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SIMULTANEOUS EXTRACTION AND SEPARATION OF TRACE AMINES OF BIOLOGICAL INTEREST

D.F. LeGATT*, G.B. BAKER and R.T. COUTTS

Neurochemical Research Unit, Faculty of Pharmacy and Pharmaceutical Sciences and Department of Psychiatry, University of Alberta, Edmonton, Alberta T6G 2N8 (Canada)

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SUMMARY

A method is described for the simultaneous extraction and separation of the trace amines 2-phenylethylamine, *m*-tyramine, *p*-tyramine, *p*-octopamine, normetanephrine, and 3-methoxytyramine. The method involves acetylation in aqueous solution, specific hydrolysis of phenolic acetate groups, derivatization with trifluoroacetic anhydride and analysis on a gas chromatograph equipped with an electron-capture detector. Analyses utilizing both packed glass columns and glass capillary columns are described.

The method possesses the potential for quantitative as well as qualitative analysis, with one or more of the following amines employed as internal standards: benzylamine, 3-phenylpropylamine, tranylcypromine, and 2-(4-chlorophenyl)ethylamine.

INTRODUCTION

Neurochemical research in recent years has focused on a group of amines normally present in urine, but detectable in only trace quantities in the central nervous system (CNS). The functional role of these trace amines in the CNS has not been unequivocally determined. However, a number of these substances have been implicated in several nervous disorders. Migraine [1], epilepsy [2], Parkinsonism [3], schizophrenia [4], and depression [5] have been cited as neurological disorders in which the *para*-isomer of tyramine (*p*-TA) may be involved. A decrease in the urinary excretion of the acid metabolite of *para*-octopamine (*p*-OA) has been exhibited in depressed patients [5]. The CNS symptoms of hepatic failure (tremor, coma) may be explained in terms of *p*-OA accumulation in central adrenergic nerves [6]. A role for the trace amine 2-phenylethylamine (PEA) in the aetiology of depression, mania, and schizophrenia has been suggested [7—9].

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In addition, clinical interest in the involvement of catecholamines in psychiatric and neurological disease states has led to a research interest in the O-methylated metabolites of noradrenaline and dopamine, normetanephrine (NME) and 3-methoxytyramine (3-MT), respectively. The latter has been suggested as a reliable indicator of dopaminergic neuron activity in brain, as formation of this metabolite arises from the action of catechol-O-methyl transferase on dopamine released into the synaptic cleft [10, 11].

Methods of analysis developed for qualitative and quantitative determination of the trace amines in biological fluids and brain tissue include thin-layer chromatography [12], colorimetric determination [13], electrophoresis [14], high-performance liquid chromatography [15, 16], gas chromatography (GC) [17—19], spectrophotofluorometric methods [20—25], radioenzymatic procedures [26—29], combined GC—mass spectrometry [30—33], and integrated ion-current mass spectrometry [34].

Although the three latter procedures represent the most widely used methods for trace amine analysis at present, disadvantages are inherent in these techniques. Such disadvantages include complexity of methodology, the requirement for expensive instrumentation (mass spectrometric methods) and an inability to differentiate *m*-tyramine (*m*-TA) and *p*-TA (radioenzymatic procedures), which are both present in brain and body fluids [35, 36]. In addition, radioenzymatic techniques are not used presently for the simultaneous quantitative determination of a spectrum of trace amines in the picogram range.

A GC procedure reported in the literature [18] documents the separation of *p*-TA, octopamine (OA), 3-MT and NME with application to urine. This procedure, however, was not developed to provide sufficient sensitivity for analysis of these amines in brain tissue and no mention is made of the ability of the procedure to separate *p*-TA from the structurally related *meta*-isomer. In our laboratory, we have recently developed an analytical technique for the quantitation of *m*- and *p*-TA in urine [37] using GC with a packed column. We have now initiated efforts for simultaneous extraction and separation of additional trace amines, and the results of these efforts with packed and capillary columns are described in this paper.

