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Determination of the total concentration of highly protein-bound drugs in plasma by on-line dialysis and column liquid chromatography: application to non-steroidal anti-inflammatory drugs

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Abstract

The potential of on-line dialysis as a sample preparation procedure for compounds highly bound to plasma proteins is evaluated, using non-steroidal anti-inflammatory drugs as model compounds and column liquid chromatography as the separation technique. Different strategies to reduce the degree of drug–protein binding and so increase the analyte recovery are systematically explored and discussed: alteration of the conformation of the binding protein by changing the pH of the sample or by adding an organic solvent, addition of several displacing compounds and combinations of such approaches. A fully automated method is presented for the determination of ketoprofen, ibuprofen, flurbiprofen, fenoprofen and naproxen in human plasma, in which the absolute analyte recoveries are increased from 0–1% (untreated samples) to 40–65%. Relevant analytical data are given to demonstrate the reliability of the proposed procedure.

1. Introduction

The determination of traces of analytes in complex matrices by column liquid chromatography (LC) often requires extensive sample preparation to remove interfering compounds and/or to enrich the analytes. As sample preparation often is labour-intensive and easily becomes the rate-limiting step in the total procedure, a number of automated sample-preparation methods have been developed over the last decade. For protein-containing samples one of the most attractive approaches is on-line dialysis, since the use of a semi-permeable membrane offers the possibility of removing macromolecular sample components in an automated way, thus protecting the chromatographic column and often increasing the sample throughput considerably. In addition, if a trace-enrichment precolumn is incorporated in the set-up to overcome the dilution of the sample caused by the dialysis step, efficient sample clean-up and analyte enrichment can be performed with the same system in a fully automated way.

Although on-line dialysis has been successfully

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applied to a variety of biomedical, food and environmental sample types [1,2], a problem commonly encountered when using dialysis for biological samples is an often substantial decrease in recovery, caused by protein binding of the analytes. Since dialysis is a non-destructive technique, which leaves the protein structure intact, and only the unbound analyte fraction can diffuse through the membrane, large reductions in analyte recovery are often observed when biological samples are dialysed instead of aqueous samples, the extent of which depends on the degree of analyte–protein binding. On the other hand, with conventional sample preparation methods the total amount of analyte can usually be recovered, because the binding sites are destroyed by the denaturation of the proteins. Therefore, to improve the analyte recovery and, consequently, the sensitivity of a bioanalytical method using dialysis, a means of efficiently releasing highly bound analytes from their binding sites should be found.

Biological fluids contain a wide variety of proteins but the most important drug-binding one in man is human serum albumin (HSA), which is the most abundant plasma protein, accounting for about 58% of the total plasma protein concentration (equivalent to 40 g/l or 0.6 m*M*). HSA binds acids, bases and neutral compounds and is undoubtedly the main carrier of small molecules and drugs in the body [3]. Although the three-dimensional structure of HSA has not yet been fully elucidated, it is generally accepted that there are six major high-affinity sites, which bind both endogenous and exogenous compounds, and a large number of sites of much lower affinity. At least two of the major sites are involved in drug binding; they are most commonly known as the warfarin site (or site I) and the diazepam site (or site II) [4]. The warfarin site presumably is a large and rather flexible binding region, probably consisting of several subsites, which has a variable affinity for many different drugs [5]. The diazepam site has been described as a hydrophobic cleft with a cationic group (probably histidine or arginine) located near the surface [6]. Neutral and negatively charged compounds can bind to this site, but positively charged ones can not. Besides diazepam, a wide variety of compounds of very different chemical structure including most benzodiazepines and other analgaesic, hypoglycaemic and non-steroidal anti-inflammatory drugs as well as fatty acids of appropriate chain length (C_7–C_{11}), have been proven to bind to this site with very high affinity [7]. In addition, there is no doubt that at least one globulin, the α_1-acid glycoprotein (AGP) is important in drug binding, particularly for basic and neutral compounds. AGP has a mean plasma concentration of 0.9 g/l (22 μM), but this value can be much higher in certain diseases such as inflammations. There is also evidence that most drugs bind to only one site [8]. Some drugs are known to bind to both HSA and AGP, and the ratio in which a drug binds to these two proteins is determined by their concentrations and the binding affinities.

