ELSEVIER

Journal of Chromatography B, 673 (1995) 299–305

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Solid-phase microextraction for the determination of the free concentration of valproic acid in human plasma by capillary gas chromatography

Mette Krogh*, Karianne Johansen, Finn Tønnesen, Knut E. Rasmussen

Institute of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, Oslo, Norway

First received 10 March 1995; revised manuscript received 29 May 1995; accepted 15 June 1995

Abstract

The potential of solid-phase microextraction in the bioanalysis of drugs is demonstrated. The free concentration of valproic acid in human plasma was determined by equilibrium dialysis followed by solid-phase microextraction and capillary gas chromatography. Human plasma samples were dialysed at room temperature. To the dialysate was added an internal standard and the pH was adjusted to 2.5. The polymethylsiloxane-coated fused-silica fibre of the solid-phase microextraction device was inserted into the dialysate for 3 min. The sorbed analytes were then thermally desorbed at 210°C in the split–splitless injection port of the gas chromatograph, separated on a Nukol capillary column and detected with a flame ionization detector. The method was shown to be highly reproducible with a detection limit of 1 μg/ml of free valproic acid in human plasma.

1. Introduction

Solid-phase microextraction (SPME) is a technique for the extraction of organic compounds in aqueous samples which was first described by Belardi and Pawliszyn [1]. SPME has successfully been used for the extraction of volatile compounds in environmental samples and foodstuffs [2–15], but to our knowledge has not been applied for the extraction of drugs from biological samples. In bioanalysis, much time and effort are spent on sample preparation as it is often necessary to isolate, purify and concentrate the analyte from the biological matrix prior to chromatographic analysis. Solid-phase microextraction integrates sampling, extraction, concentration and sample introduction in a single step. SPME combines partitioning of analytes between the coating and the sample with thermal desorption of concentrated analytes into the injection port of an analytical instrument, e.g., a gas chromatograph.

Valproic acid (2-propylpentanoic acid) is an antiepileptic agent used to control several types of seizures. It has been associated with drug interactions resulting in both decreased and increased plasma concentration. Over 90% of valproic acid is bound to plasma proteins (mainly albumin) [16] and the binding is saturable when the total plasma concentration is above 80 μg/ml [17–20]. Determination of the free concentration rather than the total drug concentration should be considered for drugs for which the usefulness of therapeutic drug monitoring has been estab-

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(95)00273-1

lished, which are highly bound to plasma proteins and which exhibit a variable free concentration. The chromatographic determination of valproic acid in biological matrices is usually performed by immunological methods, high-performance liquid chromatography (HPLC) with UV detection [21], postcolumn reaction detection, e.g., fluorescence labelling [22–24], or by gas chromatography and gas chromatography–mass spectrometry (GC–MS) [25,26].

We recently reported the application of an automated on-line equilibrium dialysis procedure for the determination of the free concentration of three antiepileptic drugs in plasma [27,28]. The method was based on the ASTED (automated sequential trace enrichment of dialysate) system, which combines on-line dialysis and trace enrichment of the dialysates. Fully automated analysis can be performed when the ASTED system is coupled on-line to an HPLC system through a column-switching device. A modified dialysis cell which maintained the drug–protein equilibrium was developed and evaluated for the determination of free drug concentrations.

In the present study, automated equilibrium dialysis was to be performed with the ASTED system with a modified dialysis cell to ensure the determination of the non-protein-bound drug in addition to the removal of proteins and any particulate matter from the plasma samples prior to introduction of the coated fibre into the sample. The purpose of the study was to demonstrate the potential of the SPME technique in bioanalysis and to develop a simple, specific chromatographic method for the determination of the free concentration of valproic acid in human plasma for therapeutic drug monitoring.

2. Experimental

2.1. Chemicals

Valproic acid was obtained from Sigma (St. Louis, MO, USA), caprylic acid [internal standard (I.S.)] from Fluka (Buchs, Switzerland) and sodium hydroxide, sodium acetate, sodium chloride, sodium citrate and potassium chloride from Merck (Darmstadt, Germany). Human plasma with added heparin and plasma samples from epileptic patients were supplied by Ullevål Hospital (Oslo, Norway). Deionized water was obtained from a Milli-Q water-purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of standards

Stock standard solutions (10 mg/ml) of valproic acid and caprylic acid (10 mg/ml) (I.S.) were prepared in 0.1 *M* NaOH. Working standard solutions of valproic acid (2–20 μg/ml) in water and plasma samples spiked with 50–150 μg/ml of valproic acid (total concentration) were prepared from the stock standard solution. The spiked plasma samples were prepared freshly.

