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Automated analysis of free and total concentrations of three antiepileptic drugs in plasma with on-line dialysis and high-performance liquid chromatography

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Abstract

A fully automated method for determination of the free and total concentration of drugs with a varying degree of protein binding is described. The antiepileptic drugs phenytoin, carbamazepine and phenobarbitone were chosen to demonstrate the utility of this technique. The method was based on the ASTED system and combined on-line equilibrium dialysis at 37°C with concentration of the dialysate on a trace enrichment column and HPLC determination with UV detection. The dialysis cell was a modification of the ASTED dialysis cell and 22% of the free concentration of the drugs were recovered in the recipient channel of the dialyser after 10 min of dialysis at 37°C. The free concentration, the total concentration as well as the drugs protein binding could be determined. The method was shown to be well suited for routine monitoring of the free and the total concentrations of the drugs in plasma from epileptic patients.

1. Introduction

One of the primary goals of measuring drugs plasma concentration is to relate the measurements to pharmacologic response, adverse drug reactions and toxicity. As only the free concentration of a drug is assumed to be pharmacologically active assays of the free concentration and plasma protein binding are important factors in establishing the pharmacokinetic and pharmacodynamic properties of a drug. Traditionally used techniques for measuring free drug concentrations are equilibrium dialysis and ultrafiltration [1,2]. These techniques are time consuming, tedious and manually performed. Consequently it would be of great interest to develop rapid automated methods for determination of the free concentration as well as the total concentration of drugs in plasma. Therapeutic drug monitoring of phenytoin, carbamazepine and phenobarbitone is well documented in epileptic patients and the total therapeutic plasma concentration ranges are well established [3]. Particularly for phenytoin it may be beneficial to monitor free concentration rather than the total concentration since the protein binding of this

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drug is affected by a range of factors such as renal diseases, hypoalbuminemia, age and displacement by other drugs sharing the same protein binding site on albumin.

Phenobarbitone, carbamazepine and phenytoin are 50–90% bound to proteins in human plasma and were selected as test substances for the development of an automated method for free and total concentration measurements. The ASTED system (Automated Sequential Trace Enrichment of Dialysate) deproteinates biological matrices in a flat bed dialyser. By connecting ASTED to high-performance liquid chromatography (HPLC) in a column-switching system fully automated analysis of drugs in plasma can be performed. The ASTED in combination with HPLC has been used for analysis of drugs in biological matrices [4–20], and is today a well accepted technique for automated bioanalysis of drugs. Recently, an automated method for the determination of free phenytoin in human plasma based on ASTED and HPLC was reported [14]. The paper described a new dialysis cell which was able to maintain the drug–protein equilibrium during on-line dialysis. In this report properties of the new dialysis cell were further evaluated and used to develop a fully automated method for routine monitoring of the free and total concentrations of phenytoin, phenobarbitone and carbamazepine in plasma from epileptic patients.

2. Experimental

2.1. Chemicals and reagents

Phenytoin sodium and carbamazepine were obtained from Sigma (St. Louis, MO, USA). Phenobarbitone sodium, hexobarbitone and potassium chloride were purchased from Norsk Medisinal Depot (Oslo, Norway). HPLC-grade acetonitrile and methanol were supplied by Rathburn (Walkerburn, UK). HPLC-grade tetrahydrofuran, trichloroacetic acid (TCA), trisodiumcitrate-2-hydrate, sodium acetate, sodium chloride, diammoniumhydrogen phosphate, sodium hydroxide and Triton X-100 were all obtained from Merck (Darmstadt, Germany). Sodium azide was of AnalaR grade, supplied by BHD (Poole, UK). HPLC-grade water was prepared from a Milli-Q water purification system (Millipore, MA, USA).