EXPERIMENTAL

Chemicals and reagents

The following were used: trifluoroacetic anhydride (TFAA), benzylamine (BZA), *p*-tyramine (*p*-TA) hydrochloride, *p*-octopamine (*p*-OA) hydrochloride, cyclohexane (spectrophotometric grade) (Aldrich, Milwaukee, WI, U.S.A.); pentafluoropropionic anhydride (PFPA) (Pierce, Rockford, IL, U.S.A.); tranylcypromine (TCP) hydrochloride, normetanephrine (NME) hydrochloride, 3-methoxytyramine (3-MT) hydrochloride, 2-phenylethylamine (PEA) hydrochloride (Sigma, St. Louis, MO, U.S.A.); *m*-tyramine (*m*-TA) hydrochloride (Vega Biochemicals, Tucson, AZ, U.S.A.); 3-phenylpropylamine (PPA) and 2-(4-chlorophenyl)ethylamine (CPEA) were obtained as the free bases (Aldrich), and the hydrochloride salts were synthesized in our laboratories; sodium bicarbonate (reagent grade) (Amachem, Portland, OR, U.S.A.); ethyl acetate

(glass distilled) (Caledon Laboratories, Georgetown, Canada); ammonium hydroxide (reagent grade) (J.T. Baker, Phillipsburg, NJ, U.S.A.); and acetic anhydride (analytical grade) (BDH Chemicals, Toronto, Canada).

Extraction and derivatization

All agitations and centrifugations of immiscible liquid systems, unless specified, were performed with an Evapo-Mix Shaker (Buchler Instruments) and a GLC-1 Centrifuge (Sorvall), respectively. Solutions each containing 500 ng to 2 μg of the four amines, *p*-TA, *p*-OA, NME, and 3-MT and 500 ng of the internal standard CPEA were prepared in 0.5 *M* hydrochloric acid (2.5 ml). Two amines, *m*-TA and PEA and three potential internal standards, BZA, TCP and PPA, were added for GC analysis utilizing a glass capillary column. Solid sodium bicarbonate was added to basify each solution and acetic anhydride (300 μl) was added with shaking. Additional amounts of sodium bicarbonate were added intermittently with vortexing until all effervescence had ceased [17]. The aqueous phase was extracted with ethyl acetate (4 ml for 3 min), followed by agitation of the isolated organic phase with ammonium hydroxide (10 *N*, 400 μl) for 40 min and subsequent neutralization with 8 *M* hydrochloric acid (300 μl). After the ethyl acetate layer was evaporated to dryness under nitrogen, ethyl acetate (25 μl) and TFAA (75 μl) were added to the residue. Derivatization was allowed to proceed for 30 min at room temperature. Cyclohexane (300 μl) and saturated aqueous sodium tetraborate (3.0 ml) were added to each tube followed by a 15-sec vortex. The organic layer was isolated after a brief centrifugation.

In addition to the above extraction and derivatization procedure, modifications to this procedure were undertaken for analysis of the amines on the SP 2100 GC capillary column. One modification involved omitting the hydrolysis step (agitation of the organic layer with ammonium hydroxide) and derivatizing the acetylated compounds directly with TFAA. For the second modification, PFPA was substituted for TFAA as the derivatizing agent with the hydrolysis step either included or omitted. For PFPA derivatization, ethyl acetate (25 μl) and PFPA (75 μl) were added to each residue, and reaction proceeded at 60°C for 30 min.

Gas—liquid chromatography

GC analyses were performed on a Hewlett-Packard Model 5835A gas chromatograph equipped with a 15 mCi ^{63}Ni source linear electron-capture detector. The following glass columns (1.8 m × 4 mm I.D.), packed with stationary phase in our laboratory, were used: 3% OV-1 on Gas-Chrom Q (100—120 mesh) and 3% OV-17 on Gas-Chrom Q (100—120 mesh) (Serva, Heidelberg, G.F.R.); 10% OV-1 on Chromosorb W HP (100—120 mesh) (Terochem Laboratories, Edmonton, Canada); 5% OV-7 on Chromosorb 750 (100—120 mesh) (Chromatographic Specialties, Brockville, Canada); 3% OV-3 on Chromosorb W HP (80—100 mesh) (Pierce). The carrier gas was argon—methane (90:10) maintained at a flow-rate of 30 or 40 ml/min. The column temperature ranged between 150 and 195°C. The temperature of both the injection port and detector was 250°C. For capillary analysis, a Grade AA WCOT SP 2100 glass capillary column (10 m) (Supelco, Bellefonte, PA, U.S.A.) was

used. Helium at 7 p.s.i. was used as carrier gas, with argon—methane (90:10) at a flow-rate of 35 ml/min used as make-up gas to the detector. The initial column temperature of 80°C was maintained for 0.6 min and increased to temperatures between 120 and 170°C at rates of 10–30°C/min. The temperature of both the injection port and detector remained at 250°C. Injection volumes of 1 μl and 2 μl were used for analysis on capillary and packed columns, respectively.

RESULTS AND DISCUSSION

Initial efforts in the development of a sensitive, specific GC technique for the simultaneous analysis of trace amines was directed towards resolution of *p*-TA, *p*-OA, NME and 3-MT on glass columns packed with various stationary phases. Two potential internal standards, TCP and/or CPEA, were initially included in the assay.

