ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT

Journal of Chromatography B, 784 (2003) 69–75

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Synthesis of [$^{18}O_2$]valproic acid and its use as an internal standard for the quantitative measurement by gas chromatography–electron ionization mass spectrometry

Hans Jörg Leis*, Werner Windischhofer, Gerald N. Rechberger, Günter Fauler

University Children's Hospital, Division of Analytical Biochemistry and Mass Spectrometry, Auenbruggerplatz 30, A-8036 Graz, Austria

Received 9 July 2002; received in revised form 20 September 2002; accepted 23 September 2002

Abstract

A specific method for the quantitative determination of valproic acid in human plasma is presented. Valproate was extracted from acidified plasma by hexane extraction and converted to its trimethylsilyl derivative without sample concentration. The derivatives were analyzed without any further purification. Using gas chromatography–electron ionization mass spectrometry, diagnostic useful fragment ions at m/z 201 and 205 were obtained for valproic acid and [$^{18}O_2$]valproic acid internal standard, respectively. [$^{18}O_2$]Valproic acid was synthesized from unlabeled valproate by acid-catalyzed exchange reaction in $H_2^{18}O$. The method was validated in the expected concentration range of a pharmacokinetic study. Thus, calibration graphs were linear within a range of 0.47–120 μg/ml plasma. Intra-day precision was 2.29% (0.47 μg/ml), 2.93% (4 μg/ml), 3.22% (20 μg/ml) and 4.40% (80 μg/ml), inter-day variability was found to be 1.49% (0.47 μg/ml), 3.79% (20 μg/ml), 2.74% (40 μg/ml) and 3.03% (80 μg/ml). Inter-day accuracy showed deviations of 1.94% (0.47 μg/ml), 0.53% (4 μg/ml), −0.32% (20 μg/ml) and 0.06% (80 μg/ml). The method is rugged and robust and has been applied to the batch analysis of valproate during pharmacokinetic profiling of the drug.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Internal standards; [$^{18}O_2$]Valproic acid

1. Introduction

Valproic acid (VPA; 2-*n*-propylpentanoic acid) is a C_8-branched carboxylic acid widely used for the treatment of seizure disorders [1]. Several methods for the quantitation of VPA in plasma have been described. Due to the volatile nature of the target, most assays are based on gas chromatography–mass spectrometry (GC–MS). A variety of derivatives and detection modes have been employed: electron ionization (EI) of methyl ester derivatives [2], trimethylsilyl (TMS) derivatives [3,4] or *t*-butyldimethylsilyl derivatives [5–7]. A very sensitive method has been elaborated using negative ion chemical ionization (NICI) of the pentafluorobenzyl derivatives [8,9]. This method provides excellent sensitivity, but sample work-up is critical due to high background interference of reagent and derivatized matrix components [10]. Underivatized VPA was analyzed using GC with flame ionization detection [11]. High-per-

*Corresponding author. Tel.: +43-316-385-4037; fax:+43-316-385-3300.
E-mail address: hans.leis@kfunigraz.ac.at (H. Jörg Leis).

1570-0232/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S1570-0232(02)00755-9

formance liquid chromatography has also been used for the detection of VPA, requiring derivatization with a suitable chromophore or fluorophore [12,13].

One problem associated with volatile analytical targets like VPA is the critical loss of sample and internal standard during sample concentration steps. Although maximum sensitivity is not an ultimate requirement to follow the pharmacokinetic time-course of orally administered VPA, unequal loss of analyte and standard may cause large variations in the measurements. Thus, the use of a stable isotope-labeled standard would be essential.

For pharmacokinetic applications robustness and short analysis time is a major concern since processing of a large number of samples is usually involved. It was therefore the aim of this study to elaborate a method for the determination of VPA in human plasma that meets the requirements of sensitivity, specificity, speed and ruggedness for pharmacokinetic applications. The major objective was to elaborate a method using a stable isotope-labeled internal standard and a sample work-up and derivatization procedure avoiding any sample concentration steps.

