Journal of Chromatography, 231 (1982) 291—299
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1310

SIMULTANEOUS ANALYSES OF PHENETHYLAMINE, PHENYLETHANOLAMINE, TYRAMINE AND OCTOPAMINE IN RAT BRAIN USING FLUORESCAMINE

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(First received June 22nd, 1981; revised manuscript received April 17th, 1982)

SUMMARY

A fluorometric method for the simultaneous analyses of phenethylamine, phenylethanolamine, tyramine and octopamine has been developed. The method involves ion-exchange chromatography, derivatization with fluorescamine, solvent extraction and then separation by thin-layer chromatography. The fluorescent spots are then quantitated by scanning. The detection limits of this method are about 10 pmoles for phenethylamine, phenylethanolamine and tyramine, and 20 pmoles for octopamine. The method was used for simultaneous analyses of putative neurotransmitter amines in whole rat brain.

INTRODUCTION

Recently, it has been suggested that aromatic trace amines, including phenylalkylamines, indoleamines, and imidazoleamines, may play roles in neural transmission in the brain [1—3]. Furthermore, abnormal metabolism of these amines has been shown to be implicated in various pathologic conditions, such as migraine [4], Parkinson's disease [5], schizophrenia [6], phenylketonuria [7], depressive illness [8] and hepatic encephalopathy [9—12].

Many reports have shown that severe liver disorder results in disturbances in metabolism of putative neurotransmitter amines in the brain [9—13], which may explain the pathogenesis of hepatic encephalopathy. To clarify the relation between disturbances in metabolism of brain amines and hepatic encephalo-

0378-4347/82/0000—0000/$02.75

pathy, we needed to analyze brain neurotransmitter amines simultaneously in experimental animals. In particular, since phenethylamine, phenylethanolamine, tyramine and octopamine, which are mainly derived from phenylalanine and tyrosine in the brain [14], undergo similar metabolism and may be interconvertible [15], it was important to determine the changes in contents of these amines in the brain simultaneously.

Recently developed analytical methods, including radioenzymatic, thin-layer chromatographic—mass spectrometric and gas chromatographic—mass spectrometric methods, have revealed the presence of phenethylamine [16—18], phenylethanolamine [18—20], tyramine [21, 22] and octopamine [20, 23—25] in rat brain. These methods, however, require expensive instruments, purified enzymes, or radioactive compounds, and thus a simpler method that can be routinely used in a laboratory is desirable.

We developed a systematic analytical method for biogenic amines [26]. In this paper, we have attempted to measure phenethylamine, phenylethanolamine, tyramine and octopamine simultaneously by converting them to fluorescent fluorescamine conjugates and separating the latter by thin-layer chromatography.

EXPERIMENTAL

All chemicals and solvents were of the purest grade commercially available. Precoated silica gel plates (without fluorescent indicator, thickness 0.25 mm) were from Merck (Darmstadt, G.F.R.). 2-Phenyl[1-^{14}C]ethylamine·HCl (specific radioactivity, 60.6 mCi/mmole) and [1-^{14}C]tyramine (50.0 mCi/mmole) were purchased from Amersham International (Arlington Heights, IL, U.S.A.) and New England Nuclear (Boston, MA, U.S.A.), respectively.

Extraction of fluorescamine derivatives with ethyl acetate

A solution of 1—10 nmoles of each amine in 3 ml of 0.5 *M* sodium borate, pH 8.5, was rapidly mixed with 1.0 ml of fluorescamine solution in acetone (20 mg per 100 ml) at room temperature and stirred on a vortex mixer for 1 min. The mixture was then evaporated to 0.3 ml under nitrogen at 25° C, and the residue was dissolved in 3.0 ml of 4 *M* acetate buffer, pH 3.0—6.5. The fluorescent conjugate was extracted twice with 3.0 ml of ethyl acetate and the fluorescence of the acetate buffer phase was measured in an Hitachi fluorospectrophotometer, Model MPF-4, at 480 nm with excitation at 390 nm. The extraction rate was calculated by comparing the fluorescence before and after extraction and a correction was made with appropriate blanks.

