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Gas chromatographic determination of novel valproyl taurinamide derivatives in mouse and dog plasma

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Abstract

Valproyl taurinamides are a novel group of compounds that possess anticonvulsant activity. In this study a gas chromatographic micromethod was developed for the quantification of selected valproyl taurinamides and some of their metabolites in biological samples. Valproyl taurinamide (VTD), *N*-methyl valproyl taurinamide (M-VTD), *N*,*N*-dimethyl valproyl taurinamide (DM-VTD) and *N*-isopropyl valproyl taurinamide (I-VTD) were analyzed in mouse and dog plasma and in dog urine using gas chromatography. Flame ionization detection and mass spectrometric detection were compared. The plasma samples were prepared by solid-phase extraction using C_{18} cartridges. The urine samples were prepared by liquid–liquid extraction. The sample volume used was 100 μl of dog plasma, 50 μl of mouse plasma and 20 μl of dog or mouse urine. The quantification range of the method was 1.5–50 mg/l in dog plasma (VTD only), 2.5–250 mg/l in mouse plasma (0.7–90 pmol injected) and 0.04–2 mg/ml in dog urine (VTD only). The inter-day precision in plasma and urine samples was around 10% for all quantified concentrations except LOQ (15–20%). The accuracy for all four compounds was between 90 and 110% within the entire concentration range. The developed method was suitable for quantification of a series of CNS-active valproyl taurineamide derivatives in biological samples at relevant in vivo concentrations.

Keywords: Derivatization, GC; Valproyl taurinamide

1. Introduction

Valproyl taurinamides are a novel group of amide derivatives of valproic acid (VPA), one of the major antiepileptic drugs (AEDs; [1]). Valproyl taurinamide (VTD) is a follow-up compound to valproyl glycinamide (VGD, valrocemide) a new antiepileptic drug currently in phase II clinical trials [2]. From a

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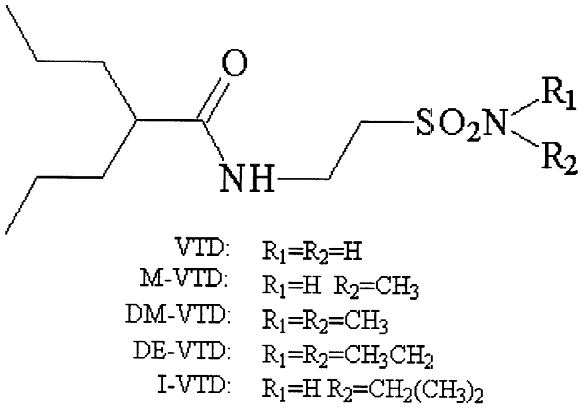

Fig. 1. Chemical structures of the valproyl taurinamide derivatives.

series of structure–activity relationship studies, three valproyltaurinamide derivatives; VTD, *N*-isopropyl valproyl taurinamide (I-VTD) and *N*,*N*-dimethyl valproyl taurinamide (DM-VTD), have shown anticonvulsant activity. The chemical structures of these compounds are presented in Fig. 1. The plausible metabolic pathway of DM-VTD and I-VTD is *N*-dealkylation leading to *N*-methyl valproyl taurinamide (M-VTD) and to VTD. As a crucial part of the design and development of new AEDs, their biological fate must be analyzed, which requires a robust analytical method with a wide quantification range to be utilized in both toxicological and pharmacological studies.

VPA and its aliphatic amide derivatives and analogs, have been analyzed using gas chromatography (GC; [3–5]), whereas the *N*-alkylated derivatives of VGD have been analyzed by HPLC [6]. However, unlike VGD and its *N*-alkylated derivatives, the valproyl taurinamides are not good candidates for HPLC analysis since the sulfonamide is a poor chromophore. Consequently, GC was chosen for method development.

There are very few reports in the literature of sulfonamide analysis using GC. Therefore the chromatographic characteristics, volatility, temperature stability and mass spectral fragmentation of these novel compounds was unknown. This study reports GC analysis of a series of valproyl taurinamides using mass spectrometric (MS) or flame ionization detection (FID).

2. Materials and methods

2.1. Chemicals

Chloroform and methanol for the GC analysis were obtained from J.T. Baker (Deventer, The Netherlands), and were of HPLC grade. All the solvents used in chemical synthesis were obtained from Frutarom, Israel, and were of analytical grade. Double-distilled water (DDW) was used throughout the study. The amines used in the chemical reactions were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Synthesis of the taurinamide derivatives

The valproyl taurinamide derivatives were synthesized by the following procedures:

Valproyl taurine: 36.5 g of taurine (0.29 mol, 1 equivalent, eq) was dissolved in 200 ml of 15% NaOH solution. 47.4 g of valproyl chloride (0.29 mol, 1 eq) was added dropwise to the solution, and the mixture was stirred overnight at room temperature. The solution was then acidified and evaporated to dryness in vacuo. The residue was mixed in 1 l boiling ethanol, dissolving the valproyl taurine produced, and the undissolved salts were filtered off. The valproyl taurine was crystallized from ethanol by adding 300 ml of diethyl ether. Recrystallization was performed from methanol yielding 51 g (0.19 mol) of the sodium salt of valproyl taurine (yield 64%). Valproyltaurine had a melting point (mp) of 245 °C.