2. Experimental

2.1. Chemical and reagents

N-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS), and silylation grade pyridine were obtained through Pierce (Rockford, IL, USA). Sodium valproate was a kind gift of Biokinet Laboratories, Vienna. $H_2^{18}O$ (96.1 atom% ^{18}O) was from Campro Scientific, (Veenendaal, The Netherlands). All other solvents and reagents of analytical grade were from Merck (Darmstadt, Germany).

2.2. Preparation of [$^{18}O_2$]valproic acid

Sodium valproate (50 mg) was dissolved in 0.3 ml of $H_2^{18}O$ and 10 μl of fuming HCl was added. The vial was closed and left at 75 °C for 4 days. After cooling, the mixture was extracted with 0.5 ml *n*-hexane and the solvent evaporated carefully at room temperature under a gentle stream of nitrogen. The residue was dissolved in methanol to yield stock solutions of the internal standard. Purity and isotopic distribution were checked by GC–MS after derivatization to the TMS ester.

2.3. Gas chromatography–mass spectrometry

A Finnigan TRACE 8000 GC coupled to a Finnigan TRACE quadrupole MS (ThermoQuest, Vienna) was used. The GC was fitted with a DB–5MS fused-silica capillary column (15 m×0.25 mm I.D., Thermoquest). The injector was operated in the split mode (split ratio 1:10) at 260 °C. Helium was used as a carrier gas at a constant flow-rate of 1.5 ml/min. Initial column temperature was 50 °C for 1 min, followed by an increase of 40 °C/min to 300 °C and an isothermal hold of 2 min. The mass spectrometer transfer line was kept at 310 °C. EI was performed at an electron energy of 70 eV and an emission current of 0.150 A. During single ion recording, m/z 201.13 and m/z 205.14 were recorded for target and internal standard, respectively, with a dwell time of 30 ms.

2.4. Sample preparation

A volume of 50 μl of the methanolic solution of the internal standard, containing 3.2 μg [$^{18}O_2$]valproic acid were pipetted into a 5-ml polypropylene tube and 100 μl of plasma was added. After short, vigorous shaking, 1 ml HCl (0.2 *M*) was added followed by 0.5 ml *n*-hexane. The tubes were stoppered and shaken at a reciprocal shaker for 15 min. After centrifugation at 4000 rpm for 10 min, 200 μl of the (upper) organic layer were transferred to a 96-well deep-well plate (polypropylene). A volume of 50 μl reagent solution (MSTFA, containing 1% TMCS in pyridine, 2:1, v/v) was added. The plate was closed with a polypropylene seal, mixed, left at room temperature for 15 min and stored at −20 °C until analysis.

2.5. Analytical method validation

Calibration graphs were established in the range of of 0.47 μg/ml plasma to 120 μg/ml plasma. For this purpose, blank plasma was spiked with the appropriate amounts of VPA by adding 50 μl of the

corresponding methanolic solution. Standard solutions of VPA were prepared by serial dilution in methanol to yield concentrations of 0.47, 0.938, 1.875, 3.75, 7.5, 15, 30, 60 and 120 μg/50 μl. A volume of 50 μl of these solutions were added to 950 μl of blank plasma to obtain calibration plasma samples. Standard solutions were stored at −20 °C. Blank plasma was checked for possible VPA content before use. For calibration, weighted linear regression analysis was applied (weighting factor: $1/s^2$).

For accuracy and precision measurements, daily calibration curves were prepared. The lower limit of quantitation (LOQ) was set to 0.47 μg/ml plasma as required for pharmacokinetic studies. LOQ must not be below the limit of detection, for which a signal-to-noise ratio of at least 3:1 is required.

Inter-day precision was determined at 0.47 (LOQ), 4, 20 and 80 μg/ml plasma by carrying one sample at each concentration level throughout the analytical sequence (sample preparation and GC–MS analysis) at 5 consecutive days. Spiked samples were prepared from blank plasma.

