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Dialysis as an on-line sample-pretreatment technique for column liquid chromatography: influence of experimental variables upon the determination of benzodiazepines in human plasma

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Abstract: An evaluation is provided of dialysis, coupled on-line to column liquid chromatography, as a sample-pretreatment procedure for macromolecule-containing biological samples. The influence of parameters such as acceptor phase flow rate, temperature, hydrophobicity of the analytes, pH, ionic strength and viscosity of the sample on the recovery and rate of dialysis is studied. In addition, methods to reduce the degree of drug–protein binding and thereby improve the recovery are reported. Diazepam, nitrazepam and oxazepam are used as model compounds. A method is reported for the fully automated determination of these compounds in human plasma using only 100 μl of sample. Data on repeatability, linearity and detectability are given.

Keywords: *On-line dialysis; column liquid chromatography; benzodiazepines, physico-chemical parameters, drug–protein binding.*

Introduction

In general, in the determination of trace amounts of analytes in complex matrices by column liquid chromatography (LC) two types of problems are encountered. Firstly, if the analyte concentration is too low, trace enrichment is necessary to improve sensitivity. Secondly, if other sample constituents interfere with analyte separation and/or detection, clean-up is required to remove these from the sample, i.e. to enhance selectivity. In particular, macromolecular constituents such as proteins can cause problems when they are not removed prior to sample injection. They are readily adsorbed onto the stationary phase which results in clogging of the analytical column, a decreased separation efficiency and a limited lifetime of the column. Therefore, sample preparation should be an integral part of the analysis of complex samples [1, 2]. Most sample-pretreatment procedures available today use off-line methods like deproteination, liquid–liquid extraction or liquid–solid sorption. However, these techniques often are performed manually and are, therefore, laborious and time-consuming and sometimes lack repeatability. Since for routine analyses high sample throughput and good accuracy and precision are required, automation of sample pretreatment often is a necessity. Nowadays, automated methods are available for the removal of interfering macromolecules. On-line precolumn techniques are most often used, but they can not always be recommended, because in many cases only a small number of samples can be analysed on one precolumn. Alternative techniques that prevent the adsorption of macromolecules onto the stationary phase and allow direct sample injection are micellar chromatography [3] and techniques using modified stationary phases, such as internal-surface reversed phases [4].

On-line dialysis is another promising alternative for sample pretreatment aimed at the removal of proteins from biological samples. A sample processor equipped with a dialysis membrane has been shown to be useful in this respect [5], especially when a trace-enrichment step is incorporated to overcome the dilution of the sample caused by dialysis. Although a number of applications have been described [6–14], dialysis has not been widely accepted as an on-line sample-pretreatment procedure,

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possibly because of the still widely held opinion that it is rather slow and neither a quantitative nor a selective technique. Analytical chemists tend to overlook the fact that the use of moving acceptor and/or donor phases combined with a suitable, selective trace-enrichment step results in a rapid and efficient clean-up plus enrichment procedure [8, 10, 13].

In the present study, several aspects of dialysis as an on-line and automated sample-pretreatment technique are evaluated. The influence of a number of relevant physicochemical parameters on the rate of analyte transport through the membrane and the final recovery is investigated. Secondly, the problem of analyte binding to plasma proteins is studied. Due to the fact that only the unbound drug fraction can actually diffuse through the membrane, protein binding of analytes gives rise to a decrease in dialysis rate. Since in this report the determination of the total drug fraction is described, several methods to disrupt the analyte–protein binding are compared and discussed. The benzodiazepines nitrazepam, oxazepam and diazepam, which display a high degree of protein binding, are used as model compounds.

Experimental

Chemicals and reagents

Nitrazepam, diazepam and oxazepam were obtained from Bufa Chemie (Castricum, The Netherlands). Methylcellulose was purchased from Brocades (Maarssen, The Netherlands). All other chemicals and solvents were of analytical grade and came from J.T. Baker (Deventer, The Netherlands). HPLC-grade water was prepared by using a Milli-Q purification system (Millipore, Bedford, MA, USA), followed by filtration over a column filled with 40 μm C_{18} material (J.T. Baker).