Preparation of the trace amine fraction

Male Wistar rats weighing 350—400 g, some subjected to surgical construction of a portacaval shunt (PCS) 4 weeks earlier [13], were decapitated between 10:00 a.m. and noon. The brain was quickly removed, weighed, and stored at —70° C until use.

The amine fraction containing polyamines, histamine and trace amines, such as phenethylamine, phenylethanolamine, tyramine, and octopamine, was prepared with Amberlite CG-50 and Sephadex G-10 chromatography as reported

previously [13, 26]. Briefly, whole brain (about 2 g) was homogenized in 6.0 ml of ice-cold 3% perchloric acid containing 0.2% disodium EDTA and 1.0 m*M* dithiothreitol in a Polytron homogenizer (Kinematica, Luzern, Switzerland) operated at the maximum setting for 1 min in an ice-bath. The homogenate was centrifuged at 10,000 *g* for 40 min at 4°C. The pH of the supernatant was adjusted to 6.0 by dropwise addition of 5 *M* potassium hydroxide solution and the precipitate was removed by brief centrifugation. The supernatant was applied to a column of Amberlite CG-50 (0.4 cm I.D. × 9.5 cm) equilibrated with 0.1 *M* sodium phosphate buffer, pH 6.0, and the column was eluted with 2.0 ml of distilled water. The resultant eluate was collected (fraction A, containing neutral and acidic amino acids and amine metabolites). The column was washed with 8.0 ml of distilled water and with 4.0 ml of 0.01 *M* sodium phosphate buffer, pH 6.0, containing 1.5% boric acid; then noradrenaline was eluted with 3.0 ml of the same solution (noradrenaline fraction) and dopamine was eluted with 3.0 ml of 4% boric acid (dopamine fraction). After elution of the dopamine fraction, basic amino acids (Lys, Orn, Arg) were eluted with 5.0 ml of 0.1 *M* sodium phosphate buffer, pH 6.9, and with 5.0 ml of 0.1 *N* hydrochloric acid (fraction B). Finally, amines were eluted with 3.0 ml of 0.5 *N* hydrochloric acid, and the eluate was applied to a column of Sephadex G-10 (0.4 cm I.D. × 9.5 cm) equilibrated with 0.1 *N* hydrochloric acid, and the column was eluted with 1.0 ml of 0.5 *N* hydrochloric acid. The resultant eluate was collected (amine fraction — containing polyamines, histamine, and trace amines such as phenethylamine, phenylethanolamine, tyramine, and octopamine) and stored at −80°C for further analysis. Indoleamines, including serotonin and tryptamine, were eluted with 3.0 ml of distilled water (serotonin fraction).

Determination of trace amines

The amine fraction was evaporated to dryness under vacuum and the residue was dissolved in 1.0 ml of 0.5 *M* sodium borate buffer, pH 8.5. The solution was promptly mixed with 1.0 ml of fluorescamine solution in acetone (75 mg per 100 ml) at room temperature with stirring on a vortex mixer for 1 min. The reaction mixture was evaporated to 0.3 ml under nitrogen at 25°C, and then acidified with 1.0 ml of 4 *M* acetate buffer, pH 4.5, and mixed with 4 ml of ethyl acetate on a vortex mixer for 1 min. The ethyl acetate layer was separated and evaporated to 0.2 ml under nitrogen at 25°C. Then, 0.1 ml of distilled water was added and the mixture was evaporated to 0.2 ml under nitrogen at 25°C. The fluorescent compounds were extracted with 20 μl of ethyl acetate and a 5-μl aliquot of extract was used for thin-layer chromatography. Two-dimensional chromatography was performed on a 10 × 10 cm silica gel plate with ethyl acetate—hexane—methanol—water (60:20:25:10) as the first solvent and benzene—dioxane—acetic acid (90:25:5) as the second. The fluorescent spots of phenethylamine, phenylethanolamine, tyramine and octopamine were located under ultraviolet light and measured with a Shimadzu chromatoscanner, Model CS900.

For recovery experiments, 5 nCi of each of [^{14}C]phenethylamine or [^{14}C]-tyramine were mixed with the supernatant fraction of brain homogenate and treated similarly as described above. The spots on the thin-layer chromatogram

corresponding to phenylethylamine and tyramine were scraped off and counted for radioactivity in 10 ml of a scintillation cocktail (ACS II, Amersham) with an Aloka liquid scintillation counter (LCS-700).

