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Note

Simultaneous determination of plasma phenethylamine, phenylethanolamine, tyramine and octopamine by high-performance liquid chromatography using derivatization with fluorescamine

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It has been suggested that aromatic trace amines play a role in neural transmission [1]. In addition, impaired metabolism of these amines has been implicated in certain pathological conditions, such as migraine, Parkinson's disease, schizophrenia, phenylketonuria and depressive illness [2]. Patients with advanced liver disease often manifest neurological disorders, encephalopathy, circulatory renal failure and hyperdynamic conditions. These symptoms have been attributed to aromatic trace amines derived from tyrosine and phenylalanine, which are normally metabolized in the liver [3,4]. Increased brain levels of phenethylamine (PEA), phenylethanolamine (PEOH), tyramine (TYR) and octopamine (OCT), as well as decreased levels of catecholamines, have been reported in animals with severe liver dysfunction [5,6]. Elevated plasma levels of PEOH, TYR and OCT have been reported in patients with hepatic encephalopathy and liver cirrhosis [7–11].

Since the trace amines undergo similar metabolism and may be interconvertible [12], simultaneous determinations are needed to clarify the etiological relationships between disturbances in amine metabolism and pathological conditions. Several fluorometric methods have been reported for the determination of trace amines in biological specimens [13], but most of these seem to be unreliable. The reported amine levels were considerably higher than those measured by more

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specific methods [14–21]. The specific methods, which include radioenzymatic assay (REA), thin-layer chromatographic–mass spectrometric (TLC–MS) and gas chromatographic–mass spectrometric (GC–MS) methods, require expensive instruments, purified enzymes or radioactive compounds. Furthermore, most of them are not suitable for the simultaneous determination of all trace amines.

Previously, we developed a method for the simultaneous determination of PEA, PEOH, TYR and OCT by TLC using derivatization with fluorescamine [22]. In order to improve recovery and sensitivity, we now propose the use of high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals and apparatus

All chemicals and solvents were of the purest grade commercially available. Sep-Pak C_{18} cartridges from Waters (Milford, MA, U.S.A.) were used for the preparation of a trace amine fraction. Two cartridges of Sep-Pak C_{18} were connected in series and washed with 5.0 ml of methanol and 5.0 ml of distilled water before use. HPLC analyses were carried out with a Shimadzu LC-4A apparatus equipped with an RF-530 fluorescence spectrometer and a C-R2A Chromatopac integrator (Tokyo, Japan). The reversed-phase chromatographic column was a 250 mm × 4.5 mm I.D. ODS-120T column from Toyo Soda (Tokyo, Japan).

Preparation of the trace amine fraction

Heparinized blood (5 ml) was centrifuged at 3000 *g* for 10 min with 50 μl of 0.2 *M* disodium EDTA and 0.2 *M* sodium metabisulphite. As an internal standard, 40 pmol of benzylamine (BA) were added to 2.0 ml of the plasma. After deproteinization with 2.0 ml of 2.5% perchloric acid, the pH of the supernatant was adjusted to 6.5 by dropwise addition of 2 *M* potassium hydroxide solution. The precipitate was removed by centrifugation at 3500 *g* for 15 min. The supernatant was applied to a column filled with Amberlite CG-50 (9.5 cm × 0.4 cm I.D.) which had previously been equilibrated with 0.2 *M* sodium phosphate buffer (pH 6.5) and washed with 10.0 ml of 5 m*M* disodium EDTA. For the elution of the catecholamines, the column was washed with 2.0 ml of 0.8 *M* lithium borate. Basic amino acids were eluted with 3.0 ml of 0.1 *M* sodium phosphate buffer (pH 6.9) containing 5 m*M* disodium EDTA. Finally, the trace amine fraction containing PEA, PEOH, TYR and OCT was eluted with 7.5 ml of 1 *M* sodium phosphate buffer (pH 8.5) containing 5 m*M* disodium EDTA.

Derivatization with fluorescamine

After the pH of the trace amine fraction had been adjusted to 8.5 by dropwise addition of 4 *M* potassium hydroxide solution, the fraction was applied to two Sep-Pak C_{18} cartridges connected in series. The cartridges were washed first with 2.0 ml of distilled water and 1.0 ml of 90% methanol. The trace amines were eluted with 4 ml of 90% methanol. After addition of 1.0 ml of 0.1 *M* sodium phosphate buffer (pH 8.5), the eluate was evaporated to 1.0 ml under a gentle stream of nitrogen at 38°C and then mixed with 0.6 ml of fluorescamine solution in acetone

(3 mg per 100 ml) at room temperature with stirring on a vortex mixer for 1 min. The reaction mixture was evaporated to 1.0 ml under nitrogen at 38°C and then acidified with 50 μl of 4 *M* sodium acetate buffer (pH 5.5). Ethyl acetate (3 ml) was added for extraction and the mixture was stirred on a vortex mixer for 1 min. After brief centrifugation, the ethyl acetate layer was separated and evaporated under nitrogen at 38°C after addition of 40 μl of distilled water. The residual water layer was mixed with 40 μl of the HPLC mobile phase by brief stirring. Aliquots (50 μl) of the mixture were used for HPLC separation.