NMR (300 MHz, H_2O): δ 3.58–3.61 (m, 2H), δ 2.81–2.97 (t, 2H), δ 2.15–2.21 (m, 1H), 1.47–1.55 (m, 2H), δ 1.25–1.36 (m, 6H), δ 0.88–0.92 (t, 6H)

Valproyl taurinyl chloride: Valproyl taurine (15 g, 0.055 mol, 1 eq) was chlorinated using 19.6 g of thionyl chloride (0.17 mol, 3 eq) in 200 ml of dry dichloromethane (DCM). The reaction was stirred under $CaCl_2$ at 60 °C for 24 h and the excess of DCM and $SOCl_2$ were distilled off under reduced pressure. The solid residue containing the valproyltaurinyl chloride was dissolved in dry DCM and stored at −20 °C until used.

Valproyl taurinamide (VTD): To 100 ml of 25% aqueous ammonia, 5 g valproyltaurinyl chloride (0.018 mol) dissolved in dry DCM was added

dropwise, and the mixture was left to react for 1 h in room temperature. Then, 50 ml of saturated aqueous NaCl were added and the reaction mixture was extracted twice with 100 ml of ethyl acetate. The organic phases were combined and washed with diluted HCl, dried over Na_2SO_4 and evaporated to dryness in vacuo. Crude yield was 4 g. Crystallization from chloroform and hexane yielded 2.9 g (0.012 mol) of pure crystals (yield 64%). The mp of the obtained sulfonamide was 131 °C

Elemental microanalysis gave:

Obtained C: 47.85% H: 8.48%, N: 10.91%

Calculated C: 47.97%, H: 8.86%, N: 11.19%

NMR (300 MHz, $CDCl_3$): δ 7.5 (bs, 1H), δ 6.3 (s, 2H), δ 3.66–3.68 (m, 2H), δ 3.22–3.26 (t, 2H), δ 2.18 (m, 1H), δ 1.47–1.55 (m, 2H), δ 1.19–1.35 (m, 6H), δ 0.89–0.90 (t, 6H)

The other amides were prepared by similar procedures using aqueous solutions of methylamine, dimethylamine, isopropylamine or diethylamine as reactants.

The obtained amides had the following parameters:

N-Methyl valproyl taurinamide (M-VTD) (reaction yield 78%):

Elemental microanalysis:

Obtained C: 49.92% H: 8.85%, N: 10.47%

Calculated C: 49.96%, H: 9.17%, N: 10.60% mp 94 °C

NMR (300 MHz, $CDCl_3$): δ 6.3 (bt, 1H), δ 4.29–4.32 (d, 1H), δ 3.66–3.70 (q, 2H), δ 3.09–3.13 (m, 2H), δ 2.81–2.83 (d, 3H), δ 1.97–2.00 (m, 1H), δ 1.47–1.55 (m, 2H), δ 1.19–1.35 (m, 6H), δ 0.89–0.90 (t, 6H)

N,N-Dimethyl valproyl taurinamide (DM-VTD) (reaction yield 73%):

Elemental microanalysis:

Obtained C: 51.68% H: 9.16%, N: 10.04%

Calculated C: 51.76%, H: 9.43%, N: 10.06% mp 135 °C

NMR (300 MHz, $CDCl_3$): δ 6.3 (bt, 1H), δ 3.66–3.70 (q, 2H), δ 3.09–3.13 (m, 2H), δ 2.873 (s, 6H), δ 1.97–2.00 (m, 1H), δ 1.47–1.55 (m, 2H), δ 1.19–1.35 (m, 6H), δ 0.89–0.90 (t, 6H)

N-Isopropyl valproyl taurinamide (I-VTD) (reaction yield 72%):

Elemental microanalysis:

Obtained C: 53.09% H: 9.35%, N: 9.54%

Calculated C: 53.39%, H: 9.65%, N: 9.58% mp 85 °C

NMR (300 MHz, $CDCl_3$): δ 6.3 (bt, 1H), δ 4.29–4.32 (d, 1H), δ 3.66–3.70 (q, 2H), δ 3.53–3.58 (m, 1H), δ 3.09–3.13 (m, 2H), δ 1.97–2.00 (m, 1H), δ 1.47–1.55 (m, 2H), δ 1.19–1.35 (m, 6H), δ 1.24–1.27 (d, 6H), δ 0.89–0.90 (t, 6H)

N,N-Diethyl valproyl taurinamide (DE-VTD) (reaction yield 68%):

Elemental microanalysis:

Obtained C: 54.57% H: 9.59%, N: 9.16%

Calculated C: 54.87%, H: 9.87%, N: 9.14% mp 88 °C

NMR (300 MHz, $CDCl_3$): δ 6.3 (bs, 1H), δ 3.71–3.75 (q, 2H), δ 3.28–3.33 (q, 4H), δ 3.11–3.21 (m, 2H), δ 2.07 (m, 1H), δ 1.47–1.54 (m, 2H), δ 1.19–1.39 (m, 14H), δ 0.89–0.90 (t, 6H)

2.3. Stock solutions

Stock solutions of VTD, M-VTD, DM-VTD, I-VTD and DE-VTD were prepared by dissolving each compound in methanol to obtain concentrations of 1 mg/ml. Aliquots from these stock solutions were taken to prepare calibration samples in plasma and urine. Spiked plasma and urine samples were prepared by adding to a test tube the appropriate amount of the stock solution, evaporating this to dryness and adding the appropriate volume of plasma or urine.

