Journal of Chromatography, 621 (1993) 189–198
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7120

Automated determination of free phenytoin in human plasma with on-line equilibrium dialysis and column-switching high-performance liquid chromatography

Alf T. Andresen* and Knut E. Rasmussen

Institute of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, N-0316 Oslo (Norway)

Hans E. Rugstad

Department of Clinical Pharmacology, Rikshospitalet, The National Hospital, N-0027 Oslo (Norway)

(First received July 6th, 1993; revised manuscript received September 13th, 1993)

ABSTRACT

Free phenytoin in human plasma was automatically determined by on-line equilibrium dialysis using the automated sequential trace enrichment of dialysate (ASTED) sample preparation system and HPLC. The dialysis cell was a modification of the cell supplied with the ASTED. Total phenytoin was analysed with the same analytical set-up and plasma protein binding was determined. Free phenytoin was determined in plasma from epileptic patients and the results were compared to those obtained by ultrafiltration. Automated determination of free and total phenytoin in plasma by the ASTED–HPLC combination was shown to be an accurate and reproducible method and the results in free phenytoin analyses were in agreement with those found with ultrafiltration. The sample throughput with the automated on-line combination of dialysis and column-switching HPLC was 75 samples in 24 h when the sample was dialysed at 37°C.

INTRODUCTION

Plasma protein binding is an important factor in establishing the pharmacokinetic and pharmacodynamic properties of a drug, as only the free fraction of a drug is pharmacologically active. Determination of free drug concentrations in plasma should be considered for drugs for which the usefulness of plasma level monitoring has been established, which are highly bound to plasma proteins and which exhibit a variable free fraction. Equilibrium dialysis and ultrafiltration are the two techniques most widely used for separation of the free drug from the protein bound drug [1–7]. Microdialysis, which has been applied to determine *in vivo* drug concentrations, has also been used to determine the extent of protein binding *in vitro* [8–10]. These techniques require time consuming manual sample handling which is a limitation for routine monitoring of the free concentration in plasma.

The ASTED (automated sequential trace enrichment of dialysate) system combines on-line dialysis, trace enrichment of dialysate and high-performance liquid chromatography (HPLC) in a column-switching system. This system considerably simplifies drug analysis in biofluids. Proteins are removed by dialysis and the analyte collected in the dialysate is concentrated on a solid-

* Corresponding author.

0378-4347/93/$06.00

phase extraction column while impurities are washed out to waste. Coupled to HPLC in a column-switching system fully automated analysis of drugs in biofluids and tissue extracts are performed [11–13,14 (review)]. High dialysis recoveries in short analysis times are obtained by using dialysis cells with a large membrane area and by maintaining a high concentration gradient across the membrane. A high concentration gradient is maintained by removing the dialysate during dialysis either in a continuous or in a pulsed mode. So far this technique has been used to determine the total concentration of drugs in biofluids.

The aim of the present study was to develop a rapid automated method for the determination of the free and the total concentrations of a drug in plasma using the ASTED system coupled to HPLC. Phenytoin was chosen as model substance because the binding of phenytoin to human plasma proteins has been extensively studied [15–17]. Two dialysis cells, the standard cell supplied with the ASTED system and a modified version of the standard cell, were evaluated. On-line equilibrium dialysis was carried out with both cells for monitoring free phenytoin in plasma. The results obtained with the modified cell were in agreement with those obtained with ultrafiltration. The modified cell was further evaluated for automated determination of the free and the total phenytoin concentrations in plasma from epileptic patients. The automated on-line dialysis method with the ASTED–HPLC combination was shown to be a reliable and time saving method as compared to methods in current use for free level monitoring of phenytoin.

EXPERIMENTAL

Chemicals

Phenytoin sodium salt (5,5-diphenylhydantoin) was supplied by Sigma (St. Louis, MO, USA). Potassium chloride and hexobarbitone were obtained from The Norwegian Medicinal Depot (Oslo, Norway). HPLC-grade acetonitrile, methanol and tetrahydrofuran were supplied by Rathburn (Walkerburn, UK). Trichloroacetic acid (TCA), trisodium citrate 2-hydrate, sodium acetate anhydrous, sodium chloride, diammonium hydrogenphosphate, ammonium dihydrogen phosphate, sodium hydroxide and Triton X-100 were obtained from Merck (Darmstadt, Germany). Sodium azide Analar, was supplied by BDH (Poole, UK). HPLC-grade water was obtained from a Milli-Q (Millipore, Bedford, MA, USA) water purification system.

