Acta Medica Okayama

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

Masahiro Kawabata[*]   Kiyofumi Kobayashi[†]

Toshikiyo Shohmori[‡]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama University,

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

Masahiro Kawabata, Kiyofumi Kobayashi, and Toshikiyo Shohmori

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 spectromutry

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

Masahiro Kawabata, Kiyofumi Kobayashi and Toshikiyo Shohmori

Department of Clinical Neurochemistry, Institute for Neurobiology, Okayama University Medical School, Okayama 700, Japan

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), heterogenous 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, how-

ever, 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.

271

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 dysthymic 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-d_7) 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) ethanolic 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-d_7 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 pentafluoro-benzyl esters of phenylacetic acid and hepta-deuterated 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 focussed 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,
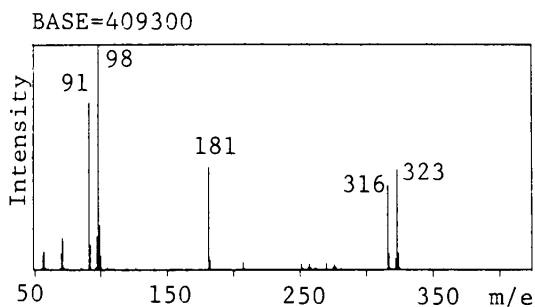
BASE=409300

Fig. 1 Mass spectra of pentafluorobenzyl esters of phenyl-acetic acid (PAA-PFB) and heptadeuterated phenylacetic acid (PAA-d$_7$-PFB). The spectra determined at 20 eV.
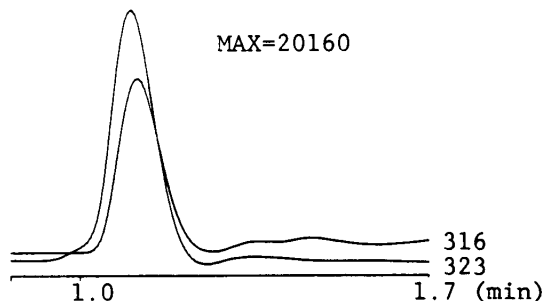
MAX=20160

Fig. 2 Typical mass fragmentogram of pentafluorobenzyl esters of phenylacetic acid (m/e 316) and heptadeuterated phenylacetic acid (m/e 323).

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
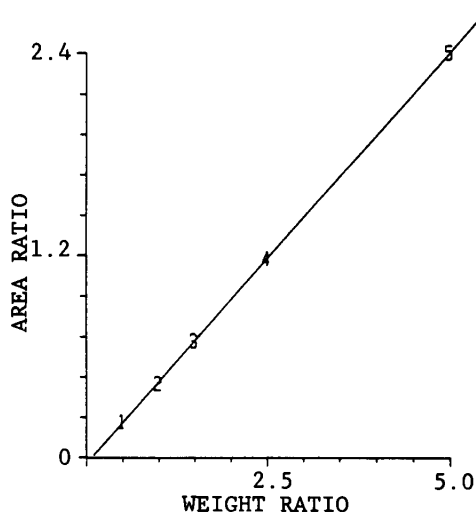

Fig. 3 Calibration curve constructed from the ratios of molecular ions of PAA-PFB* to PAA-d$_7$-PFB* with observed ratio as ordinate and known ratio as abscissa. * : See legend in Fig. 1.

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

Table 1 Free phenylacetic acid concentrations in cerebrospinal fluid

Subjects	Number	Phenylacetic acid[a]
Controls	18	26.2 ± 3.0
Schizophrenia	24	30.1 ± 3.4
Depression	6	14.8 ± 1.9*

a: Mean ± SEM (ng/ml), * $p < 0.05$ vs. controls

Table 2 Free phenylacetic acid concentrations in fractions of cerebrospinal fluid (ng/ml)

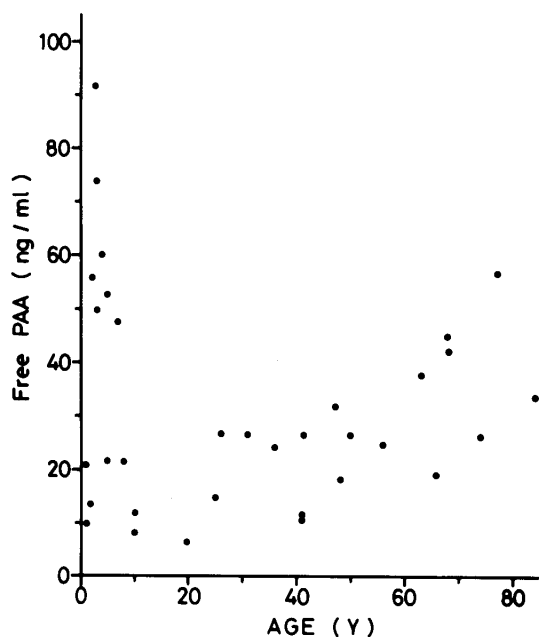| Patient	Fraction 1[a]	Fraction 2[a]
K.F.	85.01	81.46
T.K.	6.42	4.99
S.O.	26.82	28.40
K.H.	7.35	6.73
K.H.	26.75	27.13
T.T.	10.88	9.33
Y.M.	7.62	7.10
I.O.	24.33	23.80
A.T.	27.96	29.34
S.T.	14.64	16.89
T.U.	18.04	17.70
T.H.	13.28	13.50
M.T.	38.50	33.72

a: See text.

