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Preconcentration of quinolones by dialysis on-line coupled to high-performance liquid chromatography

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Abstract

The parameters influencing dialytic separation of ciprofloxacin (CF) fluoroquinolone were investigated. Dialysis with a porous cellulose acetate membrane was on-line coupled with HPLC and the analysis of dialysate was made by isocratic ion-pairing liquid chromatography using a reversed-phase analytical column and fluorescence detection. Optimisation of the experimental conditions for selective dialytic enrichment are described and explanations of some phenomena affecting dialysis efficiency discussed. By the use of a neutral donor (pH≈7) and acidic acceptor solution (pH<4) a substantial enrichment of quinolones was achieved. Accumulation of CF in the acidic acceptor phase is based on the protonation of the analyte in the acceptor compartment. Continuous-flow of donor solution and a stagnant acceptor solution gave high dialysis efficiency in 5–15 min. Effects of interfering substances present in real samples on the variation of dialysis efficiency can be minimised by successive dialysis runs of the original and spiked samples.

Keywords: Dialysis; Preconcentration; Quinolones; Ciprofloxacin

1. Introduction

Quinolones have wide use in medical treatments due to their antibacterial and pharmacological properties. Determination of fluoroquinolone antibiotics and their metabolites in body fluids is usually performed by HPLC with UV or fluorimetric detection. However, the presence of interfering macromolecules in biological samples limits the applicability of the direct determination of quinolones by HPLC. An extensive review of HPLC methods for the determination of some clinically useful fluoroquinolones in biological samples was published recently by Carlucci [1]. In the literature many clean-up preparations of biological samples prior to HPLC determination of fluoroquinolones have been reported, e.g. extraction [1,2], precipitation [1,3] or ultrafiltration [1,4]. Another promising approach consists of using dialysis as a sample preparation step prior to HPLC analysis [5]. Although dialysis is relatively slow and not a highly selective process, on-line coupling of dialysis with HPLC enables clean-up, trace enrichment and automation of the analytical procedure [6,7]. A number of papers and reviews dealing with dialytic sample pretreatment in chromatography [5] and flow injection systems [8] shows the evident importance of this separation principle for analytical practice.

It is well known that for a successful application of dialysis the membrane properties, the geometry of the dialyser unit as well as the hydrodynamic conditions must be considered. Since dialysis has not

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been yet applied for the determination of quinolones by HPLC, an investigation of the dialytic sample clean-up was undertaken. This work describes the dialytic separation and preconcentration of ciprofloxacin (CF) fluoroquinolone using a porous cellulose acetate membrane, coupled with an ion-pairing LC system with fluorimetric detection. Since the accuracy and precision of such a coupled analytical system depends mainly on the performance of the sample pretreatment step, a detailed study of the parameters affecting the dialysis process was made, and the parameters studied were optimised with respect to analyte recoveries.

2. Experimental

2.1. Instrumentation

The dialytic system schematically shown in Fig. 1 was constructed from a peristaltic pump (ISMATEC, MS-4 Reglo/8-100, Glattbrugg-Zuerich, Switzerland) and a dialytic cell (B 54535, Gilson Medical Electronics, Villiers-le-Bel, France) with a cellulose acetate membrane (B 54561, 15 kDa molecular mass cut-off); the volume of the donor channel was 175 μl and of the acceptor channel 100 μl. Sample was aspirated at a flow-rate of 0.5 ml min^{-1} into the donor channel of the dialysis cell. Recipient solution (350 μl) was injected (0.6 ml min^{-1}) into the acceptor channel by a Hamilton Microlab M Dispenser (Hamilton Bonaduz AG, Switzerland), equipped with a 1 ml syringe and dialysate (150 μl) was transferred after dialysis to the 46.3 μl sample loop of the six-port injection valve (Valco, Houston, TX, USA). By actuating the dispenser the solenoid drain valve (South Bend Control Inc, IN) automatically opened the flow path to the waste. After each dialysis the sample line and the donor channel was flushed with deionised water for 3 min. All connections on the low pressure side of the dialysis system were made from teflon tubing (0.5 mm I.D.).

The liquid chromatographic system consisted of a ConstaMetric IIIG pump (LDC Milton Roy, Riviera Beach, FL, USA, flow-rate 1.0 ml min^{-1}), a fluorescence detector (RF-535, Shimadzu Corporation, Kyoto, Japan, excitation wavelength 277 nm and emission wavelength 451 nm) and an integrator (3395, Hewlett Packard). A Rheodyne 7125 injector (Rheodyne, Cotati, CA) with a 20 μl sample loop and a LichroCART (250×4 mm I.D.) column (Knauer, Berlin, Germany), packed with 5 μm RP-18 Lichrospher packing material were used for the injection of CF standard solutions and separations, respectively.