2.4. Sample preparation

2.4.1. Preparation of plasma samples

The solid-phase extraction (SPE) cartridges (3 ml, 500 mg, Extra-Sep, Lida, Kenosha, WI, USA) were conditioned using 2 ml methanol and 1 ml DDW. For mouse plasma samples, 50 μl of plasma, 50 μl of internal standard solution (100 mg/l DE-VTD) and 0.5 ml DDW were combined and the mixture was vortexed and applied to the SPE cartridge. For dog plasma samples, 100 μl of plasma, 20 μl internal standard solution (100 mg/l I-VTD) and 0.9 ml water were combined, and the mixture was vortexed and applied to the SPE extraction cartridges. The SPE cartridge was washed using 0.5 ml (mouse plasma) or 1 ml (dog plasma) of DDW and the analytes were eluted with 2 ml of methanol. The

methanol was evaporated to dryness and the sample redissolved in 200 μl of chloroform.

2.4.2. Preparation of urine samples

To 20 μl of dog or mouse urine 10 μl of internal standard solution (I-VTD, 1 mg/ml for dog urine and DE-VTD, 1 mg/ml for mouse urine) and 1 ml chloroform was added. The solution was vortexed and centrifuged and the organic supernatant transferred to an autosampler vial, and 1 μl was injected into the GC apparatus. Dog urine samples were also treated with β-glucuronidase in order to study VTD-glucuronide formation. The enzymatic treatment was performed as follows: 200 μl urine were mixed with 200 μl phthalate buffer (0.2 *M*, pH5) and 30 μl β-glucuronidase (type H-2 from *Helix pomatia*, 89 400 units/ml, Sigma–Aldrich). The mixture was protected from light and incubated at 37 °C for 12 h. At the end of the incubation period, a 40-μl sample was taken and extracted using 1 ml of chloroform as described above for urine samples.

2.5. Gas chromatography

2.5.1. Method A: GC–FID

An HP 5890 Series II gas chromatograph equipped with a 7673A autosampler, flame ionization detector (FID) and HP chemstation was used (Hewlett-Packard, Waldbronn, Germany). The injector temperature was held at 250 °C and splitless injection was used. A quadrex 007 methyl 20% phenyl silicone capillary column (25 m, 0.25-μm film thickness, 0.25-mm I.D., Quadrex, New Haven, CT, USA) was used for separation. The temperature program included 2 min at an initial temperature of 100 °C, a gradient of 20 °C/min up to 180 °C, hold time of 1 min and a second gradient of 0.5 °C/min up to 200 °C, where held for 1 min. The detector was maintained at 300 °C. Nitrogen (99.99% pure) was used as a carrier gas at a head pressure of 70 kPa.

2.5.2. Method B: GC–MS

An HP 5890 Series II gas chromatograph equipped with a 6860 autosampler, an HP 5971 mass selective detector and HP Chemstation data analysis software was used (Hewlett-Packard). The chromatographic separation was obtained using an HP5 Trace analysis column (5% phenylmethylsiloxane, 25 m, 0.33-μm film thickness, 0.20-mm I.D. Agilent Technologies, Palo Alto, CA, USA). The injector operated on splitless mode and was maintained at 220 °C. The oven temperature program included 2 min at an initial temperature of 50 °C, a gradient of 40 °C/min up to 180 °C, hold time of 1 min, a second gradient of 0.5 °C/min up to 192 °C and a final gradient of 10 °C/min up to 220 °C/min. The detector was maintained at 300 °C. He (99.99% pure) was used as a carrier gas at a head pressure of 50 kPa.

The mass spectrometer transfer line temperature was set at 280 °C and the detector was operated on the selected ion mode scanning for ions m/z 162, 179, 208, 221–222, 234 and 264.

2.6. Calibration and method validation

Calibration curves in four replicates were prepared at a concentration range of 1.5–50 mg/l in dog plasma (VTD only), 2.5–250 mg/l in mouse plasma (VTD, I-VTD, M-VTD and DM-VTD), 60–4000 mg/l in dog urine (VTD only) and 25–1000 mg/l in mouse urine (VTD, I-VTD, M-VTD and DM-VTD). Peak area ratios of the test compound to the internal standard (DE-VTD for mouse plasma and urine and I-VTD for dog plasma and urine) were plotted as a function of the concentration of each analyte in the sample. For dog plasma, seven calibrators at concentrations 1.5–50 mg/l, and in mouse plasma 10 calibrators at concentrations 2.5–250 mg/l were used for the linear regression analysis. Calibration curves were constructed by the method of least-squares linear regression without weighting. Linearity of the calibration curves was tested by the F-test and a P value <0.1 was considered significant. In addition, the boundaries for linearity of the calibration line were also determined at the points where the slope of the line deviated from the overall slope by more than 5% [7].

The method was validated according to published guidelines [7,8]. Inter-day repeatability (measured as percent coefficient of variation) was calculated at three to nine different plasma concentrations (0.75–250 mg/l), with 8 to 12 replicate analyses of each concentration performed within 1 month in mouse and dog plasma, and six replicate analyses at each of

three different urine concentrations. The accuracy of the quantification was determined by dividing the obtained mean concentration from the replicate analyses by the known spiked concentration. Recovery was calculated by comparing the result for diluted standard samples to spiked plasma and urine (concentrations 25 and 250 mg/l in plasma and 60 and 2000 mg/l in urine) that underwent the whole process of sample preparation.

To test the applicability of the assay, plasma and urine samples taken from a dog following intravenous (i.v.) administration of VTD or from a mouse after intraperitoneal (i.p.) administration of I-VTD or DM-VTD, were analyzed.