Equipment

In all experiments a Gilson (Villiers-le-Bel, France) ASTED combined on-line with an LC system and UV detection was used. The ASTED system consisted of a Model 231 autosampling injector, equipped with two 1 ml Model 401 dilutors in slave configuration, a Model 99/55 rack for 128 sample vials of 860 μl and five reagent vials of 25 ml, and a Model 7010 Rheodyne (Berkeley, CA, USA) six-port switching valve. The dialysis cell was made of polymethylmethacrylate, with donor and acceptor channel volumes of 100 and 170 μl, respectively. A Cuprophane membrane with a molecular weight cut-off of 15 kD was used. The dialysis cell and the acceptor phase were thermostatted with a Marius (Utrecht, The Netherlands) water bath or a Savant Instruments (Hicksville, NY, USA) cryostat. For preconcentration a 10 mm × 2.0 mm i.d. stainless-steel precolumn, slurry-packed with 40 μm Baker C_{18} material in a home-made precolumn holder, was used. The LC system consisted of a Gilson Model 305 high-pressure piston pump and a 150 mm × 3.1 mm i.d. stainless-steel analytical column packed with 5 μm RoSil (Research Separation Laboratories, Eke, Belgium) C_{18} stationary phase. Various mixtures (v/v) of methanol and 20 mM sodium acetate buffer (pH 5.0) were used as mobile phase. The flow rate was 0.5 ml min^{-1} and LC was performed at ambient temperature. A Knauer (Berlin, Germany) UV photometer was used for detection at 254 nm; the signal was recorded with a Hewlett–Packard (Waldbronn, Germany) Model 3396A integrator.

Relative viscosities were determined at 20°C using an Ubbelohde viscometer.

Set-up

A schematic diagram of the system is shown in Fig. 1. Unless stated otherwise, the dialysis process was studied by measuring the recovery for a 100-μl aqueous sample containing 1 ppm of nitrazepam as a function of dialysis time. The experiments were performed in duplicate using water as the acceptor phase at a flow rate of 0.75 ml min^{-1}, and run at ambient temperature. Before each run the donor channel was flushed with 2 ml of water and the acceptor channel with 2 ml of the acceptor phase; the precolumn was subsequently conditioned with

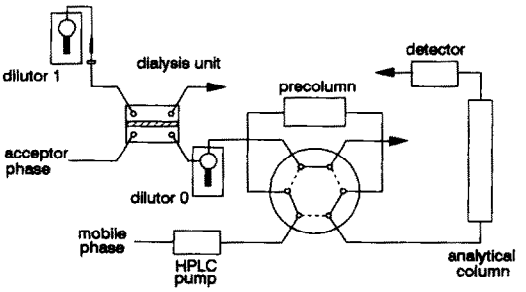

Figure 1
Schematic representation of the on-line dialysis–LC system. Sample is introduced by dilutor 1 and acceptor phase is pumped by means of dilutor 0.

1 ml of acceptor phase. All dialysis experiments were performed in the conventional static mode [14]. By switching the six-port valve, the enriched analyte was backflushed by the LC eluent to the analytical column. Finally the donor channel was washed with 2 ml of water or an aqueous 0.05% (v/v) Triton X-100 solution and the acceptor channel with 2 ml of acceptor phase.

Results and Discussion

In daily analytical practice, speed and high recovery are major requirements. Improving the rate of dialysis, i.e. the number of analyte molecules that is recovered per unit time, results in either a higher recovery in the same time or the same recovery in a shorter period of time.

Two different problems encountered in on-line dialysis, which affect the rate of dialysis, were addressed in this study. Firstly, the dialysis process itself was investigated using aqueous standard solutions as samples. Since dialysis is a diffusion-controlled process, the rate of dialysis depends on a number of physico-chemical variables, some of which will be discussed below. In addition, the effect of the interaction of the analytes with the membrane on analyte recovery was studied. The influence of the dimensions of the donor and acceptor channel and of using a moving instead of a stagnant donor phase have already been described [14] and are not further dealt with here.

Secondly, matrix effects were studied. The binding of drugs by plasma proteins inevitably diminishes the amount of free and, thus, diffusible analyte, and results in low dialysis rates. Methods to overcome this problem, i.e. to reduce the degree of protein binding have also been studied.

Influence of physico-chemical parameters

When trying to optimize a dialysis procedure, even with a simple theoretical model acceptable predictions can be made, although it is known that a rather large number of physical processes are involved with transport of analytes through membranes [15].

In the present study the most frequently used dialysis mode — conventional static dialysis — is considered. This utilizes a stagnant donor phase, a continuously flowing acceptor phase and enrichment on a precolumn. Four different processes can be distinguished during the transfer of analyte molecules from the donor phase to the precolumn (cf. Fig. 1):

(1) diffusion from the bulk solvent of the donor phase to the membrane;
(2) diffusion through the membrane;
(3) diffusion from the membrane to the bulk acceptor phase;
(4) removal from the dialysis cell by the flowing acceptor phase to the precolumn.