2.2. Instrumentation

The ASTED unit (Gilson Medical Electronics, Villiers-le Bel, France) consisted of a 231 autosampling injector equipped with two 401 dilutors fitted with 1-ml syringes. The modified dialysis cell had a donor channel volume of 400 μl and a recipient channel volume of 175 μl [14]. The donor and recipient channel were separated by a Cuprophane membrane with a 15 kDa molecular mass cut-off. An automated six-port valve (Rheodyne, Berkeley, CA, USA) connected the trace enrichment column 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 (5×1.6 mm I.D.) was packed with 10-μm particles of ODS-2 Hypersil (Gilson). The temperature was controlled by placing the autosampling injector, the dialysis cell, the two dilutors, the donor and acceptor solutions and the analytical column inside a thermostated incubator (Haerus, Germany) at 37°C. The HPLC pump, the integrator and UV-detector were placed outside the incubator connected with the ASTED through holes in the walls of the incubator.

The chromatographic system consisted of an LC 6A (Shimadzu, Kyoto, Japan) HPLC pump and an SPD-6A UV detector (Shimadzu) operated at 240 nm. Peak heights were recorded on a Chromatopac C-R4A integrator (Shimadzu). The analytical column (100×3.0 mm I.D.) was packed with 5-μm particles of Spherisorb ODS-2 Chrompack (Middelburg, Netherlands).

2.3. Donor, recipient solutions and HPLC mobile phase

The mobile phase consisted of acetonitrile–tetrahydrofuran–0.02 *M* phosphate buffer pH 6.0 (22:6.5:71.5, v/v/v) delivered at a flow-rate of 0.6 ml/min at 37°C. Isotonic donor and

recipient solutions were prepared from 5.9 g sodium chloride, 4.1 g sodium acetate, 0.3 g potassium chloride and 1.65 g sodium citrate adjusted to pH 7.4 with citric acid and diluted with water to 1 l. The donor solution was added 25 μg/ml Triton X-100.

2.4. Stock solutions, external standard and internal standard

Stock solutions of carbamazepine, phenytoin and phenobarbitone (50, 200 and 1000 μg/ml) were prepared in methanol. Free concentration of the drugs were determined by using external standard solutions prepared in 0.02 *M* ammonium phosphate buffer pH 6.0. Standard solutions were made in the concentration range of 0.25–8 μg/ml phenytoin, 1–20 μg/ml phenobarbitone and 0.25–4 μg/ml carbamazepine. In the total concentration determination an internal standard (I.S.) was used. The internal standard solution of 5 μg/ml hexobarbitone was prepared in 0.02 *M* ammonium phosphate buffer pH 6.0 with 0.02 *M* TCA and 50 μg/ml sodium azide.

2.5. Plasma standards

Citrated plasma from healthy donors was obtained from The Blood Centre at Ullevaal Hospital (Oslo, Norway). Citrated plasma samples from epileptic patients were collected at The National Hospital (Oslo, Norway). The plasma standards concentration range was based on the therapeutic plasma concentration range (10–20 μg/ml for phenytoin, 10–30 μg/ml for phenobarbitone and 5–10 μg/ml for carbamazepine). The desired amount from the stock solutions were transferred into volumetric glasses and the methanol evaporated with nitrogen under careful heating. Plasma standards in the concentration range of 0.25–20 μg/ml phenytoin, 1–30 μg/ml phenobarbitone and 0.25–10 μg/ml carbamazepine were prepared. To validate the method plasma spiked with 2, 10 and 20 μg/ml phenytoin, 5, 10 and 30 μg/ml phenobarbitone and 2, 5 and 10 μg/ml carbamazepine were analysed ($n = 6$). The plasma samples were stored at −20°C and the free and total concentration were determined weekly during 6 weeks of storage.

