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A METHOD FOR MEASUREMENT OF TRANYLCYPROMINE IN RAT BRAIN REGIONS USING GAS CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION

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Abstract—A gas chromatography method, using electron capture detection, is described for measurement of the monoamine oxidase inhibitor tranylcypromine (TCP) in rat brain regions. The analytical method involves extraction of the drug from tissue homogenate with a liquid ion exchange resin and back extraction into acid. TCP is then acetylated and derivatized with pentafluoropropionic anhydride or trifluoroacetic anhydride for gas chromatographic analysis. Conditions for analysis on both packed and capillary columns are described. The method has been used to quantify TCP in rat brain regions after three different dosage/time schedules of TCP administration. The presence of *N*-acetyl-TCP has been demonstrated in brain tissue from rats treated with TCP.

Tranylcypromine (TCP) is a clinically efficacious antidepressant of the monoamine oxidase (MAO) inhibitor type. The mechanism of action of this type of compound in the treatment of depression was originally considered to be inhibition of MAO, which produces increases in the concentrations of biogenic amines at synaptic sites in the brain. In 1968, however, Hendley and Snyder [1] pointed out that, within this group of compounds, efficiency as an antidepressant correlated better with degree of inhibition of norepinephrine (NE) uptake than with potency as an MAO inhibitor. It was later shown that (−)-TCP, which is three to four times more potent than (+)-TCP as an inhibitor of NE uptake [2], is also a more effective antidepressant than the (+)-isomer [3]. These results, however, have been questioned, and it has been suggested that the racemic mixture in clinical use might be most effective because it combines the inhibitions of MAO and of NE uptake [4]. (±)-TCP has been shown to facilitate dopamine (DA) release [5–8] and to inhibit DA uptake [2, 5, 9] in rat striatal tissue. More recently (±)-TCP has been shown to cause dramatic increases in the brain levels of the "trace amines" β-phenylethylamine and tryptamine [10, 11], both of which have been implicated in depression [12, 13]. In our studies on the effects of (±)-TCP on the levels of the catecholamines and their *O*-methylated metabolites and 5-hydroxytryptamine in various regions of rat brain, it became apparent that the effects of the drug often differed from region to region. It therefore became desirable to determine the concentration of tranylcypromine in these different brain regions.

Described here is a simple gas chromatographic method utilizing electron capture detection for the measurement of TCP extracted from rat brain tissue. The method involves modification of a procedure developed by Martin and Baker [14, 15] for the measurement of β-phenylethylamine in rat brain tissue. Conditions for measurement on both packed and capillary columns are presented.

MATERIALS AND METHODS

Reagents

All reagents were used without further purification. The internal standard, 3-phenylpropylamine (PPA, Aldrich Chemical Co., Milwaukee, WI), was converted to its HCl salt by dissolving the amine in ether through which HCl gas was bubbled. Chemicals were from ACS Fisher Scientific (Edmonton, Canada) except as follows: di-(2-ethylhexyl)phosphoric acid (DEHPA) and (±)-TCP–HCl, Sigma Chemical Co. (St. Louis, MO): chloroform, ACS, Anachemia (Toronto, Canada); pentafluoropropionic anhydride (PFPA), Pierce (Rockford, IL); trifluoroacetic anhydride (TFAA), Aldrich Chemical Co. and Koch-Light (Buckinghamshire, England); and cyclohexane, AnalaR (BDH Chemicals, Toronto, Canada).

Gas chromatography

The gas chromatograph used was a Hewlett–Packard 5830 model with a ^{63}Ni electron-capture detector. The packed glass column used was 6 ft long, 4.0 mm i.d., and contained 3% OV-1 on 100–200 mesh Gas-Chrom Q (Serva, Heidelberg, Germany). For packed columns, the carrier gas was 10% methane in argon at a flow rate of 40 ml/min. Injection port temperature was 250°, detector temperature 250°, and the column temperature 140°. The capillary column used was a 10 m WCOT column (J & W Scientific, Orangeville, CA), 0.8 mm o.d., 0.25 mm i.d.,

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coated with SP-2100. Helium at 7 psi was used as carrier gas, and 10% methane in argon at a flow rate of 36 ml/min was used as make-up gas at the detector. Injection port and detector temperatures were as for packed columns. Column temperature was programmed from 60 to 155° at a rate of 30°/min.

