AMINO ACID CONJUGATES: METABOLITES OF 2-PROPYPENTANOIC ACID (VALPROIC ACID) IN EPILEPTIC PATIENTS

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ABSTRACT:

In this study, spectroscopic and chromatographic evidence is presented for the identification and characterization of the metabolites, valproyl glutamate (2-propylpentanoyl glutamate, VPA-GLU) and valproyl glutamine (2-propylpentanoyl glutamine, VPA-GLN) in the urine, serum, and cerebrospinal fluid (CSF) of patients on valproic acid (VPA) therapy. Moreover, the identification of valproyl glycine (2-propylpentanoyl glycine, VPA-GLY) in the serum and urine of patients on VPA, albeit in trace concentrations, is also reported here. The three amino acid conjugates excreted in urine accounted for about 1% of the VPA dose in four patients who were on VPA therapy chronically and had reached steady state. VPA-GLU was quantitatively the most prominent metabolite (0.66–13.1 µg/mg creatinine) compared with VPA-GLN (0.78–9.93 µg/mg creatinine) and VPA-GLY (trace-1.0 µg/mg creatinine) in overnight urine samples of all patients studied (n = 29). The relatively low serum concentrations of the three amino acid conjugates of VPA in six patients suggest that the metabolites are readily excreted once formed. In contrast, whereas VPA GLY was absent in the CSF of one patient on VPA, the concentrations of VPA-GLU and VPA-GLN in this CSF sample were 9 and 5 times, respectively, their corresponding serum concentrations.

The antiepileptic drug, valproic acid (VPA), is primarily used for the management of generalized and absence seizures in children. Concerns over its teratogenicity and hepatotoxicity side effect have encouraged the need to develop analogs of VPA devoid of the adverse effects. However, this task is complicated by the fact that the mechanism of action of the drug itself remains to be understood.

A large body of evidence has emerged to link the hepatotoxicity of VPA to its metabolism and has been reviewed over the years (Eadie et al., 1988; Abbott and Anari, 1999; Radatz and Nau, 1999). Moreover, a review of mass-balance studies indicated that the total recovery of a VPA dose cannot be completely accounted for in humans (Bailie and Sheffels, 1995), suggesting the existence of yet unknown metabolites. For all these reasons, despite the extensive work that has been conducted on the metabolism of VPA, the subject remains an area that warrants further investigation.

To date, the role of the amino acid conjugation pathway in the biotransformation of VPA, particularly in humans, has remained relatively unexplored. 2-Propylpentanoyl glycine (VPA-GLY) is the only amino acid conjugate of the drug that has been detected and studied in rats (Granneman et al., 1984a,b). The role of amino acid conjugation of VPA in human is significant owing to the potential for CoA consumption. The depletion of mitochondrial CoA with VPA therapy is one of several hypotheses put forth to explain VPA hepatotoxicity (Turnbull et al., 1983; Harris et al., 1991). Furthermore, the conjugation of VPA with amino acids known to be neurotransmitters involved in epilepsy also need to be explored as a potential to further understand the mechanism of action of VPA.

Although in general, bile acids form conjugates with glycine and taurine by the action of microsomal enzymes, amino acid conjugation of xenobiotics is believed to be a mitochondrial reaction occurring primarily in the liver or the kidney (Williams, 1989). Conjugation of organic acids is initiated by the obligatory activation of the acid to a CoA ester followed by the acyl transfer to an amino acid residue with concurrent release of CoA. The last step is catalyzed by N-acetyltransferase specific for each amino acid and defines the structural requirement for amino acid conjugation of xenobiotics (Hutt and Caldwell, 1990). Glycine-N-acetyltransferase (Tishler and Goldman, 1970; Mawal et al., 1997) and glutamine-N-phenylacetyltransferase
Aldrich (St. Louis, MO). 2-Fluoro-2-propylpentanoic acid was synthesized in tillled in glass grade solvents (ethyl acetate, methanol, acetonitrile) were purchased from Aldrich Chemical Co. (Milwaukee, WI); disodium L-glycine were purchased from Aldrich Chemical Co. (Milwaukee, WI); disopropylethylamine (DIPEA), triisopropylamine, TFA). Mobile phase B consisted of a gradient program that allowed 100% of TFA and mobile phase B consisted of acetonitrile. Mobile phase A was pumped isocratically for 30 min. Mobile phase B was gradiently increased from 0 to 100% at time 30.1 min and held for 5 min, followed by a sharp increase of mobile phase A to 100% at 35.1 min. This method was used for the profiling of amino acid conjugates in human serum and CSF samples.

