Determination of phenylacetic acid in cerebrospinal fluid by gas chromatography-mass spectrometry.

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Abstract

Using gas chromatography-mass spectrometry, we developed a sensitive and reliable technique to measure phenylacetic acid (PAA), an oxidatively deaminated metabolite of beta-phenylethylamine (PEA), in small amounts of cerebrospinal fluid (CSF). In a preliminary analysis, PAA concentrations in depressive patients were significantly lower than those in controls, while there were no differences in PAA levels between schizophrenic patients and controls. This suggests a possible link between the decreased PEA metabolism in the brain and the etiology of depression. However, further studies are needed to clarify the effects of neuroleptics and antidepressants on PAA levels in CSF, since the samples were obtained without regard to medication in the present study. In control subjects, a U-shaped distribution was obtained when the values of PAA were plotted as a function of age. There were no sex differences and no significant concentration gradients in CSF PAA levels.

KEYWORDS: phenylacetic acid, cerebrospinal fluid, depression, schizophrenia, gas chromatography-mass spectrometry

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Determination of Phenylacetic Acid in Cerebrospinal Fluid by Gas Chromatography-Mass Spectrometry

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Using gas chromatography-mass spectrometry, we developed a sensitive and reliable technique to measure phenylacetic acid (PAA), an oxidatively deaminated metabolite of β-phenylethylamine (PEA), in small amounts of cerebrospinal fluid (CSF). In a preliminary analysis, PAA concentrations in depressive patients were significantly lower than those in controls, while there were no differences in PAA levels between schizophrenic patients and controls. This suggests a possible link between the decreased PEA metabolism in the brain and the etiology of depression. However, further studies are needed to clarify the effects of neuroleptics and antidepressants on PAA levels in CSF, since the samples were obtained without regard to medication in the present study. In control subjects, a U-shaped distribution was obtained when the values of PAA were plotted as a function of age. There were no sex differences and no significant concentration gradients in CSF PAA levels.

Key words: phenylacetic acid, cerebrospinal fluid, depression, schizophrenia, gas chromatography-mass spectrometry

It is now well established that β-phenylethylamine (PEA) is an intrinsic amine in the mammalian brain (1, 2). While its physiological roles are not as yet fully understood, PEA is thought to act as a neurotransmitter or neuromodulator in the central nervous system (CNS) from the following characteristics: high turnover rate (3), heterogeneous distribution (1) and subcellular localization (4). On the other hand, it has been claimed that PEA may be involved in the etiology of certain mental disorders as mentioned below. Most previous clinical studies have concentrated on urinary levels of PEA and phenylacetic acid (PAA), the major deaminated metabolite of PEA, in various disease states. This approach, however, has to be viewed with great caution as only a small proportion of urinary PEA and PAA is the result of brain PEA metabolism. In order to gain a better understanding of the relationship between PEA and mental disorders, it is necessary to assay substances in the biological fluids reflecting the activity of the CNS. For this purpose, the use of PAA levels in the cerebrospinal fluid (CSF) appears more reasonable.

Recently the authors have developed a sensitive and reliable gas chromatographic-mass spectrometric method for the assay of free PAA in the CSF. In this communication we describe an assay procedure and the preliminary data on PAA levels in the CSF of patients with schizophrenia and depression.
Materials and Methods

Subjects. The subjects were 24 schizophrenic patients (ages 19 to 57 years, 14 males and 10 females) and 6 male depressive patients (ages 33 to 55 years, 5 patients with major depression and one with a dysthmic disorder). Since CSF could not be obtained from non-neurological patients except for a 26-year-old normal volunteer, 17 patients (ages 20 to 77 years, 14 males and 3 females) with a variety of neurological and psychiatric disorders (5 patients with epilepsy, 2 with Parkinson’s disease, 2 with spinocerebellar degeneration, 3 with cerebrovascular accident, one with periodic paralysis, 2 with dementia, one with hypochondriasis and one with psychogenic pain disorder) served as controls in this study. The first 3-4 ml of CSF (Fraction 1) was obtained through lumbar puncture usually performed between 9 and 10 a.m. after an overnight fast and bed rest. Samples were immediately frozen and stored at -20°C until assayed. After collecting the next 4-5 ml of CSF for routine diagnostic purposes, a second sample of CSF (8 to 10th ml; Fraction 2) was taken from 13 patients. The patients were sampled without regard to medication. All schizophrenics and five of the 6 depressives in this study received neuroleptics and antidepressants at the time of spinal tap, respectively. For examining age differences in the PAA level, additional CSF obtained from 14 children under 15 years of age for diagnostic purposes was used. The diagnosis of psychiatric disorders was made according to the DSM III (the third edition of Diagnostic and Statistical Manual of Mental Disorders) criteria (5). All investigations were performed with the patients’ or their parents’ informed consent. Data were analyzed statistically by using one-way analysis of variation (ANOVA).