3. Results and discussion

3.1. Chromatographic separation

The separation of the analytes from interfering endogenous compounds is shown in Figs. 2–4. Fig. 2 shows a chromatogram of blank mouse plasma, mouse plasma spiked with DM-VTD, I-VTD, M-VTD and VTD at the LOQ and chromatograms of mouse plasma samples obtained after administration of DM-VTD (300 mg/kg) or I-VTD (300 mg/kg) obtained using method A (GC–FID). A chromatogram of blank dog plasma, dog plasma spiked with VTD and a chromatogram of a dog plasma sample following administration of VTD (20 mg/kg) obtained using method B (GC–MS) are shown in Fig. 3. Fig. 4 depicts chromatogram of blank dog urine, dog urine spiked with VTD at the LOQ and chromatograms of dog urine after administration of VTD obtained using method B before and after β-glucuronidase treatment of the sample. The significantly greater selectivity of method B when compared to method A is demonstrated in the blank chromatograms shown in Figs. 2–3. In the GC–MS analysis (method B) no interfering peaks (or other matrix associated peaks) could be detected, whereas in the GC–FID method (method A) a number of peaks eluting relatively close to the analytes were observed. However, the greater separation of VTD and M-VTD by the 20% phenyl column (in method A) when compared to the 5% phenyl column (HP-5) in method B was clearly observed. Therefore, if M-VTD and VTD are expected in the same sample, a 20% phenyl column should be used for the separation.

A mild drawback of the chromatography was the evaluation of column capacity. At sample concentrations above 250 mg/l (100 pmol injected to column) some column overload (nonlinear chromatography) could be detected from fronting analyte peaks. Therefore, the column separation capacity is the limiting factor of analysis at the higher concentrations.

3.2. Sample preparation

Initial studies attempted using liquid–liquid extraction with organic solvents (chloroform, ethyl acetate and *tert*.-butylmethylether) gave insufficient recovery from plasma. Significant interference from matrix, that could not be separated from the analytes by chromatography was observed. Therefore, solid-phase extraction (SPE) was adapted. The developed SPE method was robust, simple to perform and demonstrated good capacity and repeatability. The efficacy of the sample preparation method is demonstrated in the GC–FID chromatograms. In urine, due to the high analyte concentrations, chloroform extraction was sufficient for sample preparation.

3.3. Mass spectrometry

The fragmentation pattern for the taurinamides investigated was slightly different. For all the compounds, the highest m/z value observed in the mass spectrum was obtained following the cleavage of the propyl group in the valproate part of the molecule (m/z values 208, 222, 236, 250 and 264 for VTD, M-VTD, DM-VTD, I-VTD and DE-VTD, respectively). Extensive fragmentation could also be observed. The fragment of m/z 162 was common to all the taurinamides monitored (abundance 14–100%) and was therefore selected as the ion monitored. In addition, ions of m/z 179 and 208 were monitored for VTD, m/z 221 and 222 for M-VTD and I-VTD, m/z 234 for DM-VTD and I-VTD and m/z 264 for DE-VTD. In the mass spectra, the characteristic M+2 peak of S^{32} and S^{34} isotopes were observed (4% natural abundance).