The binding of an analyte, A, to a protein, P, is a reversible process [9]:

$$A + P \rightleftharpoons AP \tag{1}$$

with the corresponding association constant, K_a, given by:

$$K_a = \frac{[AP]}{[P][A]} \tag{2}$$

where P, A and AP represent the unbound protein, free analyte and protein-bound analyte, respectively. Eq. (2) shows that the free fraction of an analyte can be increased by lowering K_a, which can be achieved by changing the confirmation of the analyte or the protein. Furthermore, the addition of another compound binding to the same site will lead to displacement of the analyte:

$$AP + D \rightleftharpoons A + DP \tag{3}$$

where D is the free displacer and DP the protein-bound displacer. This equilibrium is governed by K, the ratio between the association constants $K_{a,D}$ and $K_{a,A}$ for the displacer–protein and analyte–protein interactions, respectively:

$$K = \frac{K_{a,D}}{K_{a,A}} = \frac{[A][DP]}{[D][AP]} \tag{4}$$

whence

$$\frac{[A]}{[AP]} = \frac{K_{a,D}[D]}{K_{a,A}[DP]} \quad (5)$$

The unbound analyte fraction can, thus, be efficiently increased by adding a displacer with a higher affinity for the protein than the analyte ($K_{a,D} > K_{a,A}$) and/or by increasing the concentration of the displacer.

In this work, some non-steroidal anti-inflammatory drugs, which bind to the diazepam site in HSA were used as model compounds because of their very high degree of protein binding (94–99%). Several alternatives have been tested for releasing drugs from their protein binding site(s) and, thus, increase analyte recovery in on-line dialysis-LC: (i) altering the confirmation of HSA by changing the pH of the sample or by adding an organic solvent, (ii) adding several displacers and (iii) combining such approaches. On the basis of these studies, a procedure for the automated determination of non-steroidal anti-inflammatory drugs in plasma by on-line dialysis-LC has been developed.

2. Experimental

2.1. Chemicals and reagents

Fenoprofen, flurbiprofen, ibuprofen, ketoprofen, probenecid and *n*-octanoic acid were obtained from Sigma (St. Louis, MO, USA) and naproxen from UCB (Leuven, Belgium). Methanol was obtained from Rathburn (Walkerburn, UK). All other chemicals and solvents were obtained from J.T. Baker (Deventer, Netherlands). LC-grade water was prepared by using a Millipore (Bedford, MA, USA) Milli-Q purification system, followed by filtration over a column filled with 40-μm C_{18} material (J.T. Baker).

2.2. Equipment

In all experiments a Gilson (Villiers-le-Bel, France) ASTED combined on-line with an LC system was used. A schematic set-up has been published previously [10]. 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 six-port switching valve. The dialysis cell was made of polymethylmethacrylate, with donor and acceptor channel volumes of 100 and 170 μl, respectively. A Gilson Cuprophane membrane with a molecular mass cut-off of 15 kDa was used. For preconcentration a 10×2 mm I.D. stainless-steel precolumn, slurry-packed with 40-μm Bondesil (Analytichem International, Harbor City, CA, USA) C_{18} material in a home-made precolumn holder was used. The LC system consisted of an LKB (Uppsala, Sweden) Model 2248 pump and a 250×3.1 mm I.D. stainless-steel column packed with a 5-μm RoSil Research Separation Laboratories (Eke, Belgium) C_{18} stationary phase. Acetonitrile–methanol–0.02 *M* phosphate buffer (pH 3.2) (50:10:40, v/v) was used as the eluent at 1.0 ml/min. An LKB Model 2141 UV detector was used for detection at 261 nm (ketoprofen), 264 nm (ibuprofen), 247 nm (flurbiprofen) and 273 nm (fenoprofen and naproxen); the signals were registered with an LKB Model 2210 recorder. All assays were performed at ambient temperature.

2.3. Set-up

All dialysis experiments were performed in the static mode: 100-μl samples were held stagnant in the donor compartment, while the acceptor phase (0.02 *M* phosphate buffer solution at pH 7.0) was continuously pumped through the acceptor channel. Before each run the acceptor and the donor channels were flushed with 2 ml of acceptor phase and water, respectively, and the precolumn was preconditioned with 1 ml of the acceptor phase. Next, the donor channel was filled with 125 μl of sample and dialysis was carried out. By switching the six-port valve the trapped analyte was backflushed to the analytical column and, next, the LC separation was performed. Finally, after each injection, the donor channel was flushed with 1 ml of a 0.05% (v/v)

Triton X-100 solution followed by 1 ml of 1 m*M* hydrochloric acid, and the acceptor channel with 1 ml of acceptor phase. All experiments were performed in duplicate.

3. Results and discussion

3.1. Dialysis of aqueous standards

With the mode of dialysis and the set-up used in the present study, the recovery and speed of on-line dialysis depend mainly on the acceptor-phase composition and flow rate, the precolumn packing material and the time during which dialysis is carried out [10]. The influence of these parameters was initially evaluated using aqueous solutions of the model compounds.

