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Sensitive capillary gas chromatographic–mass spectrometric method for the therapeutic drug monitoring of valproic acid and seven of its metabolites in human serum Application of the assay for a group of pediatric epileptics

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Abstract

A sensitive capillary gas chromatographic–mass spectrometric method for the determination of valproic acid and 7 of its metabolites is described. It is based on the selected-ion monitoring of the *tert.*-butyldimethylsilyl derivatives using N-(*tert.*-butyldimethylsilyl)-N-methyl-trifluoracetamid (MTBSTFA) as the derivatization reagent. The limits of detection for valproic acid and its metabolites are in the low ng/ml-range, except for the 4-hydroxy metabolite with a limit of detection of 100 ng/ml. The method has been tested against an established GC method for valproic acid. The assay has been used for therapeutic drug monitoring in epileptic pediatric patients. The concentrations of the ω- and ω1-oxidation metabolites in a group of patients receiving additional antiepileptic drugs were found to be significantly enhanced compared to the levels found in a monotherapy group.

1. Introduction

Valproic acid [2-(*n*-propyl)-pentanoic acid, VPA] is an antiepileptic drug effective on a variety of seizure types and currently the drug of choice for the treatment of generalized tonic-clonic seizures and absences [1]. VPA has been applied in the treatment of psychotic disorders [2–4].

The parent compound is extensively metabolized [5,6], *e.g.* by glucuronidation, dehydrogenation, and oxidation to hydroxy- and keto-metabolites (For a complete scheme see ref. 6). Some of the metabolites also have or may have an antiepileptic activity, as described for (E)-2-en-VPA in several animal models [5,7]. A very rare, but severe hepatotoxic side effect is associated with VPA-therapy. Metabolites with hepatotoxic potential (*e.g.* 4-en-VPA [8,9]) may be the cause of this side effect.

The mechanism of this hepatopathy is still unknown as pointed out recently by Siemes *et al.* [10]. Their study on the largest number of patients so far showed correlations between the

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hepatotoxic symptoms and disturbances in the metabolic pattern. In all patients with probable VPA-associated hepatotoxicity, Siemes *et al.* found some aspects of the VPA metabolism differing distinctly from a reference group. However, the question whether these metabolic changes forebode a hepatotoxicity or if they are the consequence of liver damage, is still unanswered.

The results published by Siemes *et al.* [10] suggest that measuring several selected metabolites along with the parent drug during therapeutic drug monitoring (TDM) of valproate may be of value to indicate an existing or beginning hepatopathy. If one of the metabolic pathways is blocked or saturated somehow, then the metabolite ratios between pathways will change significantly.

Therefore, the aim of this work was to implement an assay for the simultaneous determination of valproic acid and its metabolites, and to monitor the amounts of these substances over the period of therapy.

The assays reported in the literature are usually based on the GC–MS technique with derivatization of valproic acid and its metabolites [11–22]. Most of them use trimethylsilyl derivatization [11–18] and only a few make use of the higher sensitivity of *tert.*-butyldimethylsilyl (*t*BDMS) derivatives [19–21]. There is one publication featuring a very sensitive but less stable negative-ion chemical ionization (NICI) detection [22].

We describe an assay for the TDM of VPA and 7 of its metabolites. The assay combines an extraction procedure not forcing labile compounds to extreme pH values and a derivatization technique resulting in low detection limits. The method has a long-term stability without the need for recalibration and quality control samples are used. The assay is thus suitable for prospective studies. No isotope-labelled standards are necessary for this assay. It is based on a GC–MS–computer system, uses *t*BDMS derivatives and has been applied for TDM in the routine control of the therapy of pediatric patients.

2. Experimental

2.1. Reagents

Valproic acid was obtained from Desitin (Hamburg, Germany). 2-(*n*-Propyl)-pent-4-enoic acid (4-en-VPA) and the sodium salt of 2-(*n*-propyl)-pent-(E)-2-enoic acid [(E)-2-en-VPA] were a kind gift from Prof. H. Nau (Berlin, Germany). The 4-keto-2-(*n*-propyl)-pentanoic acid (4-keto-VPA), 2-(*n*-propyl)-glutaric acid (2-PGA), 5-hydroxy-2-(*n*-propyl)-pentanoic acid δ-lactone (5-OH-VPA) and 4-hydroxy-2-(*n*-propyl)-pentanoic acid γ-lactone (4-OH-VPA) were purchased from Applichem (Gatersleben, Germany).