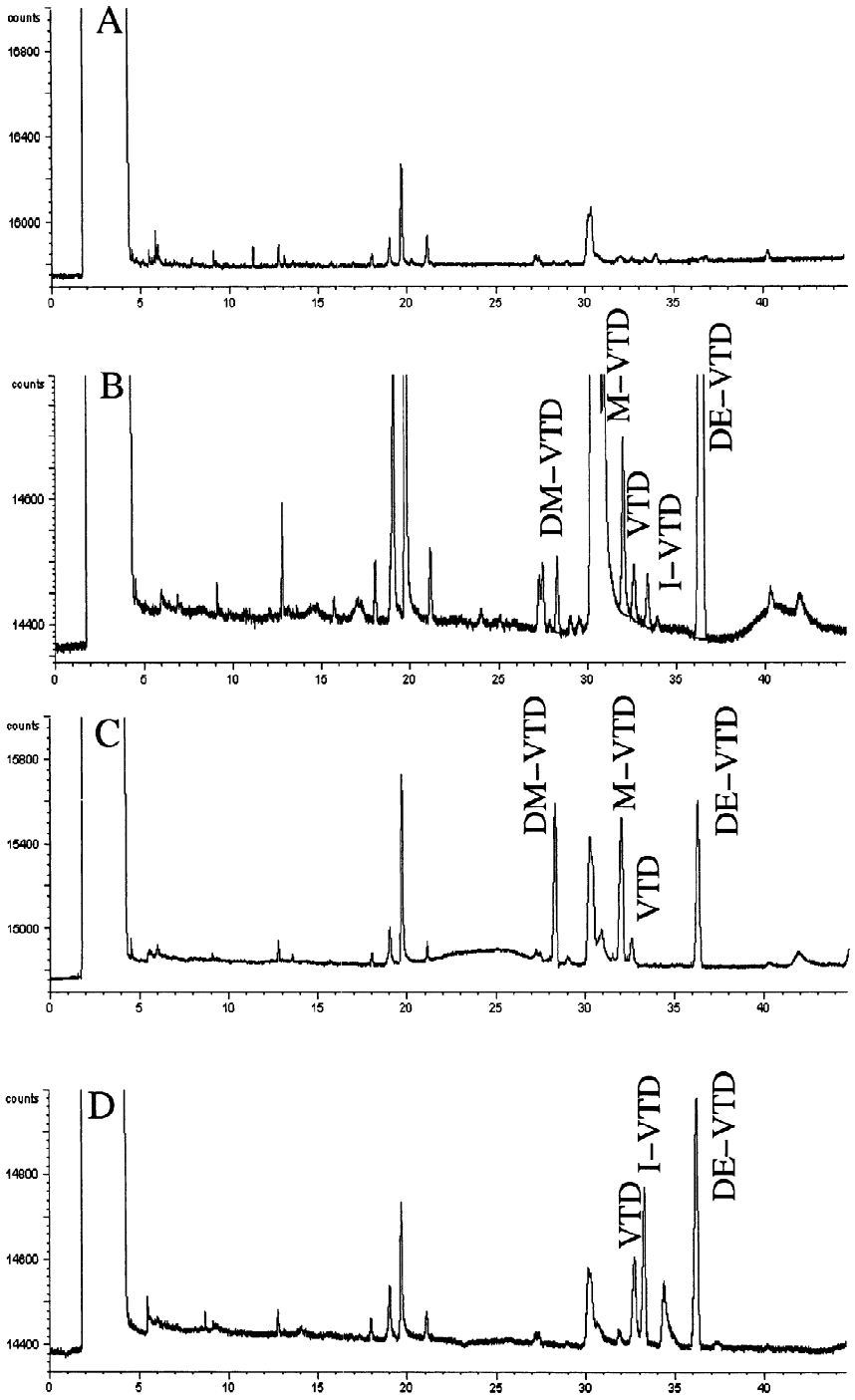

Fig. 2. Chromatograms from blank mouse plasma (A), from spiked mouse plasma at the LOQ (2.5 mg/l) (B) and from mouse plasma obtained 40 min after i.p. administration (300 mg/kg) of DM-VTD (determined concentrations 115 mg/l DM-VTD, 119 mg/l M-VTD and 26 mg/l VTD) (C) and 60 min after administration (300 mg/kg i.p.) of I-VTD (determined concentrations 74 mg/l VTD and 88 mg/l I-VTD), (D) analyzed by method A.

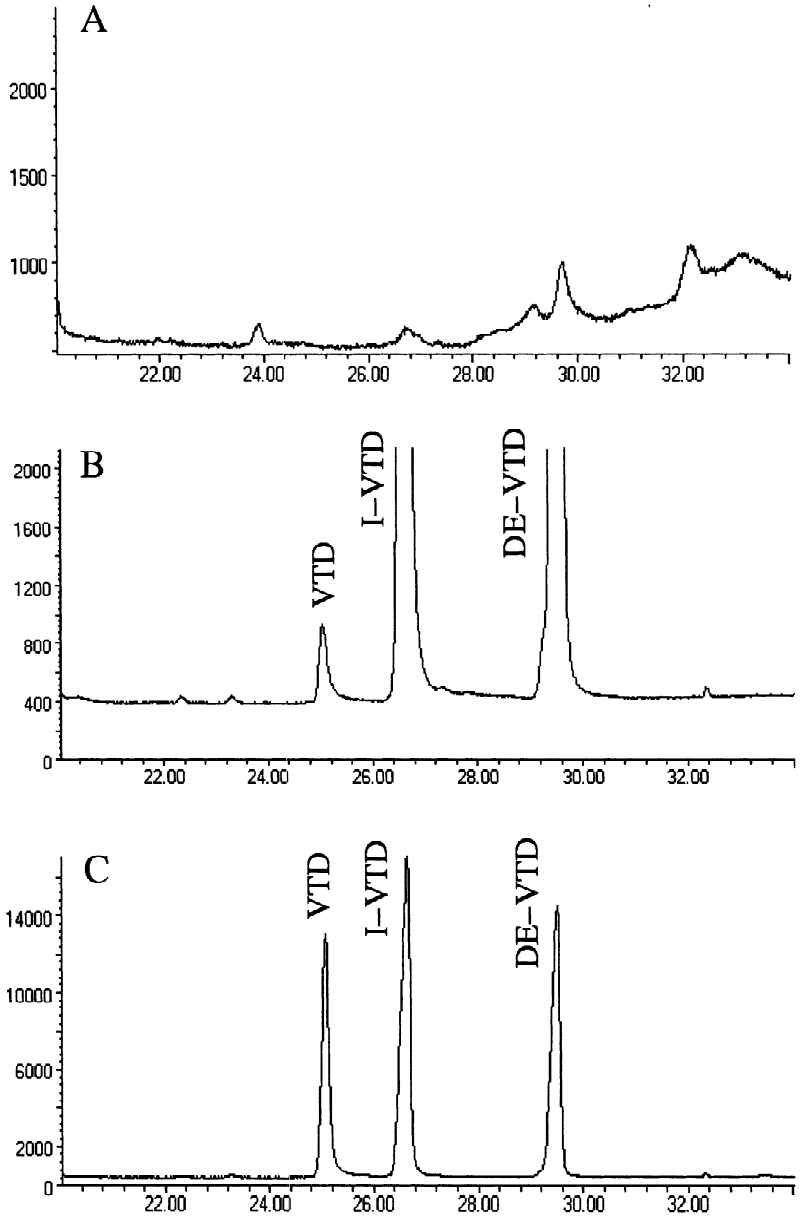

Fig. 3. Selected ion chromatograms (ions monitored at m/z 162, 179, 208, 221, 234 and 264) of blank dog plasma (A), dog plasma spiked with 1.5 mg/l VTD (LOQ) using I-VTD and DE-VTD as internal standards (B) and dog plasma 10 h after administration (20 mg/kg i.v.) of VTD to a dog, with a calculated concentration of 9.7 mg/l of VTD as analyzed by method B.