2.6. Determination of free concentration

An aliquot of 400 μl plasma was delivered to the donor channel of the dialyser. Segmentation with air bubbles prevented longitudinal diffusion of sample into the transporting donor solution. The valve was in the "load" position. The sample in the donor channel and the recipient solution in the recipient channel were held static at 37°C. After 10 min of dialysis 500 μl of the recipient solution was transported through the recipient channel of the dialyser in one pulse, to bring the analytes into the trace enrichment column. The flow-rate of the recipient solution through the dialyser was 0.71 ml/min. Upon switching the valve to inject position, the analytes retained on the trace enrichment column were brought into the analytical column by elution with the HPLC mobile phase. The recipient channel of the dialyser was then washed with 5 ml recipient solution and the donor channel of the dialyser was washed with 8 ml donor solution. By switching the six-port valve to the load position the trace enrichment column was regenerated with 1 ml of recipient solution, and the next sample injected into the dialyser. Automated injections were performed every 20 min.

2.7. Determination of total concentration

An aliquot of 200 μl plasma was automatically mixed with 600 μl internal standard solution by the autosampler, and 400 μl of the mixture were injected into the dialysis cell. Dialysis was carried out for 6.5 min with the same procedure as for the determination of free concentration, except that two aliquots of 0.5 ml of the recipient solution were pumped through the acceptor channel and into the trace enrichment column.

2.8. Validation of the procedures

The calibration graphs, accuracy and precision of the method were evaluated by analysis of plasma samples spiked with phenytoin, car-

bamazepine and phenobarbitone. The standard curves for the free concentration analysis in plasma were based on external standard solutions and peak height measurements. The calibration graphs for the total concentration analysis in plasma were based on peak height measurements versus the peak height of the I.S. for all the compounds. The protein binding was calculated by the equation:

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

where T is the total concentration of the drug in plasma and F is the free concentration of the drug in plasma. The results obtained by the ASTED–HPLC on-line dialysis method were compared with results from ultrafiltration and HPLC. Ultrafiltration was performed inside the incubator at 37°C as described in an earlier report [14].

3. Results and discussion

3.1. Dialysis

The design of the dialysis cell can affect drugs protein binding as described by Andresen et al. [14]. A dialysis cell with a larger donor channel volume than the recipient channel volume maintained the protein binding during 10 min of static–static dialysis at 37°C. The dialysis recovery (RE) of the free concentration of a drug is defined as the percentage of solute molecules present in the recipient compartment. RE depends on the $V_a/(V_a + V_d)$ ratio described as follows [15]:

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

where V_a and V_d are the recipient and donor channel volumes respectively, τ is the tortuosity of the membrane, l is the membranes thickness, t is the dialysis time, D is the diffusion coefficient of the solute and A is the membrane area available for diffusion. As time increases, the concentration gradient, and consequently, the flux slowly decreases and becomes zero. With a donor channel volume of 400 μl and a recipient channel volume of 175 μl a maximum recovery of 30.4% can be obtained with the dialysis cell used. For determination of the actual recovery the dialysis cell was replaced with a standard 50-μl loop on the injection valve and aqueous standard solutions of phenytoin, carbamazepine and phenobarbitone were injected. The results were compared with the results obtained after dialysis of plasma samples containing the same drug amounts. After 10 min of dialysis at 37°C, 3% of phenytoin, 6% of carbamazepine and 10% of phenobarbitone added to the drug-free plasma were recovered. When the protein binding of the drugs are taken into account the recovery from the free concentration can be calculated. As shown in Table 1 22% of the free concentration of phenytoin and 23% of the free concentration of carbamazepine and phenobarbitone were recovered. With a maximum of 30.4% these results show that 71–75% of the maximum

Table 1
Dialysis recoveries (RE) during determination of free concentration

Analyte	Added total concentration (μg/ml)	RE of added total concentration (%)	Protein-binding (%)	RE of free concentration (%)	RE of free concentration as a function of the maximum recovery (%)
Phenytoin	10	3	85	22	71
Carbamazepine	5	6	73	23	75
Phenobarbitone	20	10	55	23	75

recovery is achieved during 10 min of dialysis at 37°C. It has been demonstrated earlier that the dialysis recovery increases only slightly at longer dialysis times [14]. The results obtained by the ASTED–HPLC on-line dialysis method were compared with the results obtained by ultrafiltration and HPLC analysis of the ultrafiltrate as presented in Table 2. The results were in agreement with each other. One limitation of using dialysis for free drug measurements is drug–membrane interactions. Hydrophobic and ionic interactions have been reported for cellulose acetate [13,21] and a hydrophobic alkaline drug, pholcodine, has been shown to adsorb strongly to the cuprophane membrane [9]. Drug–membrane interactions introduce a new equilibrium in the system which may alter the drug–protein equilibrium. No interactions with the membrane were found for the drugs studied in this investigation and it was demonstrated that the ASTED–HPLC on-line dialysis method was a robust and accurate method for measuring the free concentrations.