Animals

Male Wistar rats, 180–280 g, were obtained from the Charles River Breeding Laboratories, Wilmington, MA, U.S.A.

Methods

Rats were injected intraperitoneally with 20 mg (118 μmoles)/kg or 4 mg (23 μmoles)/kg TCP–HCl dissolved in physiological saline. The rats were decapitated under light ether anaesthesia 1.5 or 6 hr after injection of the drug. Brains were removed immediately and the following regions were dissected out as described by McKim [16]: corpus striatum, hippocampus, mesencephalon-pons and diencephalon. The areas were frozen in dry ice-cooled isopentane and stored at −60° until used for drug analysis.

Extraction. The tissue samples were homogenized in 5 vol. (minimum volume, 1 ml) of ice-cold 0.4 N perchloric acid, using a Tri R Stir R model 563C with glass mortar and teflon pestle (0.11 to 0.15 mm clearance). PPA (1 μg) was added as internal standard prior to homogenization. The samples were centrifuged at 12,000 *g* at 4° for 15 min in an IEC B20 centrifuge. Each supernatant fraction was neutralized using solid potassium bicarbonate, and the precipitate of potassium perchlorate was removed by centrifuging at 1100 *g* for 5 min. The supernatant fraction was extracted with 2 vol. DEHPA in chloroform (2.5%, v/v) by shaking vigorously for 1 min. Following brief centrifugation, the aqueous layer was aspirated, and the chloroform layer was transferred to a clean tube and shaken for 2 min with an equal volume of 0.5 N HCl. After brief centrifugation the aqueous layer was removed, neutralized using solid sodium bicarbonate, and then subjected to acetylation as follows. Acetic anhydride (15% of sample volume) was added with stirring and further additions of solid sodium bicarbonate were made until bubbling virtually ceased (20 min). The acetylated compounds were extracted into 2 vol. of ethyl acetate by shaking for 2 min. Following brief centrifugation, the ethyl acetate layer was transferred to a 13 mm × 100 mm tube with a teflon-lined screw cap. The ethyl acetate was evaporated to dryness under nitrogen, and the residue was derivatized as described below.

Derivatization for electron-capture detection. The residue was taken up in 25 μl ethyl acetate, 75 μl PFPA or TFAA was added, and tube caps were screwed on tightly. The reaction mixtures containing PFPA were heated at 60° for 30 min on a Reacti-Therm heating module (Pierce). Those samples containing TFAA were allowed to stand at room temperature for 30 min. Following this period (and when tubes containing PFPA had cooled), 300 μl cyclohexane was added, and excess PFPA or TFAA was extracted into 3 ml saturated sodium tetraborate by mixing for 15 sec. The cyclohexane layer was removed to a capped vial and analyzed on packed columns (2 μl sample) or capillary columns (1 μl sample). Gas chromatography conditions were as described above. Retention times for both PFP and TFA derivatives of PPA and TCP were: on packed column, 3.4 and 4.1 min, and on capillary column, 3.7 and 4.7 min, respectively.

Quantification. 3-Phenylpropylamine (PPA) was used as internal standard. A standard curve was prepared after adding to aliquots of control brain