2.2. Chemicals and reagents

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid) was supplied by Krka (Krka, Novo mesto, Slovenia), analytical grade phosphoric acid was from

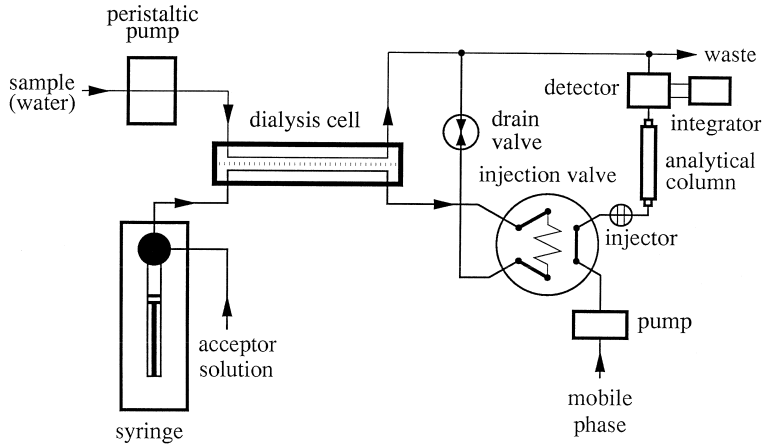

Fig. 1. Scheme of the experimental set-up for on-line coupled dialysis with HPLC.

Kemika (Kemika, Zagreb, Croatia), HPLC-grade acetonitrile and sodium dodecane-1-sulfonate (SDS) was obtained from Merck (Darmstadt, Germany). Water was prepared from a Milli-Q water purification system (Millipore, MA, USA).

A stock solution of CF (1.0×10^{-4} *M*) was prepared in deionised water and working solutions of lower concentrations were prepared by dilution with the mobile phase daily. Two mobile phases were used; first (MP1) consisted of 0.01 *M* phosphoric acid–acetonitrile (80:20, v/v) pH 2.5 and second (MP2) of 0.01 *M* phosphoric acid–acetonitrile (55:45, v/v) pH 2.65 and 0.005 *M* SDS as an ion-pairing agent.

3. Results and discussion

3.1. Stationary equilibrium dialysis

The efficiency of dialysis depends mainly on the analyte–membrane properties, the physical dimensions of the cell (ratio between membrane area and volume of the channels) and the hydrodynamic conditions (stationary or flowing solutions). Dialysis efficiency *DE*, defined as the quotient of the amounts of analyte in the acceptor channel n_a and the donor channel n_d, is generally a time- and space- (length of channels l) dependent function:

$$DE = \frac{n_a}{n_d} = f(l, t) \quad (1)$$

In stationary equilibrium dialysis, where both the donor and the acceptor solution are stagnant, *DE* is an exponential function [5], and the maximum recovery (at infinite time) is determined only by the acceptor and donor channel volumes:

$$DE^{eq}_{max}(t \to \infty) = \frac{V_a}{V_a + V_d} \quad (2)$$

DE is experimentally determined from the ratio of the peak area of dialysate (Fig. 2(B)) and the peak area of CF standard solution (Fig. 2(A)) injected directly on the analytical column.