A lack of sensitivity and poor resolution characterized the results obtained from two of the stationary phases, namely 5% OV-7 and 10% OV-1. Good separability of the four amines and internal standards was achieved with the 3% OV-1 stationary phase. However, this column proved unsuitable as metanephrine, a 3-O-methylated metabolite of epinephrine, interfered with NME regardless of the GC operating conditions employed. The 3% OV-3 column proved to be a suitable column for the spearation of NME, 3-MT, and the two internal standards, CPEA and TCP, but *p*-TA could not be resolved from its metabolite, *p*-OA. The packed column which provided the best separation was the 3% OV-17 phase. The derivatives of *p*-OA, *p*-TA, 3-MT, NME, and the internal standard CPEA gave symmetrical peaks on GC analysis with retention times of 1.8, 2.4, 5.5, 9.1, and 3.6 min, respectively. The temperature program employed was as follows. An initial temperature of 155°C was maintained for

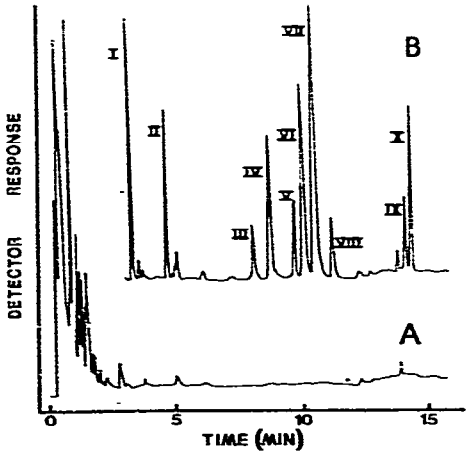


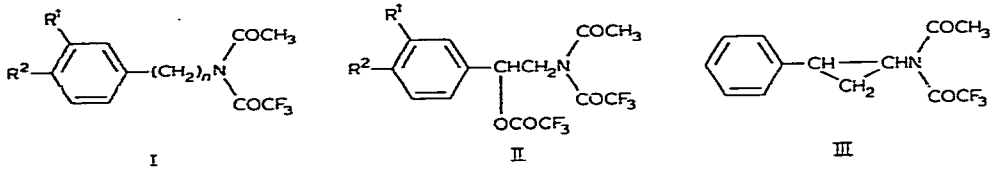
Fig. 1. GC traces of (A) hydrochloric acid blank; (B) standard prepared with 500 ng of each of the amines. Peaks are derivatives of: I, benzylamine; II, 2-phenylethylamine; III, 3-phenylpropylamine; IV, *m*-tyramine; V, tranylcypromine; VI, *p*-tyramine; VII, 2-(4-chlorophenyl)-ethylamine; VIII, *p*-octopamine; IX, normetanephrine; X, 3-methoxytyramine on an SP-2100 glass capillary column.

4.7 min followed by an increase of 5°C/min to a final temperature of 195°C. Although resolution of the amines was achieved, sensitivity of the GC assay required improvement. On both the 3% OV-3 and 3% OV-17 packed phases, the limit of sensitivity for all four amines was 170 pg on column. By extrapolating the amount to a volume of 4 ml of extracted rat brain supernatant, this corresponds to a whole brain concentration of ca. 25 ng/g, which exceeds whole brain concentrations reported for trace amines.

In an attempt to improve the sensitivity of the assay, an SP-2100 wall-coated open-tubular glass capillary column was substituted for the packed column system. Two amines, *m*-TA and PEA and three additional internal standards, BZA, PPA and TCP were included in the assay. Sharp, symmetrical peaks resulted for all amines and internal standards using the following temperature program: an initial temperature of 80°C was maintained for 0.6 min followed by a temperature increase of 30°C/min to 120°C. At a run time of 11.6 min, the column temperature was increased at 160°C at a similar rate. Retention times of the derivatized amines PEA, *m*-TA, *p*-TA, *p*-OA, NME, and 3-MT were 4.9, 8.8, 10.1, 11.2, 13.7, and 14.1 min, respectively (Fig. 1). The four internal standards BZA, PPA, TCP and CPEA chromatographed at 3.6, 7.8, 9.6, and 10.5 min, respectively. Compounds found not to interfere with the assay were *ortho*-tyramine, *meta*-octopamine, metanephrine, synephrine, phenylethanolamine, noradrenaline, dopamine, tryptamine, and 5-hydroxytryptamine. Sensitivity was substantially increased for all amines, as 3-pg amounts could now be detected on column. This on-column value corresponds to less than 1 ng/g of amine in rat whole brain. Calibration curves were also constructed by adding varying amounts (1—1000 ng) of each of the amines to 0.5 *N* hydrochloric acid, carrying the samples through the procedure described above, and comparing the peak heights to those of the internal standards. A linear relationship (r^2, coefficient of determination, $\geqslant$ 0.9903) was found between peak height ratios (amine to internal standard) and the varying quantities of the amines.