Internal standards were 2-ethyl-2-methylcaproic acid (EMCA) from Ferak (Berlin, Germany) and 3,3-dimethylglutaric acid (DMGA) from Merck (Darmstadt, Germany).

The reagents used for sample extraction and derivatization were ethyl acetate and N-trimethylsilyl-N-methyl-trifluoracetamide (MSTFA) from Merck, acetonitrile from J.T. Baker (Groß-Gerau, Germany), N-(*tert.*-butyldimethylsilyl)-N-methyl-trifluoracetamid (MTBSTFA) from Aldrich (Steinheim, Germany), and NaH_2PO_4 from Reanal (Budapest, Hungary). All reagents were of analytical grade.

2.2. Internal standards

All mono-*t*BDMS derivatives (VPA, 4-keto-VPA, 4-en-VPA, (E)- and (Z)-2-en-VPA) were quantified using EMCA as the internal standard. DMGA has been used as the internal standard for all compounds giving diderivatives (4-OH-VPA, 5-OH-VPA and 2-PGA).

The standard solution was prepared by diluting 10 mg of each of the two substances in 1 l of ethyl acetate.

2.3. Sample preparation

For extracting the sample, 100 μl of the serum were pipetted into a 1.5-ml Eppendorf microtube. Following the method introduced by

Nau *et al.* [11], 50 μl of 1 *M* NaH_2PO_4 buffer (adjusted to pH 5) and 100 μl of the standard solution were added. In this way, the samples were extracted at a pH that should lead to good recoveries [11], and the metabolites were not exposed to extreme pH values [14].

As extraction solvent a 1-ml volume of ethyl acetate was used. The vials were rotated for 20 min and centrifuged for 5 min at 500 *g*. The supernatant organic phase was transferred to a 3-ml glass tube and preconcentrated to approximately 100 μl under a stream of nitrogen at 37°C in a heating block. Meanwhile the sample was extracted a second time with another 1-ml volume of ethyl acetate. The organic phases were combined and 10 μl of acetonitrile were added to avoid uncontrolled loss of the more volatile components [11]. The ethyl acetate phase was reduced to *ca.* 20 μl. A 40-μl volume of MTBSTFA was added and the sample was shaken vigorously. The samples were transferred to automated-sampler microvials fitted with 250-μl inserts. The vials were capped tightly and heated for 1 h at 70°C in a heating block to allow VPA and its metabolites to react to their *t*BDMS derivatives. Aliquots (1 μl) of the samples were injected onto the GC system.

2.4. Instrumentation

The GC–MS–computer system (Hewlett-Packard, Waldbronn, Germany) consisted of a HP5989A MS-Engine, a HP5890 Series II-GC, a HP7673A automated sampler, and a HP Apollo 400t computer with UNIX-based Chemstation software, version C.01.01.

The GC column was a nonpolar HP1 capillary column (25m × 0.2 mm I.D., film thickness 0.33 μm) operated with a helium head pressure of 75 kPa. The split/splitless injector was used in the splitless mode with a splitless time of 1 min.

The temperatures of the injector block, GC–MS transfer line, the source and the mass analyzer were 250, 250, 200 and 100°C, respectively. The samples were injected at an oven temperature of 60°C. This temperature was held during the splitless period and then raised rapidly to 90°C within 1 min. Up to 150°C, the heating rate was 5°C/min. Then the heating rate was set to 40°C/min until a temperature of 250°C was reached, which was held for 3.5 min. The total run time for the assay is 20 min.

The analyses were performed by in the selected-ion monitoring (SIM) mode after electron-impact ionization.

3. Results and discussion

In our assay, we focused on the analyses of VPA, 4-en-VPA, 4-keto-VPA, 5-OH-VPA, 4-OH-VPA, 2-PGA, (E)- and (Z)-2-en-VPA.

For derivatization, we tested the most commonly used TMS derivatives [11,13–18], and the *t*BDMS derivatives suggested by Abbott *et al.* [19]. In accordance with their results, we found an increased sensitivity especially for monounsaturated metabolites when using *t*BDMS derivatives.

