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On-column gas chromatographic–mass spectrometric assay for metabolic profiling of valproate in brain tissue and serum

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Abstract

A sensitive capillary gas chromatographic–mass spectrometric method for the determination of valproic acid and at least twelve of its metabolites in serum based on *tert.*-butyldimethylsilyl (*t*BDMS) derivatives is described. Low detection limits are achieved by using a direct on-column injection technique. The addition of dry pyridine during the derivatization step now leads to uniform formation of 3-keto-VPA di-*t*BDMS derivatives and thereby avoids the necessity of a deuterated internal standard. A novel extraction procedure for metabolic profiling of valproate in brain tissue samples is presented. Using this method, (Z)-2-en-VPA was determined in rat brain tissue for the first time.

Keywords: Derivatization, GC; Valproate

1. Introduction

Valproic acid [VPA; 2-(*n*-propyl)-pentanoic acid] is the antiepileptic drug of first choice for the treatment of generalized seizures and absences [1] and equipotent to carbamazepine for treating focal seizures [2]. VPA is known to be metabolized intensively forming numerous metabolites representing various metabolic pathways [3]. Several of these metabolites are expected to contribute to the anticonvulsive activity of VPA [4–6] or are considered to be responsible for neurotoxic [6–8] and hepatic side effects [9].

The interest in the metabolic fate of VPA and the kinetics of its metabolites led to the development of a number of assays for the quantification of these compounds in biological material. Most of these assays are based on GC–MS using trimethylsilyl (TMS) [10–17] or *tert.*-butyldimethylsilyl (*t*BDMS) derivatives [18–22]. There is one publication featuring a very sensitive but less stable negative ion-chemical ionization detection method [23]. Methods reported as suitable for analyzing brain tissue samples are rare and often limited to certain groups of metabolites [24] or by background interferences [26].

We present here a method for the analysis of VPA and twelve of its metabolites in serum or plasma and a procedure for metabolic profiling of VPA in brain tissue samples. The positive influence of pyridine as catalyst during the derivatization step and the advantages of using an on-column injector are discussed.

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2. Experimental

2.1. Reagents

VPA was obtained from AWD (Dresden, 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 H. Nau (Berlin, Germany). The 2-(*n*-propyl)-pent-(Z)-2-enoic acid ((Z)-2-en-VPA), 2-(*n*-propyl)-pent-(E)-3-enoic acid (3-en-VPA), 2-(*n*-prop-(E)-2-enyl)-pent-(E)-2-enoic acid [(E,E)-2,3′-dien-VPA], 3-hydroxy-2-(*n*-propyl)-pentanoic acid (3-OH-VPA), 4-keto-2-(*n*-propyl)-pentanoic acid (4-keto-VPA), 2-(*n*-propyl)-glutaric acid (2-PGA), 2-(*n*-propyl)-succinic acid (2-PSA), 4-hydroxy-2-(*n*-propyl)-pentanoic acid (4-OH-VPA) as its γ-lactone, 5-hydroxy-2-(*n*-propyl)-pentanoic acid (5-OH-VPA) as its δ-lactone and 3-keto-2-(*n*-propyl)-pentanoic acid (3-keto-VPA) as its ethyl ester were purchased from Applichem (Gatersleben, Germany).

The reagents used for sample extraction and derivatization were ethyl acetate and HCl from Merck (Darmstadt, Germany), acetonitrile from J.T. Baker (Groß-Gerau, Germany), N-(*tert.*-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA) from Aldrich (Steinheim, Germany), pyridine, Na_2SO_4 (anhydrous), KH_2PO_4 and Na_2HPO_4 from VEB Laborchemie Apolda (Apolda, Germany). All reagents were of analytical grade. The pyridine had been dried and distilled over KOH (VEB Laborchemie Apolda).

2.2. Internal standards

Internal standards were 2-ethyl-2-methylcaproic acid (EMCA) from Ferak (Berlin, Germany), 3-methylglutaric acid (MGA) from Aldrich (Steinheim, Germany) and 3-hydroxy-2-(*n*-[$^2H_7$]propyl)-pentanoic acid (3-OH[$^2H_7$]-VPA), which was a kind gift from F.S. Abbott (Vancouver, Canada).