Procedures. The method of Fellows et al. was modified in the present study (6). To each sample of 0.5 ml CSF, 20 ng of heptadeuterated PAA (PAA-d₇) was added as an internal standard. The samples were acidified with 20 µl of 6M hydrochloric acid, saturated with sodium chloride, and then extracted with 6.5 ml of ethyl acetate. Five ml of the extracts was transferred into siliconized test tubes followed by the addition of 50 µl triethylamine and dried in vacuo at 50°C by using a centrifugal evaporator. After the residues were dissolved in 0.15 ml ethanol and transferred into 0.3 ml Reacti-vials (Pierce Chemical Co., Rockford, IL), 10 µl triethylamine was added to prevent the loss of PAA, and the solutions was evaporated to dryness in a stream of nitrogen. To the residues was added 10 µl of 1% (w/v) ethanoletic potassium hydroxide and 50 µl of 2% pentafluorobenzyl bromide (PFB) in ethanol. The vials were capped and heated at 75°C for 40 min. After cooling, excess reagent was evaporated in a stream of nitrogen. Just before the analysis, the dried residues were finally dissolved in 10 µl distilled water and 20 µl n-heptane. After the vials were shaken well and centrifuged, 1-2 µl of the upper organic phase was injected into the gas chromatograph-mass spectrometer inlet. Standard solutions were prepared containing the same amounts of deuterated PAA and various amounts of PAA. A pooled CSF sample was used for the determination of assay precision.

A gas chromatograph-mass spectrometer (Shimadzu 9020-DF, Kyoto, Japan) equipped with a Shimadzu SCAP 1123 data system was used for the mass fragmentographic analysis employing an electron impact ionization mode. A 2.0 m × 0.3 cm (i.d.) glass column packed with 3% OV-1 on Uniport HP (80-100 mesh, Gasukuro Kogyo Inc., Tokyo, Japan) was operated isothermally at 200°C with a helium flow rate of 30 ml/min. The temperatures at the jet separator and in the ion source were 220°C and 240°C, respectively. The ionization voltage was adjusted to 20 eV. For routine analysis the fragments m/e 316 (pentafluorobenzyl ester of phenylacetic acid) and m/e 323 (pentafluorobenzyl ester of heptadeuterated phenylacetic acid) were recorded. The concentration of PAA was calculated by comparing the peak areas of the deuterated PAA internal standard with those corresponding to the appropriate fragment derived from PAA.

Reagents. PAA-d₇ was purchased from MSD Isotopes (Quebec, Canada). PFB was obtained from Pierce Chemical Co. (Rockford, IL). All other chemicals were of the purest grade commercially available.

Results

The conditions selected in the present study were found to give maximal detector
responses. The mass spectra of pentafluorobenzyl esters of phenylacetic acid and heptadeuterated phenylacetic acid are shown in Fig. 1. Relative ion intensity of m/e 316 and m/e 323 to the total (m/e 50-400) was 8.1% and 9.6%, respectively. When the mass spectrometer was focused on these ions, a mass fragmentogram free of interfering peaks was obtained as illustrated in Fig. 2. Since the retention time was about 1 min for the peak, it is possible to make a rapid analysis. The PAA added to the pooled CSF was recovered at a rate of 62.5%. Fig. 3 shows the calibration curve of the pure compound demonstrating the linearity of the response following injection of various amounts of PAA (10-100 ng/ml). Correlation coefficients, determined by linear regression, were always over 0.997. The minimum detectable quantity of PAA was 300 pg. In the pooled CSF, PAA was determined with a ‘within batch’ and ‘between batches’ coefficient of variation of 1.4% and 5.8%, respectively.

Free PAA levels in the lumbar CSF of controls and patients with schizophrenia and depression are shown in Table 1. The concentrations of PAA in depressive patients were significantly lower (p<0.05) than those in controls. There were no significant differences in PAA levels between schizophrenics and controls, whereas PAA concentrations were more than 40 ng/ml in six out

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**Table 1** Free phenylacetic acid concentrations in cerebrospinal fluid

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number</th>
<th>Phenylacetic acid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>18</td>
<td>26.2 ± 3.0</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>24</td>
<td>30.1 ± 3.4</td>
</tr>
<tr>
<td>Depression</td>
<td>6</td>
<td>14.8 ± 1.9*</td>
</tr>
</tbody>
</table>