ASTED

The sample preparation system was a Gilson ASTED unit (Gilson Medical Electronics, Villiers-le Bel, France) consisting of a Model 231 auto-sampling injector, two Model 401 dilutors equipped with 1-ml syringes and a planar dialyser described below. The auto-sampler with the dialysis cell, dilutors, the donor and recipient solutions and the analytical column were kept inside a thermostatted incubator (Heraeus, Hanau, Germany) to control the temperature. Dialysis was performed at 20°C and 37°C. The HPLC tubing and the electrical connections were run through holes in the walls of the incubator. The incubator had a glass door for observing the process and for controlling the temperature. An automated six-port Model 7010 valve (Rheodyne, Berkeley, CA, USA) connected a trace enrichment column (or a 100-μl sample loop) either with the recipient channel of the dialyser or with the mobile phase used in the analytical column of the HPLC system. The trace enrichment column (10 × 2 mm I.D.) from Chrompack (Middelburg, The Netherlands) was packed with 36-μm particle size polystyrene–divinylbenzene from Dyno Particles (Lillestrøm, Norway).

Dialysis cells

The standard dialysis cell supplied with the ASTED had a donor channel volume of 100 μl and a recipient channel volume of 175 μl fitted with a cuprophan (cellulose acetate) membrane, with a molecular mass cut-off of 15 000. The membrane surface area was 4 cm^2 and the donor area/donor volume ratio was 40 cm^2/ml. The modified dialysis cell had an increased donor channel volume of 400 μl and a recipient channel colume of 175 μl fitted with a cuprophan mem-

brane, with a molecular mass cut-off of 15'000. The membrane surface area was 4 cm^2 and the donor area/donor volume ratio was 10 cm^2/ml.

High-performance liquid chromatography

The ASTED was coupled to an LC 6A (Shimadzu, Kyoto, Japan) HPLC pump. The UV detector was a Model SPD-6A (Shimadzu) with a 8-μl flow cell, operated at 220 nm. The Sperisorb ODS-2 analytical column (100 × 3.0 mm I.D.) from Chrompack (Middelburg, The Netherlands) was packed with 5-μm particles. The mobile phase was acetonitrile–tetrahydrofuran–0.02 *M* phosphate buffer pH 5 (25:7.5:67.5, v/v/v) at a flow-rate of 0.7 ml/min when operated at 20°C, and acetonitrile–tetrahydrofuran–0.02 *M* phosphate buffer pH 5 (22:6.5:71.5, v/v/v) at a flow-rate of 0.7 ml/min when operated at 37°C. Peak heights were recorded on a Chromatopac C-R4A integrator (Shimadzu).

Plasma standards

Citrated bovine plasma used for method development was obtained from the Veterinary Institute (Oslo, Norway). Citrated human plasma from healthy donors and patient plasma samples were obtained from The National Hospital (Oslo, Norway). Plasma standards of phenytoin were prepared in drug free plasma from stock solutions (50 and 200 mg/l) of phenytoin in methanol. The desired amounts from the stock solutions were dispensed into volumetric glasses and the methanol evaporated with nitrogen under careful heating. Phenytoin was then dissolved in plasma in the concentration range 0.25–20 μg/ml. In order to validate the method plasma spiked with 2, 10 and 20 μg/ml phenytoin was examined.

External standard solutions for determination of free phenytoin

A stock standard solution of phenytoin (200 mg/l) was prepared in 0.05 *M* phosphate buffer containing 25 mg/l sodium azide, adjusted to pH 8.0 with sodium hydroxide to give standards in the concentration range 0.5–4.0 μg/ml.

Donor and acceptor solutions

The isotonic donor and acceptor solutions were prepared from 5.9 g sodium chloride, 4.1 g sodium acetate, 0.3 g potassiumchloride and 1.65 g sodium citrate adjusted to pH 7.4 with citric acid and diluted with water to 1 litre. To the donor solution was added 50 mg/l Triton X-100.

Determination of free phenytoin

Sample loading. Plasma was injected into the dialysis cell by filling the donor channel of the dialyser. Segmentation with air bubbles prevented forward or backward diffusion of the sample into the transporting donor solution. The six-port valve was in the load position.

Dialysis and trace enrichment. The sample was held static in the donor channel for 25 min at 20°C and 10 min at 37°C, while the acceptor solution was held static in the recipient channel during the same time. After dialysis, 0.5 ml of recipient solution was aspirated through the recipent channel of the dialyser in one pulse to bring the analyte into the trace enrichment column, with the injection valve in the load position. The recipient solution was transported through the dialyser at a flow-rate of 0.5 ml/min.