All mono-*t*BDMS derivatives [VPA, 4-keto-VPA, 3-en-VPA, 4-en-VPA, (E,E)-2,3′-dien-VPA, (E)- and (Z)-2-en-VPA] and the 4-OH-VPA γ-lactones were quantified using EMCA as the internal standard. MGA has been used as the internal standard for all compounds reacting to diderivatives (5-OH-VPA, PGA, PSA and 3-keto-VPA). 3-OH-VPA has been quantified by using its heptadeuterated analogue. The standard solution was prepared by diluting 20 mg of each of the three substances in 1 l of ethyl acetate.

2.3. Calibration samples

A calibration sample was prepared by adding known volumes of aqueous, alkaline solutions of the reference substances to drug free serum. Previously, the 3-keto-VPA ethyl ester was treated with 0.04 *M* NaOH at 35°C for 1 h in a thermomixer to hydrolyze the ester. The sample was diluted with the drug-free serum to achieve 10 equidistant calibration samples. Calibration samples for brain tissue analyses were prepared by spiking the tissue sample prior to homogenization. Drug free brain tissue was obtained from untreated rats. The concentration ranges of the analytes used for calibration are given in Table 1.

2.4. Sample preparation

2.4.1. Serum or plasma samples

For extracting a sample, 120 μl of 1 *M* HCl, 10 μl of the internal standards solution and 1 ml of ethyl acetate were added to 100 μl of the serum or plasma. The samples were rotated for 20 min and centrifuged for 5 min at 500 *g*. The supernatant organic phase was transferred to a conical glass tube and preconcentrated to approximately 100 μl under a stream of nitrogen. Meanwhile the sample was extracted a second time with another 1 ml of ethyl acetate. The organic phases were combined, dried over Na_2SO_4, transferred to another conical glass tube and 10 μl of acetonitrile was added. The ethyl acetate phase was reduced to about 20 μl; 20 μl of pyridine and 40 μl of MTBSTFA were then added and the sample was shaken vigorously. The sample volumes were transferred to automated-sampler micro vials fitted with 250 μl inserts. The vials were capped tightly and heated for 1 h at 50°C. Aliquots (1 μl) of the samples were injected onto the GC system.

2.4.2. Brain tissue samples

The same amount of 0.067 *M* phosphate buffer (pH 6.8) was added to the brain tissue samples and the mixture was treated for 3 min using a homogenizer with a Teflon piston. About 400 μl of the

Table 1
Concentration ranges used for the calibration of VPA and its metabolites in serum and brain tissue samples and inter-day precision of the assays

Substance	Ranges for calibration		R.S.D. (%)[a]	
	Serum (μm/ml)	Brain tissue (μg/g)	Serum	Brain
VPA	14.93–149.3	14.93–149.3	11.5	14.1
(E)-2-en-VPA	0.40–4.0	0.020–0.20	9.8	19.9
(Z)-2-en-VPA	0.046–0.458	0.018–0.183	9.3	19.8
(E)-3-en-VPA	0.252–2.52	0.025–0.252	10.0	21.4
4-en-VPA	0.050–0.50	0.010–0.10	16.8	20.9
(E,E)-2,3'-dien-VPA	0.4–4.0	0.020–0.20	9.9	19.7
3-OH-VPA	0.472–4.72	0.024–0.236	12.9	23.0
4-OH-VPA	0.2–2.0	0.050–0.50	11.2	79.5
5-OH-VPA	0.093–0.925	0.093–0.925	10.1	27.0
3-keto-VPA	1.06–10.6	0.042–0.424	17.8	22.2
4-keto-VPA	0.19–1.90	0.019–0.190	15.4	21.0
PGA	0.021–0.209	0.021–0.209	12.9	20.0
PSA	0.002–0.021	0.002–0.021	16.4	20.3

[a] R.S.D. at the lowest point of the calibration curve ($n=10$).

homogenate was weighed into an Eppendorf microtube. According to the method described above, the samples were extracted twice with ethyl acetate after adding 120 μl of 1 *M* HCl and 10 μl of the I.S. solution and the volume of the organic phase was reduced to about 20 μl.