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 structually 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

1. Durden DA, Philips SR and Boulton AA: Identification and distribution of β-phenylethylamine in the rat. Can J Biochem (1973) **51**, 995-1002.
2. Inwang EE, Mosnaim AD and Sabelli HC: Isolation and characterization of phenylethylamine and phenylethanolamine from human brain. J Neurochem (1973) **20**, 1469-1473.
3. Durden DA and Philips SR: Kinetic measurements of the turnover rates of phenylethylamine and tryptamine *in vivo* in the rat brain. J Neurochem (1980) **33**, 1725-1732.
4. Boulton AA and Baker GB: The subcellular distribution of β-phenylethylamine, *p*-tyramine and tryptamine in rat brain. J Neurochem (1975) **25**, 477-481.
5. Amarican Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders (3rd ed.). American Psychiatric Association, Washington, DC (1980).
6. Fellow LE, King GS, Pettit BR, Goodwin BL, Ruthven CRJ and Sandler M: Phenylacetic acid in human cerebrospinal fluid and plasma: selected ion monitoring assay. Biomed Mass Spectrom (1978) **5**, 508-511.
7. Sandler M, Ruthven CRJ, Goodwin BL, King GS, Pettit BR, Reynolds GP, Tyrer SP, Weller MP and Hirsch SR: Raised cerebrospinal fluid phenylacetic acid concentration: preliminary support for the phenylethylamine hypothesis of schizophrenia? Commun Psychopharmacol (1978) **2**, 199-202.
8. Karoum F, Torrey EF, Murphy DL and Wyatt RJ: The origin, drug interaction, urine, plasma and CSF concentrations of phenylacetic acid in normal and psychiatric subjects; in Neurobiology of the Trace Amines, Boulton, Baker, Dewhurst and Sandler eds, Humana Press, Clifton (1984) pp. 457-473.
9. Jeste DV, Doonagaji DR, Panjwani D, Datta M, Potkin SG, Karoum F, Thatte S, Sheth AS, Apte

Kawabata *et al.*

JS and Wyatt RJ: Crosscultural study of a bio-
chemical abnormality in paranoid schizophrenia.
Psychiatr Res (1981) **3**, 341-352.

10. Potkin SG, Karoum F, Chuang L, Cannon-Spoor HE,
    Phillips I and Wyatt RJ: Phenylethylamine in paranoid
    chronic schizophrenia. Science (1979) **206**, 470-471.
11. Beckmann H, Reynolds GP, Sandler M, Waldmeier
    P, Lauber J, Riederer P and Gattaz WF: Phenyl-
    ethylamine and phyenylacetic acid in CSF of schizo-
    phrenics and healthy controls. Arch Psychiatr Nerve-
    nkr (1982) **232**, 463-471.
12. Sandler M, Ruthven CRJ, Goodwin BL and Coppen
    A: Decreased cerebrospinal fluid concentration of
    free phenylacetic acid in depressive illness. Clin
    Chim Acta (1979) **93**, 169-171.
13. Fischer E and Heller B: Phenylethylamine as a
    neurohumoral agent in brain. Behav Neuropsychiatry
    (1972) **4**, 8-11.
14. Mosnaim AD, Inwang EE, Sugerman JH, DeMartini
    WJ and Sabelli HC: Ultraviolet spectrophotometric
    determination of 2-phenylethylamine in biological
    samples and its possible correlation with depression.
    Biol Psychiatry (1973) **6**, 235-257.
15. Sabelli H, Fawcett J, Gusovsky F, Edwards J,
    Jeffriess H and Javaid J: Phenylacetic acid as an
    indicator in bipolar affective disorders. J Clin
    Psychopharmacol (1983) **3**, 268-270.
16. Sandler M, Ruthven CRJ, Goodwin BL, Lees A and
    Stern GM: Phenylacetic acid in human body fluids:
    high correlation between plasma and cerebrospinal
    fluid concentration values. J Neurol Neurosurg Psy-
    chiatry (1982) **45**, 366-368.
17. Pedemonte WA, Mosnaim AD and Bulat M: Penetra-
    tion of phenylacetic acid across the blood-cerebrospinal
    fluid barrier. Res Commun Chem Pathol Pharmacol
    (1976) **14**, 111-116.
18. Young SN, Davis BA and Gauthier S: Precursors
    and metabolites of phenylethylamine, *m* and *p*-tyramine
    and tryptamine in human lumbar and cisternal cere-
    brospinal fluid. J Neurol Neurosurg Psychiatry
    (1982) **45**, 633-639.
19. Durden DA and Boulton AA: Identification and dis-
    tribution of phenylacetic acid in the brain of the rat.
    J Neurochem (1982) **38**, 1532-1536.
20. Fulton TR, Triano T, Rabe A and Loo YH: Phenyl-
    acetate and the enduring behavioral deficit in experi-
    mental phenylketonuria. Life Sci (1980) **27**, 1271-
    1281.