The ionization energy for LC-MS/MS experiments was in positive electrospray (ES+). The HPLC eluent was introduced to the stainless steel capillary sprayer held at 3.5 kV. The mass spectrometer was operated in either precursor ion scanning, product ion scanning or multiple reaction monitoring (MRM) mode. Multipliers 1 and 2 were set at 650 V for all three experiments; cone voltage was set at 30 V with skimmer offset by 5 V. The low and high mass resolution was set at 12.5 for both precursor ion scanning and product ion scanning and at 5 for MRM. The product ion scanning experiment determined the product ions of MH+, and the precursor ion scanning confirmed the pseudomolecular ion or the protonated molecular ion (MH+) of the metabolite of interest. Argon, the target gas, was set at a pressure of 3.0 x 10−3 mbar for collision-induced dissociation (CID) and with a collision energy of 40 eV. During MRM, the pseudomolecular ion for each analyte was selected in the first quadrupole and subjected to CID with the target gas and collision energy set at 50 eV. Specific fragment ions were then selected by the third quadrupole. For all the experiments, the source temperature was at 140°C. Data analyses were processed using Mass Lynx software (Micromass).

**Materials and Methods**

**Materials.** 1-Bromo-2,3,4,5,6-pentafluorotoluene (PFBBr), disopropylethylamine (DIPEA), triisopropylamine, TFA. 1-fluoro-2-propylpentanoic acid was synthesized in the alcohols of ethanol, methanol, acetonitrile, and 1-glycine were purchased from Aldrich Chemical Co. (Milwaukee, WI); Bond-Elut C8 solid phase extraction cartridges (500 mg/3 ml) were purchased from Varian Sample Preparation Products (Harbor City, CA); polyethylene tubing PE-10 was purchased from Clay Adams ( Parsippany, NJ).

**Instrumentation and Analytical Conditions.** 1H NMR spectra were obtained on a Bruker WH-200 spectrometer (Bruker, Neware, DE) or a Bruker WH-400 spectrometer (Department of Chemistry, University of British Columbia).

**Gas chromatography in negative ion chemical ionization (GC-MS NICI) was performed on a Hewlett Packard (Avondale, PA) 5895 mass spectrometer (MS) engine coupled to a 5890 series II gas chromatograph (GC) fitted with a quadrupole mass spectrometer (Micromass, Montreal, QC) interfaced to a Hewlett Packard 1090 II liquid chromatograph. Chromatography was conducted on a Phenomenex (Torrance, CA) C18 column, (100 mm x 2.1 mm, 5 μm). Three HPLC methods were used in this study.

**Method 1.** Mobile phase A consisted of methanol/water (43:57, 0.05% TFA), and mobile phase B consisted of methanol. At time 0, mobile phase A was pumped isocratically for 30 min. Mobile phase B was gradiently increased from 0 to 100% at time 30.1 min and held for 5 min, followed by a sharp gradient increase of mobile phase A to 100% at 35.1 min. This method was used initially for the identification of the amino acid conjugates of VPA in human urine. Flow rate was at 0.1 ml/min.

**Method 2.** Mobile phase A consisted of acetonitrile/water (30:70, 0.05% TFA) and mobile phase B consisted of acetonitrile. Mobile phase A was pumped for 25 min followed by a gradient increase of mobile phase B to 100% at 26 min, held for 5 min and reverted to 100% A at 30.1 min. Flow rate was at 0.1 ml/min. This method was used to detect the amino acid conjugates of VPA in urine where a longer run time program was required to separate the peaks of interest from interfering chromatographic peaks.