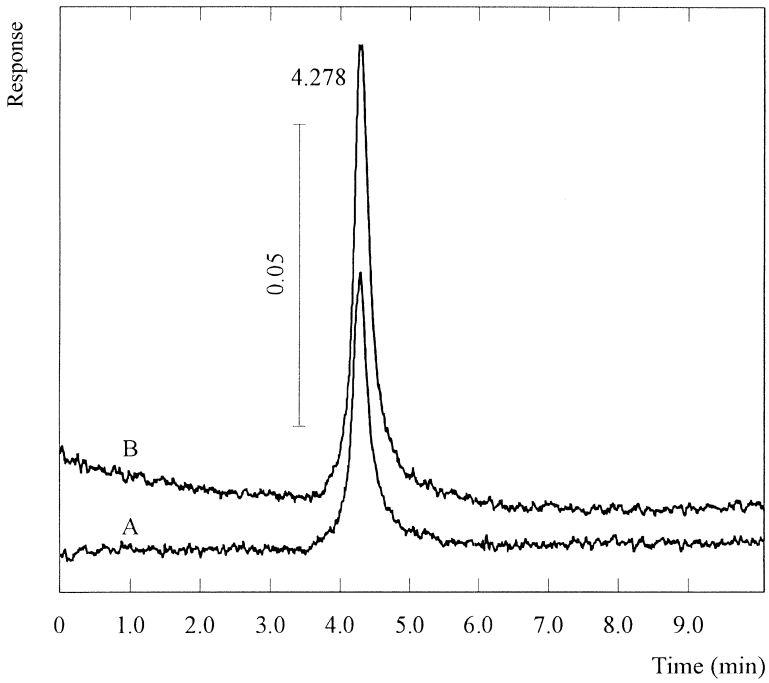

Fig. 2. Chromatograms of ciprofloxacin in phosphoric acid–acetonitrile–SDS mobile phase obtained by injection of (A) 46 μl of acceptor solution (MP2) after 5 min of stationary dialysis of 1×10^{-6} *M* aqueous solution of CF, and (B) by direct injection of 20 μl CF standard solution (1×10^{-6} *M* in MP2).

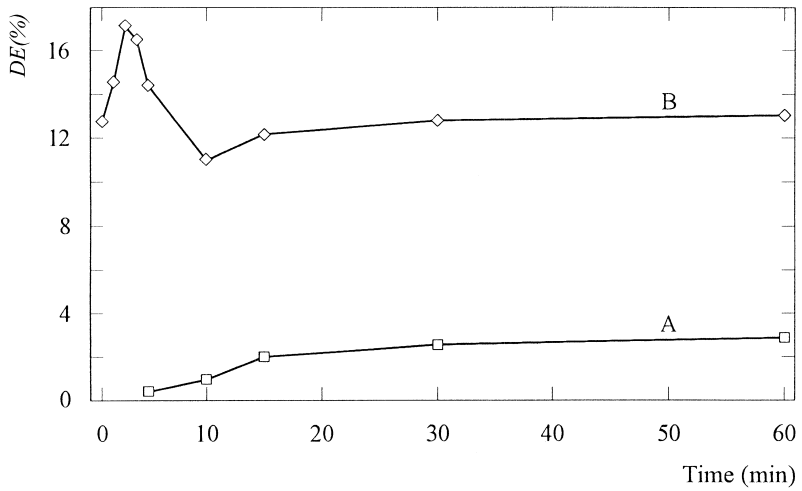

Fig. 3. Equilibrium dialysis efficiency as a function of dialysis time in (A) deionised water and (B) MP2 as acceptor solution. The donor solution was 1×10^{-6} *M* CF in water.

Fig. 3 shows the time dependence of the dialysis efficiency expressed as a ratio between amount of ciprofloxacin in the acceptor channel, $n_a(t)$, and its initial amount in the donor channel $n_d(0)$:

$$DE(t) = \frac{n_a(t)}{n_d(0)} \quad (3)$$

It can be seen that *DE* asymptotically approaches a limiting value as predicted by theory; however, for both recipient solutions the final value is well below 36% as calculated from Eq. (2) and the channel volumes given in Section 2. One of the reasons for the low permeation of CF through the porous cellulose membrane is the adsorption of quinolone on the cellulose membrane. It is known that neutral molecules and charged species are adsorbed by a cellulose acetate membrane [9,10]. Murata and Tanioka recently showed [10] that adsorbed compounds penetrate the membrane pores and decrease their porosity.

As evident from Fig. 3 the dialysis efficiency of CF strongly depends on the pH of the donor and the acceptor solutions and as seen, its final concentration in an acidic acceptor solution (curve B) is about five time higher than when water was used as the recipient solvent. The main reason for this effect in our opinion is the protonation of CF in acidic media. Namely quinolones contain one acidic and one basic functional group and between pH 7 and 8 a mixture of zwitterionic $CF^{\pm}$ and neutral CF^{0} species predominates. In acid media below pH 5.5 the analyte in the acceptor phase is converted to its protonated form

$$CF^{0}, CF^{\pm} + H^{+} \rightarrow HCF^{+} \quad (4)$$

At first sight it was surprising that this protonated species accumulated in the acceptor channel and did not diffuse back to the donor compartment and reach a final equilibrium as in the case when water was used as the acceptor. Since the MW of CF is much lower than MWCO of the membrane (15 kDa), the accumulation of HCF^{+} in the acceptor channel can be explained by the electrostatic repulsion between HCF^{+} and the excess of positive charge on the acceptor side of the membrane and inside their pores. Adsorption of protons and HCF^{+} on the acceptor side of the membrane therefore hinders back-diffusion of HCF^{+} into the donor channel and enables accumulation of CF in the recipient channel.