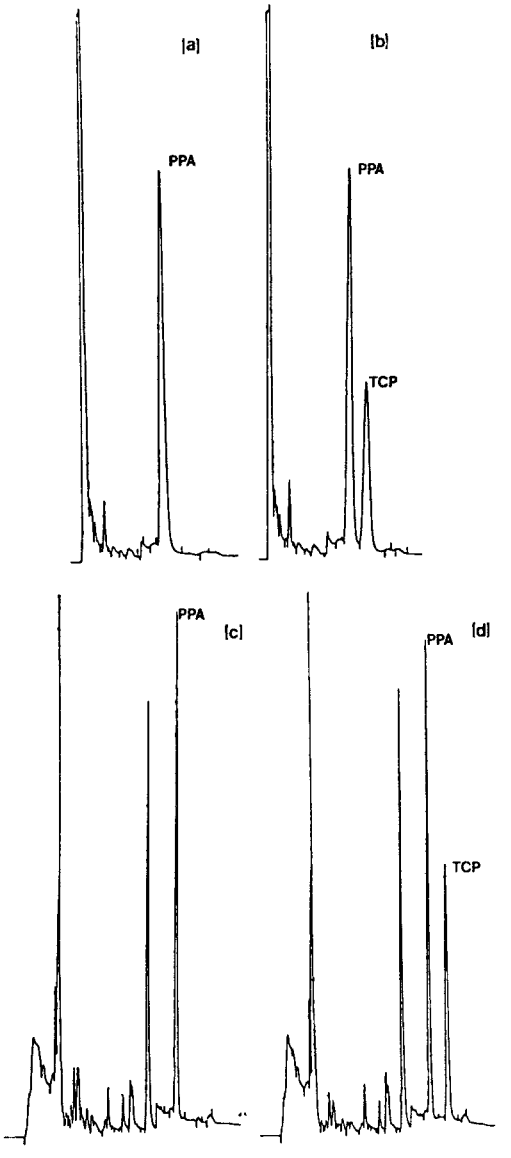
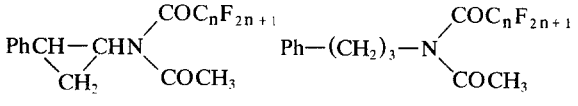


Fig. 1. Gas chromatograph scans of rat brain mesencephalon-pons, extracted and derivatized as described in text. Scans a and c were from control rats, and scans b and d from rats treated with tranylcypromine (TCP), 118 μmoles/kg, 1.5 hr before death. Derivatives of phenylpropylamine (PPA) and TCP are indicated. Scans a and b, of PFP derivatives, were obtained using a packed column (3% OV-1 on Gas-Chrom Q) and scans c and d, of TFA derivatives, using a WCOT capillary column (SP-2100). Gas chromatography conditions are described in the text. The peak appearing before PPA in the bottom trace is an impurity present in the TFAA reagent.

homogenate a fixed quantity of PPA (1 μg) and various amounts of TCP (150 ng to 10 μg). The ratios of TCP to PPA g.c. peak areas were plotted against quantities of TCP originally added. PPA (1 μg) was added to homogenates of brain tissue samples from TCP-treated animals, and the ratio of TCP to PPA g.c. peak areas was compared to the standard curve to determine the quantity of TCP. Typical chromatograms of tissue from control and TCP-treated animals are shown in Fig. 1. Use of such a standard curve instead of absolute peak areas had the following advantage: although the derivatives of TCP and PPA decomposed slowly with time, the rate of breakdown of the PFP derivatives of the two compounds was similar, so that ratios obtained after samples had been frozen for 3 days were consistent with analyses performed the day the samples were prepared.

RESULTS AND DISCUSSION

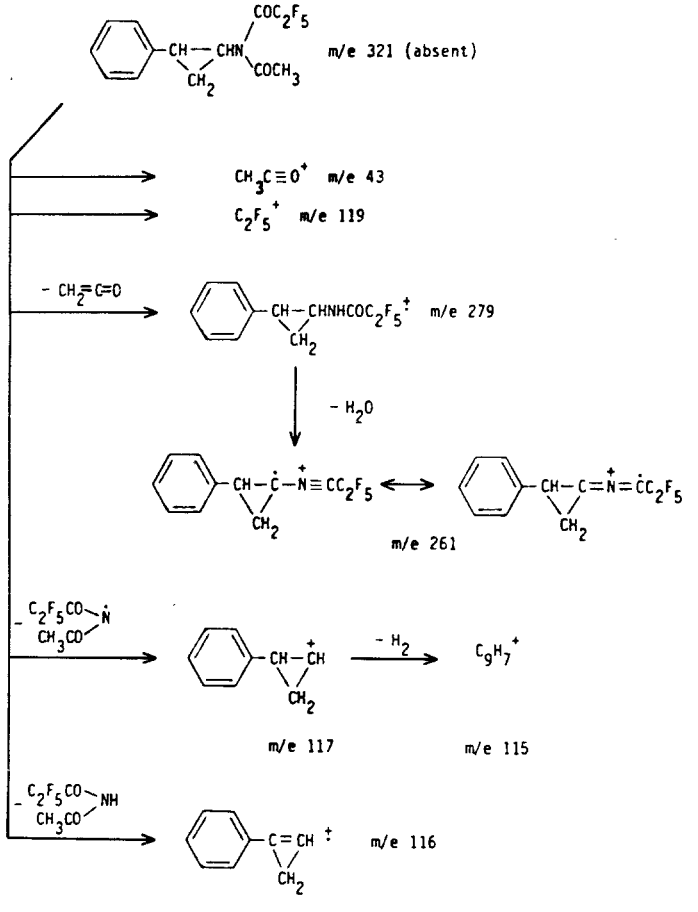
Identification of the derivatives using combined gas chromatography–mass spectrometry (GC–MS)