**Method 3.** Mobile phase A consisted of acetonitrile/water (40:60, 0.05% TFA). Mobile phase B consisted of a gradient program that allowed 100% of mobile phase A to be pumped for 14 min at a flow rate of 0.1 ml/min. Mobile phase B was then increased to 100% at a flow rate of 0.2 ml/min at 15 min and held for 5 min at 0.2 ml/min. Subsequently, mobile phase A was increased to 100% at time 20 min and at 0.1 ml/min. This method was used for the profiling of amino acid conjugates in human serum and CSF samples.

**Human Studies.** Overnight urine samples (n = 29), 24-h urine samples (n = 4), serum samples (n = 6), and one CSF sample were obtained from epileptic and pediatric patients who have been on VPA therapy for more than 6 months and have reached steady state. The patients were attending the Seizure Clinic at Vancouver Children’s Hospital, British Columbia, Canada. The average dose of VPA received by each patient was 23.3 ± 13.2 mg/kg, the average age of the patients was 12.6 ± 5.9 years. Control urine and serum samples were obtained from healthy volunteers (n = 4). Control CSF were obtained from nonepileptic subjects (n = 10), who were either suffering from multiple sclerosis and attending the University of British Columbia Hospital, Canada or were healthy volunteers. Approval for all human studies was issued by the U.B.C. Human Ethics Committee (U.B.C. C05-0412).

**Chemical Synthesis of Amino Acid Conjugates of VPA.** The amino acid conjugates were synthesized according to the procedure described for 2-fluoro VPA-GLN (NPAVL-GLN) (Tang et al., 1997). VPA was converted to the corresponding N-hydroxyxysuccinimide ester in the presence of dicyclohexylcarbodiimide followed by coupling with t-glutamic, t-glycine, t-glutamic acid, or L-serine to form 2-propylpentanol glutaminyl (VPA-GLN), VPA-GLY, 2-propylpentanol glutamate (VPA-GLU), or valproyl aspartate (VPA-ASP), respectively.
to 10 μl of a solution (0.01 mg/ml) of VPA-GLU, VPA-GLN, VPA-GLY, and VPA-ASP in ethyl acetate and allowed to react at 50°C for 1 h.

Preparation of Urine Samples for the Identification and Profiling of Amino Acid Conjugates of VPA by Solid Phase Extraction. One milliliter of urine sample was adjusted to pH 3 with HCl and applied to a C<sub>2</sub> Bond-Elut cartridge that was preconditioned with water and methanol. The cartridges were washed once with water/methanol (95:5) and water. The amino acid conjugates of VPA were eluted with 6 ml of methanol. The organic layers were...
dried and reconstituted with water. Ten microliters of the reconstituted solvent were then injected into the LC-MS/MS system and analyzed using LC-MS/MS methods 1 and 2.

For the identification and characterization experiments, the chromatographic and mass spectral characteristics of the conjugates from biological fluids were compared with those of the synthesized compounds using precursor ion scanning, product ion scanning or MRM. MS/MS dwell times were set at 1 s:100 amu. In MRM mode, the following transitions were monitored: VPA-GLU: m/z 274 → m/z 57, m/z 274 → 99, m/z 274 → 127, and m/z 274 → 148; VPA-GLN: m/z 273 → 57, m/z 273 → 99, m/z 273 → 127, m/z 273 → 129, m/z 273 → 147; VPA-GLY: m/z 202 → 57, m/z 202 → 99, m/z 202 → 127; VPA-ASP: m/z 260 → 57, m/z 260 → 99, m/z 260 → 127, m/z 260 → 134.

For the quantitation of VPA-GLU, VPA-GLN, and VPA-GLY in urine, 1 ml of each sample was spiked with 25 μl of 0.08 mg/ml of FVPA-GLN (IS) and VPA-ASP (IS). The samples were prepared and analyzed with a set of calibration standards ranging from 0.10 to 5.0 μg/ml. Calibration standards and all samples of patients were extracted as described above. The LC-MS/MS transitions monitored were as follows: m/z 291 → 130 for FVPA-GLN; m/z 274 → 148 for VPA-GLU; m/z 273 → 147 for VPA-GLN, m/z 260 → 127 for VPA-ASP (IS), and m/z 200 → 57 for VPA-GLY.