It is assumed that neither diffusion to, nor diffusion from, the membrane is rate limiting [15]; processes 1 and 3 can, therefore, be neglected. Other assumptions are that the membrane is inert and that a concentration gradient is the only driving force for diffusion through the membrane. This means that process 2 can be described by Fick's law:

$$j_i = -\frac{D_{ij}\,A}{\tau}\,\frac{dc_i}{dx}\,, \qquad (1)$$

where j_i is the flux (or dialysis rate) of analyte i from the donor to the acceptor channel (mol s^{-1}), D_{ij} the diffusion coefficient of analyte i in medium j (m^2 s^{-1}), A the membrane area available for diffusion (m^2), c_i the concentration of analyte i (mol m^{-3}), x the coordinate direction along which diffusion is taking place (m) and τ the tortuosity of the membrane, a parameter that takes all membrane effects into account. Tortuosities usually range between 2 and 6, with an average of about 3 [15].

D_{ij} can be expressed by the Stokes–Einstein relation:

$$D_{ij} = \frac{kT}{6\pi\eta_j r_i}\,, \qquad (2)$$

where k is the Boltzmann constant (J K^{-1}), T the absolute temperature (K), η_j the viscosity of medium j (kg m^{-1} s^{-1}) and r_i the molecular radius of the analyte (m).

Finally, no serious error is introduced if the acceptor phase is assumed to move as a plug [16], which means that process 4 can be described by:

$$j_i^* = fc_i, \qquad (3)$$

where j_i^* is the analyte flux out of the acceptor channel (mol s^{-1}) and f the volumetric flow velocity (m^3 s^{-1}).

Processes 2 and 4 are interrelated, since j_i^* determines the concentration in the acceptor phase and, thus, the concentration gradient over the membrane, which also affects j_i (cf. equation 1). This means, for example, that at high values of j_i^* diffusion through the membrane (j_i) becomes the rate-limiting step.

The information provided by equations (1)–(3) can be used when optimizing a dialysis process, because it shows which parameters influence the rate of dialysis. Process 2 can be accelerated by increasing the diffusion coefficient (e.g. by raising the temperature, or by reducing the viscosity of the solvent). Furthermore, a large membrane area, a low tortuosity and a high concentration gradient are favourable. Process 4 can be accelerated by increasing the acceptor phase flow rate.

Acceptor phase flow rate. The influence of the acceptor phase flow rate on the dialysis rate of nitrazepam is shown in Fig. 2(A and B). Increasing the flow rate from 0.36 via 0.75 to 1.5 ml min^{-1} results in an increasing recovery per unit time, caused by a higher analyte flux through the membrane. A further increase to 3.0 ml min^{-1} does not effect a further improvement of the dialysis rate. The final recovery is the same in all cases. This result is in agreement with equations (1) and (3). The concentration gradient over the membrane is determined by the velocity, f, with which the analytes are removed from the acceptor channel. A high flow rate results in rapid removal and helps to maintain a high concentration gradient, giving rise to a high analyte flux. At a certain flow rate, however, all diffusing molecules are immediately flushed away. Diffusion through the membrane becomes the rate-limiting step and higher acceptor phase flow rates do not further improve the rate of dialysis.

Figure 2(B) also illustrates a potential disadvantage of increasing the acceptor phase flow rate. At higher values (1.5 and 3.0 ml min^{-1}), breakthrough of the analytes on the precolumn is seen to occur. This indicates the necessity to carefully choose an appropriate acceptor phase flow rate or, more correctly, an appropriate acceptor phase flow rate/pre-concentration column combination. In further experiments an acceptor phase flow rate of 0.75 ml min^{-1} was used as a compromise.