The total drug concentrations in plasma were determined after static-pulsed dialysis for 6.5 min with the same dialysis cell as used for the determination of the free concentrations. Plasma samples were mixed with I.S. solution containing TCA which displaces the drugs from the proteins. The static-pulsed mode of dialysis increases the concentration gradient across the membrane and increases the dialysis recovery. The dialysis recoveries were 20, 22 and 22% for phenytoin, phenobarbital and carbamazepine, respectively. These dialysis recoveries are considerably lower than the ones generally obtained when the standard dialysis cell supplied with the ASTED are used [6,8–11]. The standard cell is designed for high dialysis recoveries with a donor channel volume of 100 μl and a recipient channel volume of 175 μl. When 400 μl sample is dialysed on the modified cell the recoveries obtained were sufficiently high to detect subtherapeutic concentrations of the drugs, and both free and total concentrations could be determined under the same analytical conditions.

3.2. Trace enrichment

Several packing materials were investigated for trace enrichment. The reversed-phase materials polystyrene–divinylbenzene and silica-based Bond-Elut C_2, C_8, C_{18} with 40-μm particles gave either unsatisfactory clean-up, poor recovery or peak broadening. The problems encountered were eliminated with a ODS-2 trace enrichment column packed with 10-μm particles from Gilson. The breakthrough volume of phenobarbi-

Table 2
Determination of free concentration of phenytoin, carbamazepine, and phenobarbitone in human plasma after ultrafiltration (UF)–HPLC at 37°C and on-line equilibrium dialysis with ASTED and HPLC

Analyte	Total Concentration (μg/ml)	Measured free concentration (mean ± S.D., n = 6) (μg/ml)	
		UF–HPLC	ASTED–HPLC
Phentoin	2.00	0.27 ± 0.04	0.29 ± 0.01
	10.00	1.58 ± 0.10	1.62 ± 0.04
	20.00	3.33 ± 0.16	3.26 ± 0.05
Carbamazepine	2.00	0.53 ± 0.02	0.53 ± 0.01
	5.00	1.49 ± 0.03	1.36 ± 0.02
	10.00	2.91 ± 0.08	2.73 ± 0.02
Phenobarbitone	5.00	2.35 ± 0.03	2.36 ± 0.01
	10.00	4.95 ± 0.13	4.59 ± 0.04
	30.00	14.02 ± 0.65	13.18 ± 0.3

S.D. = standard deviation; dialysis temperature = 37°C.

tone was 2 ml when recipient solution was pumped through the trace enrichment column. No breakthrough of phenytoin, carbamazepine or hexobarbitone were observed when up to 10 ml of recipient solution were pumped through the trace enrichment column. No breakthrough of the drugs were observed under the conditions used.

3.3. *Viability of the dialysis membrane and trace enrichment column*

Peak broadening and unsatisfactory clean-up were observed after 250 analysis, so the trace enrichment column was replaced every 200 analysis. The dialysis membrane was changed after every 1000 samples as recommended by the ASTED users' guide.

3.4. *HPLC*

Satisfactory separation of all the analytes and the internal standard were obtained with no interferences from plasma. Typical chromatograms from a drug-free plasma and from a patient plasma sample are shown in Fig. 1. The active metabolite of carbamazepine, carbamazepine -10,11- epoxide co-eluted with the front. With minor modifications of the mobile phase determination of carbamazepine -10,11-epoxide can be performed with the same analytical set up.