RESULTS

Extraction of fluorescamine derivatives of trace amines into organic solvent

To subject fluorescamine derivatives of trace amines to thin-layer chromatography, it is necessary to extract them into an organic solvent. Therefore, the optimal pH for extraction of the derivatives was examined. As shown in Fig. 1, the lower the pH, the better the extraction. However, as fluorescamine derivatives were labile in acidic conditions, pH 4.5 was used in the routine experiments.

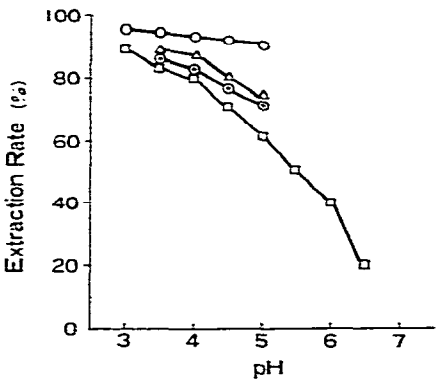

Fig. 1. Effects of pH on extraction rate of fluorescamine derivatives with ethyl acetate. Fluorescamine derivatives of various amines were dissolved in 3 ml of 4 *M* acetate buffer at different pH values and were extracted with 3 ml of ethyl acetate twice. The fluorescence of the acetate buffer phase was measured at 480 nm with excitation at 390 nm as described under Experimental. ○, Phenethylamine; △, phenylethanolamine; ◎, tyramine; □, octopamine.

Determination of trace amines by thin-layer chromatography

Fig. 2 shows the thin-layer chromatogram of fluorescamine derivatives of trace amines on a silica gel plate when developed by ethyl acetate—hexane—methanol—water (60:20:25:10) as the first solvent and benzene—dioxane—acetic acid (90:25:5) as the second. The four major trace amines — phenethylamine, phenylethanolamine, octopamine and tyramine — were separated completely under these conditions. Fluorescent spots were scanned with a Shimadzu chromatoscanner for quantitation. Fig. 3 shows scans of spots corresponding to the four major trace amines from the brains of normal (A) and PCS (B) rats. The minimum amounts detectable by this procedure were 10 pmoles of phenylethylamine, phenylethanolamine and tyramine, and 20 pmoles of octopamine. Fig. 4 shows a standard curve of these trace amines, indicating a linear relationship between fluorescence intensities and the amounts of trace amines over the range from 10—20 to 300 pmoles.