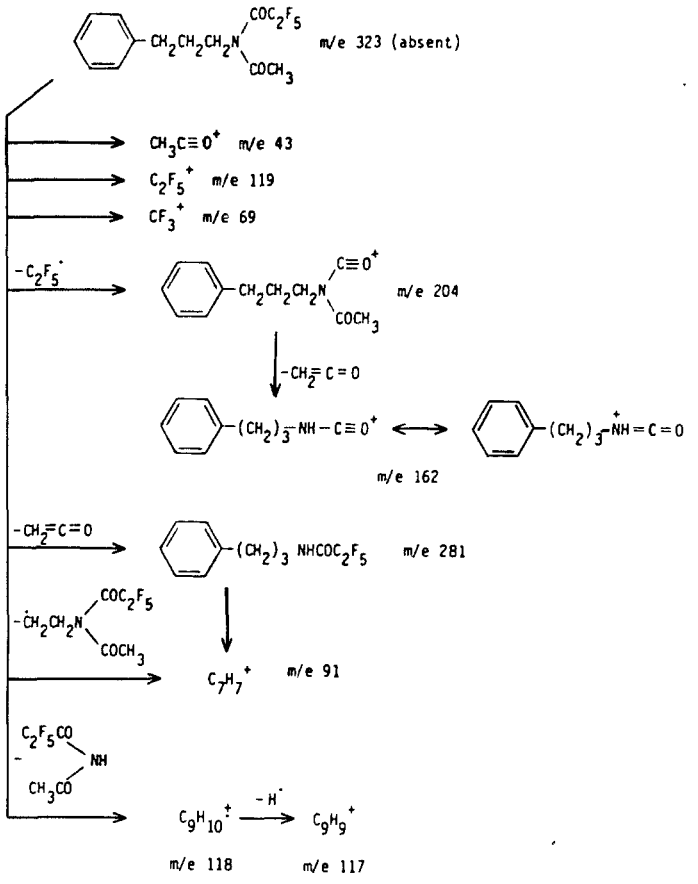
I (a) n = 2 (b) n = 1 II (a) n = 2 (b) n = 1

Mass spectra; *m/e* (per cent relative abundance): Ia, 279(13), 261(25), 119(23), 117(22), 116(100), 115(40), and 43(26); Ib, 229(10), 211(29), 117(24), 116(100), 115(57), 69(29), and 43(93); IIa, 281(4), 204(48), 162(17), 119(22), 118(72), 117(100), 91(68), 69(11), and 43(81); IIb, 231(4), 204(41), 162(16), 118(60), 117(100), 91(81), 69(16), and 43(75).

The structures of the *N*-perfluoroacylated derivatives of *N*-acetyl-TCP (Ia and Ib) and the equivalent derivatives (*N*-acetyl-*N*-perfluoroacyl-3-phenylpropylamine, IIa and IIb) of the reference compound were confirmed by gas chromatography–mass spectrometry with a Hewlett–Packard model 5710A gas chromatograph coupled to a Hewlett–Packard model 5981A mass spectrometer using the same packed column as described above with helium as carrier gas. An ionization potential of 70 eV was used. The structures of all major ions in the spectrum of Ia are identified in Scheme I. The mass spectrum of *N*-acetyl-*N*-trifluoroacetyl-TCP (Ib) contained ions that were equivalent to those illustrated in Scheme 1, except that the fragments which contained a C_2F_5 group in the mass spectrum of Ia were replaced with fragments possessing a CF_3 group in the spectrum of Ib. Fragmentations of the derivatized reference compounds (IIa and IIb) were similarly related and were consistent with the proposed structures. The spectrum of the *N*-pentafluoropropionyl derivative (IIa) is interpreted in Scheme 2.