The fragments usually detected in the SIM of *t*BDMS derivatives and used for quantitation are the intense m/z $[M-57]^+$ ions, which correspond to the loss of the *tert.*-butyl fragment. We measured these ions except in two cases. The m/z $[M-57+1]^+$ ion is selected for VPA. The reasons for this decision are described below. In the second case, the m/z 199 ion was selected for the 4-OH-VPA *t*BDMS diderivatives. As can be seen from Fig. 1, this ion is more intense than the $[M-57]^+$ ion (m/z 331). The ions detected for the compounds of interest are listed in Table 1. Other characteristic ions are cited in refs. 19–21. For ions detected in the SIM (see Table 1), no interfering peaks from endogenous compounds or the derivatization reagent were seen. This has been tested by analyzing blank serum samples.

A problem in the analysis of valproic acid and its metabolites is the wide range of concentrations to be covered. Whereas VPA can be found in amounts up to 200 μg/ml in serum, the concentrations of some metabolites are in the low ng/ml range. To measure VPA and its metabolites simultaneously in one chromato-

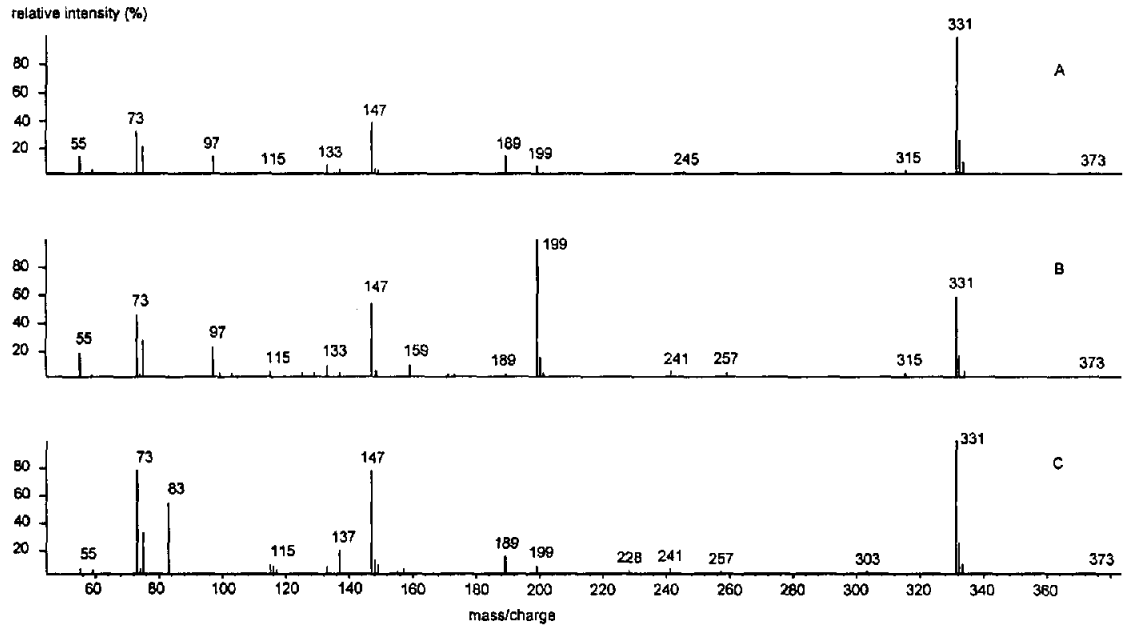

Fig. 1. Mass spectra of the *t*BDMS diderivatives of 5-OH-VPA (A), 4-OH-VPA (B), and DMGA (C).

graphic run, it was preferred to detect the *m/z* 202 ion for valproic acid, which is the isotopic ion of the $[M-57]^+$ fragment usually analyzed. The detected fragment has an abundance of *ca.* 15% of that of the *m/z* 201 ion. So, even for the highest VPA concentrations, the maximum peak lies in the linear range of the detector, and the analysis parameters could be optimized for the low-level metabolites like 4-en-VPA.

The huge concentration difference between VPA and 4-en-VPA also caused another problem: sufficient resolution is necessary to separate the very close eluting VPA and metabolite peaks. As reported by others [17], slow temperature

Table 1
Characteristic ions of *t*BDMS derivatives used in the SIM, slopes of the calibration curves and their relative standard deviation

Compound	Ion	Slope	R.S.D.(%)	I.S.
VPA	202	0.0204	6.9	EMCA
4-en-VPA	199	0.1008	1.2	EMCA
(E)-2-en-VPA	199	0.1265	3.9	EMCA
(Z)-2-en-VPA	199	0.0925	2.2	EMCA
4-keto-VPA	215	0.0359	4.7	EMCA
4-OH-VPA	199	0.0108	11.0	DMGA
5-OH-VPA	331	0.0554	3.1	DMGA
2-PGA	345	0.0731	7.0	DMGA
EMCA	215			
DMGA	331			

The slopes are mean values obtained by repeated calibration on 5 consecutive days. The I.S. column shows the internal standard used for the compound.

programming and long capillary columns are required to provide adequate resolution and to prevent an isobaric ion peak resulting from the mass spectrum of the VPA-*t*BDMS derivative from overlapping with the fragment peak of the 4-en-VPA-*t*BDMS ester. Otherwise, a correct quantitation of the metabolite is difficult.

