phosphoric acid through the membrane, since the donor phase is held static. Due to the decrease of pH the concentration gradient of CF across the membrane additionally decreases when the time of dialysis increases. Consequently prolongation of the dialysis period does not result in an increased sensitivity and lower detection limit as was observed in continuous-flow dialysis. The lowest concentration of CF in aqueous samples and serum spiked samples which could still be reliably quantified ($S/N=10$) after 10 min of dialysis was $\sim1\cdot10^{-9}$ *M*.

Fig. 7 shows a typical chromatogram obtained by static dialysis (2 min) of 1:10 diluted serum, spiked with $5\cdot10^{-8}$ *M* CF (chromatograms a) and subsequent dialysis of water (chromatograms b), both in triplicate. For one dialysis cycle 0.15 ml and for a sequence of three consecutive runs 0.4 ml of sample should be available, respectively. As evident, peak area/height slightly increases with each repetition of dialysis of the same CF standard solution and decreases during the following wash cycle exponentially. Consequently, for the evaluation of the concentration of CF in a sample a single peak area value (RSD 8%) or the average peak area of three subsequent dialysis runs was used (RSD 4%). Between dialysis of each sample the donor channel was washed with 0.3 ml of water, i.e. an amount equal to $3V_d$.

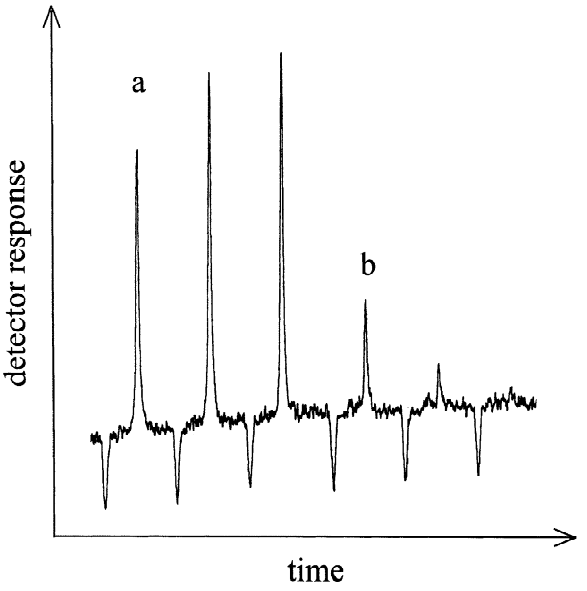

Fig. 7. Chromatograms obtained by (a) three replicate dialysis of diluted (1:10) human blood serum spiked with $5\cdot10^{-8}$ *M* CF and (b) three replicate dialysis of water; dialysis time 2 min; column 1, mobile phase 70:30 (v/v), flow-rate 1 ml/min, fluorescence detection.

In this dialysis mode, a high sample throughput was achieved using a concurrent sequential sample clean-up process and chromatographic separation. If beside the analyte, there are no other coeluting and/or detector responses affecting substances present in the sample, the dialysis–wash cycles and LC process can be performed continuously. In this case the rate limiting stage of the analysis is the dialysis time (Fig. 7). To further increase the sensitivity of this dialysis mode, multiple loading of the TEC with the analyte during subsequent dialysis runs of the same sample should be used. To diminish the influence of variation of DE on the accuracy of the results, multiple dialysis of the original and spiked samples is recommended.

4. Conclusions

Different modes of dialysis coupled on-line with liquid chromatography were investigated and methods for automated sample clean-up and determination of ciprofloxacin at the trace level developed. Preconcentration of CF was performed on C_{18} ma-

terial in 0.01 *M* phosphoric acid and desorption from the TEC was achieved with 0.01 *M* phosphoric acid–acetonitrile eluent with the volume fraction of acetonitrile between 0.15 and 0.3. It was shown that the trace enrichment column combined with the reversed-phase HPLC system with fluorescence detection enables detection of femtomolar amounts of CF. When the system is coupled on-line with continuous-flow dialysis, reliable measurement of CF concentration in human blood serum at the 0.1-n*M* level is possible.

Incorporation of a TEC into the liquid chromatographic system improves the peak shape of CF and eliminates band broadening caused by dissolution of CF in solvents different from those of the mobile phase. On-line coupling of a TEC with the LC system causes a substantial increase in retention of CF on the C_{18} analytical column. It was demonstrated that this phenomenon was caused by a plug of phosphoric acid released from the TEC together with the analyte and the consequent on-column change in the eluent microcomposition around the analyte.

Despite the high selectivity of the LC technique with fluorescence detection and sample clean-up by dialysis and TEC, it was found that phthalates leached from plastic materials might cause a serious error in analysis of human blood serum samples. Coelution of phthalates and CF can be avoided by the eluent containing 18 vol.% of acetonitrile. To prevent macromolecular constituents of biological samples influencing the dialysis efficiency, determination of the analyte concentration by the standard addition technique, i.e. multiple analysis of the original and spiked samples, is recommended.

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