Injection and purging. Upon switching of the six-port valve to the inject position the analyte on the trace enrichment column was brought into the analytical column with the HPLC mobile phase. The recipient channel of the dialyser was simultaneously washed with 5 ml of recipient solution and the donor side of the dialyser was washed with 8 ml of donor solution.

Regeneration. The six-port valve was switched back to load position to bring the pre-column back to the recipient channel of the dialyser. After regeneration of the trace enrichment column with 1 ml of recipient solution the next sample was injected into the dialyser. Automated injections were performed every 35 min (20°C) or every 20 min (37°C).

Internal standard solutions for determination of total phenytoin

A solution of hexobarbitone (2 μg/ml) was prepared in ammonium phosphate buffer (0.5 *M*)

with TCA (0.2 *M*) and sodium azide (50 mg/l) adjusted to pH 7.4 with ortho phosphoric acid.

Determination of total phenytoin in plasma

For total phenytoin analysis 200 μl plasma was automatically mixed with 600 μl internal standard solution by the autosampler and 400 μl of the mixture was injected into the dialysis cell. Dialysis was performed for 7.5 min according to the procedure described above but in the static-pulsed mode of dialysis with 2 pulses of 0.5 ml of recipient solution.

Ultrafiltration

Ultrafiltration was performed with an Amicon micro-ultrafiltration system 8 MC (Amicon, Beverly, USA). The Diaflo YM 30 (Amicon, Danvers, USA) ultrafiltration membranes, were rated at 30 000 molecular mass cut-off. The operating pressure of nitrogen above the membrane (25 mm in diameter) was 304 MPa. From a 5-ml plasma sample 1 ml of filtrate was collected. The sample was under continuous stirring for 15 min (20°C) and 10 min (37°C) during ultrafiltration. Ultrafiltration at 37°C was performed inside the incubator.

Validation of the procedures

The standard curves, accuracy and precision of the free and total phenytoin analyses were evaluated by analysis of spiked and real plasma samples. The calibration graph for the analysis of the free phenytoin concentrations in plasma was based on peak-height measurements *versus* concentrations in the aqueous external standard solutions. The calibration graph for total phenytoin was based on peak-height ratios relative to the internal standard in plasma. The protein binding (*PB*) in plasma was calculated by the equation:

$$PB\,(\%) = (T - F)\,100\% / T$$

where T is the concentration of total phenytoin in plasma and F is the concentration of free phenytoin in plasma.

RESULTS AND DISCUSSION

Dialysis

Assuming that diffusion through the membrane is the rate limiting process of dialysis and that the concentration gradient is the only driving force for diffusion, the process can be described by Fick's law:

$$J = -\frac{DA}{\tau} \cdot \frac{dc}{dx} \quad (1)$$

where J is the flux or dialysis rate from the donor to the acceptor channel, A is the membrane surface area available for diffusion, dc/dx is the concentration gradient across the membrane and τ is the tortuosity of the membrane.

The diffusion coefficient, D can be expressed by the Stokes–Einstein relation:

$$D = \frac{kT}{6\pi\eta r} \quad (2)$$

where k is the Boltzmann constant, T the absolute temperature, η the viscosity of the medium, and r the molecular radius of the analyte.

In equilibrium dialysis both the donor and acceptor phase are stagnant. The dialysis recovery *RE* of the free fraction of a drug defined as the percentage of solute molecules present in the acceptor compartment was described by Merbel *et al.* [14]:

$$RE = \frac{V_a}{V_a + V_d}\left[1 - \exp\left(-\frac{DA}{\tau l}\left(\frac{1}{V_d} + \frac{1}{V_a}\right) t\right)\right] \quad (3)$$

where V_a and V_d are the compartment volumes of the acceptor and donor channels, respectively, τ is the tortuosity, l is the membrane thickness and t is the dialysis time. This equation shows how the cell design affects the dialysis recovery. When the acceptor compartment is decreased relative to the donor compartment the dialysis recovery will decrease because of the smaller $V_a/(V_a + V_d)$ ratio.