In contrast to the procedure for serum samples, an additional clean-up step was introduced here. For this purpose, 0.5 ml chloroform was added together with 200 μl of phosphate buffer (0.067 *M*, pH 8.6). The samples were extracted on a roller shaker for 20 min and centrifuged for 5 min at 500 *g*. The upper layer (ethyl acetate+buffer) was transferred to a 3-ml conical glass tube. Another 200-μl volume of phosphate buffer was added to the sample and the extraction procedure was repeated. The combined aqueous phases were adjusted to pH 2 by adding 1 *M* HCl. Then the procedure of extracting with ethyl acetate and reducing the organic phase in volume was repeated as at the beginning of the sample treatment. The resulting 20-μl volume of the organic phase was derivatized in the same way as that of the serum samples. Aliquots (1 μl) of the samples were injected onto the GC system.

2.5. Instrumentation

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

The GC column was a DB1701 capillary column [30 m×0.25 mm I.D., film thickness 0.25 μm, Fisons Instruments, (Mainz-Kastel, Germany)] equipped with a retention gap (5 m×0.25 mm I.D., Hewlett-Packard) and operated with helium at a constant flow of 1 ml/min. An on-column injector was used in connection with a kit for direct automatic injection onto the capillary column (Hewlett-Packard).

The temperatures of the GC–MS transfer line, the ion source and the mass analyzer were 260, 200 and 100°C, respectively. The injector temperature followed the oven temperature program but was always 3°C higher. The temperature started at 70°C (held for 0.10 min) and then was raised to 100°C at a rate of 20°C/min. Up to 135°C, the heating rate was 2°C/min. Then the rate was set to 15°C/min until an upper temperature of 270°C, which was held for 1.9 min. The total run time for the assay was 30 min.

2.6. Selected-ion monitoring (SIM)

The analyses were performed by SIM after electron impact ionization. The ions selected for the monitoring represented the intense m/z $[M-57]^+$

ions of the *t*BDMS derivatives. For VPA, the $[M-57+1]^+$ ion has been selected [22]. 4-OH-VPA isomers were detected as γ-lactones. The most intense ion of their mass spectra, m/z 100, was used for the monitoring.

3. Results and discussion

The method for serum sample analyses represents a modified version of the two published in Refs. [21] and [22]. In contrast to our previous assay [22], the pH during the extraction was lowered (pH 2 instead of pH 5) resulting in higher recoveries for the dicarboxylic acids. 3-Keto-VPA is known to decarboxylate partially under these conditions. But, it was shown, that the major portion remains intact [18]. We took the good linearity of the calibration curves and precision of the determinations (see Table 1) as evidence that the decarboxylation is either negligible or, at least, reproducible.

We added pyridine in the derivatization step forcing the 3-keto-VPA to form diderivatives exclusively and thereby avoiding the necessity of a deuterated internal standard. The keto function of 3-keto-VPA enolized to yield Z- and E-isomers which are separated on the GC column used. As has been stated by other authors [21], 3-keto-VPA could be quantified using only the larger peak of the two. This holds true for using MGA as the internal standard instead of a deuterated analogue. The use of pyridine as a catalyst for the silylation forces the 3-OH-VPA isomers to form mainly but not exclusively diderivatives. Therefore a deuterated standard was found to be essential for the quantification of the 3-OH-VPA isomers. 3-OH-VPA could be forced to form only diderivatives by the addition of dry crystals of sodium or potassium acetate. Such a procedure had been suggested for silylation of corticosteroids at their hydroxy groups [27]. But this led to a loss of the derivatives of PGA and PSA, the dicarboxylic metabolites of VPA. In our assay the diderivatives of 3-OH-VPA exhibited the more intensive signals and better peak shapes as compared with the mono-*t*BDMS derivatives. The latter show a significant tailing [21]. So, in contrast to Yu et al. [21], we used the diderivatives for quantification.

The use of the on-column injector provides a better sample transfer onto the column compared with a split/splitless injector. This might be due to the broad range of volatility of the analytes injected. Any temperature chosen for a split/splitless injector would represent a compromise leading to discrimination against either low or high volatile compounds. In comparison to our former split/splitless method [22], we experienced at least 2-fold higher peaks. For PSA, a metabolite occurring at low ng/ml concentrations, a detection limit of 0.5 ng/ml was achieved in serum (defined as $S/N > 3$).