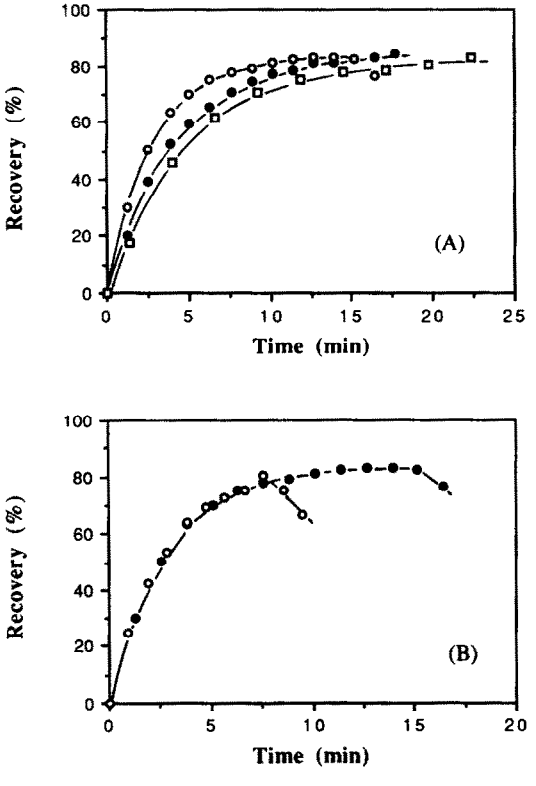

Figure 2
Per cent recovery of nitrazepam vs dialysis time, (A) at 0.36 (□), 0.75 (●) and 1.5 ml min^{-1} (○), and (B) at 1.5 (●) and 3.0 ml min^{-1} (○). For other conditions see text.

Temperature. The effect of temperature on the dialysis rate of nitrazepam is shown in Fig. 3. The observed increase in analyte flux at elevated temperature can be explained by means of equation (2). This equation shows that, since D_{ij} is directly proportional to the temperature, the analyte molecules move more rapidly through the membrane at higher temperatures, which results in a higher recovery per unit time. Clearly, the temperature also influences the solvent viscosity; this parameter

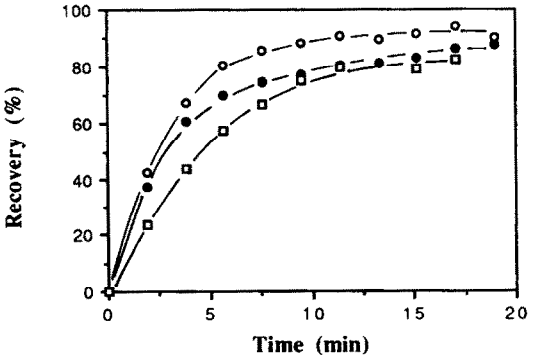

Figure 3
Per cent recovery of nitrazepam vs dialysis time, at 10 (□), 35 (●) and 50°C (○). For other conditions see text.

will be dealt with in more detail below. For the sake of convenience, all further experiments were performed at ambient temperature (here, one should realize that a temperature fluctuation of 5°C can cause errors of up to 5%; Fig. 3).

Hydrophobicity. The possibility of a hydrophobic interaction taking place between analyte and membrane was tested by dialysing compounds with different hydrophobicities (expressed below as log P, the logarithm of the octanol–water partition coefficient). Figure 4 shows that the final recovery is different for the three model compounds, indicating that some binding to the membrane occurs and that the three model compounds do not interact to the same extent with the membrane. The most hydrophobic compound, diazepam (log P = 2.66), clearly binds more strongly than oxazepam (log P = 2.17) and nitrazepam (log P = 2.12). These results suggest a hydrophobic interaction between the benzodiazepines and the membrane material (cellulose acetate). This is in agreement with the findings of Kiso [17], who found a positive correlation between the hydrophobicity of various sets of analytes and the interaction with cellulose acetate.

pH. Benzodiazepines are bases and are positively charged at low pH values. The influence of charge on the recovery was studied for pH values between 4.0 and 7.0; both the sample and acceptor phase were buffered with a 20 mM phosphate buffer. The experiments were performed using oxazepam (pK_a = 1.8), which is non-charged in this pH range, and nitrazepam (pK_a = 3.4), which will become

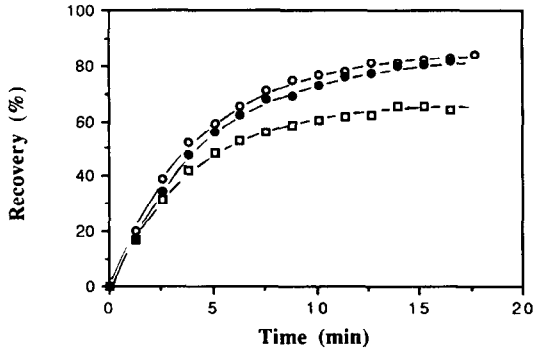

Figure 4
Per cent recovery of nitrazepam (○), oxazepam (●) and diazepam (□) vs dialysis time. For other conditions see text.