For the precolumn selected, breakthrough volumes higher than 50 ml were obtained for all analytes in the pH range of 3–7, when a 0.02 *M* phosphate solution was used as acceptor phase. In other words, the retention is adequate even for the highest acceptor phase flow-rates and dialysis times used in further studies. Increasing the acceptor-phase flow-rate from 0.36 via 0.75 to 1.5 ml/min resulted in an increase in the analyte recoveries per unit time; however, almost no difference between acceptor-phase flow-rates of 1.5 and 3.0 ml/min was found (data not shown). This is in agreement with earlier findings [10] and indicates that at these high flow-rates molecules diffusing through the membrane are immediately flushed to the precolumn and that diffusion of the analytes through the membrane is the limiting step in the dialysis process. Recoveries ranging from 60–85% were typically found after performing dialysis for 16 min (Table 1).

Since the analytes all have pK_a values of 4–5, they are negatively charged at high pH values and may electrostatically interact with the membrane, which will cause lower recoveries. This effect was studied using acceptor phases in the 3–7 interval, but no significant differences in analyte recovery were found. In further experiments, an acceptor phase of pH 7 was used.

3.2. Dialysis of untreated plasma samples

Next, plasma samples spiked with the analytes of interest were processed using different times of dialysis. The acceptor-phase flow-rate was kept at 3.0 ml/min to induce a maximum release of bound analytes by maintaining a high transport rate of the analytes through the membrane. In Table 1 the analyte recoveries so obtained are compared with those found for aqueous samples, using 16 min of dialysis. As expected, the recoveries observed for plasma samples are much lower. This is due to the high degree of protein binding rather than to parameters such as the viscosity or the ionic strength of the sample [10]. Since analyte recoveries invariably were only 1% or less, dialysis as such can not be applied for the determination of these highly bound compounds in plasma. In further experiments, ketoprofen was taken a as model compound to systematically explore the possibilities to release the analytes from HSA.

Table 1
Dialysis recoveries for five anti-inflammatory drugs from water and plasma samples and their degrees of protein binding [11]; dialysis time 16.0 min

Compound	Degree of protein binding (%)	Concentration (μg/ml)	Recovery in water (%)	Recovery in plasma (%)
Ketoprofen	<94	2.5	73	1
Ibuprofen	99	50.0	63	<1
Flurbiprofen	>99	5.0	62	<1
Fenoprofen	>99	25.0	87	<1
Naproxen	99	50.0	85	<1

3.3. Change of sample pH

Various authors have reported that a simple change of the sample pH can release some drugs from their binding site [11–13]. In view of the limited pH stability of the dialysis membrane (pH 2–8), the effect of adjusting the sample pH was studied in the 3–7 interval. The results of Fig. 1 show that the recovery of ketoprofen significantly increased only at the lowest pH value tested (pH 3). However, even in that case only a relatively small fraction was recovered (< 15%). Probably, the interaction of neutral ketoprofen (pH 3) with HSA is weaker than that of the negatively charged form (pH 4–7) and/or the structure of site II on HSA is only sufficiently altered to induce an increase in the free ketoprofen concentration at low pH values. On the other hand, the reduction of the sample pH also released many endogenous compounds which diffused through the membrane and interfered in the LC-UV chromatogram, causing the selectivity to become rather poor.

3.4. Addition of organic solvent

In order to avoid membrane deterioration, the amount of organic solvent to denaturate plasma proteins should be limited. Moreover, since the organic solvent also diffuses through the membrane into the acceptor phase, a high percentage can easily cause losses of analyte due to early breakthrough on the precolumn. Therefore, 20% (v/v) was taken as the maximum organic solvent content. Plasma proteins are (partly) precipitated at this organic solvent content. Results for acetonitrile and methanol, which can be considered as typical solvents are shown in Fig. 2.

The efficiency obtained in the presence of acetonitrile is slightly higher than that obtained when using methanol, but in both cases the analyte recovery is low (10%). In addition, when methanol was used, the recoveries found by performing dialysis for 16 min were lower than those observed after 12.8 min, which indicates breakthrough of the analyte on the precolumn due to the presence of the organic solvent in the acceptor.

3.5. Addition of displacing agent

Two groups of displacers with a high affinity for site II on HSA were studied, viz. drugs with a chemical structure similar to that of the analyte and fatty acids of appropriate chain length.

Ibuprofen was used to study the displacement of ketoprofen because it has a very similar structure and because it binds to the same site in HSA for 99%; final concentrations in the plasma samples were in the 1–8 m*M* range. Fig. 3 shows that ibuprofen effects an efficient release of ketoprofen; absolute analyte recoveries of about

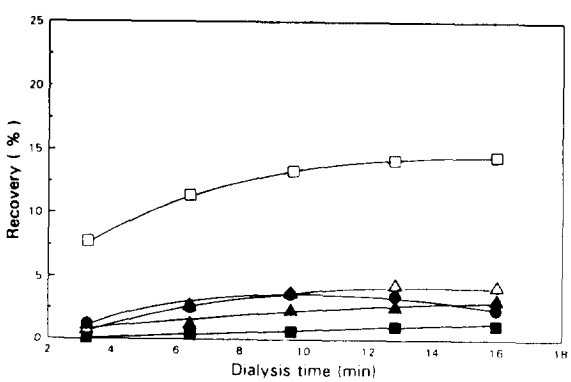

Fig. 1. Percent recovery vs. dialysis time for ketoprofen in plasma obtained with acceptor phases buffered at pH 7 (■), pH 6 (▲), pH 5 (△), pH 4 (●) and pH 3 (□). Concentration of ketoprofen, 2.5 μg/ml. For experimental details, see text.