Intra-day precision was determined at 0.47 (LOQ), 4, 20 and 80 μg/ml plasma by carrying five samples at each concentration level throughout the analytical sequence and analyzing the samples at 1 day. Spiked samples were prepared from blank plasma.

Accuracy of the methods was also tested at the above mentioned concentrations. Thus, the data from inter- and intra-day precision measurements were used to calculate the deviation of the values measured from the actual spiked values.

Specificity was tested by analyzing 10 different blank plasma samples.

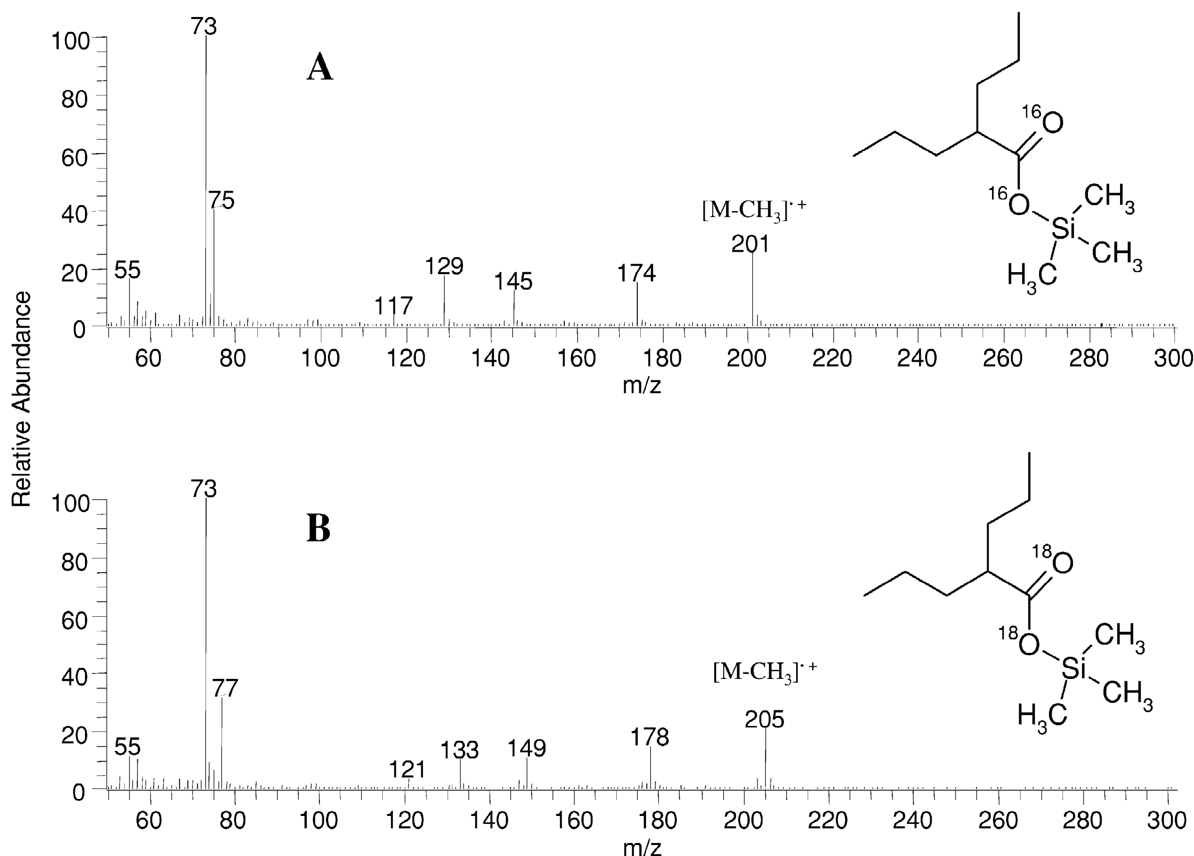

Fig. 1. EI mass spectrum of (A) native and (B) $^{18}O_2$-labeled valproic acid TMS ester.