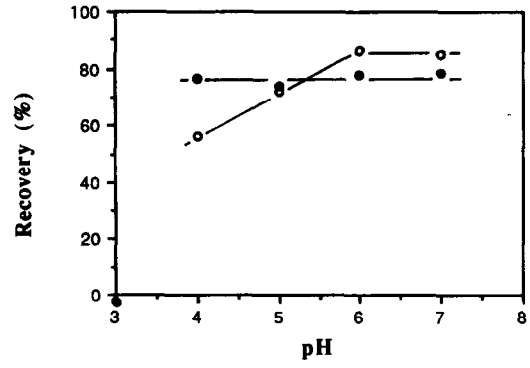

Figure 5
Per cent recovery of nitrazepam (○) and oxazepam (●) vs pH, dialysis time is 19 min. For other conditions see text.

partly protonated at pH ≤ 5 (Fig. 5). The observed decrease in the recovery of positively charged analytes may be explained by an electrostatic interaction with the negatively charged groups on the membrane. These results demonstrate that dialysis should preferably be performed with non-charged compounds. The limited pH stability of cellulose acetate (pH 2–8) is a disadvantage in this respect and other membrane materials should be tested to try to overcome this limitation. Further experiments were done at pH 7.0, using water as the acceptor phase.

Influence of matrix effects

Dialysis of an analyte frequently proceeds less efficiently when working with protein-containing biological samples instead of aqueous standards, which is usually attributed to protein binding of the analyte. The concentration of diffusible analyte in the donor channel and, thus, the concentration gradient over the membrane is influenced by the presence of a binding species, for example, a protein. Generally, the binding of a solute, S, by a protein, P, according to

$$P + S \rightleftharpoons PS \qquad (4)$$

can be described by means of an association constant K_a:

$$K_a = \frac{[PS]}{[P]\,[S]}, \qquad (5)$$

where [PS] is the concentration of the solute–protein complex, [P] the concentration of the free protein and [S] the concentration of the free solute. The value of K_a and, therefore, the

concentration of the free solute, depends on the mechanism of interaction between solute and protein. Normally, hydrophobic, electrostatic and/or van der Waals forces are involved, and the relative contribution of these forces is different for each solute–protein combination and can also differ between separate binding sites on a specific protein. Other factors affecting K_a are temperature, and the presence of compounds binding to the same site.

The primary goal of this study was to establish if the observed decrease in dialysis rate is due to protein binding only. Since ionic strength and viscosity differ markedly between aqueous standards and plasma samples, their influence was also studied. Furthermore, it was attempted to reduce the degree of analyte–protein binding and so improve the rate of dialysis with plasma samples.

Ionic strength and viscosity. The ionic strength of blood plasma corresponds to a sodium chloride concentration of *ca* 150 mM; the viscosity of plasma is about twice that of water. The effect of sample ionic strength was therefore tested in the range of 0–400 mM sodium chloride and the influence of sample viscosity in the range of $\eta_{sample}/\eta_{water} = 1–3$, using methylcellulose as viscosity-inducing agent. In both cases, no effect on either the dialysis rate or the recovery was observed. Two conclusions can be drawn from these results. Firstly, a decline in the dialysis rate of plasma samples compared with water cannot be ascribed to the larger ionic strength or viscosity of plasma. This suggests that protein binding in fact is the major reason for the decrease in dialysis rate often observed in practice. Secondly, a greater viscosity of the donor phase does not result in slower dialysis, which indicates that diffusion through the membrane rather than diffusion to the membrane is the rate-limiting step. This confirms that the processes of diffusion to and from the membrane can be neglected, as has been assumed above.

Protein binding. A large number of drugs exhibit at least some degree of protein binding. It is evident that only the free fraction of a drug can diffuse through a dialysis membrane. In order to increase the analyte transport rate, a method should therefore be found to release the drug from its protein binding site(s). The most abundant plasma protein is albumin, which is known to bind acids and bases as well as neutral compounds. There is now evidence for the existence of several structurally specific and independent binding sites, as well as less specific sites, that bind both endogenous and exogenous compounds [18]. At least six binding sites have been proposed, of which the so-called diazepam site is the most important one in the present context. It has been reported to bind various classes of drugs, of which the benzodiazepines are the best known. Since the binding of benzodiazepines to albumin is strong, a high percentage of the drug will be bound; the figures for nitrazepam, diazepam and oxazepam are 87–90, 97–99 and 86%, respectively [19].