As can be seen from Figs. 2 and 3, sufficient baseline resolution was achieved for these two peaks.

The parent drug and those of its metabolites reacting only to their mono- or di-*t*BDMS esters, *i.e.* 4-en-VPA, 2-en-VPA and 2-PGA, gave good and reproducible results after 1 h of derivatization. Analyses of samples derivatized for 2 and 4 h showed that derivatization is already completed after 1 h. The more problematic metabolites for derivatization are those that are capable of forming ethers as well as esters. That is the case for the hydroxy- and keto-metabolites. Whereas 5-OH-VPA reacts readily to its diderivative within 1 h (Fig. 4), and 4-keto-VPA only produces the mono-*t*BDMS ester, three types of derivatives were found to result from 4-OH-VPA. The first one is the 4-OH-VPA lactone, the second the mono-*t*BDMS ester, and the third the mixed *t*BDMS ester–*t*BDMS ether derivative. Because the diastereomeric forms of all these different derivatives of 4-OH-VPA were separated on the column used, six peaks can be detected altogether. In contrast to the data reported by Abbott *et al.* [19], all three forms were seen in all patient samples analyzed. The lactone form of 4-OH-VPA is the dominant one, but its peaks were disturbed by an unknown but obviously endogenous substance. The best linearity was observed for the diderivatives, but due to their low intensity, the limit of detection was 100 ng/ml when using 100-μl samples. Until now, no attempts have been made to use a larger serum volume for sample preparation. The main reason is that getting more blood from neonates and small children, *i.e.* the high-risk group with respect to hepatopathy [23,24], is usually problematic. Although it is obviously not possible to monitor normal levels of 4-OH-VPA, markedly