Dialysis cells

Dialysis cells used in an automated system for on-line equilibrium dialysis must maintain the

drug–protein equilibrium during dialysis, the donor-channel volume must be sufficiently large to detect low concentrations of highly-protein-bound drugs, and a reproducible determination of the free drug should be obtained in as short a time as possible. Equilibrium dialysis was performed with the standard cell and with the pilot cell by operating the ASTED in the static–static mode of dialysis. The dialysis recovery as a function of time is dependent on the $V_a/(V_a + V_d)$ ratio (eqn. 3). The standard cell had a $V_a/(V_a + V_d)$ ratio of 0.64 and the pilot cell had a $V_a/(V_a + V_d)$ ratio of 0.30. The membrane area available for diffusion was the same for both cells (4 cm^2). A higher dialysis recovery should therefore be obtained with the standard cell than with the pilot cell.

Bovine plasma was used to reduce the consumption of human plasma during method development. As shown in this article phenytoin is stronger bound to human plasma proteins than to bovine plasma proteins. It is therefore assumed that if the protein binding is maintained in the dialysis of bovine plasma it will also be maintained in the dialysis of human plasma.

Fig. 1 shows the dialysis recovery as a function of dialysis time using bovine plasma spiked with 10 μg/ml of phenytoin. The dialysis recovery increased rapidly during the first 10 min of dialysis and increased only slightly at longer dialysis times. The dialysis recovery was 5.5% on the standard cell after 10 min of dialysis and 3% on the pilot cell. A higher dialysis recovery was obtained on the pilot cell during the first 10 min of dialysis at 37°C compared to 20°C because the diffusion coefficient increases with temperature (eqn. 2).

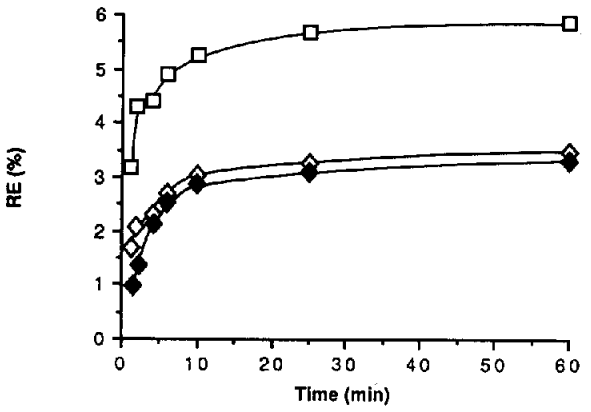

Fig. 1. Dialysis recovery (*RE*) as a function of dialysis time using static–static dialysis of bovine plasma spiked with 10 μg/ml of phenytoin with the standard cell at 20°C (□), and the pilot cell at 20°C (◆) and at 37°C (◇). For experimental conditions see text.

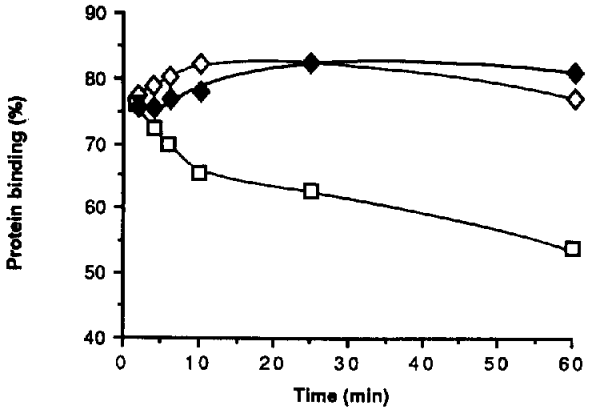

Fig. 2. Protein binding as a function of dialysis time using static–static dialysis of bovine plasma spiked with 10 μg/ml of phenytoin with the standard cell at 20°C (□), and the pilot cell at 20°C (◆) and at 37°C (◇). For experimental conditions see text.

Fig. 2 shows the protein binding measured as a function of dialysis time. The protein binding of phenytoin in bovine plasma measured after ultrafiltration was 82% both at 20°C and 37°C. The protein binding at 20°C measured with the standard cell was lower than both the protein binding measured with the pilot cell at 20°C and the result obtained by ultrafiltration. After 1 min of dialysis the protein binding was 75% and the protein binding decreased with increasing dialysis time. At 60 min the protein binding had decreased to 54%. These results demonstrate that cell design may affect protein binding. The acceptor channel volume of the standard cell is larger than the donor channel volume [$V_a/(V_a + V_d) = 0.64$] which results in an increased concentration gradient across the membrane and an increased dialysis rate as compared to the pilot cell. Dialysis under these conditions reduced the protein binding. Cooper *et al.* [18] used the ASTED with the standard cell to examine protein binding isotherms of phenytoin to human serum albumin in a buffer solution and obtained values in general agree-

ment with the established equilibrium dialysis values. However, Cooper *et al.* [18] concluded that the ASTED might be capable of measuring an estimate of free analyte concentrations and that much work remained on this assay potential.