Using 100 μl of drug-free human plasma, spiked with either 100 ppb nitrazepam or 200 ppb oxazepam or diazepam, the effect of protein binding on the rate of dialysis was investigated (Figs 6–8). Several conclusions can be drawn from these figures. Firstly, comparison of the data with those of Fig. 4 shows that the recovery per unit time is much lower than in water, which is obviously due to the decreased amount of free analyte. Secondly, the effects of acceptor phase flow rate and of temperature are much more pronounced than with aqueous samples. This is best illustrated for nitrazepam (Fig. 6). By increasing the acceptor phase flow rate up to 3.0 ml min^{-1} and raising the temperature up to 50°C, the recovery could be improved from 25 to 90% (dialysis time, 10 min). This increase can be explained by considering the bound drug fraction as a reservoir of analyte molecules. If the membrane passage is slow compared to the shift in the drug–protein equilibrium, an increase in acceptor phase flow rate will cause a higher transport rate through the membrane and a rapid release of bound drug molecules. The gain will be higher than with aqueous samples, where there is no such reservoir present, although in that case the initial concentration gradient will be steeper. As for the temperature, the interaction between analyte and protein is weaker at high temperatures, which will result in a shift of the equilibrium and a higher concentration of free analyte.

There are several other ways to reduce the degree of drug–protein binding [7]: modification of either analyte or protein can result in a decrease of K_a (equation 5), but displacement of the drug by a competing species is more selective and elegant. This displacement

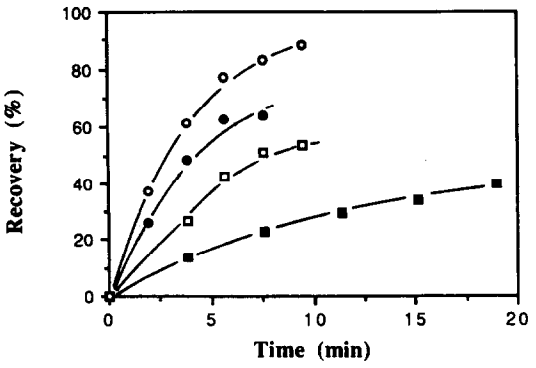

Figure 6
Per cent recovery of nitrazepam from plasma vs dialysis time. Conditions: 0.75 ml min^{-1}, 25°C (■); 3.0 ml min^{-1}, 25°C (□); 3.0 ml min^{-1}, 25°C, 1 mM *n*-octanoic acid added (●); 3.0 ml min^{-1}, 50°C (○).

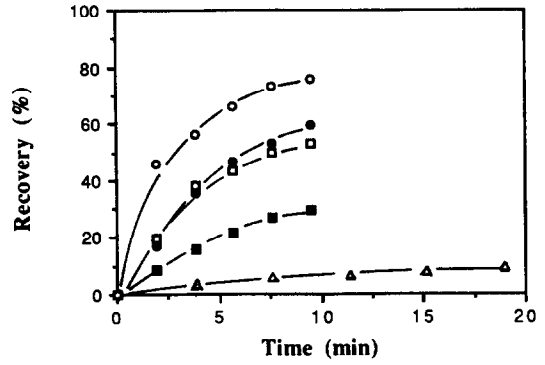

Figure 7
Per cent recovery of oxazepam from plasma vs dialysis time. Conditions: 0.75 ml min^{-1}, 25°C (△); 3.0 ml min^{-1}; 50°C (■); 3.0 ml min^{-1}, 25°C, 1 mM *n*-octanoic acid added (□); 3.0 ml min^{-1}, 25°C, 2 mM *n*-octanoic acid added (●); 3.0 ml min^{-1}, 50°C, 1 mM *n*-octanoic acid added (○).