The retention gap was shown to be essential for sufficient peak form and resolution. At higher concentrations a fronting of the VPA peak could be observed, indicating an overloading of the column (Fig. 1). This did not influence the quantification of VPA significantly. Correlation coefficients of the calibration curves usually exceeded 0.995. The inter-day and intra-day precision at the lowest point of the calibration curves were within $\pm 18\%$ for all metabolites in the serum sample assay.

The method reported for plasma or serum samples was shown not to be suitable for extracting brain tissue samples. Experiments with brain tissue homogenates resulted in colored samples contaminated by coextracted endogenous compounds. We also experienced the formation of a precipitate during the last step of reducing the solvent volume. In the chromatograms, large late eluting peaks appeared. The column was deteriorated after a couple of runs. Extraction at a higher pH (5.0) as proposed by Nau et al. [10] did not lead to significantly better results. This is consistent with observations by Semmes and Shen [24]. On the other hand, the methods favored by them and Adkinson et al. [25] using chloroform as the solvent for extracting VPA and some unsaturated metabolites and sample clean-up by back-extraction, is not suitable for the analysis of more polar metabolites (with hydroxy-, keto- or a second carboxylic moieties). So we tried to combine the advantage of extracting with ethyl acetate with the ability of chloroform to provide extracts from tissue samples suitable for injection onto a GC column.

Almost all metabolites detectable in the serum assay could be quantified in the brain tissue samples as well. So, for the first time the concentration of

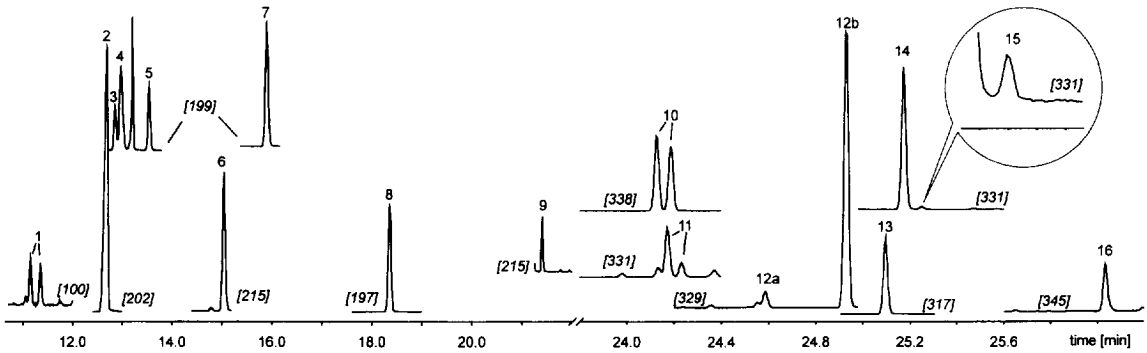

Fig. 1. SIM chromatograms of a patient serum sample. Numbers in brackets represent m/z values. The chromatograms of the ions m/z 100, m/z 199 (from 12.6 to 14 min), m/z 331 (from 25 to 25.6 min) and m/z 345 are multiplied by 10, m/z 215 (from 14.4 to 15.2 min) and m/z 338 by 2, m/z 215 (from 21.3 to 22 min) by 4 and m/z 331 (from 23.8 to 24.4 min) by 5. Peaks: (1) 4-OH-VPA γ-lactones, (2) VPA, (3) 4-en-VPA, (4) (E)-3-en-VPA, (5) (Z)-2-en-VPA, (6) EMCA (I.S.), (7) (E)-2-en-VPA, (8) (E,E)-2,3′-dien-VPA, (9) 4-keto-VPA, (10) 3-OH[$^2H_7$]-VPA, (11) 3-OH-VPA, (12) 3-keto-VPA, (13) MGA (I.S.), (14) 5-OH-VPA, (15) PSA, (16) PGA.