2.3. Dialysis

Dialysis was performed using the ASTED system (Gilson Medical Electronics, Villiers-de-Bel, France) consisting of a Model 231 autosampling injector, two Model 401 diluters equipped with 1-ml syringes and a modified flat-bed dialyser with a donor channel volume of 400 μl and a recipient channel volume of 175 μl, fitted with a Cuprophane membrane of molecular mass cut-off 15 000. Isotonic donor and recipient solutions were prepared by dissolving 5.9 g of sodium chloride, 4.1 g of sodium acetate, 1.65 g of sodium citrate and 0.3 g of potassium chloride in 1 l of water. Both the donor (sample) and the recipient solution were held static during dialysis. Dialysis was performed at room temperature for 25 min, by which time equilibrium had been obtained, as reported previously [27,28]. Subsequently the dialysate (200 μl) was collected manually in a glass vial, 5 μl of caprylic acid (200 μg/ml) were added as I.S. and the pH was adjusted to 2.5 by addition of 5 μl of 7.4 *M* phosphoric acid.

2.4. Solid-phase microextraction (SPME)

The SPME device equipped with a polymethylsiloxane-coated fibre (film thickness 100 μm) was supplied by Supelco (Bellefonte, PA,

USA). To prevent the injection of impurities on to the capillary column, cleaning of the fibre was performed by heating it at 250°C for 5 min in the injection port of a second gas chromatograph. The cleaning procedure was performed at the beginning of each day. The SPME assembly was clamped in place above the glass vials and the coated fibre was inserted into the dialysate to allow sorption of valproic acid and caprylic acid (I.S.). After 3 min of exposure, the uncoated fibre was withdrawn from the dialysate and into the SPME holder. The tip of the SPME device was thereafter inserted into the heated (210°C) split–splitless injector for 1 min to desorb the adsorbed analytes thermally into the capillary GC system for chromatographic separation and determination. The fibre was extended only during sampling or desorption of the analyte.

2.5. Capillary GC analysis

Chromatographic analysis was performed on a Shimadzu (Kyoto, Japan) GC-14A capillary gas chromatograph equipped with a 30 m × 0.2 mm I.D. Nukol column (0.25 μm film thickness) (Supelco) and a flame ionization detector. During the splitless desorption step, the tip of the SPME holder was inserted through the septum and the coated fibre was exposed inside the liner above the inlet to the analytical column. After the desorption was completed, the fibre was withdrawn into the SPME holder and removed from the injection port and the split vent was opened. The chromatographic separation was achieved with temperature programming. The temperature was held at 60°C for 2 min, then increased at 30°C/min to 150°C and at 10°C/min to 190°C, and held constant at 190°C for 4 min. The chromatograms were recorded on a Shimadzu CR3-A integrator.

2.6. Validation of the method

The calibration graph for the determination of the free concentration of valproic acid in plasma was based on peak-height measurements versus the peak height of the I.S. which was added after dialysis. The limit of detection was determined at a signal-to-noise ratio of 3.

2.7. Analysis of patients' samples

The applicability of the method was demonstrated by the analysis of plasma samples from epileptic patients. The total concentration of valproic acid was determined at Ullevål Hospital by FPIA, Abbot TD_x (fluorescence polarization immunoassay system; Abbot Laboratories, Irving, TX, USA), prior to determination of the free concentration by the method developed in this work. The degree of protein binding was calculated from the results obtained.

3. Results and discussion

3.1. Solid-phase microextraction

SPME is an extraction technique for organic compounds in aqueous samples in which the analytes are adsorbed directly from the aqueous sample on to a fused-silica fibre coated with an appropriate stationary phase, in this case polymethylsiloxane. While the fibre is inserted in the sample, the analytes partition from the sample matrix into the stationary phase until equilibrium is reached. Each fibre can be used for 100 analyses or more, depending on the particular application. A single fibre was used throughout this investigation. The amount of analyte adsorbed by the coated fibre is dependent on time, the distribution constant of the analyte, the volume of the solvent phase and the stirring of the solution [2,3]. To enhance sorption, the dialysate was placed in conical glass inserts with a volume of 200 μl and stirred during partitioning. The optimum sorption time was determined by comparison of peak heights after insertion of the fibre in a standard solution of 10 μg/ml of valproic and caprylic acid in 3 m*M* phosphoric acid (pH 2.5), as shown in Fig. 1. A sorption time of 3 min was chosen as it was sufficient to be able to detect concentrations below the therapeutic free concentration of valproic acid in human plasma, although an increased time resulted in