3.4. Method validation

The method validation results are presented in Tables 1–8. The calibration curve characteristics for each analyzed compound in mouse plasma are summarized in Table 1 (method A) and Table 2 (method B). The main observation in the calibration curves in mouse plasma was that the response was not linear over the whole analyzed concentration range 1–250 mg/l in the FID for all the compounds.

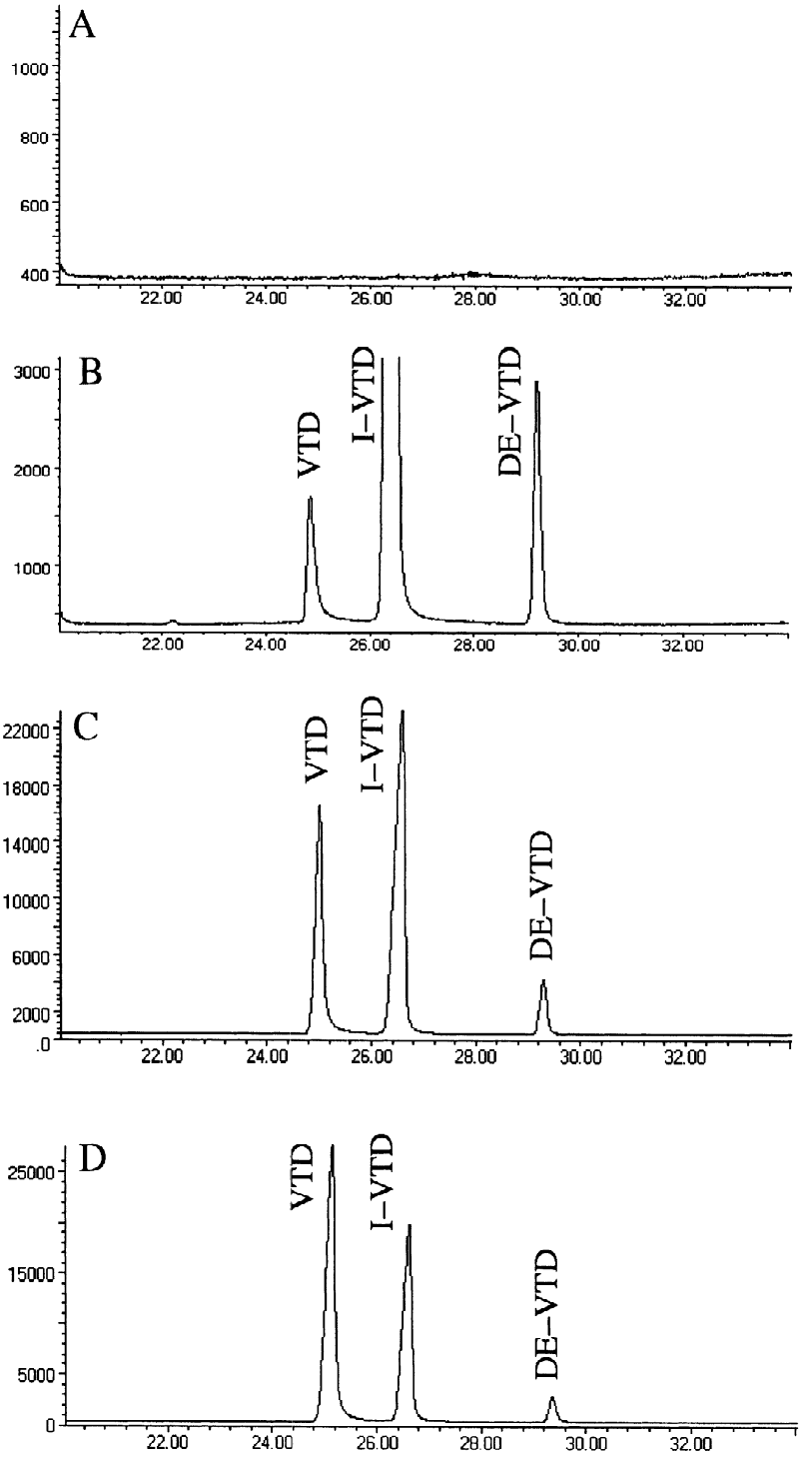

Fig. 4. Selected ion chromatograms of blank dog urine (A), dog urine spiked with VTD (60 mg/l) (B), a dog urine sample with a VTD concentration of 0.46 mg/ml obtained 2 h after administration of VTD (20 mg/kg i.v.) (C) and the same dog urine sample after treatment by β-glucuronidase having VTD concentration of 0.92 mg/ml (D) as analyzed by method B.