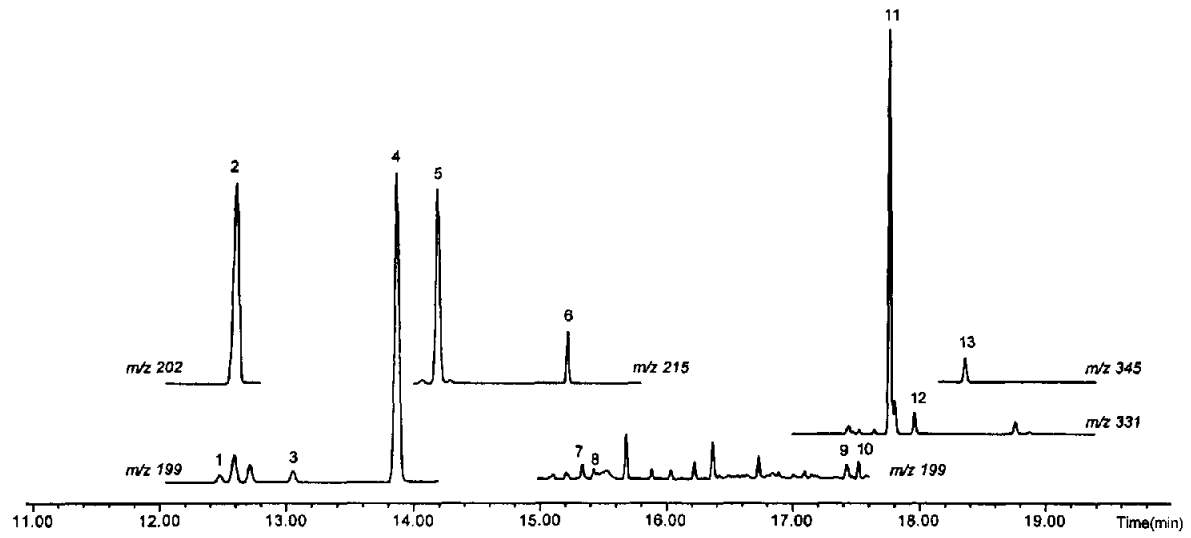

Fig. 2. Ion chromatograms of a patient serum sample. The chromatograms of the ions m/z 199 (from 12 to 14 min), m/z 199 (from 15 to 17.6 min), m/z 331 and m/z 345 are multiplied by 2, 20, 4 and 30, respectively. Peaks: 1 = 4-en-VPA (0.278 μg/ml), 2 = VPA (103.1 μg/ml), 3 = (Z)-2-en-VPA (0.416 μg/ml), 4 = (E)-2-en-VPA (10.4 μg/ml), 5 = EMCA (I.S. 1), 6 = 4-keto-VPA (0.998 μg/ml), 7,8 = 4-OH-VPA (monoderivates), 9,10 = 4-OH-VPA (diderivates, 0.31 μg/ml), 11 = DMGA (I.S. 2), 12 = 5-OH-VPA (1.26 μg/ml), 13 = 2-PGA (0.167 μg/ml).

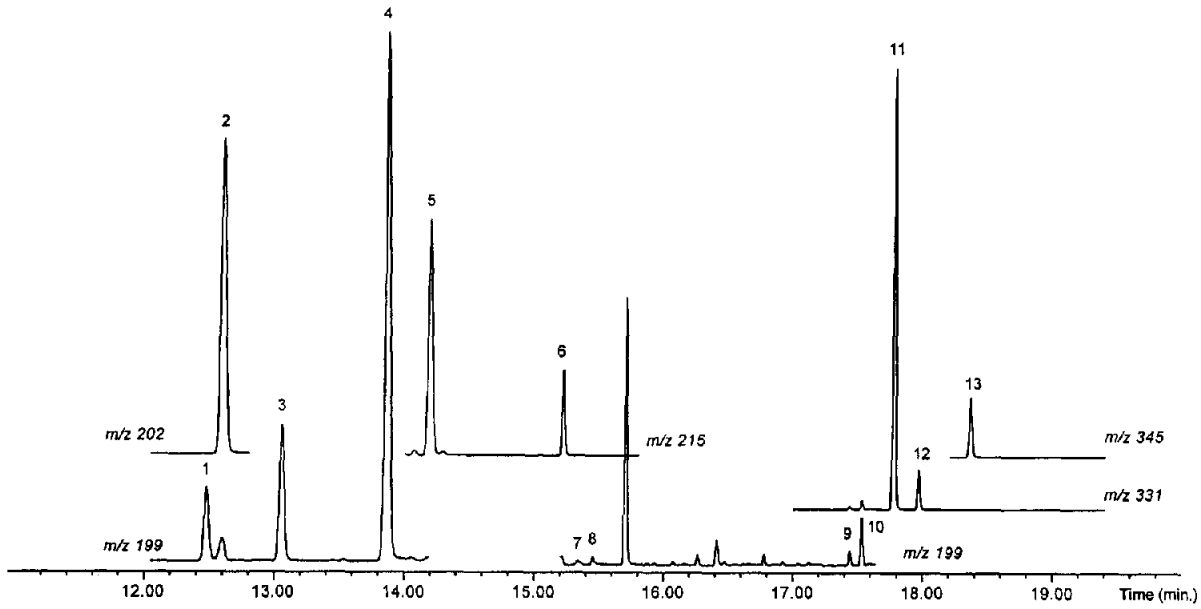

Fig. 3. Ion chromatograms of a quality control sample QCS 3 (blank serum spiked with VPA and metabolites, see text). The chromatograms of the ions *m/z* 199, *m/z* 331 and *m/z* 345 are multiplied by 5, 3 and 2, respectively. Peaks: 1 = 4-en-VPA, 2 = VPA, 3 = (Z)-2-en-VPA, 4 = (E)-2-en-VPA, 5 = EMCA (I.S. 1), 6 = 4-keto-VPA, 7,8 = 4-OH-VPA (monoderivates), 9,10 = 4-OH-VPA, 11 = DMGA (I.S. 2), 12 = 5-OH-VPA, 13 = 2-PGA. For concentrations see Table 2.