Recovery and reproducibility of the assay

To estimate the recovery and the reproducibility of the present assay meth-

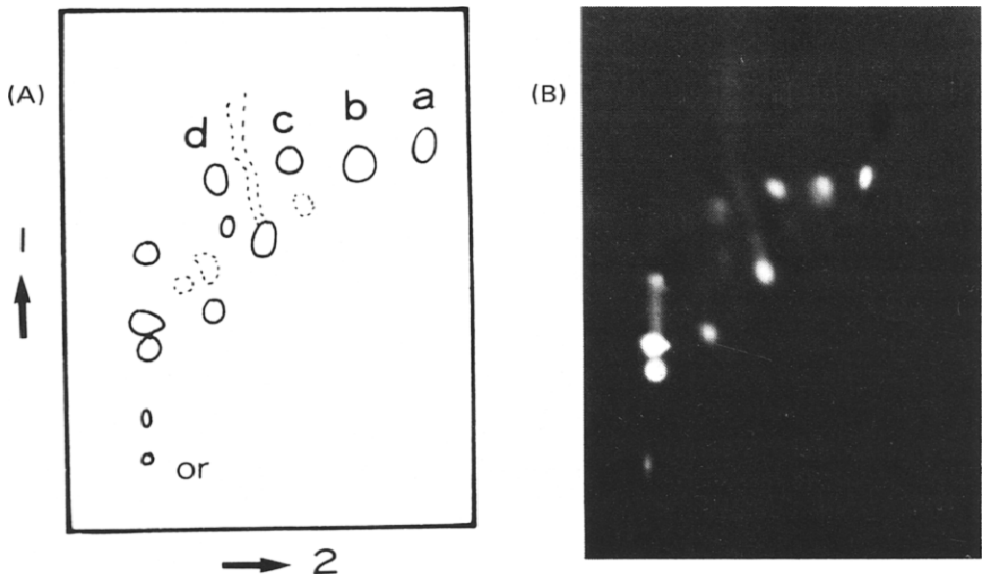

Fig. 2. Two-dimensional thin-layer chromatography of the fluorescamine derivatives of trace amines on silica gel plate (B) and its tracing (A). The sample was applied at the point "or" and developed in two dimensions. Solvent system 1 = ethyl acetate—hexane—methanol—water (60:20:25:10), and solvent system 2 = benzene—dioxane—acetic acid (90:25:5). Spots were located under an ultraviolet lamp. The spots a, b, c, and d show the locations of the fluorescamine derivatives of phenethylamine, phenylethanolamine, tyramine and octopamine, respectively.

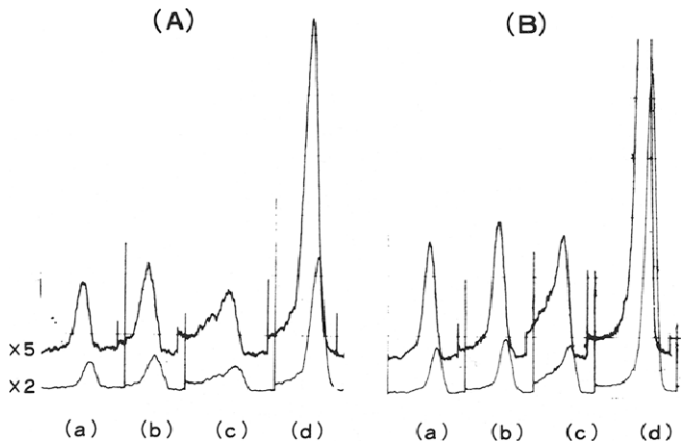

Fig. 3. Typical scanning curves of spots corresponding to phenethylamine (a), phenylethanolamine (b), tyramine (c), and octopamine (d) with a Shimadzu chromatoscanner, Model CS-900 (excitation 365 nm, filter 500 nm). (A) normal rat brain; (B) PCS rat brain.

od, ^{14}C-labelled phenethylamine and tyramine were processed through the same procedure and the corresponding spots on the thin-layer plate were cut out and counted in a liquid scintillation counter as described under Experimental. As shown in Table I, the recoveries of phenylethylamine and tyramine through the whole procedure were 30 ± 5 and 48 ± 18%, respectively, and the

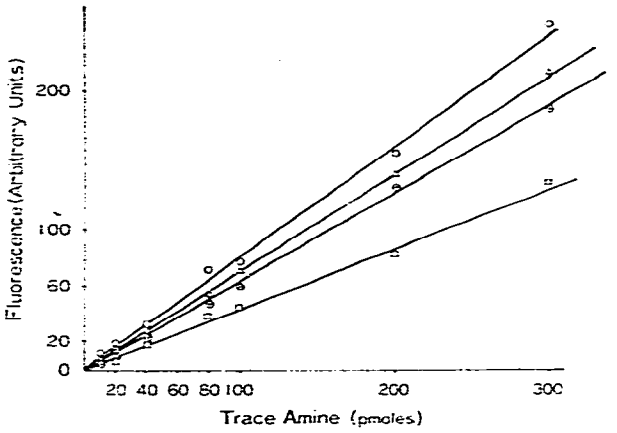

Fig. 4. Standard curves of fluorescamine derivatives of phenetylamine (○), phenylethanolamine (△), tyramine (⊖) and octopamine (□).

TABLE I

RECOVERY AND REPRODUCIBILITY OF THE ASSAY OF ^{14}C-LABELLED PHENYLETHYLAMINE AND TYRAMINE

	Phenylethylamine	Tyramine
Initial radioactivity (dpm)	9508	16,593
Radioactivity after thin-layer separation (dpm)	722 ± 124	1998 ± 783
Recovery* (%)	30 ± 5	48 ± 18
Coefficient of variation (%)	17	39

*The recovery was calculated by multiplying by 4 the counts of spots on the thin-layer plate because the fluorophores were extracted with 20 μl of the ethyl acetate and 5 μl were spotted.

coefficients of variation were 17 and 39% for phenylethylamine and tyramine, respectively.