Table 1
Calibration curve characteristics obtained using method A (GC–FID) in mouse plasma ($n=4$)

Compound (concentration range)	Slope±SE	Intercept	r^2	P value for nonlinearity
VTD (2.5–150 mg/l)	0.0048±0.0005	−0.0054±0.025	0.997	0.999
M-VTD (2.5–250 mg/l)	0.0072±0.00053	0.098±0.047	0.998	0.995
DM-VTD (2.5–100 mg/l)	0.0097±0.0003	−0.0098±0.013	0.998	0.718
DM-VTD (50–250 mg/l)	0.0082±0.0013	−0.086±0.192	0.999	0.848
I-VTD (2.5–100 mg/l)	0.0094±0.0002	−0.0078±0.0083	0.998	0.444

Table 2
Calibration curve characteristics obtained using method B (GC–MS) in mouse plasma ($n=4$)

Compound (concentration range)	Slope±SE	Intercept±SE	r^2	P value for nonlinearity
VTD (10–250 mg/l)	0.0197±0.0011	−0.3363±0.1661	0.998	0.723
M-VTD (2.5–250 mg/l)	0.0109±0.0005	−0.0337±0.0499	0.998	0.999
DM-VTD (2.5–250 mg/l)	0.0077±0.0006	0.0451±0.061	0.999	0.872
I-VTD (2.5–50 mg/l)	0.0167±0.0005	−0.0405±0.0132	0.999	0.569
I-VTD (2.5–250 mg/l)	0.0150±0.0009	−0.0047±0.0986	0.996	0.999

The mass selective detector had a wider quantification (linear) range than GC–FID, but did not have a lower LOD or LOQ than the GC–FID method. The unexpected equal sensitivity of the two methods is probably due to the extensive fragmentation of the valproyl taurinamides and consequent poor availability of major fragments for selected ion monitoring. Only for M-VTD a single calibration curve for the whole tested range from 2.5 to 250 mg/l was acceptable in both methods. I-VTD could be quantified in the range of 2.5–250 mg/l in both methods using single calibration equation, but the accuracy at low concentrations was greatly improved in GC–MS by use of a separate calibration curve for concentrations of 2.5–50 mg/l. DM-VTD had a linear calibration curve for the whole range, but only in the GC–MS method. In GC–FID two calibration curves were prepared, as the deviation in the slope was over 10% (Table 1). In the GC–MS method (method B), probably due to the incomplete separation between VTD and M-VTD, VTD concentrations below 10 mg/l should not be quantified in samples that M-VTD and VTD are both possibly present. In the GC–FID method (method A), there was a significant

Table 3
Method validation in mouse plasma for DM-VTD ($n=8$)

	GC–FID	GC–MS
Plasma spiked at 2.5 mg/l:		
Measured concentration	2.3±0.3	2.8±0.4
Accuracy (%)	92	111
Precision (%)	14	15
Plasma spiked at 50 mg/l:		
Measured concentration	50.6±4.6	51.1±5.4
Accuracy (%)	101	102
Precision (%)	9	11
Plasma spiked at 250 mg/l:		
Measured concentration	248.2±17.1	251.5±12.1
Accuracy (%)	99	101
Precision (%)	7	5

Table 4
Method validation in mouse plasma for M-VTD ($n=8$)

	GC–FID	GC–MS
Plasma spiked at 2.5 mg/l:		
Measured concentration	2.67±0.45	2.2±0.4
Accuracy (%)	107	89
Precision (%)	18.2	19.2
Plasma spiked at 50 mg/l:		
Measured concentration	49.0±4.29	49.0±1.22
Accuracy (%)	98	98
Precision (%)	8.8	2.2
Plasma spiked at 250 mg/l:		
Measured concentration	260.8±32.6	270.2±22.0
Accuracy (%)	104	108
Precision (%)	12.5	8.1

Table 5
Method validation in mouse plasma for I-VTD ($n=8$)

	GC–FID	GC–MS
Plasma 2.5 mg/l:		
Measured concentration (mg/l)	2.5±0.3	2.8±0.3
Accuracy (%)	99	115
Precision (%)	12	11
Plasma 50 mg/l:		
Measured concentration (mg/l)	50.6±4.0	48.2±5.4
Accuracy (%)	101.3	96
Precision (%)	8	11
Plasma 250 mg/l:		
Measured concentration (mg/l)	240.1±27.9	258.7±18.5
Accuracy (%)	96	103
Precision (%)	11	7

Table 6
Method validation in mouse plasma for VTD ($n=8$)

	GC–FID	GC−MS
Plasma 2.5 mg/l:		
Measured concentration (mg/l)	2.8±0.6	2.8±0.4[a]
Accuracy (%)	112	112[a]
Precision (%)	20	13[a]
Plasma 50 mg/l:		
Measured concentration (mg/l)	50.2±6.0	50.2±2.6
Accuracy (%)	100	100
Precision (%)	12	5.1
Plasma 250 mg/l:		
Measured concentration (mg/l)	250.2±9.6[b]	279.9±34.1
Accuracy (%)	100.1[b]	111
Precision (%)	3.9	12.2

[a] Determined for samples not containing M-VTD.
[b] Separate calibration curve was used for quantification.

Table 7
Method validation using gas chromatography–mass selective detection in dog plasma for VTD using I-VTD as an internal standard ($n=8$)

Spiked concentration (mg/l)	Measured concentration ±SD (mg/l)	Accuracy (%)	Precision
1.56	1.71±0.19	110	11
3.13	3.28±0.30	105	9
6.25	6.20±0.71	99	11
12.5	12.5±1.5	100	12
25	28±1.3	110	7
50	54.1±3.7	108	7

Table 8
Method validation using gas chromatography-mass selective detection in dog urine for VTD using I-VTD as an internal standard ($n=6$)

Spiked concentration (mg/ml)	Measured concentration ±SD (mg/ml)	Accuracy (%)	Precision (%C.V.)
0.06	0.05±0.005	81	10
0.5	0.48±0.07	97	15
2	2.20±0.22	110	10

deviation of linearity for the VTD calibration curve at concentrations above 150 mg/l. Consequently, the higher concentrations should be analyzed using a separate calibration curve. A separate experiment was performed to evaluate the linearity of GC–FID method in VTD analysis at concentrations of 100–450 mg/l with dilution of the samples to a final volume of 0.5 ml chloroform. The response was linear in this range. The precision and accuracy at the limit of detection (LOD) in mouse plasma (1 mg/l with S/N of 9/1) was unacceptable. Consequently, the LOQ of all four compounds using method A (GC–FID) and of M-VTD, DM-VTD and I-VTD using method B (GC–MS) was determined to be 2.5 mg/l in mouse plasma. This LOQ is sufficient for the pharmacological applications in mice and dogs.