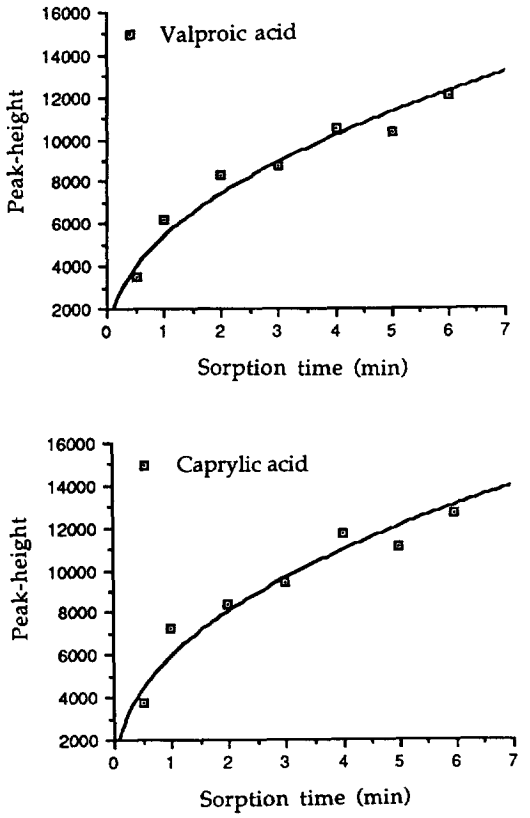

Fig. 1. Peak height versus SPME sorption time in a standard solution of 10 μg/ml each of valproic acid and caprylic acid in 3 m*M* phosphoric acid (pH 2.5).

enhanced adsorption. The peak height of valproic acid versus the peak height of caprylic acid (I.S.) was constant within the sorption time range 0.5–6.0 min with an R.S.D. of 2%. Complete desorption was achieved when the fibre was held in the injection port of the GC for 1 min at 210°C.

Prior to microextraction the pH was adjusted to 2.5. At this pH, both valproic acid and caprylic acid will be completely protonated ($pK_a = 5.0$) and the sorption on the polymethylsiloxane fibre is enhanced. The extraction efficiency and selectivity can be controlled by the pH of the sample. By simple pH adjustments of the aqueous solution, selective extraction and concentration are achieved by the SPME technique. Fig. 2 demonstrates the effect of pH on both the recovery and selectivity of SPME extraction. At pH 7.4 both valproic acid and caprylic acid are fully ionized and are not sorbed on the SPME fibre, whereas at pH 5 valproic and caprylic acid are partly ionized and some sorption is observed.

The SPME technique permits injections into the GC system without the introduction of solvents as no water adheres to the fibre because of its high surface tension. The microextraction step ensured further clean-up of the dialysate and concentration of the analytes. By comparison of peak heights it was estimated that the amount of sorbed analytes during the SPME procedure would be equivalent to the injection of 8 μl of a solution with the same concentration of the analytes as the dialysate.

Touching the needle with bare hands resulted in significant contamination and the appearance of several peaks in the chromatogram from the biological acids, as reported previously [4], and should be avoided.

3.2. GC analysis

The separation was performed on a Nucol column, which is specially designed for the separation of free fatty acids without the need for derivatization. Variations in the front of the chromatograms were observed, which were caused by contact between the septum piercing needle of the SPME device and the dialysate. Separation was achieved within 12 min and no interfering peaks were detected in the analysis of drug-free plasma samples.

3.3. Dialysis

Automated equilibrium dialysis was performed with the ASTED system. We recently reported the application of a dialysis cell with an increased donor channel volume used for the determination of the free concentrations of three antiepileptic drugs [27,28]. This dialysis cell maintained the drug–protein equilibrium and was suitable for the determination of the free concentrations. Equilibrium was found to be achieved when dialysis was performed for 25 min at room temperature (20°C) or 10 min at 37°C [27]. Dialysis was performed at room tempera-