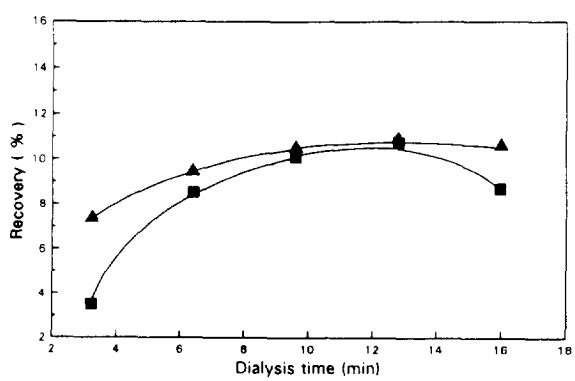

Fig. 2. Percent recovery vs. dialysis time for ketoprofen in plasma containing 20% (v/v) acetonitrile (▲) or methanol (■). Concentration of ketoprofen, 2.5 μg/ml. For experimental details, see text.

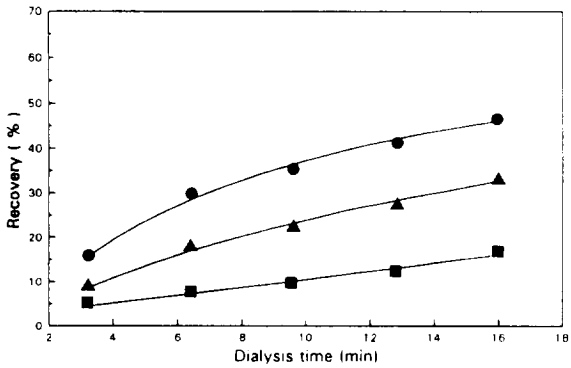

Fig. 3. Percent recovery vs. dialysis time for ketoprofen in plasma containing 1 m*M* (■), 4 m*M* (▲) and 8 m*M* (●) ibuprofen. Concentration of ketoprofen, 2.5 μg/ml. For experimental details, see text.

50% could be obtained in 16 min in the presence of 8 m*M* of ibuprofen. There are, however, also some disadvantages; diffusion of ibuprofen itself gave rise to an intense peak in the LC-UV chromatogram and its addition resulted in the release of several other, endogenous compounds, although none of their peaks interfered with that of ketoprofen. The drug probenecid was also tested as a displacer, because it has been reported to displace ketoprofen from HSA [14], but the displacement effected by this compound was less effective than that provided by ibuprofen. Recoveries lower than 8% were obtained in the presence of 1 m*M* of probenecid; in addition, concentrations of displacer higher than 1 m*M* could not be used because the large peak of probenecid in the LC-UV chromatograms overlapped with that of ketoprofen.

As an alternative, *n*-octanoic, *n*-decanoic and *n*-dodecanoic acid were added to plasma spiked with ketoprofen at final concentrations of 2–6 m*M*, which is equivalent to a displacer:protein concentration ratio between 2.3 and 10. The results are shown in Fig. 4. Under otherwise identical conditions the best efficiencies were obtained with *n*-decanoic acid. A concentration of 6 m*M* of this compound effected an absolute recovery of about 35% when dialysis was performed for 9.6 min, which is comparable to the result obtained after addition of 8 m*M* of ibuprofen. Due to the low water solubility of the