The structures of the acetylated and trifluoroacetylated derivatives of the internal standards and *p*-TA have been described elsewhere [37—39]. Derivatized PEA, *m*-TA, *p*-OA, NME and 3-MT have now been studied using combined GC—mass spectrometry with both electron-impact (EI) and chemical ionization (CI) sources [40]. The structures are shown in Fig. 2.

The amphoteric character of phenolic amines predisposes these substances to incomplete extraction from aqueous media. Acetylation in aqueous solution resulted in alleviation of this problem, with formation of lipophilic compounds possessing N-acetylated and phenolic O-acetylated functions [37, 38]. Alcoholic hydroxyl groups were not acetylated. Extraction of the derivative into ethyl acetate was quantitative (> 95% recovery). The subsequent hydrolysis step freed the phenolic OH moieties for perfluoroacylation with TFAA (see Fig. 2). The N-acetyl group was not hydrolyzed under these conditions, so the resultant compound was still favourably soluble in ethyl acetate; in addition, there was still a free hydrogen on the nitrogen, and in the case of some substances, a side-chain alcoholic hydroxyl hydrogen atom, which could be replaced by TFAA (Fig. 2). Reaction of the N-acetylated, phenolic O-acetylated derivatives directly with TFAA (omitting the hydrolysis step)



I II III

Product	R^1	R^2	n	Derivative of:
I a	H	H	1	Benzylamine
I b	H	H	2	2-Phenylethylamine
I c	H	H	3	3-Phenylpropylamine
I d	H	Cl	2	2-(4-Chlorophenyl)ethylamine
I e	CF_3COO	H	2	*m*-Tyramine
I f	H	CF_3COO	2	*p*-Tyramine
I g	CH_3O	CF_3COO	2	3-Methoxytyramine
II a	H	CF_3COO	—	*p*-Octopamine
II b	CH_3O	CF_3COO	—	Normetanephrine
III	—	—	—	Tranylcypromine

Fig. 2. Proposed structures for the derivatives of some amines of biological interest as formed by acetylation with acetic anhydride, hydrolysis with ammonium hydroxide, and perfluoroacylation with trifluoroacetic anhydride.

proved to be unsatisfactory as the resultant compounds (N-acetyl, N-TFAA, phenolic O-acetyl; and alcoholic O-TFAA in derivatized *p*-octopamine and normetanephrine) had extended retention times and decreased sensitivity as compared to the derivatives formed in the assay including the hydrolysis step. Furthermore, the derivative formed from tryptamine (another amine present in urine and brain) had the same retention time as the derivative for *p*-TA. Derivatization of the amines with PFPA did not improve the situation, and this procedure was subsequently abandoned as a derivatization method.

Although one of the packed GC columns (3% OV-17) appeared to successfully separate some of the specific amines, an increased sensitivity was required. A new GC technique with the glass capillary column, SP-2100, has been described and shown to provide not only specificity for analysis of the amines but also an on-column sensitivity which approaches that of the radioenzymatic or mass spectrometric procedures. The major advantage of this GC method for analysis of trace amines is the simultaneous extraction and separation achieved. We have previously applied the basic procedure to the quantitation of *p*-TA in rat whole brain after extracting this amine from brain homogenate by using liquid ion exchange and back extraction with 0.5 *N* hydrochloric acid, and a preliminary report of this procedure for *p*-TA has been published [38]. With the modifications and refinements made since that time and noted in the present report, separation of six amines and four potential internal standards in a single injection is now possible. Utilization of this new procedure for simultaneous analysis of PEA, *m*-TA, *p*-TA, *p*-OA, NME and 3-MT in biological samples is presently being investigated.