The cellulose acetate membrane modifications [10] and their acid–base behaviour described recently by Rosa and Pinho [11] seem to be very important for the applications of dialysis. A cellulose membrane behaves therefore as an ion-exchange membrane and its protonation in acid media hinders permeation of positively charged species. As a consequence the concentration gradient between donor and acceptor

compartments increases and an enrichment of the protonated analyte results, as in the case of nonporous membranes. Hence to achieve preconcentration of CF quinolone by dialysis, it must be in the active form as $CF^{\pm}$ or CF^{0} in the donor phase and in the inactive form as HCF^{+} in the acceptor phase. Accordingly, the pH of the donor phase must be close to the p*I* of the quinolone and the pH of the acceptor phase well below K_{a1}

$$\mathrm{pH}\begin{cases}\text{donor} & \mathrm{pH} \approx \mathrm{p}I \\ \text{acceptor} & \mathrm{pH} < \mathrm{p}K_{a1}\end{cases}$$

where K_{a1} is defined by the equation

$$K_{a1} = \frac{([CF^{0}] + [CF^{\pm}])[H_3O^{+}]}{[HCF^{+}]} \quad (5)$$

The acid ionisation constants of fluoroquinolones have been published by Ross and Riley [12] and Lee et al. [13], and the pK_{a1} values for CF equal 6.09 and 6.14, respectively. Therefore, if the pH of the donor solution is kept close to physiological pH, i.e. about 7 and the pH of the acceptor is below 4, the above conditions are fulfilled.

Considering all these facts there still remain the question why the transfer of CF at these optimal conditions is not complete, i.e. why *DE* in MP2 does not approach 100%. As seen in Fig. 3(B) in the first minutes of dialysis *DE* rapidly increases and then after 3 min starts to decrease and approaches a final value of about 12%. We found that the reason for this lies in the acidification of the donor solution under stationary conditions of dialysis. Transfer of protons from the acceptor channel to the donor channel causes the protonation of CF in the donor solution and the flux through the membrane decreases. Fig. 4 shows the result of an experiment where water in the acceptor channel contained methylorange (MO) indicator and was held in contact with 0.01 *M* H_3PO_4 (MP1) in the donor channel.

At different times the acceptor solution was injected into an UV detector set at 535 nm where the acid form of MO absorbs. As evident the pH of the acceptor is substantially lower (higher absorbance) already in the first minute of dialysis (curve B) than that when the donor contains pure water (curve A). This confirm that protons diffused very quickly through the membrane pores and an appropriate change in pH and species distribution also appeared on the other side of the membrane.

All these effects influence the mass flux of the analyte through the dialysis membrane and should be considered, especially when stationary dialysis or dialysis with the stationary donor and moving acceptor phase coupled to a preconcentration column is used. Some of these drawbacks could be eliminated by use of continuous dialysis as described below.

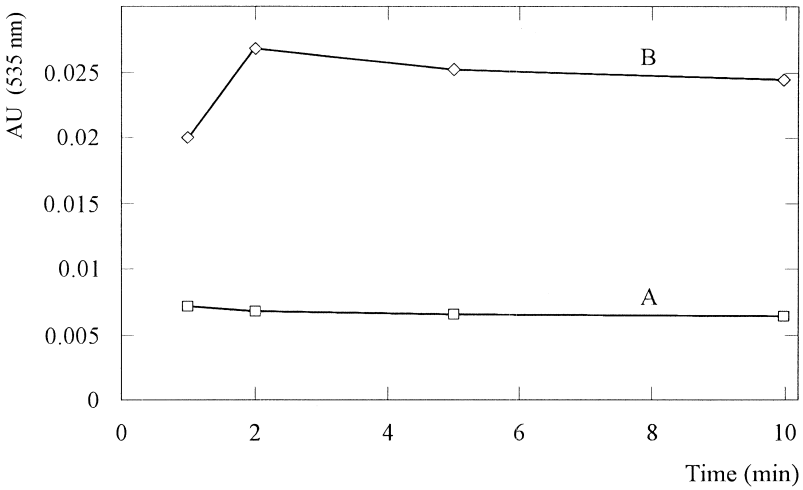

Fig. 4. Colorimetric measurement of pH change of water as the acceptor solution containing 0.1% solution of methylorange indicator for different donor solutions: (A) water, and (B) 0.01 *M* phosphoric acid.