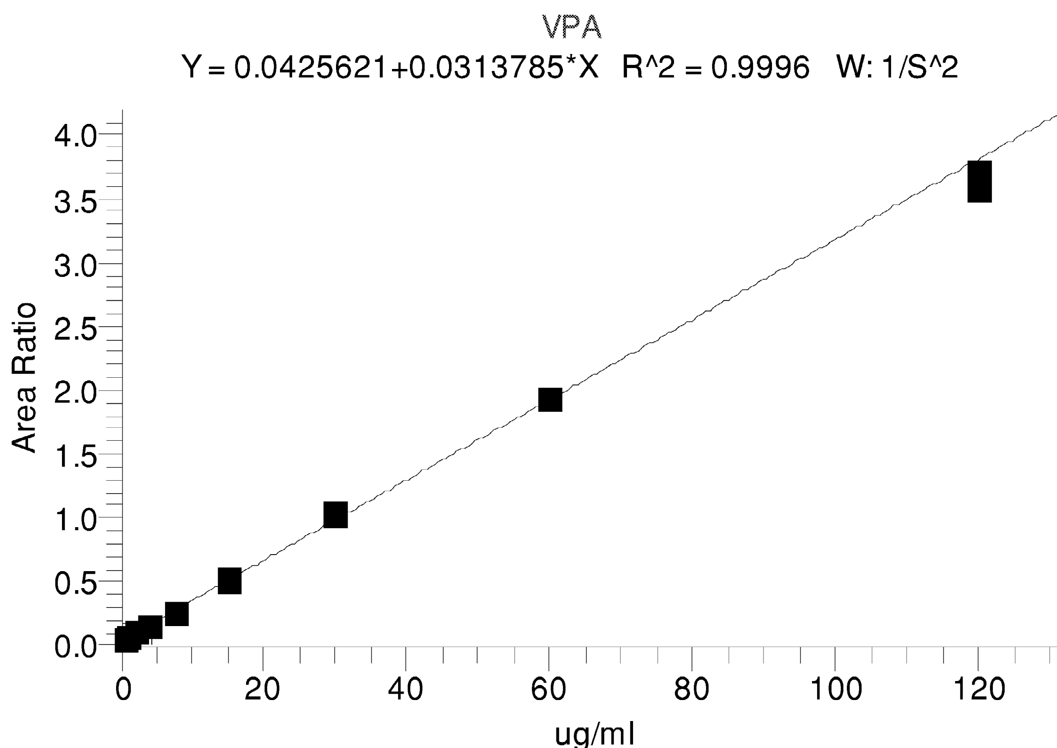

Fig. 2. Typical calibration graph obtained after analysis of valproic acid as its TMS ester derivative.

To measure freeze–thaw stability plasma samples were analyzed immediately after spiking with the indicated amounts of VPA and after three freeze–thaw (F–T) cycles.

For assessment of short-term stability, five spiked samples at 2 and 10 μg/ml concentration levels are melted to room temperature and left to stand 3 h at room temperature. After that another five identical samples of the same concentrations are melted and all samples are spiked with the internal standard and analyzed. Concentrations are calculated and the means are compared.

For determination of long-term stability, five spiked samples at 4 and 80 μg/ml concentration levels are analyzed immediately. Another five identical samples of the same concentrations are analyzed after 4 weeks storage time at −20 °C. Area ratios are calculated and the means are compared.

Autosampler stability (i.e. the precision of the GC–MS measurements under reproducibility conditions) is measured by analyzing five spiked samples at 2 and 10 μg/ml concentration levels immediately after work-up and repeating the analysis 24 h later. Concentrations are calculated and the means are compared.

3. Results and discussion

3.1. Isotopic labeling of the internal standard

The labeling procedure described herein offers a rapid way to stable isotope-labeled valproic acid. Under the conditions described, no unlabeled species was present, as confirmed by mass spectrometry. The procedure yielded the desired product in quantitative yield, showing an isotopic composition of 9.8% single-labeled and 90.2% double-labeled product. No side reactions or decomposition was observed by GC–MS. The isotopic purity with regard to unlabeled VPA is demonstrated in Fig. 3B.