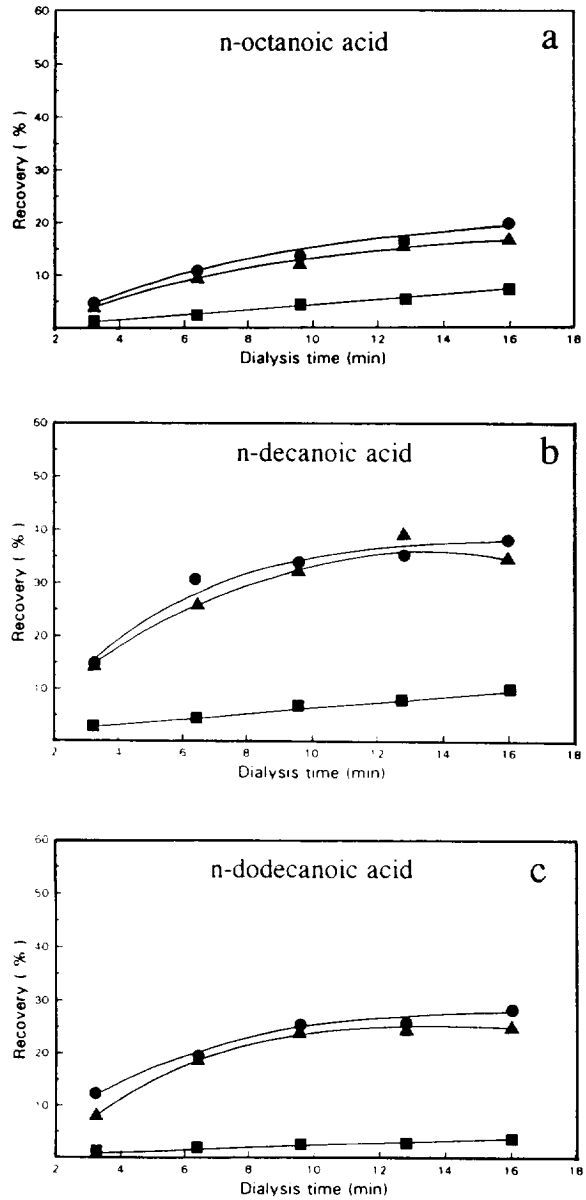

Fig. 4. Percent recovery vs. dialysis time for ketoprofen in plasma containing 2 m*M* (■), 4 m*M* (▲) and 6 m*M* (●) *n*-octanoic (a), *n*-decanoic (b) and *n*-dodecanoic (c) acid. Concentration of ketoprofen, 2.5 μg/ml. For experimental details, see text.

fatty acids, concentrations higher than 6 m*M* could not be used. In contrast with the results found with ibuprofen, increasing the dialysis times above ca. 10 min did not significantly

improve the recovery, which means that for longer dialysis times ibuprofen is a more effective displacer. This effect may be due to the diffusion of relatively high concentrations of the acids into the acceptor phase and subsequent competition with the analyte for binding on the precolumn; apparently, the competition of large amounts of ibuprofen is less effective. In all cases tested, much less interfering compounds were observed in the LC-UV chromatograms than when changing the sample pH, adding an organic solvent or adding ibuprofen; this indicates a more selective release of ketoprofen.

Similar results as those shown in Figs. 3 and 4 were found after dialysis of ketoprofen dissolved in aqueous solutions of HSA in the same concentration as in plasma. This means that, at the therapeutical concentration level, ketoprofen does not significantly bind to any other plasma protein, either before or after its release from HSA.

3.6. Addition of displacer and organic solvent

Among the possible combinations of the approaches described above, the simultaneous addition of a displacer and an organic solvent was selected, because the presence of the organic solvent also increases the solubility of the displacer in the samples, thus allowing the use of higher displacer concentrations. Based on previous results, acetonitrile was selected as the organic solvent and *n*-decanoic acid and ibuprofen as displacers. The recoveries of ketoprofen in water both before and after the addition of acetonitrile and these displacers were initially tested, but no significant differences were observed.

Fig. 5a shows the effect of the addition of 20% acetonitrile (v/v) plus different concentrations of *n*-decanoic acid. Comparison of Figs. 4b and 5a shows that the addition of 20% acetonitrile to plasma containing 4 m*M* of *n*-decanoic acid resulted in a slight improvement of the analyte recovery (45% vs. 35%; 16 min dialysis), which seems to be due to a simple addition of both effects. More importantly, the presence of the modifier allows the dissolution of distinctly higher concentrations of the displacer. A maximum analyte recovery of 58% (90% of the amount recovered from water samples) was obtained with 20 m*M* *n*-decanoic acid and a dialysis time of 12 min. However, the recovery drastically decreased (to 34%) at 16 min. A further increase of the concentration of the displacer to 40 m*M* resulted in recoveries which were much lower than expected, except for very short dialysis times. As has been suggested earlier, this may be due to the competition of the fatty acids with the analyte for binding on the precolumn, which leads to analyte breakthrough for high fatty acid concentrations and/or long dialysis times. Therefore, the concentration of this compound in the samples was limited to 8 m*M*.