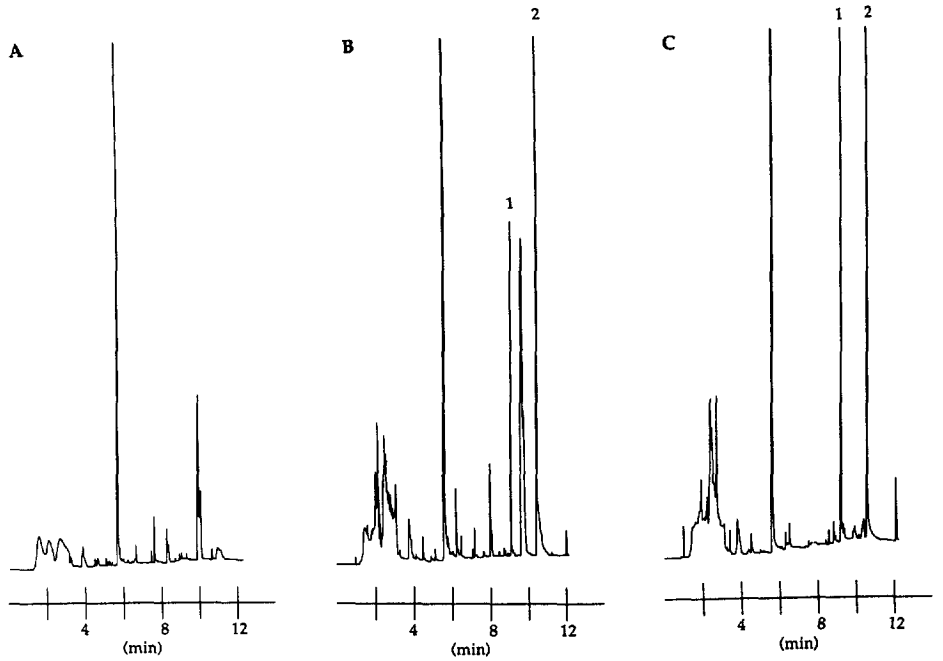

Fig. 2. Chromatograms of spiked plasma samples with 150 μg/ml of valproic acid added by the ASTED–SPME procedure at (A) pH 7.4, (B) pH adjusted to 5.0 and (C) pH adjusted to 2.5. 1 = Valproic acid, 2 = caprylic acid (I.S.).

ture for 25 min. No adsorption of valproic acid to the dialysis membrane was observed.

3.4. Validation of the method

The calibration graph was linear in the concentration range 2–20 μg/ml with correlation coefficients $r = 0.9998–0.9999$. The relative standard deviations were between 1.3 and 5% ($n = 6$) and the method was found to be highly reproducible. The protein binding was found to be 92 and 93% ($n = 6$) for plasma spiked with a total concentration of 50 and 100 μg/ml, respectively, which is in agreement with the literature. A protein binding of 85% ($n = 6$) was found for the plasma samples spiked with a total concentration of 150 μg/ml, which demonstrated the saturation of the binding to plasma albumin occurring at a high total concentration of valproic acid.

3.5. Limit of detection

The limit of detection is determined by the sample size injected into the dialyser, the dialysis time and SPME sorption time. The limit of detection at a signal-to-noise ratio of 3 was a free concentration of 1 μg/ml for valproic acid.

3.6. Analysis of patients' samples

The free concentration of valproic acid in plasma samples from epileptic patients was measured by the ASTED–SPME method. Table 1 shows the results from the analyses of eight samples and the calculated degree of protein binding at room temperature. These results demonstrate the individual variations in protein binding and therefore in the free concentration of a highly protein-bound drug such as valproic acid. Chromatograms of a drug-free plasma sample and a patient's plasma sample are shown in Fig. 3.

4. Conclusions

A simple and specific chromatographic method for the determination of the free concentration

Table 1
Analysis of pateints' samples

Patient No.	Total concentration measured by FPIA (μg/ml)	Free concentration measured by SPME (μg/ml)	Protein binding (%)
1	71.5	5.45	92.4
2	31.7	1.89	94.0
3	19.2	1.62	91.6
4	34.8	3.82	89.0
5	56.2	5.92	89.5
6	48.3	4.55	90.6
7	43.8	4.15	90.5
8	70.9	10.05	85.8

of valproic acid in human plasma has been developed. The results demonstrated the potential of the SPME technique for the determination of drugs in biological samples and the method was shown to be highly reliable. The extraction efficiency and selectivity could be governed by simple adjustments of the sample pH. The method was shown to be well suited for the analysis of plasma samples from epileptic patients.

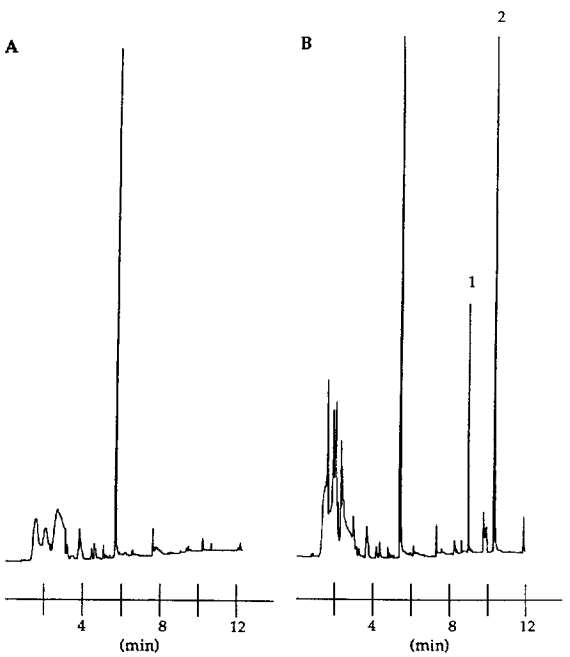

Fig. 3. Chromatograms of (A) a drug-free plasma sample and (B) a plasma sample from patient No. 8 (see Table 1). 1 = Valproic acid, 2 = caprylic acid (I.S.).