As shown in Fig. 2 the protein binding measured with the pilot cell increased as a function of time until an equilibrium was obtained after 25 min of dialysis at 20°C and after 10 min of dialysis at 37°C. The increase in protein binding is due to delayed diffusion of the free drug from the sample compartment to the membrane because of the larger donor channel volume (400 μl) of the pilot cell. At equilibrium the protein binding was 82% both at 20°C and at 37°C which is in agreement with the result obtained by ultrafiltration. However, dialysis for 60 min at 37°C caused reduction in protein binding to 77%. This reduction is caused by diffusion of phenytoin from the acceptor compartment into the recipient solution in the connecting tubing of the cell. This leads to an increased acceptor volume and an increased $V_a/(V_a + V_d)$ ratio. The donor channel was bracketed by air bubbles at either end of the sample which prevented forward or backward diffusion. Bracketing of the acceptor channel was not possible with the instrumental set-up. Equilibrium dialysis with the pilot cell should therefore be carried out only until the equilibrium in the protein binding measurements is obtained, *i.e.* for 10 min at 37°C and for 25 min at 20°C, to prevent diffusion into the connecting tubing of the acceptor channel. Under these conditions the drug–protein equilibrium is maintained. For the analysis of free drug concentrations in human plasma, dialysis at body temperature (37°C) is preferred and at this temperature 75 samples can be analysed in 24 h.

Operation of the ASTED

At the end of dialysis the analyte which diffused into the acceptor channel is transferred into the trace enrichment column by aspirating 0.5 ml of acceptor solution through the dialysis cell when the sample is still present in the donor channel. This transfer creates a static–continuous dialysis of the sample. The volume of acceptor solution used for the transfer must therefore be evaluated carefully so that only the drug which diffused into the acceptor channel during the static–static dialysis is brought into the trace enrichment column. In the instrumental set-up used in the investigation 0.5 ml of acceptor solution corresponds to the volume of acceptor solution present in the acceptor channel and in the connecting tubing from the dialysis cell to the trace enrichment column. The analyte which diffused into the acceptor solution during transfer is washed out to waste.

During method development several dialysis modes were tested using the standard cell. In the static–pulsed and static–continuous modes of operation the protein binding was greatly reduced. In the pulsed–static and continuous–static modes of operation measurements of protein binding in agreement with those obtained by ultrafiltration were found in some of the experiments. The results were dependent on the experimental conditions, and static–static dialysis with the pilot cell was prefered because of the simplicity and robustness of the procedure.

Trace enrichment and HPLC

A polystyrene–divinylbenzene pre-column was used for trace enrichment of phenytoin. No break-through of phenytoin was observed when 10 ml of recipient solution was pumped through the pre-column.

Fig. 3 (A) shows a chromatogram of drug-free human plasma after on-line dialysis and HPLC; Fig. 3 (B) shows a chromatogram of a free phenytoin analysis in human plasma at 37°C spiked with 10 μg/ml of phenytoin, and Fig. 3 (C) shows a chromatogram of a total phenytoin analysis of the same plasma after addition of TCA and hexobarbitone as internal standard. Dialysis was carried out for 10 min at 37°C. Narrow fronts and no interferences from plasma were observed after on-line dialysis and concentration of the dialysates. Measurements of total phenytoin were performed with a total dialysis time of 7.5 min. Dialysis was carried out in the static–pulsed mode with 2 pulses of 500 μl each of acceptor solution. Under these conditions the dialysis recovery of