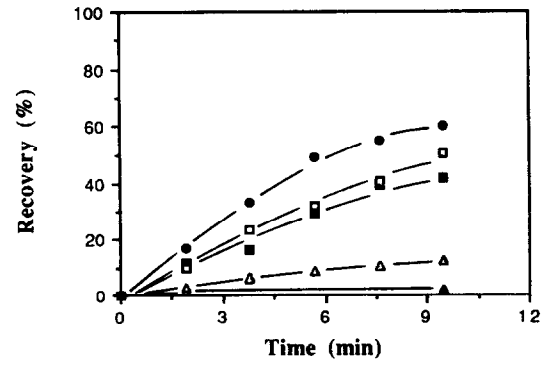

Figure 8
Per cent recovery of diazepam from plasma vs dialysis time, at 3.0 ml min^{-1}. Conditions: 25°C (▲); 50°C (△); 25°C, 1 mM *n*-octanoic acid added (■); 25°C, 2 mM *n*-octanoic acid added (□); 50°C, 1 mM *n*-octanoic acid added (●).

can be competitive (competition for a common binding site) or non-competitive (the displacer alters the tertiary structure of the protein and induces release of the drug). For a successful displacement of drugs from plasma proteins the displacer and the displaced drug must share a common binding site (competitive displacement) or a common protein (non-competitive displacement). In addition, the binding sites must be limited in number, hence the displacer concentration must approach the binding site concentration and, finally, the concentration of the displacer must be higher than that of the displaced drug and/or its binding site affinity must be higher [20].

The diazepam site has been described as a hydrophobic cleft of about 12–16 Å deep and 6–8 Å wide, with a cationic group located near the surface. Neutral compounds, such as most benzodiazepines at physiological pH, as well as negatively charged compounds bind to it, whereas positively charged ones do not. Aliphatic carboxylic acids of appropriate chain length (C_6–C_{11}) have been shown to be strong competitive displacers of typical ligands such as benzodiazepines [21]. The K_a of the most strongly bound model compound, diazepam, to the diazepam site is $4.9.10^5$ M^{-1}; the K_a values of most aliphatic carboxylic acids to this site range between 10^6 and 10^7 M^{-1} [22].

In the present study *n*-octanoic acid was used to displace the model compounds from their binding sites. A concentration of around 1 mM was chosen, because — assuming one site per albumin molecule — the concentration of the diazepam sites will be equal to the concentration of albumin, which is about 0.6 mM. The displacer concentration is therefore higher than that of the binding sites and obviously also higher than the concentration of the drugs.

The effect of the addition of *n*-octanoic acid is included in Figs 6–8. The recovery of oxazepam and diazepam is seen to be significantly enhanced. For example, in the case of diazepam, the recovery increased from 10 to 60% upon the addition of 1 mM of the displacer (10 min dialysis, 50°C). In contrast, the recovery of nitrazepam was only slightly affected by the addition of *n*-octanoic acid. This suggests that oxazepam and diazepam are predominantly bound to the diazepam site and are competitively displaced, but that nitrazepam is preferentially bound to another binding site. The release of nitrazepam upon the addition of *n*-octanoic acid may be due to non-

competitive effects. Probably, the interaction of nitrazepam with its binding site is weaker than the interaction of the other two compounds with the diazepam site, since the effects of acceptor phase flow rate and temperature are stronger for nitrazepam (see Figs 6 and 7). In Figs 7 and 8 only a small difference can be seen between the addition of 1 and 2 mM of *n*-octanoic acid; that is, a concentration of 1 mM is sufficient to displace most of the compounds from their binding sites.

In the literature, a trichloroacetic acid solution buffered at pH 7, has been successfully used to displace other drugs from plasma proteins [6, 7, 13]. This displacer was also tested, but did not induce release of any of the model compounds. This agrees with the fact that trichloroacetic acid binds to another site, the so-called warfarin site, that is also known to bind various exogenous compounds [20].

Automated determination of benzodiazepines in plasma

On the basis of the above studies, a method for the determination of nitrazepam, oxazepam and diazepam in plasma samples was set up. Despite the fact that an increase in temperature positively affected the benzodiazepine recovery in plasma samples, the actual analyses were performed at room temperature because some interfering peaks were observed in the LC chromatograms at elevated temperature. These are possibly caused by the release of endogenous compounds from plasma proteins. After the addition of 1 mM of *n*-octanoic acid to a 100 μl plasma sample (no addition in the case of nitrazepam), dialysis was carried out for 7.6 min using an acceptor phase flow rate of 3.0 ml min^{-1}. Next, after valve switching, the preconcentrated benzodiazepine was desorbed and the LC separation carried out on a RoSil C_{18} (150 mm × 3.1 mm i.d., 5 μm) column. For nitrazepam and oxazepam the LC eluent was methanol–sodium acetate (20 mM, pH 5.0) (60:40, v/v), for diazepam it was methanol–sodium acetate (20 mM, pH 5.0) (70:30, v/v). UV detection was carried out at 254 nm. The total time of analysis was 18 min. The whole procedure, including the addition of *n*-octanoic acid, was fully automated and at least 150 analyses could be performed without deterioration of membrane, precolumn or analytical column. Relevant analytical data are summarized in Table 1. It should be realized, that by increasing the sample volume and applying the pulsed dialysis mode [14], substantially higher sensitivities can be achieved. As stated earlier, this aspect was outside the scope of the present work and was not investigated. As an illustration, LC chromatograms of a plasma blank and plasma samples spiked with diazepam, with and without added *n*-octanoic acid, are shown in Fig. 9.