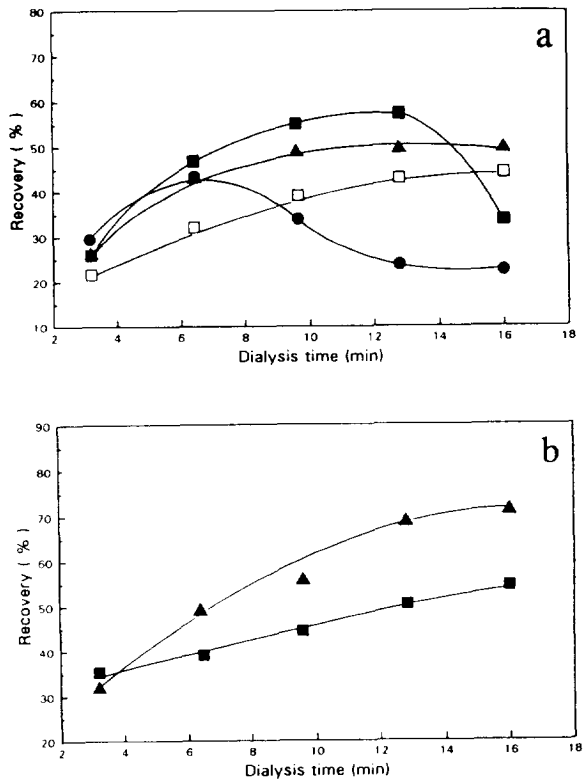

Fig. 5. Percent recovery vs. dialysis time for ketoprofen in plasma containing both 20% (v/v) acetonitrile and 4 m*M* (□), 8 m*M* (▲), 20 m*M* (■) and 40 m*M* (●) *n*-decanoic acid (a), or 10 m*M* (■) and 20 m*M* (▲) ibuprofen (b). Concentration of ketoprofen, 2.5 μg/ml. For experimental details, see text.

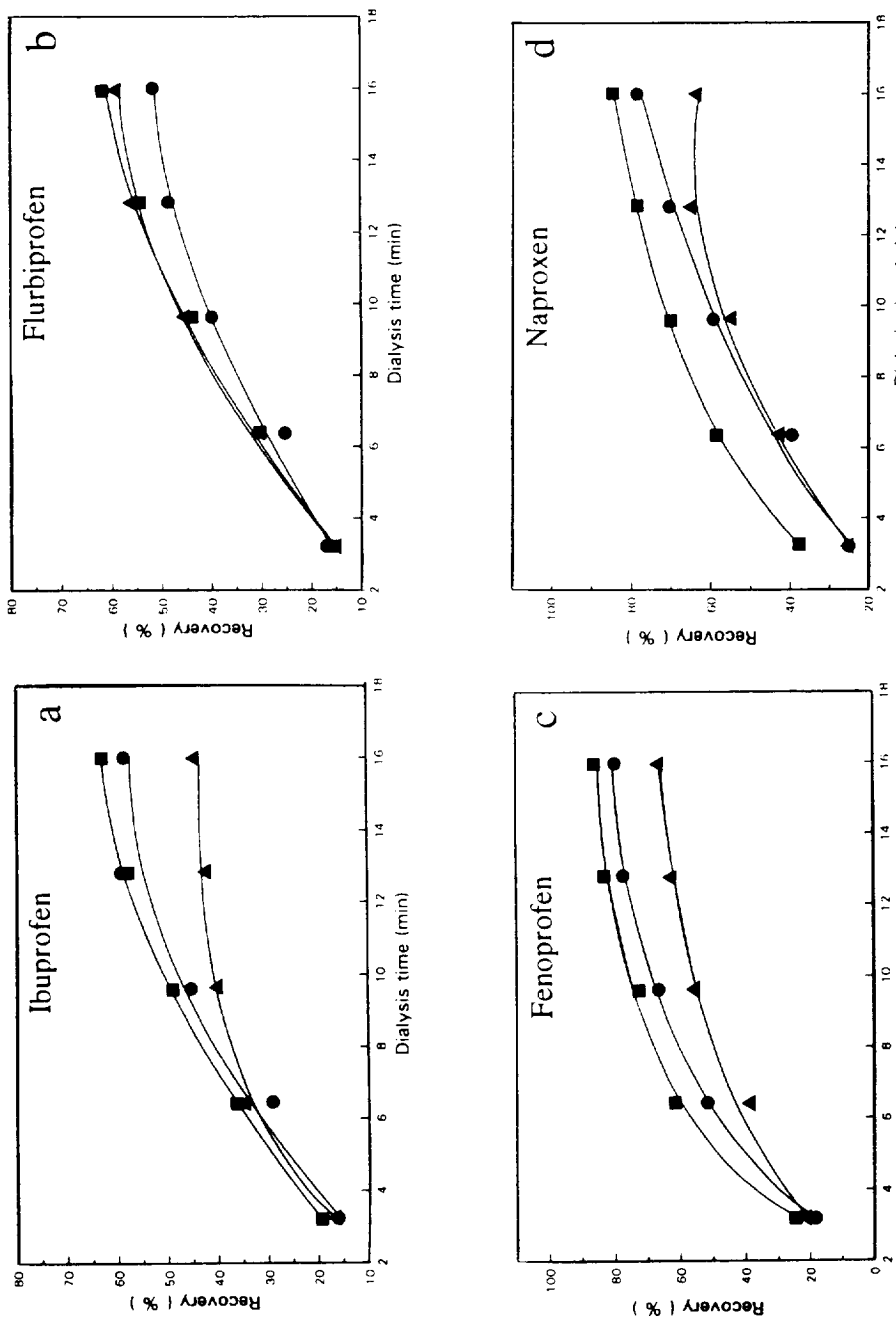

Fig. 6. Percent recovery vs. dialysis time for (a) ibuprofen, (b) flurbiprofen, (c) fenoprofen and (d) naproxen in aqueous samples (■), and in plasma containing both 20% acetonitrile (v/v) and 8 m*M* *n*-decanoic acid (▲) or 20 m*M* ibuprofen (●) (20 m*M* ketoprofen in case of ibuprofen). The concentration of drug in the samples is indicated in Table 1. For experimental details, see text.