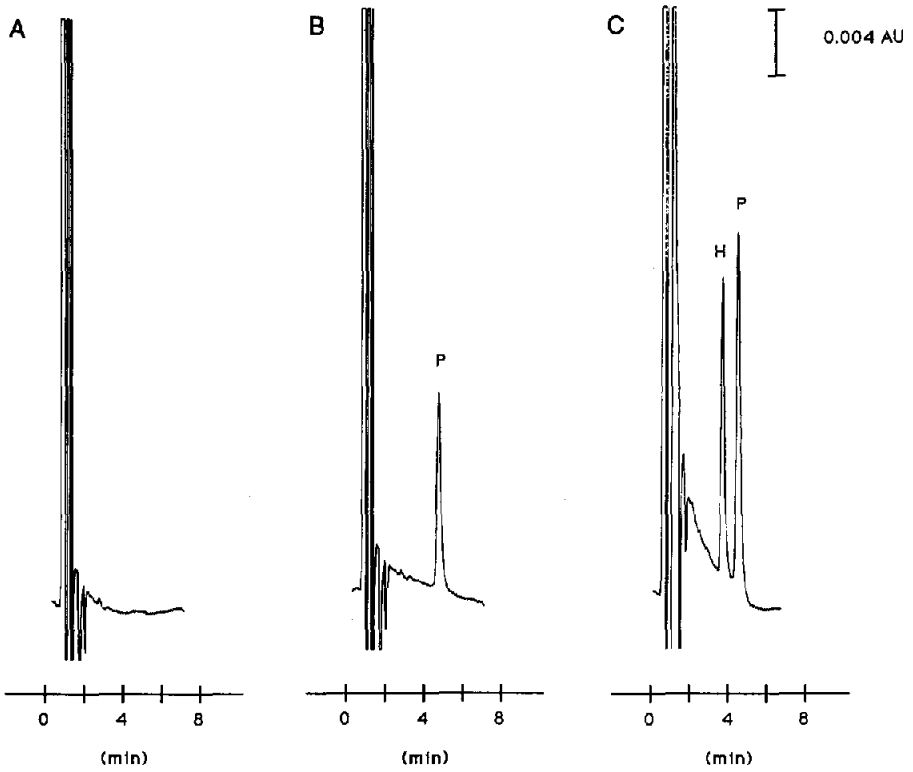

Fig. 3. Chromatograms of (A) a drug-free human plasma after on-line dialysis and HPLC, (B) a free phenytoin analysis in human plasma spiked with 10 μg/ml of phenytoin after on-line dialysis and HPLC, and (C) a total phenytoin analysis in human plasma spiked with 10 μg/ml of phenytoin and hexobarbitone as internal standard in TCA buffer after on-line dialysis and HPLC. Dialysis temperature: 37°C. Detection: UV at 220 nm. Peaks: H = hexobarbitone, P = phenytoin. For chromatographic conditions see text.

phenytoin was 22%. TCA in phosphate buffer adjusted to pH 7.4 was added to the sample before dialysis to reduce the protein binding of phenytoin.

Validation of the method

Linear calibration graphs were obtained in the concentration range 0.25–8 μg/ml for the analysis of free phenytoin and in the concentration range 2–20 μg/ml for the analysis of total phenytoin in human plasma. This is the concentration range usually monitored in plasma from epileptic patients. The correlation coefficients were 0.998 or better. Table I shows the intra-assay variations of free phenytoin in human plasma at 20°C and 37°C and the intra-assay variations of total phenytoin. The results in Table I also demonstrate that the free fraction of phenytoin increases with temperature in human plasma due to the temperature dependence of the protein binding [15]. The relative standard deviations of the free phenytoin analyses are satisfactory considering the low dialysis recovery of 3%. The limit of detection is determined by the donor channel volume of the dialysis cell and the dialysis recovery. Under the conditions used in this procedure free phenytoin could be detected at a signal-to-noise ratio of 3 ($S/N = 3$) in a plasma with a total concentration of 0.6 μg/ml of phenytoin. This is far below the therapeutic concentration range of 10–20 μg/ml of phenytoin.

Analysis of plasma from epileptic patients

Free phenytoin in plasma from epileptic patients was measured at 37°C by the ASTED–HPLC on-line dialysis method and the results were compared to ultrafiltration and HPLC analysis of the ultrafiltrate. Total phenytoin concen-

TABLE I

INTRA-ASSAY VARIATIONS OF FREE AND TOTAL PHENYTOIN IN HUMAN PLASMA DETERMINED WITH ASTED DIALYSIS AND HPLC, EXPRESSED AS SAMPLE ± STANDARD DEVIATION (S.D.), PERCENTAGE RELATIVE STANDARD DEVIATION (R.S.D.) AND PERCENTAGE PROTEIN BINDING

Added total (μg/ml)	Dialysis temperature 20°C			Measured total (mean ± S.D., n = 6) (μg/ml)	R.S.D. (%)	Dialysis temperature 37°C		
	Measured free (mean ± S.D., n = 6) (μg/ml)	R.S.D. (%)	Protein binding (%)			Measured free (mean ± S.D., n = 6) (μg/ml)	R.S.D. (%)	Protein binding (%)
2.00	0.17 ± 0.01	7.9	91	1.97 ± 0.08	4.0	0.30 ± 0.03	10.7	85
10.0	0.91 ± 0.03	3.1	91	10.01 ± 0.20	2.0	1.43 ± 0.10	7.0	86
20.0	1.83 ± 0.06	3.5	91	19.83 ± 0.39	2.0	3.07 ± 0.20	6.6	85

trations were also determined by ASTED–HPLC and the protein binding was calculated. The results of these analysis are shown in Table II. All but three of the patients were administered other antiepileptic drugs in addition to phenytoin as shown in Table II. No significant difference in the determination of free phenytoin in the samples was observed between the ASTED–HPLC method and ultrafiltration–HPLC method. The protein binding was in the range of 82–90% which is in agreement with published results [19–21].