Acknowledgements

Dr. Jan Erik Bredesen and Ullevål Hospital (Oslo, Norway) are gratefully acknowledged for their collaboration and the provision of plasma samples from patients.

References

[1] R.P. Belardi and J. Pawliszyn, Water Pollut. Res. J. Can., 24 (1989) 179.
[2] C.L. Arthur and J. Pawliszyn, Anal. Chem., 62 (1990) 2145.
[3] D. Louch, S. Motlagh and J. Pawliszyn, Anal. Chem., 64 (1992) 1187.
[4] S.B. Hawthorne, D.J. Miller, J. Pawliszyn and C.L. Arthur, J. Chromatogr., 603 (1992) 185.
[5] C.L. Arthur, L.M. Killam, S. Motlagh, M. Lin, D.W. Potter and J. Pawliszyn, Environ. Sci. Technol., 26 (1992) 979.
[6] C.L. Arthur, D.W. Potter, K.D. Bucholtz, S. Motlagh and J. Pawliszyn, LC · GC, 10 (1992) 656.
[7] D.W. Potter and J. Pawliszyn, J. Chromatogr., 625 (1992) 247.
[8] C.L. Arthur, L.M. Killam, K.D. Buchholz, J. Pawliszyn and J.R. Berg, Anal. Chem., 64 (1992) 1960.
[9] B.D. Page and G. Lacroix, J. Chromatogr., 648 (1993) 199.
[10] R. Kubinec, P. Kuran, I. Ostrovsky and L. Sojak, J. Chromatogr., 653 (1993) 363.
[11] K.D. Buchholz and J. Pawliszyn, Environ. Sci. Technol., 27 (1993) 2844.
[12] S. Motlagh and J. Pawliszyn, Anal. Chim. Acta, 284 (1993) 265.
[13] L.P. Sarna, G.R. Barrie Webster, M.R. Friesen-Fischer and R.S. Ranjan, J. Chromatogr., 677 (1994) 201.

[14] Z. Zhang, M.G. Yang and J. Pawliszyn, Anal. Chem., 66 (1994) 844.
[15] J.Y. Horng and S.D. Huang, J. Chromatogr., 678 (1994) 313.
[16] H. Liu, J.L. Montoya, L.J. Forman, C.M. Eggers, C.F. Barham and M. Delgado, Ther. Drug Monit., 14 (1992) 513.
[17] T.C. Kwong, Clin. Chim. Acta, 151 (1985) 193.
[18] G. Zaccara, A. Messori and F. Moroni, Clin. Pharmacokinet., 15 (1988) 367.
[19] R.D. Scheyer, J.A. Cramer, B.R. Toftness, J.M. Hochholer and R.H. Mattson, Ther. Drug Monit., 12 (1990) 117.
[20] J.A. Cramer, R.H. Mattson, D.M. Bennett and C.T. Swick, Ther. Drug Monit., 8 (1986) 411.
[21] L.J. Lovett, G.A. Nygard, G.R. Erdmann, C.Z. Burley and S.K.W. Khalil, J. Liq. Chromatogr., 10 (1987) 687.
[22] H. Liu, L.J. Forman, J. Montoya, C. Eggers, C. Barham and M. Delgado, J. Chromatogr., 576 (1992) 163.
[23] C. Lucarelli, P. Villa, E. Lombardi and A. Brega, Chromatographia, 33 (1992) 37.
[24] R. Gatti, V. Cavrini and P. Roveri, Chromatographia, 33 (1992) 13.
[25] W. Lin and A.R. Kelly, Ther. Drug Monit., 7 (1985) 336.
[26] A.A. Acheampong, F.S. Abbott, J.M. Orr, S.M. Ferguson and R.W. Burton, J. Pharm. Sci., 73 (1984) 489.
[27] A.T. Andresen, H.E. Rugstad and K.E. Rasmussen, J. Chromatogr., 621 (1993) 189.
[28] K. Johansen, M. Krogh, A.S. Christophersen, G. Lehne and K.E. Rasmussen, J. Chromatogr. B, 669 (1995) 281.