The presence of acetonitrile also allows the addition of relatively large amounts of ibuprofen, and the recovery obtained for plasma was nearly identical to that obtained for water. Apparently, an almost complete release of ketoprofen from HSA could be effected by adding ibuprofen at a concentration of 20 m*M* (Fig. 5b).

Similar behaviour was observed for the other non-steroidal anti-inflammatory drugs. Fig. 6 shows the plots obtained for each analyte in water and those obtained in plasma after the addition of both acetonitrile (20%, v/v) and 8 m*M* *n*-decanoic acid or 20 m*M* ibuprofen. For the samples spiked with ibuprofen, ketoprofen was selected as a displacer and, although the affinity of HSA for ibuprofen is higher than that for ketoprofen (cf. Table 1), a concentration of 20 m*M* of ketoprofen provided an almost complete release of ibuprofen.

The combined data show that, for most of the analytes dialysis proceeds more efficiently when ibuprofen or ketoprofen rather than *n*-decanoic acid is used as a displacer; only for flurbiprofen the addition of *n*-decanoic acid gives better results. In all cases, the selectivity in LC-UV is better when a fatty acid is used for displacement, indicating that the addition of ketoprofen or ibuprofen induces the release of endogenous compounds from HSA. Most probably, although non-steroidal anti-inflammatory drugs exclusively bind to site II in HSA under therapeutical-concentration conditions, at much higher concentrations (such as those when they are used as displacers) they also bind to other sites, which normally bind endogenous compounds. Alternatively, binding of the anti-inflammatory drugs to site II may induce a change in the three-dimensional structure of HSA, thereby provoking the release of bound endogenous compounds from other sites. This seems less probable, however, because the binding of fatty acids is also known to alter the structure of HSA [3].

3.7. Analysis of non-steroidal anti-inflammatory drugs in plasma

Because of its higher selectivity, *n*-decanoic acid in combination with acetonitrile was selected as the best option to release the analytes from HSA. The final procedure is as follows: 125 μl of 40 m*M* decanoic acid in acetonitrile are added automatedly to 500 μl of plasma (final concentrations: displacer, 8 m*M*, and acetonitrile, 20% v/v) and dialysis is performed for 9.6 min, which represents a compromise in terms of analyte detectability and time of analysis. In addition, there is almost no difference in analyte recovery between the use of *n*-decanoic acid and ibuprofen or ketoprofen at a dialysis time of 9.6 min (see Figs. 3–6). Fig. 7 shows typical chromatograms obtained after on-line dialysis-LC-UV of plasma samples spiked with ketoprofen and illustrates both the dramatic gain in sensitivity after the addition of a displacer and the better selectivity obtained with *n*-decanoic acid rather than ibuprofen.

The practicability of the procedure was tested with samples spiked with the analytes in their respective therapeutical intervals [15]. The analytical data are summarized in Table 2. The linearity is satisfactory in all instances, which indicates that the protein binding is fairly constant over the therapeutic interval. The absolute recoveries of 40–65% correspond to 77–102% of the values found with aqueous standard solutions. They easily allow quantitation of the compounds of interest with satisfactory sensitivity (the detection limits are shown in Table 2). With the proposed procedure, the total analysis takes about 15–20 min (depending on the retention time of the analyte on the LC column). The procedure, including mixing of the displacer and the plasma sample, can be fully automated. The reproducibility is fully satisfactory (Table 2). As has been reported previously, in plasma from patients with a disease, the precision of dialysis can be affected due to changes in plasma protein concentrations [17]. It should be noted that the studied non-steroidal anti-inflammatory drugs (except naproxen) are clinically used as a racemate, but the plasma concentration can be different for each enantiomer. In addition, protein binding of a chiral drug is potentially stereoselective. However, since the degree of protein binding for these compounds is very high, no important differences in the recovery