3.2. Continuous dialysis

Conversion of CF in the acceptor solution to its inactive form HCF^+ enables selective enrichment of the analyte, as in the use of nonporous liquid membranes [14]. To prevent the acidification of the donor phase as described above, dialysis with a moving donor and stagnant acceptor solutions was used. In this case the total amount of analyte in the donor channel is equal to the flow-rate F_d of an incoming concentration of analyte C_i during the period of time t, and DE given by Eq. (1) can be expressed as:

$$DE = \frac{n_a}{C_i t F_d} = \frac{C_a(t)V_a}{C_i t F_d} \quad (6)$$

As shown by Jönsson et al. [15] for liquid membranes with a continuously pumped donor solution and a stagnant acceptor solution, both concentrations are complex time- and space-dependent functions:

$$C(x, t) = \begin{Bmatrix} C_d = C_i f_d(x, t) \\ C_a = C_i f_a(x, t) \end{Bmatrix}$$

Generally the concentration in the acceptor channel C_a increases with time when the analyte in the acceptor phase is completely transformed to its inactive form. Fig. 5 shows the dependence of CF concentration in the acceptor channel as a function of the dialysis time. Since from Eq. (6) it follows that the amount (concentration) of CF is linearly proportional to time

$$n_a(t) = C_i F_d t DE$$

the nonlinearity of the relationship shown in Fig. 5 indicates that DE decreases with time. While peak area (height) is linearly proportional to the concentration of CF between 5×10^{-8} M and 5×10^{-5} M ($r=0.9995$), there are two main reasons for this deviation from the predicted behaviour. The first lies in the imperfect permselectivity of the protonated membrane and secondly at increased concentrations of the analyte in the acceptor channel the back-diffusion rate of HCF^+ into the donor channel also increases. As a consequence the separation efficiency decreases with time (Fig. 6). This is understandable if it is remembered that after 5 min of dialysis the concentration of CF in the acceptor channel is twice as high, and after 10 min about five times higher than that in the incoming donor solution. Considering this a dialysis time between 5 and 15 min is a reasonable compromise.

Other reasons for the decrease of DE with time are diffusion and convection of the accumulated analyte out of the acceptor channel during dialysis, as well as the dispersion of the dialysate plug during transfer to the injector loop. In our experiments we observed a large variation of CF concentration in the recipient

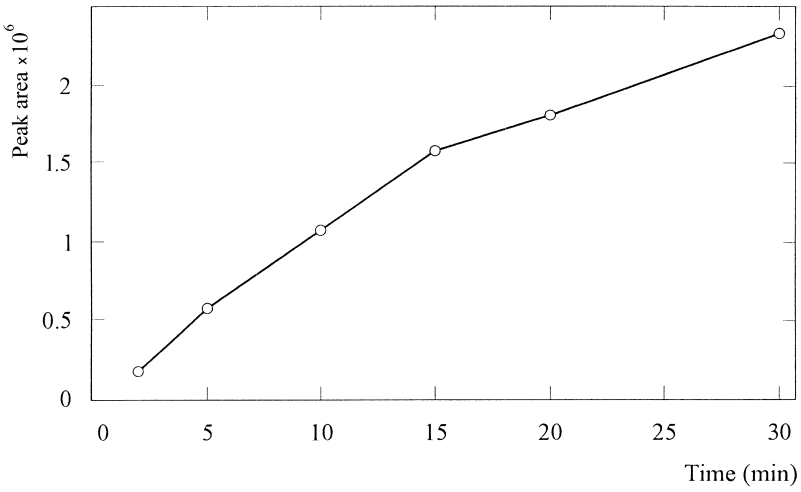

Fig. 5. Increase of CF peak area (concentration) in the stagnant acceptor solution (MP2) as a function of dialysis time. A standard solution of CF (1×10^{-7} M) was pumped continuously through the donor channel at a flow-rate of 0.5 ml min^{-1}.