At the higher concentrations analyzed (25–250 mg/l original concentration), no significant difference could be observed between the two different methods in quantification parameters. Since the same samples were analyzed in both methods, any differences in the validation parameters are due to chromatographic and detection differences.

In dog plasma, the LOQ for VTD was 1.5 mg/l. The obtained linear regression equation was $y=0.0801x-0.1116$ with $r^2=0.999$. The validation parameters are presented in Table 7 across the analyzed concentration range. In dog plasma the quantification range could have been lowered easily by concentrating and using a separate calibration curve for concentrations below 2.5 mg/l. Table 8 shows the method validation for VTD in dog urine. The calibration equation in dog urine was $y=1.796x-0.420$ with $r^2=0.999$. No interference was observed in the urine samples following treatment of the urine with β-glucuronidase, and satisfactory

reproducibility was obtained between 0.06 and 4 mg/ml initial urine concentrations. In mouse urine, a calibration curve was established for a concentration range of 25–1000 mg/l for all the four valproyl taurinamides. The calibration equations were: VTD, $y=0.0015x-0.008$ with $r^2=0.998$; M-VTD, $y=0.0019x+0.0016$ with $r^2=0.997$; DM-VTD, $y=0.0011x+0.0244$ with $r^2=0.999$ and I-VTD, $y=0.0013x-0.0004$ with $r^2=0.996$.

3.5. Stability

All reference solutions were stable for at least a month if stored at 4 °C determined by repeated injections of the stock solutions. The spiked plasma samples were stable for at least five repeated freezing and thawing cycles as determined by repeated analyses of plasma samples spiked at concentrations 5, 50 and 250 mg/l during 1 month and did not show any chromatographically detectable signs of degradation during storage at room temperature during 72 h.

3.6. Application

The method was applied in a pharmacokinetic study of VTD. Fig. 5 shows the plasma concentration versus time curve of VTD and the cumulative excretion of VTD in urine, before and after treatment of the urine with β-glucuronidase, from a dog following i.v. administration of 20 mg/kg of VTD. The method was also successfully applied to a pharmacokinetic study of DM-VTD, M-VTD, I-VTD and VTD in mice. Chromatograms of mouse plasma after administration of DM-VTD and I-VTD are shown in Fig. 2, which also demonstrates that M-VTD and VTD could be detected as a metabolites of DM-VTD in mice and VTD could be quantified as a metabolite of I-VTD. In mouse urine, DM-VTD and I-VTD could not be detected following their i.p. administration.

4. Conclusions

This study presents a novel GC method for analyzing sulfonamide derivatives of VPA. It demonstrated the possibility of analyzing taurinamides by use of GC and characterized their mass spectral fragmentation. The described method is suitable for the simultaneous quantification of a series of CNS-active valproyl taurinamides and their metabolites at a wide concentration range relevant for the concentrations found in pharmacokinetic and pharmacodynamic studies of these compounds in animals. No

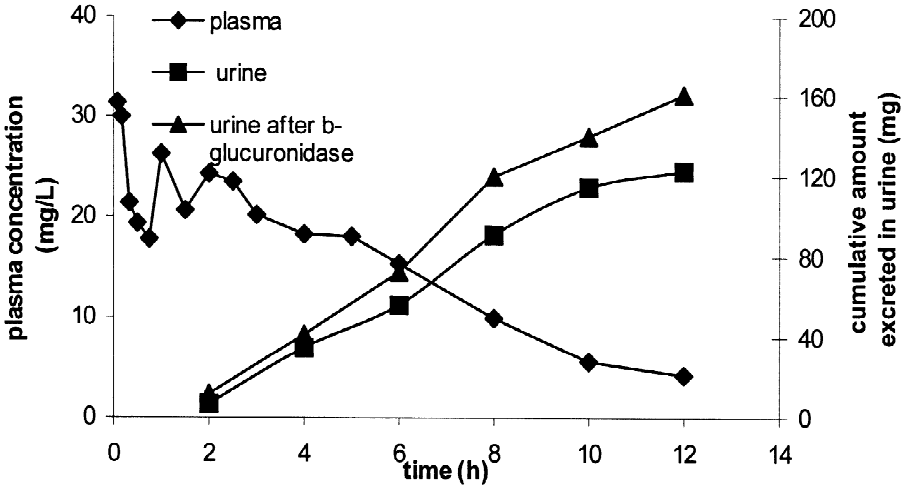

Fig. 5. Plasma concentration versus time curves of VTD in a dog obtained following i.v. administration (20 mg/kg) of VTD and the urinary excretion of VTD (cumulative amount excreted in urine) in the same dog before and after β-glucuronidase treatment.

significant differences were observed in the accuracy and/or precision in the quantification between the analyzed compounds. Surprisingly there was no significant difference in the quantification characteristics for these compounds between the GC–MS and GC–FID methods. However, the GC–MS method demonstrated its significantly greater selectivity in analysis of biological samples. The developed GC methods are both suitable for analyzing plasma and urine samples of the above compounds and their metabolites.

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