Conclusions

The performance of on-line dialysis as a sample-pretreatment technique for LC has been studied systematically, using three benzodiazepines as test compounds. Optimization of the speed and/or recovery of dialysis depends mainly on a proper combination of the acceptor phase flow rate and the preconcentration column, and on the proper choice of sample temperature and pH. In addition, the sample matrix (plasma) can also exert considerable influence, drug–protein binding being much more important than the ionic strength or viscosity of plasma. For highly bound analytes reducing the degree of drug–protein binding is the most important feature for accelerating the dialysis. The release of even highly bound analytes from plasma proteins can be significantly enhanced by the addition of a suitable displacer (*n*-octanoic acid in the case of the benzodiazepines). To effect

Table 1
Analytical data on the automated analysis of benzodiazepines in plasma samples

	Nitrazepam	Oxazepam	Diazepam
Limit of detection ($S/N = 3$)	25 ng ml^{-1}	20 ng ml^{-1}	25 ng ml^{-1}
Absolute recovery	48%	50%	37%
Linearity ($n = 6$)	25–1000 ng ml^{-1} $y = 3.35 + 0.48x$ $R^2 = 0.9998$	20–1000 ng ml^{-1} $y = 0.51 + 0.50x$ $R^2 = 0.9989$	25–1000 ng ml^{-1} $y = -2.2 + 0.37x$ $R^2 = 0.9999$
Within-day precision ($n = 10$)	3.7%	5.5%	3.9%

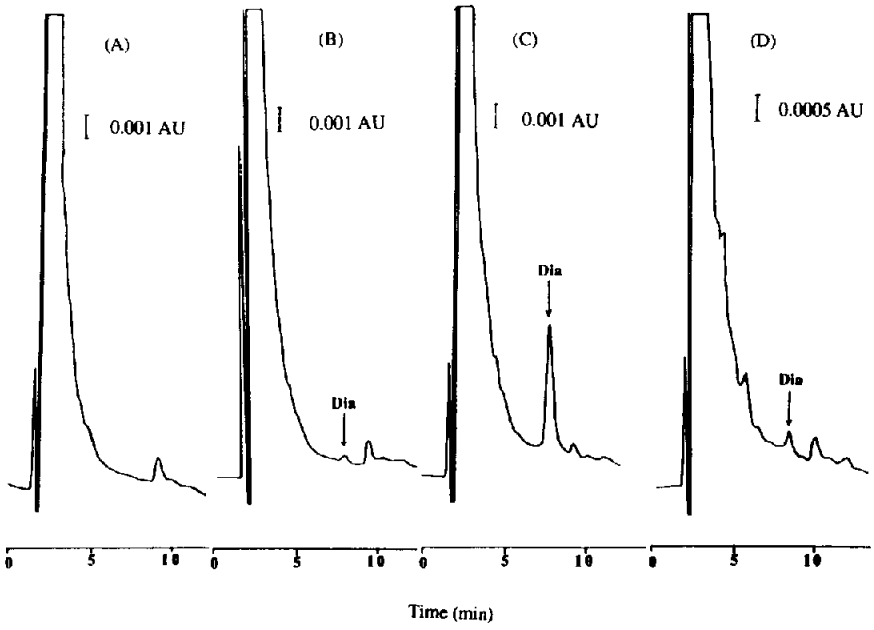

Figure 9
On-line dialysis–LC of 100 μl plasma samples. Dia = diazepam. (A) Blank, (B) plasma spiked with 200 ng ml^{-1} diazepam, (C) plasma spiked with 200 ng ml^{-1} diazepam, 1 mM of *n*-octanoic acid added, (D) plasma spiked with 25 ng ml^{-1} diazepam, 1 mM of *n*-octanoic acid added.

such a release detailed knowledge of the interaction of analyte and protein is a necessity. The rapid and fully automated determination of the benzodiazepines serves to illustrate the usefulness of on-line dialysis–LC for the routine analysis of plasma samples.

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