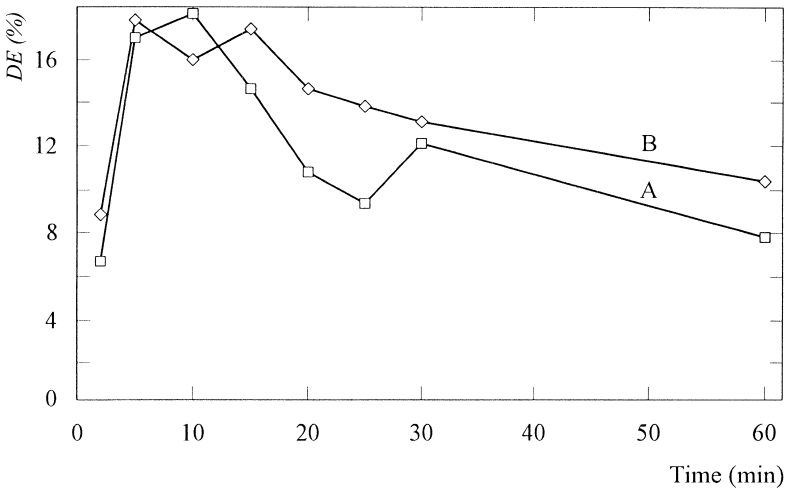

Fig. 6. Dependence of dialysis efficiency on time with stagnant acceptor solutions MP1 (A) and MP2 (B), and continuously pumped (0.5 ml min^{-1}) 1×10^{-7} *M* CF in water as the donor solution.

solution when dialysis was performed with an open-ended acceptor channel. The cause was membrane compression due to the continuous flow of donor and a corresponding decrease (variation) of the acceptor compartment volume. Therefore we decided to close the recipient side of the dialysis manifold by a valve during the time of dialysis as shown in Fig. 1. Membrane bending/compression is a serious problem often underestimated in the design of membrane-based separations. To achieve acceptable repeatability it must be minimised and/or avoided, especially when gas–liquid membrane separations [16] or high flow-rates are used [17]. In our opinion the isolation of the acceptor channel by segments of air, as usually applied to prevent losses of the accumulated dialysate, is not an optimal solution.

To minimise losses of the analyte during transfer to the sample loop its distribution in the recipient compartment and the transfer flow velocity must also be considered and optimised [15,18]. We found that dispensing 150 μl of acceptor solution with a flow-rate of 0.6 ml min^{-1}, i.e. similar to that in the donor channel (0.5 ml min^{-1}), gives the maximal response for the 46 μl sample loop used. It is to be mentioned that the use of mobile phase as the acceptor solution enables injection of relatively large volumes (up to 100 μl) without an overloading of the analytical column.

Fig. 7 shows the dependence of dialysis efficiency on the flow-rate of the donor solution. As can be seen *DE* decreases when the flow-rate increases, and from the shape of the curve it can be supposed that the mass-transfer kinetics are controlled by the membrane permeability. It was observed that *DE*, as well as *DE* vs. flow-rate dependence, changes with membrane ageing. When a new membrane was used an exponential decrease of *DE* vs. F_d was observed as predicted by theory for liquid membranes [14,15]. When the membrane was used for a longer time, e.g. some weeks or months, a slow decrease of *DE* was observed (a few percent per week).

From Fig. 7 it is evident that when small volumes of sample are available for analysis only low donor flow-rates give the required enrichment and reliable analysis. When the volume of sample is not limited, a larger flow-rate enables a higher enrichment per time unit [14]. Dialysis efficiency per time unit DE_t is defined as the ratio between the concentration of the analyte in the acceptor phase C_a and the incoming concentration of the analyte C_i in a given time *t*:

$$DE_t = \frac{C_a(t)}{tC_i}$$

According to Eq. (6) dialytic enrichment per time unit is given by

$$DE_t = \frac{DE\ F_d}{V_a} \quad (7)$$

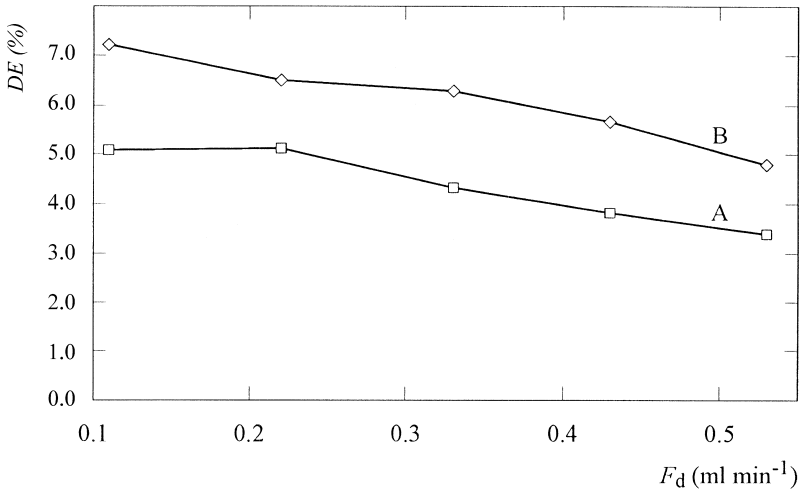

Fig. 7. Influence of the donor flow-rate F_d on *DE* at (A) 1×10^{-7} *M* and (B) 1×10^{-6} *M* aqueous donor solutions of CF using stagnant MP2 as the acceptor solution. Time of dialysis was 10 min.