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REFERENCES

1 M.B.H. Youdim, S. Bonham-Carter, M. Sandler, E. Hannington and M. Wilkinson, Nature (London), 230 (1971) 127.
2 M. Swash, A.M. Moffet and D.R. Scott, Nature (London), 258 (1975) 749.
3 A.A. Boulton and G.L. Marjerrison, Nature (London), 236 (1972) 76.
4 A.A. Boulton, G.L. Marjerrison and J.R. Major, J. Med. Acad. Sci. (U.S.S.R.), 5 (1971) 68.
5 M. Sandler, C.R.J. Ruthven, B.L. Goodwin, G.P. Reynolds, V.H.R. Rao and A. Coppen, Nature (London), 278 (1979) 357.
6 J.E. Fischer and R.J. Baldessarini, Lancet, ii (1971) 75.
7 M. Sandler, M.B. Youdim and E. Hannington, Nature (London), 250 (1974) 335.
8 W.G. Dewhurst, Nature (London), 218 (1968) 1130.
9 S.G. Potkin, F. Karoum, L.W. Chuang, H.E. Cannon-Spoor, I. Philips and R.J. Wyatt, Science, 206 (1979) 470.
10 A. Carlsson and M.V. Lindqvist, Acta Pharmacol. Toxicol., 20 (1963) 140.
11 W. Kehr, A. Carlsson and M. Lindqvist, in D.B. Calne, T.M. Chase and A. Barbeau (Editors), Advances in Neurology, Raven Press, New York, 1975, p. 185.
12 R.J. Head, J.A. Kennedy, I.S. De La Lande and G.A. Crabb, J. Chromatogr. Sci., 16 (1978) 82.
13 E. Finger and E. Kreppel, Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol., 266 (1970) 321.
14 R.L. Wolf, C.E. Gherman, J.D. Lauer, H.L. Fish and B.R. Levey, Clin. Sci. Mol. Med., 45 (1973) 263s.
15 R.E. Shoup and P.T. Kissinger, Clin. Chem., 23 (1977) 1268.
16 S. Harapat and P. Rubin, J. Chromatogr., 163 (1979) 77.
17 I.L. Martin and G.B. Baker, Biochem. Pharmacol., 26 (1977) 1513.
18 L.M. Bertani, S.W. Dziedzic, D.D. Clarke and S.E. Gitlow, Clin. Chim. Acta, 30 (1970) 227.
19 M.T. Wang, K. Imai, M. Yoskioka and Z. Tamura, Chem. Pharm. Biol., 22 (1974) 970.
20 F. Geissbuhler, Clin. Chim. Acta, 30 (1970) 143.
21 W. Kehr, Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol., 284 (1974) 149.
22 B.H.C. Westerink and J. Korf, J. Neurochem., 29 (1977) 697.
23 B.H.C. Westerink, J. Pharm. Pharmacol., 31 (1979) 94.
24 A. Carlsson, M. Lindqvist and W. Kehr, Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol., 284 (1974) 365.
25 L.B. Bigelow and H. Weil-Malherbe, Anal. Biochem., 26 (1968) 92.
26 N.D. Vlachakis and V. DeQuattro, Biochem. Med., 20 (1978) 107.
27 F. Rossi-Fanelli, C. Cangiano, A.R. Smith, A. Bozzi, J.H. James, L.A. Kay, B.A. Perelle, L. Capocaccia and J.E. Fischer, Ital. J. Biochem., 27 (1978) 450.

28 N.D. Vlachakis, N. Alexander and R.F. Maronde, Life Sci., 26 (1980) 97.
29 J.F. Tallman, J.M. Saavedra and J. Axelrod, J. Neurochem., 27 (1976) 465.
30 N. Narasimhachari, J. Chromatogr., 90 (1974) 163.
31 C.L. Galli, F. Cattabeni, T. Eros and P.F. Spano, J. Neurochem., 27 (1976) 795.
32 F. Karoum, H. Norrallah, S. Potkin, L. Chuang, J. Moyer-Schwing, I. Phillips and R.J. Wyatt, J. Neurochem., 33 (1979) 201.
33 A. Groppetti, M. Parenti, C.L. Galli, A. Bugatti, F. Cattabeni, A.M. Di Giulio and G. Racagni, Life Sci., 23 (1978) 1763.
34 S.R. Philips, D.A. Durden and A.A. Boulton, Can. J. Biochem., 52 (1974) 366.
35 G.S. King, B.L. Goodwin, C.R.J. Ruthven and M. Sandler, Clin. Chim. Acta, 31 (1974) 105.
36 A.A. Boulton, in K.F. Tipton (Editor), Physiological and Pharmacological Biochemistry, University Park Press, Baltimore, MD, 1979, p. 179.
37 R.T. Coutts, G.B. Baker and D.G. Calverley, Res. Commun. Chem. Pathol. Pharmacol., 28 (1980) 177.
38 G.B. Baker, R.T. Coutts and D.F. LeGatt, Can. J. Neurol. Sci., 7 (1980) 235.
39 D.G. Calverley, G.B. Baker, R.T. Coutts and W.G. Dewhurst, Biochem. Pharmacol., 30 (1981) 861.
40 R.T. Coutts, G.B. Baker, F.M. Pasutto and S.-F. Lui, in preparation.