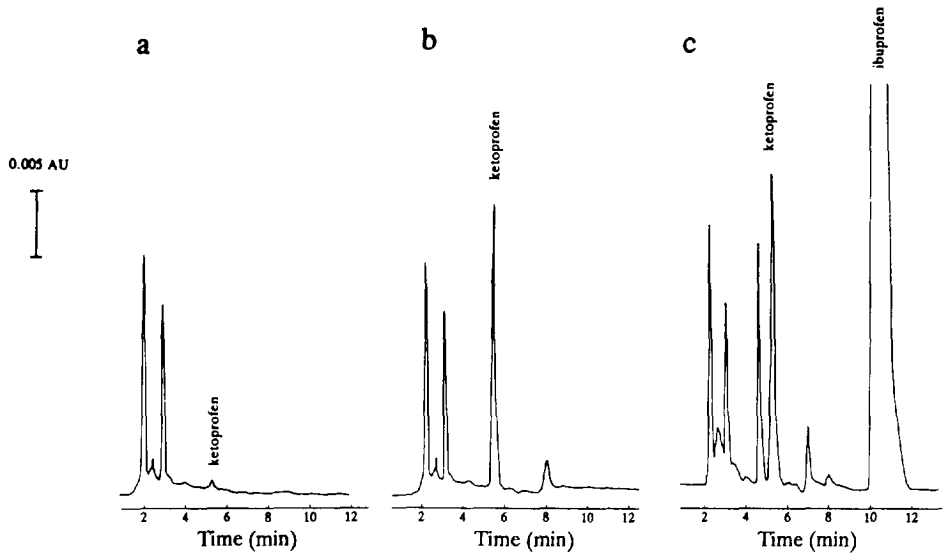

Fig. 7. LC-UV chromatograms obtained after dialysis of samples spiked with 2.5 μg/ml of ketoprofen; (a) untreated plasma, (b) plasma containing both 20% acetonitrile (v/v) and 8 m*M* *n*-decanoic acid, (c) plasma containing both 20% acetonitrile (v/v) and 20 m*M* ibuprofen. Dialysis time: 9.6 min. For experimental details, see text.

between the isomers can be expected. If the concentration of the separated enantiomers is required, the recovery from plasma should be enantioselectively investigated.

4. Conclusions

On-line dialysis can be successfully used as a sample-preparation technique for highly protein-bound drugs in plasma prior to their LC determination, if precautions are taken to release the analytes from their binding sites. Since alteration of the protein structure by a drastic pH change or high concentrations of modifiers is restricted due to stability problems of the membrane, the addition of a selective displacer to the plasma samples is a better approach to release tightly bound compounds. The presence of up to 20% of an organic solvent allows the dissolution of higher concentrations of displacer and has been proven to be an adequate strategy. For site

Table 2
Analytical data for the automated determination of five anti-inflammatory drugs by on-line dialysis-LC

Analyte	Wavelength (nm)	Concentration interval (μg/ml)	Linearity[a]			Limit of detection (μg/ml)	Within-day precision ($n = 10$)	Recovery (%)	
			Slope (± S.D.)	Intercept (± S.D.)	R^2			Absolute	Relative[b]
Ketoprofen	261	0.5–6.0	6.39 (± 0.09)	−0.25 (± 0.08)	0.9992	0.1	5.8	49	78
Ibuprofen	264	20–60	0.35 (± 0.01)	−0.02 (± 0.01)	0.9990	2.0	3.8	40	82
Flurbiprofen	247	1–15	1.71 (± 0.03)	3.30 (± 1.70)	0.9999	0.01	3.0	48	102
Fenoprofen	272	10–60	0.37 (± 0.002)	0.40 (± 0.20)	0.9992	0.5	2.4	56	77
Naproxen	272	10–80	0.95 (± 0.02)	0.40 (± 0.10)	0.9998	0.1	3.2	65	91

[a] Five data points in duplicate.
[b] Recovery in plasma relative to recovery in water.

II-bound analytes, one can use either fatty acids (such as *n*-decanoic acid) or drugs of similar structure as the analytes. In this study, the fatty acids generally caused a slightly less efficient release of the analytes than the structurally related drugs. However, with the acids displacement was more selective, i.e. it resulted in less interfering peaks due to endogenous compounds in the LC-UV chromatogram.

As an example, the combined addition of 8 m*M* *n*-decanoic acid and 20% acetonitrile to plasma provided recoveries of five non-steroidal anti-inflammatory drugs closely similar to those obtained by processing aqueous solutions, even for drugs featuring a protein-binding of over 99%. In this way, the analysis of plasma samples containing therapeutical concentrations of such drugs can be performed without problems, using a dialysis time of ca. 10 min. Since non-steroidal anti-inflammatory drugs are amongst the drug classes most tightly bound to site II on human serum albumin, a similar procedure can probably be applied to most drugs which bind to this site. For drugs bound to other binding sites, a suitable displacer should be found. For example, trichloroacetic acid has been reported to displace compounds bound to the warfarin site or site I in HSA [13,16], which besides site II, is one of the most important sites with respect to drug–plasma proteins binding, a vast majority of drugs being bound only to these sites.

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