Trace amine levels of whole brains of normal and PCS rats

The levels of trace amines in whole brains of normal and PCS rats are summarized in Table II. The levels were higher in PCS rats than in normal rats; the levels of phenethylamine, phenylethanolamine, tyramine and octopamine increased by 1.5, 3.0, 2.5 and 4.0 times, respectively, after construction of a portacaval shunt.

DISCUSSION

5-Dimethylaminonaphthalene-1-sulphonyl (Dns) derivatives have been used for determination of trace amines [16, 20—22], but they are not suitable for simultaneous fluorometric analyses of these amines because all the trace amines except phenethylamine form several fluorogenic Dns derivatives [27]. Fluorescamine has been introduced as a highly sensitive fluorogenic reagent for primary amino groups [28—30], but it has been used less extensively than Dns

TABLE II

THE LEVELS OF TRACE AMINES IN WHOLE BRAINS OF SHAM-OPERATED AND PCS RATS

Values are means ± S.D. in pmoles per g wet weight. Numbers of animals are shown in parentheses.

	Normal (5)	PCS (7)
Phenethylamine	7.5 ± 0.4	11.5 ± 2.1*
Phenylethanolamine	7.2 ± 0.4	21.9 ± 1.9**
Tyramine	7.3 ± 1.5	18.5 ± 2.8**
Octopamine	20.6 ± 4.8	80.0 ± 20.3*

*$P<0.05$, **$P<0.01$: significant difference from the value for normal rats.

reagent for determination of primary amines in biological specimens, partly because it is difficult to obtain derivatives of primary amines in biological specimens on a microscale [31]. We resolved this problem by extracting the fluorescamine derivatives with ethyl acetate. Another problem is that fluorescamine derivatives are unstable in acidic solution [32]. Moreover, use of a high temperature in the evaporation of ethyl acetate and complete drying of the fluorescamine derivatives decreased the fluorescence. To avoid destruction of the fluorescamine derivatives, we used 4 *M* acetate buffer, pH 4.5, for extraction. These conditions may be responsible for the low sensitivity of the method for octopamine.

Since the procedure is so long and complex, the recovery and reproducibility was estimated using ^{14}C-labelled phenylethylamine and tyramine: their recoveries were 30 and 48%, respectively. These values are fairly good when the long procedure is considered. The coefficients of variation were 17 and 39% for phenylethylamine and tyramine, respectively, indicating that the reproducibility is not so good but that rough estimation is possible.

There have been many reports on the content of trace amines in biological specimens determined by fluorometric methods [33—35]. However, most of them seem to be unreliable, because the reported levels of amines were considerably higher than those measured by more specific methods. The endogenous levels of trace amines in whole rat brains reported here (Table II) are in good accordance with those [16—25] determined by more sophisticated means, such as radioenzymatic, thin-layer chromatographic—mass spectrometric, and gas chromatographic—mass spectrometric methods. Although an attempt to measure simultaneously trace amines by gas chromatography—mass spectrometry was made by Karoum et al. [36], only phenethylamine, and *m*- and *p*-tyramine were detected in measurable quantities in rat brain. The method reported here is suitable for use in most laboratories as a routine method.

A considerable increase in brain octopamine content has been reported in PCS rats, which have been extensively used as a model of chronic hepatic encephalopathy [37]. Our data (Table II) show that the brain levels of phenethylamine, phenylethanolamine and tyramine as well as octopamine are significantly increased in PCS rats. Although our method cannot distinguish between

m- and *p*-tyramine [21, 22] and *m*- and *p*-octopamine [20], it enabled us to perform simultaneous analyses of phenethylamine, phenylethanolamine, tyramine and octopamine as well as of amino acids, dopamine, noradrenaline and serotonin in whole rat brain. The changes in these parameters after construction of a portacaval shunt have been reported [13, 38].

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