Chromatography

The mobile phase was 0.05 *M* citrate buffer (pH 2.5)–methanol–ethyl acetate (42:40:18, v/v/v); the flow-rate was 0.8 ml/min. Fluorescene was measured at 475 nm with excitation at 390 nm. As external standard, a mixture of reference compounds including PEA, PEOH, TYR, OCT and BA (40 pmol of each) was added to the eluate of the Sep-Pak C_{18} column after chromatography of 2.0 ml of 2.5% perchloric acid (blank). It was treated with fluorescamine in the same way as the plasma samples. In order to estimate recovery, the same amount of amines was added to plasma (internal standard). Standard curves were constructed from four parallel determinations (2, 5, 10, 20, 40 and 80 pmol of each amine), and the values were based on the peak area of 40 pmol of BA. In order to investigate the potential effect of other compounds on the determination of the fluorescamine derivatives of trace amines, 300 nmol of basic amino acids and 10 nmol of catecholamines, metanephrine, normetanephrine, histamine, serotonin, cadaverine and putrescine were added to plasma samples.

Determination of trace amines in plasma

Venous blood samples were collected into heparinized tubes with disodium EDTA and sodium metabisulphite after overnight fasting from sixteen healthy volunteers (seven females and nine males; age, 23–45 years) and six patients with liver cirrhosis. The blood samples were centrifuged and stored until use at −70°C after addition of BA (20 pmol/ml). The concentration of each amine was calculated relative to the peak area of BA. The results were compared using the Student's *t-test.*

RESULTS

A chromatogram of plasma containing a mixture of reference compounds is shown in Fig. 1. The fluorescamine derivatives of OCT, TYR, PEOH, BA and PEA were separated within less than 20 min. OCT and PEOH each produced two separate peaks, the second of which were used for determinations because of their higher intensities. Also, the first peak of OCT was often obscured by unknown peaks. Addition of basic amino acids, metabolites of catecholamines, histamine, diamines and serotonin to plasma produced no interferences. The minimum detectable amounts were below 0.2 pmol/ml for each amine. Standard curves were linear over a concentration range from 2 to 80 pmol/ml. The recovery of trace amines and BA during extraction from plasma and purification by chromato-

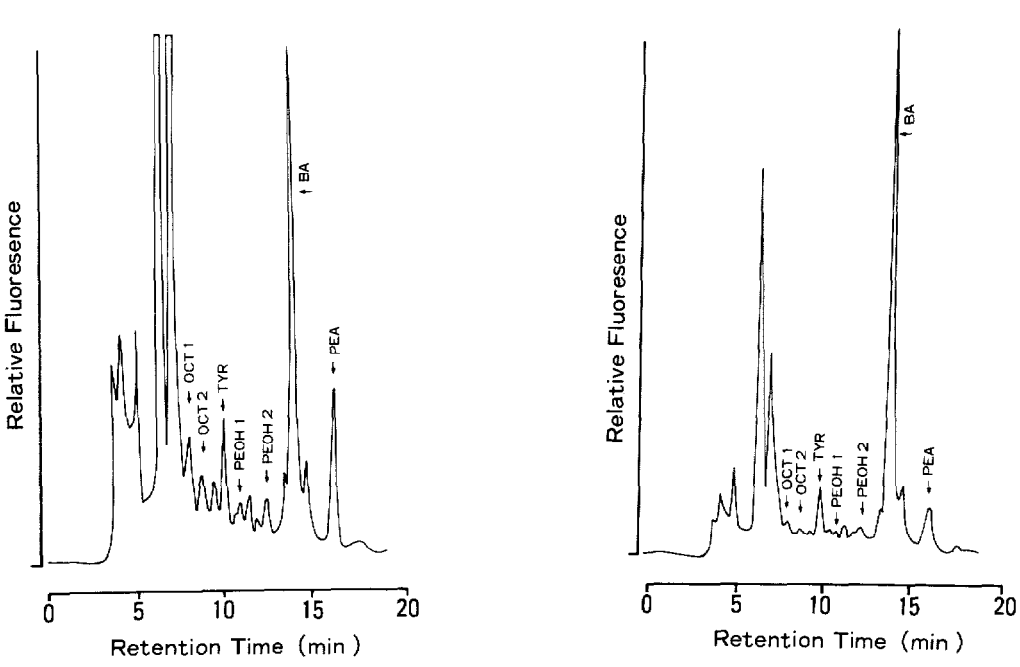

Fig. 1. Chromatogram of a standard mixture of trace amines and benzylamine (BA) added to plasma. A standard mixture of trace amines (2 pmol of each) and BA (40 pmol) was added to 2.0 ml of plasma drawn from a healthy volunteer and was taken through the entire procedure. Octopamine (OCT) and phenylethanolamine (PEOH) produced two separate peaks (OCT 1 and OCT 2, PEOH 1 and PEOH 2, respectively). Other peaks: TYR = tyramine; PEA = phenethylamine.