In Fig. 8 DE_t is plotted vs. flow-rate for two CF solutions under the same experimental conditions as in Fig. 7. It is seen that enrichment per time unit increases with donor flow-rate and asymptotically approaches its maximum value under given experimental conditions. At a sample flow-rate of 0.5 ml min^{-1} therefore, a substantial enrichment of the analyte can be achieved, despite lower efficiencies of dialysis at higher flow-rates.

Fig. 9 shows the effect of donor solution composition on the dialysis efficiency of ciprofloxacin. As is evident the presence of inorganic salts and macromolecular compounds markedly affects *DE*. It decreases to about 50% with respect to that in deionised water when phosphate buffer or 0.9% KCl was used as donor solution and to about 10% when the donor contains proteins or is acidic (MP2). The low *DE* in acidic donor solutions is a consequence of the membrane and CF protonation as explained above. Suppression of *DE* by macromolecules like albumin is at first instance unusual since the presence of charged nondialysable polyelectrolytes should

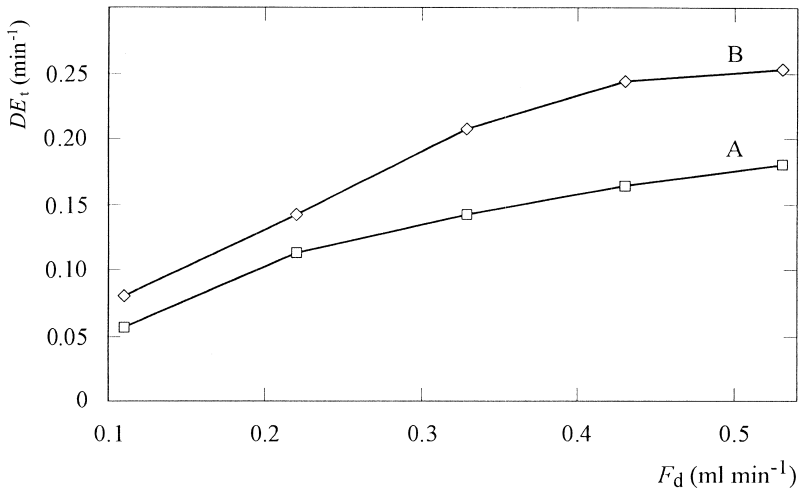

Fig. 8. Effect of donor flow-rate F_d on the dialytic enrichment per time unit DE_t. Parameters for curves (A) and (B) as in Fig. 7.

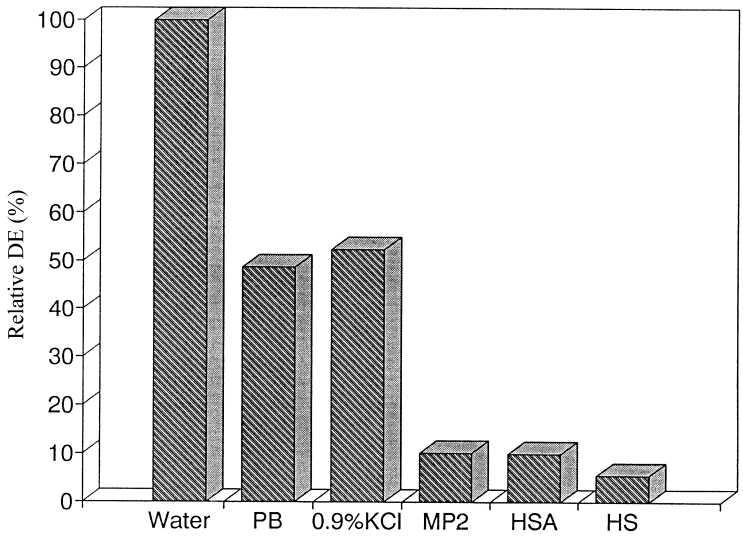

Fig. 9. Effect of donor solution composition on dialysis efficiency relative to that in deionised water for 1×10^{-7} *M* CF and 10 min of dialysis. PB, 0.01 *M* phosphate buffer of pH 7.4; HSA, 0.2% (v/v) human serum albumin; HS, human serum diluted 10 times with water.