Scheme 1. Proposed electron impact mass spectrometric fragmentation pattern for *N*-acetyl-*N*-pentafluoropropionyltranylcypromine.



Scheme 2. Proposed electron impact mass spectrometric fragmentation pattern for *N*-acetyl-*N*-pentafluoropropionylphenylpropylamine.

Levels in rat brain regions

All of the quantitative data presented are based on analyses performed on packed columns. TCP levels were determined in four different rat brain regions (hippocampus, corpus striatum, mesencephalon-pons, and diencephalon) after three different treatment schedules: (1) 118 μmoles/kg, i.p., 1.5 hr before death; (2) 118 μmoles/kg, i.p., 6 hr before death; and (3) 23 μmoles/kg, i.p., 1.5 hr before death. Tissues from two animals were combined for analysis after the latter two treatments (tissue from a single animal would be sufficient for capillary column analysis). In the four brain regions, TCP concentrations for the three treated groups ranged from 12.1 to 15.3 μg/g, 1.10 to 1.65 μg/g, and 1.60 to 2.20 μg/g respectively. On the basis of studies reported *in vitro* on the effects of TCP on transport of the catecholamines [2, 5, 8, 9], the concentrations found *in vivo* in the present study would be sufficient for this drug to exert marked effects on uptake and release of NE and DA, in addition to inhibiting MAO. These levels, at least at 1.5 hr, are not inconsistent with those reported by Fuentes *et al.* [17] who used an enzymic assay involving transfer of the ^{14}C-methyl of *S*-adenosyl-L-methionine[^{14}C] to TCP by rabbit lung *N*-methyltransferase. They showed that the concentrations of (+)- and (−)-TCP at 1.5 hr were about 30 per cent of the peak levels that occurred 15 min after i.p. injection. We found that the concentrations of TCP at 6 hr were 13–14 per cent of the corresponding levels 1.5 hr after the 118 μmoles/kg dose of TCP.

Significant differences in TCP concentrations in the different brain regions were found after all three treatments (Fig. 2). In each case the concentration of TCP was significantly higher in the hippocampus than in all other regions and the pattern of relative concentrations was similar after the three drug treatments: hippocampus > corpus striatum = diencephalon ≥ mesencephalon-pons. Fuentes *et al.* [18] reported area differences in Sprague–Dawley rats 15 min after a 59 μmoles/kg i.p. injection of TCP and suggested that the differences were due to differences in blood circulation. The pattern of distribution they reported was unlike that observed by us, which may reflect differences in species of rats used, or, more likely, differences in distribution at the longer time intervals after injection used in this study. The significance of this differential distribution is unclear at present.

Sensitivity and recovery

Acetylation of TCP produces a lipophilic molecule that readily extracts into ethyl acetate. PFP and TFA derivatives of *N*-acetyl-TCP are more stable than the corresponding derivatives of TCP. The method as