Fig. 2. Chromatogram of plasma trace amines of a healthy volunteer. As an internal standard, 40 pmol of benzylamine (BA) were added to 2.0 ml of plasma, as described in the text. Other abbreviations are as in Fig. 1.

TABLE I

RECOVERY AND REPRODUCIBILITY OF THE ASSAY OF PHENETHYLAMINE (PEA), PHENYLETHANOLAMINE (PEOH), TYRAMINE (TYR), OCTOPAMINE (OCT) AND BENZYLAMINE (BA)

Recovery and coefficient of variation were calculated from four determinations, as described in the text.

Compound	Recovery (%)	Coefficient of variation (%)
PEA	63.1	3.91
PEOH	66.2	4.15
TYR	83.3	4.06
OCT	81.0	7.77
BA	62.0	8.73

graphy was between 62.0 and 83.3%. The coefficients of variation (C.V.) were 3.91–8.73% (Table I).

The plasma levels of trace amines in healthy volunteers (Fig. 2) and patients

TABLE II

PLASMA LEVELS OF TRACE AMINES IN HEALTHY VOLUNTEERS AND PATIENTS WITH LIVER CIRRHOSIS

Trace amine	Concentration (mean ± S.D.) (pmol/ml)	
	Healthy volunteers ($n=16$)	Patients with liver cirrhosis ($n=6$)
PEA	0.33 ± 0.23	0.52 ± 0.52
PEOH	0.70 ± 0.53	4.58 ± 3.02*
TYR	0.49 ± 0.37	1.78 ± 0.88*
OCT	0.75 ± 0.50	2.64 ± 2.46*

*$P < 0.05$: significant difference between healthy volunteers and cirrhotic patients.

with liver cirrhosis are summarized in Table II. In healthy volunteers, no significant differences were noted between the amine levels of men and women. The plasma levels of PEOH, TYR and OCT in the patients with liver cirrhosis were significantly higher than those in healthy volunteers.

DISCUSSION

The recovery and reproducibility of our TLC method [22] were poor, because the procedure required a long time and because the fluorescamine derivatives were not stable [23]. We have now modified the isolation method for the trace amines, and the fluorescamine derivatives were analysed by HPLC. These modifications shortened the analysis time, but recoveries were still insufficiently high during extraction from plasma and chromatographic purification; this is especially true for PEA and PEOH (Table I). β-Hydroxylated amines, PEOH and OCT, produced two separate peaks with fluorescamine (Fig. 1), as was reported previously [22], but separation by HPLC gave a higher sensitivity and reproducibility than the previous method (Table I). Since we were unable to obtain reference samples of *m*- and *p*-tyramine and *m*- and *p*-octopamine, the separation of these amines was not attempted.

Among previously published methods for trace amines, only GC–MS allowed the simultaneous determination of PEA, PEOH, TYR and OCT, though plasma levels of PEOH and OCT could not be determined [21]. Plasma levels of trace amines as obtained by different methods differed considerably: PEA, 5.3 pmol/ml (GC–MS [21]), 35.3 pmol/ml (HPLC [24]); PEOH, not determined (GC–MS [21]), 8.0 pmol/ml (REA [10]); TYR, 5.0 pmol/ml (GC–MS [21]), 9.5 pmol/ml (radioimmunoassay (RIA) [9]); OCT, not determined (GC–MS [21]), 30.1 pmol/ml (REA [8]). These variations have been attributed to contamination with materials that may react with the reagents. The plasma levels measured by our method (Table II) were considerably lower for PEA and TYR than those mentioned above, and were closest to the lowest values [21] with regard to PEOH

and OCT. Further investigations may, however, be required to assess the true levels of these amines.

The plasma levels of PEOH, TYR and OCT have been reported to be elevated in patients with liver dysfunction [7–11]. The plasma level of PEA, however, did not increase in patients with liver cirrhosis (Table II). Since our method may also be applicable to the analysis of trace amines in other biological samples as well, including brain and cerebrospinal fluid, it should be useful to investigate abnormal amine metabolism in various pathological conditions.

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