Figs. 4A–C show chromatograms of free phenytoin in plasma from an epileptic patient (patient number 3 in Table II) after (A) ultrafiltration and HPLC analysis of the ultrafiltrate, (B) on-line dialysis for free phenytoin with ASTED–HPLC, and (C) a chromatogram of the total phenytoin analysis after on-line dialysis with AST-

TABLE II

DETERMINATION OF FREE AND TOTAL PHENYTOIN IN HUMAN PLASMA FROM EPILEPTIC PATIENTS AT 37°C AFTER ULTRAFILTRATION (UF) AND HPLC AND ON-LINE EQUILIBRIUM DIALYSIS WITH ASTED AND HPLC

Patient No.	Other antiepileptica administered	UF–HPLC Free phenytoin (μg/ml)	ASTED–HPLC		
			Free phenytoin (μg/ml)	Total phenytoin (μg/ml)	Protein binding (%)
1	Carbamazepine, lamotrigine	1.5	1.4	11.2	88
2	Valproic acid, oxcarbazepine	2.0	2.0	11.7	83
3	Phenobarbitone, carbamazepine	1.2	1.3	10.6	88
4	Carbamazepine, lamotrigine	1.2	1.4	12.4	89
5	–	1.3	1.3	12.6	90
6	–	1.3	1.4	10.2	86
7	Valproic acid, oxcarbazepine	1.3	1.2	7.1	83
8	Valproic acid, oxcarbazepine	1.0	1.0	5.5	82
9	–	0.4	0.4	3.4	88

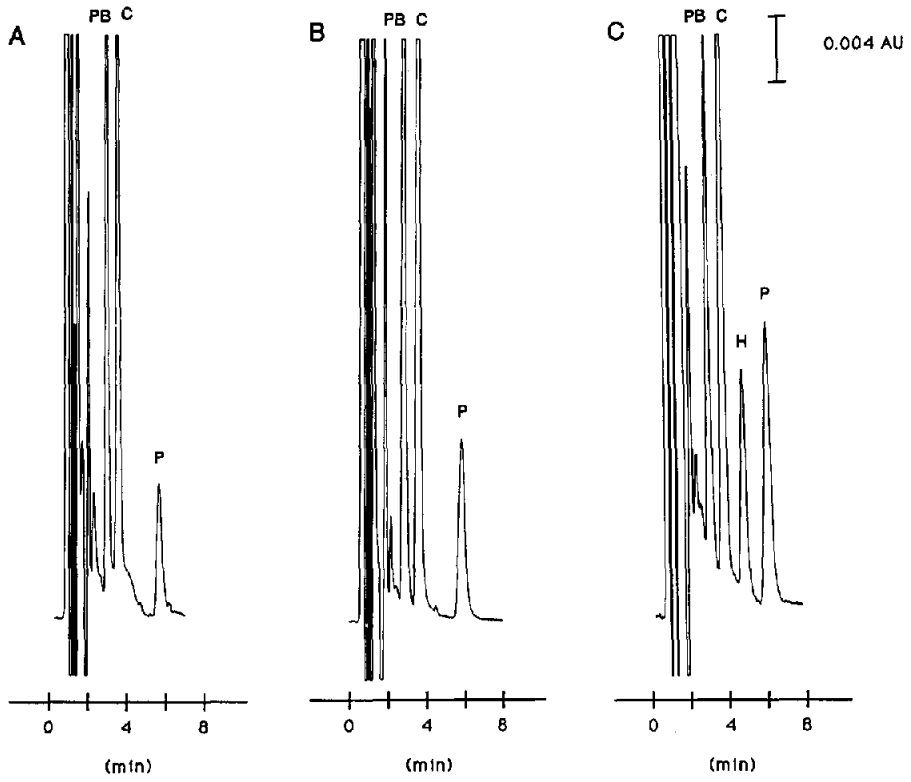

Fig. 4. Chromatograms of a patient plasma after (A) ultrafiltration and HPLC analysis of the ultrafiltrate, (B) on-line dialysis for free phenytoin with the ASTED–HPLC method, and (C) on-line dialysis for total phenytoin with the ASTED–HPLC method. Dialysis and ultrafiltration temperature: 37°C. Detection: UV at 220 nm. Peaks: P = phenytoin, H = hexobarbitone, C = carbamazepine, PB = phenobarbitone. For chromatographic conditions see text.