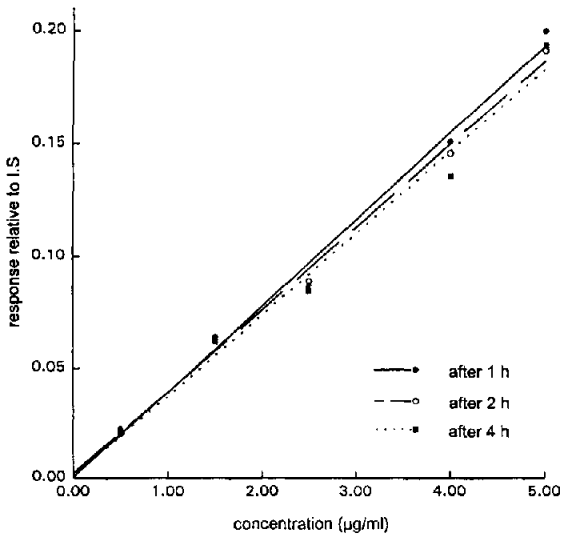

Fig. 4. Calibration graphs of 5-OH-VPA after 1, 2 and 4 h of derivatization. The slopes and correlation coefficents are: after 1 h: slope 0.0639, $r = 0.995$; after 2h: slope 0.0634, $r = 0.997$; after 4h: slope 0.0621, $r = 0.991$.

elevated amounts of this metabolite can be detected in serum. The quantitation of these diderivatives in urine should be easier due to the elevated concentrations found there. It must be noted here, that two important compounds are still missing in the assay, 3-keto-VPA and (E,E)-2,3′-dien-VPA. However, the assay should easily be extendable to these metabolites. Only the lack of adequate reference compounds hindered their quantification so far. Under the conditions described and using MTBSTFA as the derivatizing agent, 3-keto-VPA is converted to its diderivatives. Two peaks, obviously corresponding to the different isomers, having the mass spectra of di-*t*BDMS-3-keto-VPA [19] have been observed in the serum samples.

3.1. Calibration and use of quality control samples

The calibration samples were prepared by adding small volumes of the metabolite solutions in water to drug free serum to yield samples with known amounts of up to 4 metabolites per

calibration batch. So, VPA, 4-en-VPA, (E)-2-en-VPA and 2-PGA were calibrated in the first batch, the remaining four metabolites in the second.

All calibrations were performed as ten-point calibration curves over the range of concentrations cited in the literature as measured in patients under therapy [10,16,17]. The calibration graphs gave correlation coefficients exceeding 0.995 and limits of detection below 10 ng/ml, except for 4-OH-VPA. For the latter, the limit of detection is around 100 ng/ml (signal-to-noise ratio 3:1 for m/z 199). The slopes of the calibration graphs are listed in Table 1 together with the relative standard deviations obtained from analyses performed on five consecutive days.

On each day, three quality control samples (QCS) corresponding to three different concentration levels were analyzed prior to the patient samples to test the system performance concerning peak resolution and derivatization. The QCS were prepared by spiking drug free serum with known amounts of VPA and the metabolites measured in this assay. The 10-ml volume of each sample was divided into 1-ml portions which were stored frozen at −21°C.

The standard deviations for the concentrations in each QCS were determined by repeating the analysis ten times. So, a control range was defined as mean ± 3 times the standard deviation. The results are presented in Table 2. A system check or recalibration of the whole assay was performed when the amounts of one or more compounds were outside this range. The assay was found to be stable over more than one month without recalibration. This procedure seems to be preferable to an every-day calibration accompanied by an every-day tuning of the mass spectrometer as described in ref. 17. The use of the automated sampling device gave an extremely good reproducibility from injection to injection, and it enabled the analysis of more than 30 samples per day.

The assay was compared with a GC–FID method [25] for VPA which has been used in our laboratory for several years. VPA concentrations in patient samples were determined with both assays. The results of the parallel analyses are shown in Fig. 5. The correlation coefficient of 0.98 shows the good comparability of the two methods. A paired t-test did not show a significant difference on the 95% level.