Table 1
Intra-day precision and accuracy of VPA determination in human plasma

Spiked amount (μg/ml)	0.47	4	20	80
Mean	0.481	3.921	19.828	77.698
SD	0.011	0.115	0.639	3.421
n	5	5	5	5
C.V. (%)	2.29	2.93	3.22	4.40
Accuracy (%)	2.52	−1.99	−0.86	−2.88

Table 2
Inter-day precision and accuracy of VPA determination in human plasma

Spiked amount (μg/ml)	0.47	4	20	80
Mean	0.478	4.021	19.937	80.047
SD	0.007	0.152	0.546	2.425
n	5	5	5	5
C.V. (%)	1.49	3.79	2.74	3.03
Accuracy (%)	1.94	0.53	−0.32	0.06

3.2. Sample preparation

The extraction procedure described here offers a rapid way to isolate VPA from the plasma matrix. Thus, we have achieved extraction and derivatization of batches with 250 samples routinely within 5 h, with two technicians working on it. For volatile targets like VPA, loss of compounds during sample concentration steps is a serious problem. This method avoids any of these and thus assures high reproducibility for the measured response. Additionally, time-consuming solvent evaporation steps are omitted and thus analysis speed is significantly enhanced. No increase in signal intensity was observed after derivatization for 15 min at room temperature, thus indicating complete conversion to the derivative. No interference from polypropylene vial components were detected, as shown in Fig. 3B. Hexane extracts were by far cleaner than extracts using more polar solvents, like ethyl acetate. Additionally, hexane was superior to other solvents regarding the formation of emulsions at the phase boundary. Extraction recovery was above 90%, as determined by extracting a defined amount of VPA and adding the internal standard after the extraction step.

The TMS derivative was stable for at least 4 weeks at −20 °C and 6 days at room temperature. Samples were stored at −20 °C to assure unattenuated possibility of sample re-analysis over a wide time period during analysis of large sample numbers.

3.3. Mass spectrometry

For the detection of VPA we have used EI mass spectrometry of the TMS derivative. The EI mass spectrum of the TMS derivative of VPA is shown in Fig. 1a. Fig. 1b shows the corresponding mass spectrum of the $^{18}O_2$-labeled internal standard. The EI mass spectrum shows a fragment ion at m/z 201, resulting from α-cleavage of the Si–C bond by loss of a methyl radical. For $^{18}O_2$-labeled VPA, the corresponding ion is found at m/z 205. These fragment ions were used for quantification after selected ion recording.

As expected, fragment ions arising from alkyl chain cleavage are shifted by four mass units in the spectrum of $^{18}O_2$VPA.

3.4. Analytical method validation

The calibration graphs established were linear within the tested range of 0.47 μg/ml plasma to 120 μg/ml plasma, using 3.2 μg [$^{18}O_2$]VPA internal standard. A typical calibration curve with the statis-

Table 3
Freeze–thaw stability of plasma samples containing VPA

Spiked amount (μg/ml)	Amount found before 3 F–T cycles (μg/ml)	Amount found after 3 F–T cycles (μg/ml)	% found after 3 F–T cycles
2	2.000	2.008	100.40
10	10.244	10.201	99.58
40	41.142	41.682	101.31

Plasma samples were analyzed immediately after spiking with the indicated amounts of VPA and after three freeze–thaw (F–T) cycles.