The assay for the simultaneous analysis of amino acid conjugates of VPA by LC-MS/MS was linear over a range of 0.1 of 5.0 μg/ml for all three conjugates. The coefficients of determination (r²) of the calibration curves were 0.99 or better.

The interassay variation was assessed from the slopes of five calibration curves run consecutively and was less than 7% for each identified metabolite. The intra-assay variation was assessed by three replicate analyses of two samples. At 0.12 μg/ml, the intra-assay variation was 11.4, 28.8, 18.5% for VPA-GLU, VPA-GLN, and VPA-GLY, respectively. At 1.0 μg/ml, the intra-assay variation was less than 10% for all three metabolites. The larger variation observed at the lower limit of quantitation was acceptable in view of the fact that the metabolites were detected at concentrations well above the lowest standard (0.1 μg/ml). At both concentrations, each metabolite was measured within 83 to 120% of their expected values.

The concentration of each amino acid conjugate of VPA measured in overnight urine samples was normalized to creatinine. The % recovery of VPA as amino acid conjugates of VPA was calculated for 24-h urine samples (n = 4). Creatinine was measured in urine according to the routine Jaffe method (1886).

Preparation of Serum Samples for the Identification and Quantitation of the Amino Acid Conjugates of VPA. The amino acid conjugates of VPA were extracted from the serum samples of human by liquid-liquid extraction. Two milliliters of each specimen and control serum was adjusted to pH 3 with HCl and extracted with 5 ml of ethyl acetate, twice. The organic layers were reconstituted in distilled water and analyzed by LC-MS/MS method 3 for quantitation of VPA-GLU, VPA-GLN, and VPA-GLY to yield concentrations ranging from 0.01 to 0.10 μg/ml. The calibration standards, 100 μl of patient’s CSF and control CSF were spiked with 5 μl of FVPA-GLN (0.08 μg/ml) and filtered through a filter membrane as described for the serum samples. Then, 60 μl of the filtrate was injected into the LC-MS/MS system to be analyzed using LC-MS/MS method 3 as described for the serum samples.

The range of the calibration curve for each conjugate was 10 to 100 ng/ml. Standard curves afforded r² of 0.993, 0.979, and 0.999, for VPA-GLU, VPA-GLN, and VPA-GLY, respectively.

Results

Synthesis of VPA-GLU, VPA-GLN, VPA-GLY, and VPA-ASP. The synthesis of the amino acid conjugates of VPA was facile and involved the conversion of VPA to N-hydroxysuccinimide ester in the presence of dicyclohexylcarbodiimide followed by coupling with the appropriate amino acid.

Proton nuclear magnetic resonance spectroscopy of all the conjugates served as a primary tool to verify the chemico-selectivity of the conjugation reaction and to determine the structure of the four conjugates synthesized. Spectra were characterized by signals of a VPA backbone and those belonging to the amino acid moiety. The signals for the proton(s) of the α-methylene of the amino acid confirmed the structure of the conjugates was consistent with that of the amide of valproic acid. In the case of VPA-GLU, VPA-GLN, and VPA-ASP, this proton was coupled to the amino proton and the neighboring –CH₂-group and was split into a doublet of doublets at 4.45, 4.40, and 4.80 ppm, respectively. In VPA-GLY, the protons on the tertiary carbon were detected as a doublet at 4.1 ppm. The structures of VPA-GLU, VPA-GLN, VPA-GLY, and VPA-ASP are shown in Fig. 1.

LC-MS scanning showed the [MH]⁺ of VPA-GLU, VPA-GLN, VPA-GLY, and VPA-ASP to be m/z 274, m/z 273, m/z 201, and m/z 50.

TABLE 2
HPLC retention times (tR) of amino acid conjugates of VPA with different mobile phases.