3.2. Patient samples

Samples of pediatric, epileptic patients were analyzed as a routine control of antiepileptic therapies. The patients received valproic acid

Table 2
Spike values, mean measured values and their relative standard deviations of VPA and its metabolites in the quality control samples (QCS)

Compound	QCS 1			QCS 2			QCS 3		
	Spiked value (μg/ml)	Mean (μg/ml)	R.S.A. (%)	Spiked value (μg/ml)	Mean (μg/ml)	R.S.D. (%)	Spiked value (μg/ml)	Mean (μg/ml)	R.S.D. (%)
VPA	18.00	17.71	5.5	36.00	38.50	3.2	72.00	72.77	1.4
4-en-VPA	0.10	0.10	9.1	0.30	0.30	8.0	0.60	0.61	9.1
(E)-2-en-VPA	0.41	0.42	2.8	1.90	1.96	2.6	3.80	3.74	2.0
(Z)-2-en-VPA	0.10	0.11	6.5	0.50	0.52	8.7	1.00	0.99	5.7
4-keto-VPA	0.72	0.75	4.0	3.15	3.29	5.7	6.30	6.29	5.3
4-OH-VPA	0.30	0.35	15.2	0.60	0.64	10.5	1.20	1.12	8.9
5-OH-VPA	0.28	0.24	10.2	1.30	1.28	9.7	2.60	2.74	7.6
2-PGA	0.29	0.24	8.6	1.59	1.60	4.3	2.90	2.88	9.3

The mean values and their relative standard deviations (R.S.D.) are received from a ten-fold repetition of the analyses of the quality control samples.

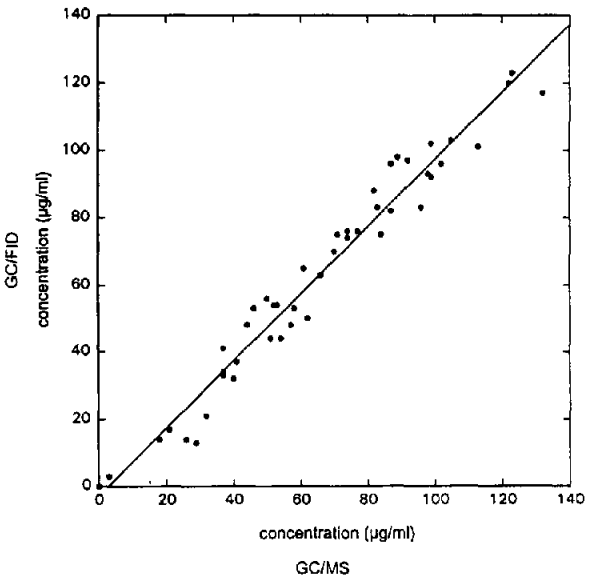

Fig. 5. Comparison between a GC–FID method [25] and the GC–MS method described here. VPA concentrations were measured in 46 patient samples. The correlation coefficient was $r = 0.98$. A paired t-test did not show significant differences at the 95% level.

either as monotherapy ($n = 58$) or as polytherapy ($n = 40$). The results are summarized in Table 3. The measured concentrations of VPA and its metabolites match the results published by others [14,16].

Table 3
Serum concentrations of VPA-metabolites in a group of pediatric patients

Compound	Monotherapy ($n = 58$)	Polytherapy ($n = 40$)
4-en-VPA	0.207 ± 0.105	0.255 ± 0.089[a]
(Z)-2-en-VPA	0.458 ± 0.158	0.445 ± 0.178
(E)-2-en-VPA	8.29 ± 3.85	9.32 ± 5.18
5-OH-VPA	0.820 ± 0.328	1.43 ± 0.57[a]
4-keto-VPA	1.01 ± 0.65	1.84 ± 1.11[a]
2-PGA	0.116 ± 0.062	0.185 ± 0.073[a]

[a]Significantly different from the monotherapy group (t-test, $p < 0.05$).

The mean age of the group was 8.7 ± 5.9 years. The mean VPA levels were 78.9 ± 31.1 µg/ml for monotherapy group and 69.0 ± 33.4 µg/ml for the polytherapy group. The concentrations of the metabolites are percent of the VPA concentrations. All values are given as means ± S.D.

In accordance with Kondo *et al.* [16] we found an elevated concentration of 4-en-VPA in the polytherapy group. However, as can be seen from Table 3, all products of VPA-ω- and ω1-oxidation (5-OH-VPA, 4-keto-VPA, 2-PGA) have higher levels in the polytherapy group. Similar results for 5-OH-VPA and 2-PGA are reported by other authors [10], [22]. This elevation may be due to an enzyme induction by coadministered drugs as reported by Perucca *et al.* [26]. On the other hand, a significantly decreased (E)-2-en-VPA concentration, as reported in refs. 16 and 22, could not be detected. The differences in the results may be due to the age and number of patients investigated and the different normalizations of the results (to VPA level [ref. 16 and this work], or to VPA dose [22]). To verify such results, a broader database and more detailed analyses of the patient group seem to be necessary.

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