favour the exchange of small ions to maintain electroneutrality according to Donnan equilibria. It seems that membrane fouling due to the adsorption of macromolecules and protein binding of CF are the main reasons for decreased membrane permeability and the low *DE* in the presence of human serum and albumin. As described by van de Merbel et al. [5], any binding of the analyte to proteins affects *DE* and for the determination of the total analyte concentration the bonded fraction should be released. This can be achieved by dilution, changing the pH or by the addition of an appropriate displacer, i.e. a compound with high affinity for the analyte binding site on the protein [5]. Johansen et al. [7] determined total concentration of antiepileptic drugs in dialysed plasma samples by the use of an internal standard. From these alternatives only dilution can be recommended for the determination of the total concentration of CF in plasma samples. Although the reduction of the dialysis efficiency in the presence of the proteins is at the first instance disappointing, it is often necessary to determine the free fraction of the analyte and not the total concentration, especially in the analysis of biologically important substances. Since only the free fraction of CF passes through the dialysis membrane, low *DE* in comparison to that in pure water (Fig. 9), reflects the concentration of the biologically active fraction of CF in serum samples. Dialysis, therefore, enables determination of non-bounded fraction of CF in the presence of proteins and can be also applied for speciation purposes.

The effect of phosphate buffer on *DE* can be explained by diffusion of phosphate to the acceptor compartment and a consequent partial neutralisation of the positively charged membrane. Diminution of an excess of positive charge on the acceptor side of the membrane enables back-diffusion of HCF^+ to the donor channel and *DE* decreases. We suppose that KCl acts similarly and the about 50% lower *DE* in its presence than in water is ascribed to the neutralisation of the excess of positive charge by chloride adsorption on the membrane surface.

Due to the above mentioned effects on the separation efficiency, every application of dialysis in analytical practice therefore needs careful examination and optimisation. Since for an accurate HPLC determination of an unknown concentration of analyte in the sample via dialytic clean-up a constant and repeatable separation efficiency is a prerequisite, the main parameters affecting *DE* must be identified and controlled. When dialysis efficiency is conditioned by sample constituents it cannot be considered constant. To avoid the necessity of knowing the actual *DE* value and/or its sample to sample varia-

tions, we suggest that the determination of fluoroquinolones in biological fluids be performed using the standard addition method. Although in this case every sample should be dialysed and chromatographed at least twice, only consecutive dialysis of the original and the spiked sample can ensure a constant enough *DE* and enable accurate and reliable evaluation of the unknown concentration of analyte from the chromatographic data without knowledge of the actual dialysis efficiency.

4. Conclusions

Separation of fluoroquinolones by dialysis is a quite efficient and convenient clean-up procedure for determination of these pharmaceuticals in biological fluids by HPLC. Besides elimination of macromolecular interfering substances, dialysis also enables effective preconcentration of quinolones by use of neutral donor and acidic acceptor solutions. Enrichment of quinolones in the recipient phase is ascribed to the protonation of the analyte in the acceptor phase and the electrostatic repulsions between HQ^+ species and the positively charged acceptor side of the microporous cellulose acetate membrane. To achieve a high flux of analyte to the stagnant acceptor phase, continuous or pulsed-flow of the donor phase is preferred. When small amounts of sample are available for analysis pulsed donor flow or a slowly moving continuous flow of donor solution should be used. If the sample volume is not limited a continuous sample flow-rate of 0.5 $ml\ min^{-1}$ gives a higher dialysis efficiency per time unit and results in about a five times higher concentration of the analyte in the acceptor channel than that in the inflowing sample; a preconcentration column is therefore superfluous.

By continuous or pulsed dialysis with a stagnant acceptor the flow of the donor compresses the acceptor chamber, thus decreasing its volume. To ensure a constant *DE* and reproducible results the acceptor channel should be closed by a valve during the dialysis.

Dialysis efficiency depends strongly on the composition of the dialysed solution. The presence of low molecular weight inorganic and organic compounds as well as macromolecular compounds affects *DE*. To ensure an acceptable accuracy in analysis of biological samples replicate (at least two) dialysis runs of spiked samples is highly recommended.

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