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-MS/MS</td>
<td>(tR = min)</td>
<td>(tR = min)</td>
<td>(tR = min)</td>
</tr>
<tr>
<td>VPA-GLN</td>
<td>22.97</td>
<td>14.70</td>
<td>7.86</td>
</tr>
<tr>
<td>VPA-GLU</td>
<td>30.70</td>
<td>23.20</td>
<td>10.53</td>
</tr>
<tr>
<td>VPA-GLY</td>
<td>30.30</td>
<td>24.0</td>
<td>11.99</td>
</tr>
<tr>
<td>VPA-ASP</td>
<td>23.99</td>
<td>9.93</td>
<td>9.33</td>
</tr>
</tbody>
</table>

The assay was linear over a concentration range of 5 to 100 ng/ml with r² of 0.99 or better for the calibration curves of each VPA-GLU, VPA-GLN, and VPA-GLY.

Preparation of CSF Samples for the Identification, and Quantitation of Amino Acid Conjugates of VPA in Human CSF. A CSF sample (100 μl) of a patient on VPA, a control human CSF sample (100 μl) spiked with 0.025 μg/ml of VPA-GLU, VPA-GLN, VPA-GLY, and VPA-ASP, and 10 control CSF samples (100 μl per sample) were filtered through a membrane filter as described for the serum samples and analyzed under LC-MS/MS method 3 for VPA-GLU, VPA-GLN, VPA-GLY, and VPA-ASP as performed for the urine samples.

A CSF sample (100 μl) of a patient on VPA, a control CSF sample spiked with VPA-GLU (0.025 μg/ml), and a control CSF sample were subjected to solid phase extraction as described for the urine samples. The residues were dissolved in 500 μl of ethyl acetate and derivatized with 10 μl of 40% PFBBr and 10 μl of DIPEA at 50°C and 1 μl of the mixture was analyzed by GC-MS NCI.

For quantitation, human control CSF samples (100 μl per sample) were spiked with the appropriate amounts of VPA-GLU, VPA-GLN, and VPA-GLY to yield concentrations ranging from 0.01 to 0.10 μg/ml. The calibration standards, 100 μl of VPA-GLU, VPA-GLN, and VPA-GLY were linear over a concentration range of 5 to 100 ng/ml.
protonated VPA-ASP in any of the patients’ samples studied. The search for VPA taurine and VPA alanine was approached by MRM of the corresponding transition [MH]+ to m/z 57, the most abundant product ion shared by the identified amino acid conjugates of VPA. Neither of the two conjugates were detected in the urine extracts analyzed.

In the patients’ urine samples analyzed, VPA-GLU was found at a concentration range of 0.66 to 13.1 μg/mg creatinine; VPA-GLN was found at lower concentration range of 0.78–9.93 μg/mg creatinine, whereas VPA-GLY was the minor metabolite at a concentration range of trace-1.0 μg/mg creatinine (Table 3). The total recoveries of the three conjugates, VPA-GLU, VPA-GLN, and VPA-GLY in urine samples of patients on VPA therapy were calculated for a 24-h period and found to range from 0.53 to 1.45% (0.75 ± 0.41) of a VPA dose (Table 4). The urinary VPA concentration was available for three of the patients, and the mean concentration was found to be 420 μg/mg of creatinine.

Identification of Amino Acid Conjugates of VPA in Serum Samples of Patients. Analysis of serum samples by LC-MS/MS MRM provided strong evidence for the identification of VPA-GLU, VPA-GLN, and VPA-GLY in the serum extracts of all six patients but not in any control serum. The retention times and area ratio of all product ions of [MH]+ of the metabolites were authenticated by comparison with synthetic standards. The mean serum concentration of VPA-GLU in six patients was 8.0 ± 2.0 ng/ml. VPA-GLN was estimated at 5.0 ± 3.0 ng/ml, and VPA-GLY was detected but was below the limit of quantitation of the assay.

Identification of Amino Acid Conjugates of VPA in Human CSF. The retention times and area ratio of all the product ions of the [MH]+ of VPA-GLU (Fig. 3) and VPA-GLN in a human CSF sample were the same as those observed for both synthesized amino acid conjugates of VPA by LC-MS/MS. The identification of VPA-GLU was further investigated by GC-MS under NICI by monitoring for the [M-181]+ fragment carboxylate anion of the di-PFB derivative of the conjugate at m/z 452. The total ion current of the PFB-derivatized CSF extract (Fig. 4) and its corresponding PFB-derivatized serum extract showed the elution of VPA-GLU at tR = 16.36 min, the identical retention time observed for the synthetic standard. The peak was absent in the PFB-derivatized control CSF and serum extract.