ED–HPLC. The dialysis temperature was 37°C. No interferences from the other drugs taken were seen in the chromatograms.

Both the ultrafiltrates and the patient plasma samples were also analysed by fluorescence polarization immuno assay (FPIA). FPIA is often used for routine monitoring of phenytoin in patient plasma. The results obtained with the ASTED–HPLC method were in agreement with those obtained with the FPIA method.

Future perspectives

The automated method for on-line dialysis and HPLC described in this report has the potential to determine free concentrations of many drugs in plasma or serum. With a sample throughput of 75 samples in 24 h routine therapeutic monitoring of free drug concentrations in plasma from patients can be established and dosage regimens can rapidly be adjusted for variation in protein binding due to disease or interactions with other drugs.

CONCLUSION

The results presented in this paper show that HPLC analysis of free and total phenytoin in plasma can be automated by on-line dialysis and column-switching combined with HPLC. The method is validated for monitoring therapeutic concentrations of free and total phenytoin in human plasma and is well suited for routine monitoring of free phenytoin in plasma from epileptic patients.

REFERENCES

1 J. W. Melten, A. J. Wittebrood, H. J. J. Willems, G. H. Faber, J. Wemer and D. B. Faber, *J. Pharm. Sci.*, 74 (1985) 692.
2 A. S. Troupin and P. Friel, *Epilepsia*, 16 (1975) 223.
3 R. K. Verbeeck and J. Cardinal, *Arzneim. Forsch. Drug Res.*, 35 (1985) 903.
4 Y. Koike, A. Magnusson, E. Steiner, A. Rane and F. Sjöqvist, *Ther. Drug Monit.*, 7 (1985) 461.
5 J. C. Argyle, D. W. Kinniburgh, R. Costa and T. Jennison, *Ther. Drug Monit.*, 6 (1984) 117.
6 N. Ratnaraj, V. D. Goldberg and M. Hjelm, *Clin. Biochem.*, 22 (1989) 443.
7 W. Godolphin, J. Trepanier and K. Farrell, *Ther. Drug Monit.*, 5 (1983) 319.
8 A. M. Herrera, D. O. Scott and C. E. Lunte, *Pharm. Res.*, 7 (1990) 1077.
9 M. Ekblom, M. Hammarlund-Udenaes, T. Lundqvist and P. Sjøberg, *Pharm. Res.*, 9 (1992) 155.
10 S. Sarre, K. van Belle, I. Smolders, G. Krieken and Y. Michotte, *J. Pharm. Biomed. Anal.*, 10 (1992) 735.
11 D. C. Turnell and J. D. H. Cooper, *J. Chromatogr.*, 395 (1987) 613.
12 A. T. Andresen, M. Krogh and K. E. Rasmussen, *J. Chromatogr.*, 582 (1992) 123.
13 A. T. Andresen and K. E. Rasmussen, *J. Liq. Chromatogr.*, 13 (1990) 4051.
14 N. C. van de Merbel, J. J. Hageman and U. A. Th. Brinkman, *J. Chromatogr.*, 634 (1993) 1.
15 P. K. M. Lunde, A. Rane, S. J. Yaffe, L. Lund and F. Sjøqvist, *Clin. Pharm.*, 11 (1970) 846.
16 I. Odar-Cederlöf and O. Borgå, *Clin. Pharm. Ther.*, 20 (1976) 36.
17 T. D. Miller and T. C. Pinkerton, *Anal. Chim. Acta*, 170 (1985) 295.
18 J. D. H. Cooper, D. C. Turnell, B. Green and F. Verillon, *J. Chromatogr.*, 456 (1988) 53.
19 L. Lund, A. Berlin and K. M. Lunde, *Clin. Pharm.*, 13 (1971) 196.
20 B. Rambeck, R. Schnabel, T. May, U. Jürgens and Villagran, *Ther. Drug Monit.*, 12 (1990) 533.
21 M. Oellerich and H. Müller-Vahl, *Clin. Pharmacokin.*, 9 (1984) 61.