(Z)-2-en-VPA was determined in rat brain. 4-Keto-VPA and PGA, mentioned by Löscher et al. [26] as not being quantified in their assay because of peak interferences from the background, exhibited sharp and undisturbed peaks (Fig. 2). Problems occurred for the quantification for 4-OH-VPA. Under the acidic conditions during the sample preparation, the 4-OH-VPA enantiomers form γ-lactones. In contrast to the 5-OH-VPA δ-lactone which returns to its open-chain form on pH-changes or during derivatization, the 4-OH-VPA γ-lactones remain stable. Therefore the 4-OH-VPA had an insufficient extraction recovery in the back-extraction step.

For all other metabolites, the detection limits (and the limits of quantification as well) are about 2–3-fold higher as compared with the serum assay. Due to its low concentrations in brain tissue, PSA could not be found in amounts exceeding its limit of quantification (0.003 μg/g). This limited our assay to the quantification of 10 metabolites in brain tissue samples. The assays for brain tissue and serum samples should be easily extended to metabolites like (Z)-3-en-VPA, (E,Z)-2,3′-dien-VPA, (E)- and (Z)-2,4-dien-VPA. These have been resolved by Yu et al. [21] under similar chromatographic conditions and may have been seen in our assay but could not be quantified due to the lack of appropriate reference substances.

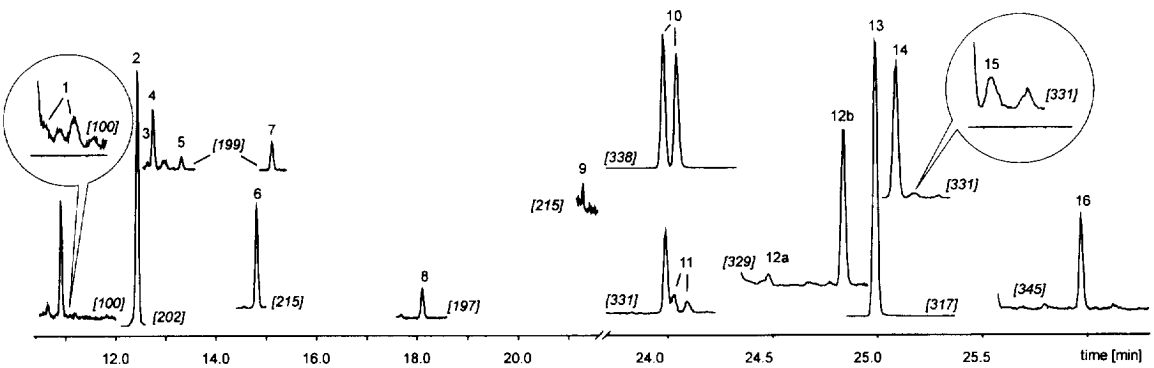

Fig. 2. SIM chromatograms of a rat brain tissue sample (striatum). Numbers in brackets represent m/z values. The chromatogram of the ion m/z 100 is multiplied by 10, m/z 197, m/z 199, m/z 329 and m/z 331 (from 25.1 to 25.4 min) by 30, m/z 215 (from 14.4 to 15 min) and m/z 338 by 3, m/z 215 (from 21.2 to 21.6 min) by 100, m/z 331 (from 23.8 to 24.4 min) and m/z 345 by 50. Peaks: (1) 4-OH-VPA γ-lactones, (2) VPA, (3) 4-en-VPA, (4) (E)-3-en-VPA, (5) (Z)-2-en-VPA, (6) EMCA (I.S.), (7) (E)-2-en-VPA, (8) (E,E)-2,3′-dien-VPA, (9) 4-keto-VPA, (10) 3-OH[$^2H_7$]-VPA, (11) 3-OH-VPA, (12) 3-keto-VPA, (13) MGA (I.S.), (14) 5-OH-VPA, (15) PSA, (16) PGA.

4. Conclusions

An improved GC–MS assay for analyzing VPA and up to 12 of its metabolites in serum/plasma or brain tissue was presented. For the brain tissue samples, a novel back-extraction was reported. Usage of the on-column injection technique provided significantly better sample transfer leading to lower detection limits. The derivatization step was modified resulting in an exclusive formation of 3-keto-VPA di-*t*BDMS derivatives and in larger amounts of the respective 3-OH-VPA diderivatives.

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