The quantitation of VPA-GLU, and VPA-GLN in CSF by LC-MS/MS was relatively facile because no extraction procedure was required. The assay was designed to monitor one characteristic MRM transition for each amino acid conjugate of VPA identified in human and using VPA-ASP as the IS. A relatively high volume (60 μl) of sample was introduced onto the HPLC column, but the resulting slight broadening of the peaks was not chromatographically significant. The concentration of VPA-GLU in the CSF of one patient was 60 ng/ml, and that of VPA-GLN was 34 ng/ml of CSF. The corresponding serum concentrations of VPA-GLU and VPA-GLN of the same patient were 7.0 ng/ml and 6.0 ng/ml, respectively. The concentration of CSF VPA of the patient was less than 15 μg/ml of CSF.

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>Summary of results for the profiling of VPA-GLU, VPA-GLN, VPA-GLY in the urine samples of patients on VPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPA-GLU (μg/mg creatinine)</td>
<td>VPA-GLN (μg/mg creatinine)</td>
</tr>
<tr>
<td>Range</td>
<td>n = 29</td>
</tr>
<tr>
<td>0.66–13.1</td>
<td>0.78–9.93</td>
</tr>
<tr>
<td>Mean</td>
<td>4.31</td>
</tr>
<tr>
<td>S.D.</td>
<td>2.76</td>
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</tbody>
</table>

| TABLE 4 | The % of VPA dose recovered as VPA-GLU, VPA-GLN, and VPA-GLY in four patients over 24 h |
|----------------|-----------------|-----------------|-----------------|-----------------|
| Patient No. | VPA GLU | VPA GLN | VPA GLY | Total % Recovered |
| 1 | 0.45 | 0.32 | 0.03 | 1.45 |
| 2 | 0.20 | 0.20 | 0 | 0.40 |
| 3 | 0.22 | 0.18 | 0.02 | 0.61 |
| 4 | 0.17 | 0.15 | 0 | 0.53 |
| Mean ± S.D. | 0.75 ± 0.41 |
Discussion

We report here the identification of valproyl glutamate and valproyl glutamine, both conjugates of VPA in humans. Valproyl glycine was also identified in the biological samples of patients on VPA. Preliminary accounts of these findings were reported (Gopaul et al., 1997a,b).

The discovery of VPA-GLU, in the biological fluids of humans, is intriguing due to its extreme rarity. To the best of our knowledge, the glutamate conjugate of benzoic acid in the Indian fruit bat (Idle et al., 1975) and p-nitrobenzoic acid in spiders (Smith, 1962) are the only two glutamate conjugates of a xenobiotic carboxylic acid reported in the literature. Conjugates of amino acids that are normally found are species-dependent and vary according to the structure of the carboxylic acid involved.

In humans, the conjugation of glycine, glutamine, taurine, and ornithine with carboxylic acids are known (Caldwell et al., 1976; Webster et al., 1976; Hutt and Caldwell, 1990; Shirley et al., 1994; Kasuya et al., 1999). Reports in the literature indicate that glycine and to a lesser extent glutamine are the most likely amino acids to be involved in the conjugation reactions of carboxylic acids (Hutt and Caldwell, 1990). It should also be added that recently the conjugation of xenobiotic amines with glutamate has also been reported (Mutlib et al., 2000; 2002).

In this study, quantitative analysis of the conjugates in human urine, serum, and CSF samples revealed that VPA-GLU was consistently the predominant amino acid conjugate compared with VPA-GLN, and VPA-GLY was a minor conjugate. The more extensive conjugation of VPA with glutamic acid than either glutamine or glycine is also an unprecedented observation for the human species.

The higher abundance of the amino acid conjugates in urine samples relative to their serum concentrations suggests that the conjugates are readily excreted upon formation in the liver. These metabolites can also be formed at other sites, such as the kidney. For example, in vitro studies support the conjugation of benzoic acid with glycine in human liver and kidney tissues (Caldwell et al., 1976; Temellini et al., 1993). Alternatively, a portion of the conjugates formed may be excreted into the intestinal lumen (via biliary excretion or exsorption) and excreted in feces.