3.5. *Validation of the method*

Linear calibration graphs for determination of free concentrations were obtained over the range of 0.25–8 μg/ml phenytoin, 0.25–4 μg/ml carbamazepine and 1–20 μg/ml phenobarbitone. In the total concentration analysis linear calibration graphs were obtained over the range of 0.25–20 μg/ml phenytoin, 0.25–10 μg/ml carbamazepine and 1–30 μg/ml phenobarbitone. The correlation coefficients were 0.998 ($n = 6$) or better. Table 3 shows the intra-assay and inter-assay variation of free and total phenytoin, carbamazepine and phenobarbitone in human plasma. The relative standard deviation for free and total determination were acceptable and there was little difference between the relative standard deviations obtained in free and total concentration analysis. No changes in the free and total concentrations of the drugs were observed when plasma samples were stored for 6 weeks at −20°C.

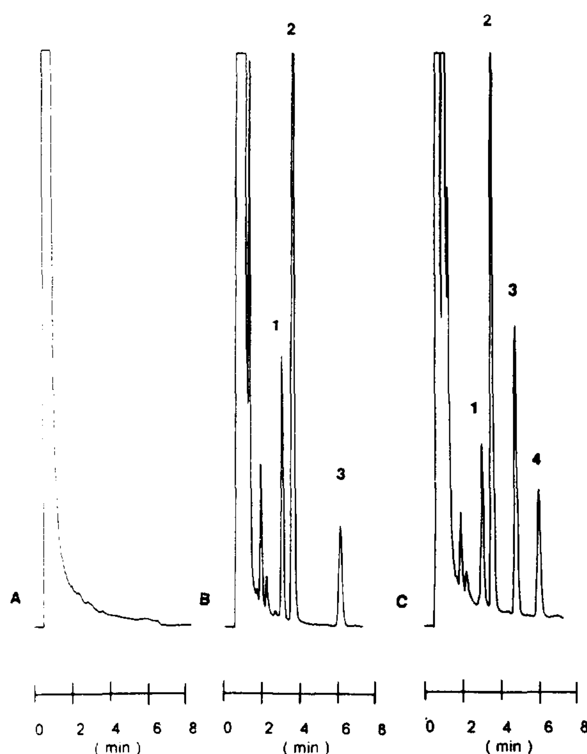

Fig. 1. Chromatograms of (A) analysis of drug-free human plasma, (B) determination of the free concentrations in a patient plasma sample. Peaks: 1 = phenobarbitone, 2.6 μg/ml; 2 = carbamazepine, 1.2 μg/ml; 3 = phenytoin, 1.4 μg/ml; (C) Determination of the total drug concentrations in the same patient plasma sample as in B. Peaks: 1 = phenobarbitone, 5.6 μg/ml; 2 = carbamazepine, 4.7 μg/ml; 3 = hexobarbitone, 5 μg/ml; 4 = phenytoin, 8.9 μg/ml. Dialysis temperature: 37°C. Detection: UV at 240 nm. For chromatographic conditions see text.

3.6. *The limit of detection*

With a protein binding of 85, 73 and 55 % for phenytoin, carbamazepine and phenobarbitone, respectively, the free concentrations in drug-free plasma spiked with a total of 0.8 μg/ml pheny-

Table 3
Intra- and inter-assay variation of free and total concentration of phenytoin, carbamazepine and phenobarbitone in human plasma determined with ASTED dialysis and HPLC