*a: Mean ± SEM (ng/ml), *p < 0.05 vs. controls
Table 2 Free phenylacetic acid concentrations in fractions of cerebrospinal fluid (ng/ml)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Fraction 1</th>
<th>Fraction 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. F.</td>
<td>85.01</td>
<td>81.46</td>
</tr>
<tr>
<td>T. K.</td>
<td>6.42</td>
<td>4.99</td>
</tr>
<tr>
<td>S. O.</td>
<td>26.82</td>
<td>28.40</td>
</tr>
<tr>
<td>K. H.</td>
<td>7.35</td>
<td>6.73</td>
</tr>
<tr>
<td>K. H.</td>
<td>26.75</td>
<td>27.13</td>
</tr>
<tr>
<td>T. T.</td>
<td>10.88</td>
<td>9.33</td>
</tr>
<tr>
<td>Y. M.</td>
<td>7.62</td>
<td>7.10</td>
</tr>
<tr>
<td>I. O.</td>
<td>24.33</td>
<td>23.80</td>
</tr>
<tr>
<td>A. T.</td>
<td>27.96</td>
<td>29.34</td>
</tr>
<tr>
<td>S. T.</td>
<td>14.64</td>
<td>16.89</td>
</tr>
<tr>
<td>T. U.</td>
<td>18.04</td>
<td>17.70</td>
</tr>
<tr>
<td>T. H.</td>
<td>13.28</td>
<td>13.50</td>
</tr>
<tr>
<td>M. T.</td>
<td>38.50</td>
<td>33.72</td>
</tr>
</tbody>
</table>

\(a: \) See text.

![Graph showing Free PAA (ng/ml) vs AGE (Y)](image)

**Fig. 4** Values of phenylacetic acid (PAA) in the lumbar cerebrospinal fluid plotted against age.

of 21 schizophrenic patients. No concentration gradients of PAA were found between Fraction 1 and Fraction 2 as shown in Table 2. There were no sex differences in the CSF PAA levels. In control subjects, on the other hand, a U-shaped curve was obtained when the values of PAA were plotted as a function of age as shown in Fig. 4. A significant correlation (\(p < 0.01\)) was found between the PAA concentration and age in the subjects over 20 years of age.

**Discussion**

The present method has been shown to offer high sensitivity and reproducibility for the determination of free PAA in small amounts of human CSF (0.5 ml or less). However, a relatively low recovery should be improved with further experimentation.

Little is known about PAA in human CSF. Sandler et al. first reported elevated free PAA levels in the CSF of schizophrenic patients (7). This finding was confirmed in paranoid schizophrenic patients by Karoum et al. who measured total PAA (8). These findings support the PEA hypothesis of schizophrenia in conjunction with the reports of elevated PEA excretion in the urine of some chronic paranoid schizophrenic patients (9, 10). In the present study no significant differences in the CSF PAA between medicated schizophrenics and controls were found. However, the metabolic influences of neuroleptics cannot be completely excluded in these CSF studies. In fact Beckmann et al. pointed out that free PAA was significantly decreased in the CSF of untreated patients as compared to controls, whereas those taking neuroleptics showed a non-significant tendency to be decreased below control values (11). They also reported no changes in PEA concentrations in the CSF of unmedicated schizophrenics, though a few patients showed extremely high levels. Thus the data on PEA metabolism in schizophrenics are still controversial. Since amphetamine-induced psychosis exhibits close similarities to paranoid schizophrenia and PEA is structurally and pharmacologically similar to amphetamine, detailed and controlled clinical investigations are needed to
clarify the roles of PEA in the pathophysiology of schizophrenia.

On the other hand, the present result that patients with depression showed significantly lowered levels of PAA compared to controls is in good agreement with the finding of Sandler et al. (12). While the control subjects in this study consisted of patients with a variety of CNS disorders, their PAA levels are comparable to the control values reported by other workers (11, 12). The urinary excretions of PEA (13, 14) and PAA (15) have been reported to be reduced in depressive patients. Since PEA is well-known as a behavioral stimulant, these findings suggest a possible link between PEA metabolism in the brain and affective disorders. However, the present data need to be confirmed with untreated depressive patients with a variety of affective disorders, since Karoum et al. reported significantly higher total PAA levels in the CSF of patients with bipolar depression (8), and our data were obtained from patients with major depression, except for one with a dysthymic disorder.

PAA is the major deaminated metabolite of PEA. It has been found that half the PAA exists in a free form in the CSF, and there are highly significant correlations between plasma and CSF concentrations of free, conjugated and total PAA (16). Since free PAA is a lipid-soluble and relatively non-polar compound, it might be expected to penetrate the blood-brain and blood-CSF barriers. In the cat, however, the $^{14}$C-labelled acid is only able to cross them with difficulty (17). The present study showed no concentration gradients of free PAA in at least 10 ml of the lumbar CSF, as was reported by Young et al. (18). PAA seems to be derived from PEA since it was demonstrated that the regional distribution of PAA parallels that of PEA (19) and, under physiological conditions, PAA mostly in the free form, accounts for more than 90% of the brain metabolites of PEA (20). It is still uncertain, however, how PAA in the CSF reflects the central PEA metabolism.

Despite these difficulties, the present data do point to the possibility of a decreased concentration or turnover of PEA in the brain of depressive patients. Because of the small number of subjects, a larger sample will be necessary before we can reach a definite conclusion. A detailed clinical study is in progress which will be published elsewhere.

References


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