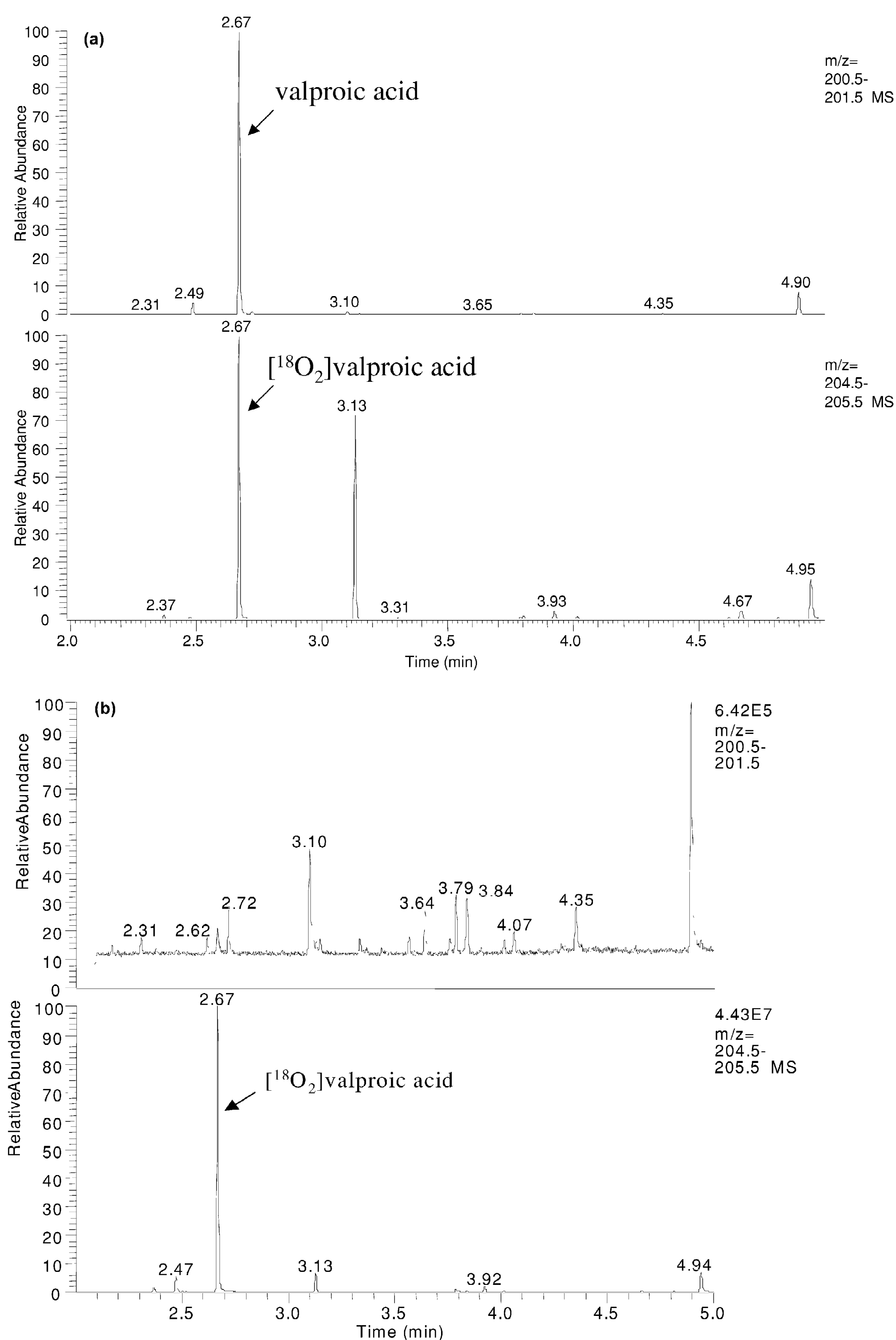

Fig. 3. Single ion recording (SIR) mass chromatogram obtained after analysis of a plasma sample from a human volunteer (A) receiving 500 mg sodium valproate orally and (B) blank sample without VPA.

tical data is shown in Fig. 2. For pharmacokinetic measurements, the limit of quantitation (LOQ) was set to 0.47 μg/ml plasma.

The coefficients of inter- and intra-day variation and accuracy of the spiked samples are presented in Table 1 and Table 2. It can be seen from these data, that the method provides a highly precise and accurate assay for VPA in human plasma. This can be attributed to the use of a stable isotope-labeled internal standard. Mass spectrometry in combination with stable isotope dilution is a very powerful tool in external quality assessment schemes, and assays based on this technique can be regarded as reference procedures to validate other analytical methods.