Added total concentration (μg/ml)	Measured free concentration (mean ± S.D., $n = 6$) (μg/ml)	R.S.D. (%)	Measured total concentration (mean ± S.D., $n = 6$) (μg/ml)	R.S.D. (%)	Protein binding (%)
Intra-assay variation					
Phenytoin					
2.00	0.30 ± 0.01	1.7	2.08 ± 0.07	3.4	86
10.00	1.54 ± 0.02	1.3	9.92 ± 0.14	1.4	84
20.00	3.22 ± 0.10	3.1	19.97 ± 0.51	2.6	84
Carbamazepine					
2.00	0.53 ± 0.01	1.9	1.94 ± 0.02	1.0	73
5.00	1.36 ± 0.01	0.7	4.91 ± 0.10	2.0	72
10.00	2.68 ± 0.05	1.9	9.92 ± 0.11	1.2	73
Phenobarbitone					
5.00	2.25 ± 0.06	2.7	5.05 ± 0.03	0.6	56
10.00	4.55 ± 0.07	1.5	10.07 ± 0.17	1.7	55
30.00	13.10 ± 0.23	1.8	29.62 ± 0.33	1.1	56
Inter-assay variation					
Phenytoin					
2.00	0.29 ± 0.01	2.6	2.04 ± 0.11	5.4	86
10.00	1.63 ± 0.07	4.3	9.93 ± 0.24	2.4	84
20.00	3.00 ± 0.12	4.0	20.34 ± 0.75	3.7	85
Carbamazepine					
2.00	0.54 ± 0.01	2.3	1.95 ± 0.13	6.7	73
5.00	1.37 ± 0.02	1.6	4.85 ± 0.13	2.7	72
10.00	2.63 ± 0.11	4.2	10.04 ± 0.39	3.9	74
Phenbarbitone					
5.00	2.22 ± 0.10	4.5	5.09 ± 0.08	1.6	56
10.00	4.51 ± 0.08	1.8	10.08 ± 0.27	2.7	55
30.00	13.28 ± 0.31	2.3	29.53 ± 0.66	2.2	55

S.D. = standard deviation, R.S.D. = relative standard deviation, dialysis temperature: 37°C.

toin, 0.3 μg/ml phenobarbitone and 0.1 μg/ml carbamazepine could be detected at a signal-to-noise ratio of 3 ($S/N = 3$). This was far below the therapeutic total plasma concentration range for all the drugs.

3.7. Analysis of plasma from epileptic patients

Plasma samples were collected from eight epileptic patients in a routine control at the National Hospital in Oslo. The dosage regimens varied greatly between the patients and were either based on single drug therapy or therapy based on combinations of phenytoin, carbamazepine and phenobarbitone. The results presented in Table 4 show free concentrations, total concentrations and protein binding of phenytoin, carbamazepine and phenobarbitone in plasma from these patients. The protein binding results were in agreement with results obtained in the literature [3,22–24]. No problems were encountered in the analysis of patient plasma samples and the method was well suited for routine monitoring of free and total concentrations of these drugs in plasma. With a sample throughput of 75 samples in 24 h, the dosage regimens can rapidly be adjusted for variations in protein binding.

Table 4
Determination of free and total concentration of phenytoin (P), phenobarbitone (P.B.) and carbamazepine (C) in human plasma from epileptic patients at 37°C with on-line equilibrium dialysis with ASTED and HPLC

Patient No.	Analyte	Dose (mg/day)	Free concentration (μg/ml)	Total concentration (μg/ml)	Protein binding (%)
1	P	200	0.9	4.4	81
2	C	1500	2.2	8.4	74
3	P	350	1.4	8.9	84
	C	800	1.2	4.7	74
	P.B.	50	2.6	5.6	53
4	C	400	0.7	3.2	76
5	C	500	1.7	5.8	71
6	P.B.	unknown	5.3	11.7	55
	C	800	1.1	4.7	76
7	P	350	0.5	2.7	83
8	C	600	1.2	4.2	72

4. Conclusion

The results presented show that determination of free phenytoin, carbamazepine and phenobarbitone in human plasma at 37°C can be fully automated by equilibrium dialysis, column-switching and HPLC. These drugs have a protein binding varying from 50–90% and 22% of the free concentration of the drugs were recovered in the dialysate after 10 min of dialysis. The total concentrations of these drugs can be determined under the same analytical conditions after static-pulsed dialysis of plasma mixed with an internal standard solution containing TCA. The results were highly reliable and the method was well suited for routine monitoring of therapeutic free and total plasma concentrations in patients.

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