Amino acid conjugation of an organic acid involves the activation of the acid to its CoA derivative prior to coupling with an amino acid by the respective amino acid transferase (Hutt and Caldwell, 1990). The mean observed recovery of amino acid conjugates of VPA in the urine samples of four patients was 0.75 ± 0.41% of the VPA dose. By comparison, the β-oxidation metabolites of VPA, namely 2-ene VPA and 3-keto VPA, that are also initiated by the activation of VPA to its CoA ester, account for more than 15% of a VPA dose in humans (Pollack et al., 1986). This would imply that the VPA CoA ester is more susceptible to β-oxidation than amino acid conjugation in mitochondria. However, whether the amino acid conjugation of VPA is a mitochondrial or microsomal-based reaction remains to be confirmed.

The identification of VPA-GLU and VPA-GLN in the CSF sample stood as the most interesting finding in this study. The availability of human CSF of patients on VPA therapy is rare, and as part of our efforts to support this human evidence, we reported the results of a preliminary study that confirmed unequivocally the identification of VPA-GLU as the only amino acid conjugate of VPA in the CSF of...
rabbıts treateď with VPA (n = 5) (Gopaul et al., 1997b). That VPA-GLN was not detected in the CSF of rabbit suggests that VPA-GLU is probably the direct result of the conjugation of VPA with glutamic acid and is not primarily the hydrolyzed product of VPA-GLN although this needs to be confirmed.

The CSF results of the patient in this study are consistent with the levels of VPA metabolites that are usually found at concentrations <10% of VPA in the CSF (Lösher et al., 1988) or <15% of VPA in the human brain (Adkinson et al., 1995). The CSF concentrations of VPA-GLU and VPA-GLN in the patient in this study were <1% of the CSF VPA level (15 μg/ml of CSF). Because, the concentrations of VPA and unsaturated VPA metabolites in the CSF are believed to correspond to those in the brain (Vajda et al., 1981; Lösher et al., 1988), it would be reasonable to suggest that the levels of the CSF VPA-GLU and VPA-GLN reflect their respective brain tissue concentrations. However, this would be inconsistent with a recent report from Scism et al. (2000), which suggests that VPA may be concentrated in the intracellular compartments of the brain parenchyma.

The CSF concentrations of VPA-GLU, and VPA-GLN were 9 and 5 times, respectively, their corresponding serum concentrations in the human subject, suggesting that the metabolites could be formed in the brain itself as a result of the conjugation of VPA with glutamic acid or its precursor, glutamine. If so, the detection of VPA-GLU and VPA-GLN in the CSF of human and rabbit provides some grounds to propose that the mechanism of action of VPA could in part be the result of the conjugation of VPA with the excitatory neurotransmitter glutamic acid believed to be responsible for seizure activity or its precursor glutamine. In support of this theory, significantly elevated concentrations of GLU are found in human epileptogenic foci (Perry et al., 1976) and in the CSF of newly diagnosed epileptic patients untreated with anticonvulsants (Kalviinen et al., 1993). Although the concentrations of human CSF VPA-GLU with respect to the concentration of VPA itself (~15 μg/ml) is low, it is consistent with the concentration of CSF glutamate that is usually found in comparatively low concentrations in the CSF of control subjects (Kalviinen et al., 1993; Jimenez-Jimenez et al., 1998). In light of the implication of VPA metabolism in the mechanism of action of VPA and the induction of hepatotoxicity, the significance of this new metabolic pathway, namely the glutamic acid conjugation with VPA in humans, needs to be pursued.

Conclusion

LC-MS/MS was used for the identification and quantitation of the novel VPA-GLU and VPA-GLN conjugates in the urine, serum, and CSF samples of patients on VPA. These amino acid conjugates of VPA in urine account for about 1% of a VPA dose. The identification of VPA-GLU and VPA-GLN in the CSF of a human subject stands as the most interesting finding with the detection of amino acid conjugates of VPA in the CSF of humans. In our opinion, the significance of these conjugates in the CNS requires further investigation. A report on the studies of the amino acid conjugates of VPA in animals is to follow.

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References