No decomposition of standard and stock solutions was measurable after 2 months of storage at −25 °C. Investigations on freeze–thaw stability of plasma samples at three different concentrations did not indicate any sample degradation after three freeze–thaw cycles. The results are shown in Table 3. Plasma samples containing VPA were stable for at least 12 weeks when stored below −20 °C. Under analysis conditions, derivatized samples were stable for at least 48 h. Ten different blank matrices were checked for interferences. In none of the samples was there background contribution above 25% LOQ.

We have applied this method to the analysis of VPA in human plasma in the course of a randomized crossover pharmacokinetic study to compare the relative bioavailability of two different formulations of VPA. Volunteers received 500 mg of VPA and blood samples were drawn at different time-points (0, 1, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, 10, 12, 15, 24, 36, 48, 72 and 84 h). Values for all samples were within the calibrated range. There was no interference from other acidic drugs like diclofenac, salicylic acid and ketoprofen.

A typical mass chromatogram obtained after analysis of a plasma sample after oral administration of 500 mg of VPA is given in Fig. 3A, together with a chromatogram of a blank sample with internal standard (Fig. 3B).

The assay proved to be useful in the batch analysis of more than 800 plasma samples. The low amounts of sample and solvents needed due to the sensitivity of the EI detection allow rapid sample preparation and assure a high throughput in analysis. Under the conditions described, the VPA derivative elutes after 2.67 min from the GC. The total analysis cycle including cooling of the GC and equilibration time is 14 min per sample.

The analysis of VPA from human plasma is of major interest in pharmaceutical research. Pharmacokinetic applications require highly specific assays with high sample throughput capacity. This is met by the presented method, since no sample concentration steps are necessary. When full scan acquisitions of plasma samples were analyzed, the whole fatty acid pattern of these plasma samples was obtained. Thus, the method can be regarded as potentially applicable to the rapid GC–MS analysis of plasma fatty acids without any sample concentration steps. The facile preparation of stable isotope-labeled standards described herein also allows access to stable isotope dilution assays of these compounds at low costs and immediate availability of the required internal standards.

References

[1] W. Loscher, Arch. Int. Pharmacodyn. Ther. 249 (1981) 158.
[2] E. Gaetani, C.F. Laureri, M. Vitto, J. Pharm. Biomed. Anal. 10 (1992) 193.
[3] H. Nau, W. Wittfoth, H. Schafer, C. Jakobs, D. Rating, H. Helge, J. Chromatogr. 226 (1981) 69.
[4] A.W. Rettenmeier, W.N. Howald, R.H. Levy, D.J. Witek, W.O. Gordon, D.J. Porubek, T.A. Baillie, Biomed. Environ. Mass Spectrom. 18 (1989) 192.
[5] D. Yu, J.D. Gordon, J. Zheng, S.K. Panesar, K.W. Riggs, D.W. Rurak, F.S. Abbott, J. Chromatogr. B 666 (1995) 269.
[6] J. Darius, F.B. Meyer, J. Chromatogr. B 656 (1994) 343.
[7] H.P.J.M. DeJong, J. Elema, B. van den Berg, Biomed. Mass Spectrom. 7 (1980) 359.
[8] K. Kassahun, K. Farrel, J. Zengh, F. Abbott, J. Chromatogr. 527 (1990) 327.
[9] M.R. Anari, R.W. Burton, S. Gopaul, F.S. Abbott, J. Chromatogr. B 742 (2000) 217.
[10] R.L. Dills, D.D. Shen, J. Chromatogr. B 690 (1997) 139.
[11] A. Carlin, J. Simmons, J. Chromatogr. B 694 (1997) 115.
[12] J.P. Moody, S.M. Allan, Clin. Chim. Acta 127 (1983) 263.
[13] J.H. Wolf, L. Veenma-van der Duin, J. Korf, J. Chromatogr. 487 (1989) 496.