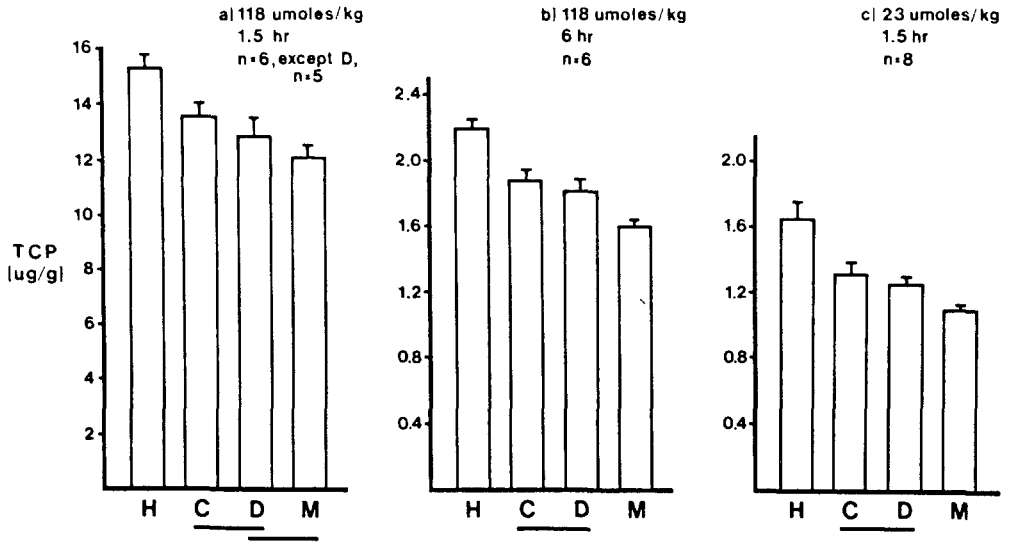


Fig. 2. Tranylcypromine (TCP) levels in rat brain regions at 1.5 or 6 hr after a single i.p. injection. The doses injected are given in the upper right corner of each graph. Vertical bars represent standard errors of the means, n = sample size, H = hippocampus, C = corpus striatum, D = diencephalon, and M = = mesencephalon-pons. Regions underlined by the same horizontal bar are not significantly different according to Student's *t*-test ($P > 0.05$).

described above has been used to quantitate amounts in tissue samples down to 75 ng using packed columns or 37.5 ng using capillary columns. This corresponds to approximately 500 pg and 125 pg, respectively, on column since 2 μl (packed column) or 1 μl (capillary columns) of the final 300 μl cyclohexane extract is used. By reducing the volume of the final cyclohexane solution, the on-column sensitivity can be further increased. Although such sensitivities are more than adequate for the drug experiments described here, it is possible to measure even lower amounts of TCP, and we have found that, when 5 ng of TCP is carried through the entire procedure (corresponding to less than 17 pg on column), a peak adequate for quantitation can be attained on capillary columns.

On packed columns, similar sensitivities were found for the TFA and PFP derivatives of acetylated TCP. The PFP derivative was found to be more stable and to give more reproducible results and, therefore, was preferred for packed column analysis. On capillary columns, however, the sensitivity for the TFA derivatives was greater than for the PFP derivatives and, therefore, TFA was used for analyses on these columns. It should be noted that capillary columns, even of the same type obtained from the same manufacturer, differed significantly regarding the sensitivity that could be achieved for these compounds.

Recovery of TCP through the procedure was tested using brain homogenates that had been spiked with 500 ng of authentic TCP. A mean recovery (±S.E.M.) of 78.1 ± 3.0 per cent (N = 6) was found.

Presence of endogenous N-*acetyl-TCP*

An attempt was made to shorten the analytical technique described by eliminating the DEHPA extraction step and the back extraction into HCl before acetylating. The neutralized perchloric acid supernatant fraction was subjected to acetylation directly and then extracted with ethyl acetate and derviatized as before. As well as shortening the technique considerably, the modified procedure, by eliminating unavoidable volume reductions during the steps that were eliminated, allowed an appreciable increase in the amount of TCP and PPA available for acetylation. However, brain concentrations of TCP obtained using this shorter method were, in general, higher than those obtained using the longer method. Therefore, the possibility that *N*-acetyl-TCP might be present in the brains of TCP-treated animals was investigated. Such endogenous *N*-acetyl-TCP would contribute to the concentration obtained for TCP using the shortened technique but would not contribute in the longer technique, because, although *N*-acetyl-TCP extracts almost quantitatively into the chloroform solution of DEHPA, only about 1 per cent extracts back into 0.5 N HCl under the conditions described.

A neutralized perchloric acid supernatant fraction of brain homogenate from a TCP-treated rat (118 μmoles/kg, 1.5 hr before death) was extracted with ethyl acetate. The ethyl acetate was removed and examined in three ways. First, the ethyl acetate was evaporated almost to dryness and a 2-μl aliquot was injected onto a 3% OV-1 column in a gas chromatograph equipped with a flame ionization detector. A peak was observed with a retention time that corresponded to that for authentic *N*-acetyl-TCP. Second, the ethyl acetate solution was evaporated to dryness and the residue was reacted with TFA and subsequently treated as described above. Using electron-capture detection, a g.c. peak was obtained with a retention time corresponding to that of *N*-acetyl-*N*-trifluoroacetyl-TCP. When control brain tissue, to which authentic TCP (3 μg) was added, was treated in these two ways, the g.c. traces obtained showed no such corresponding peaks.

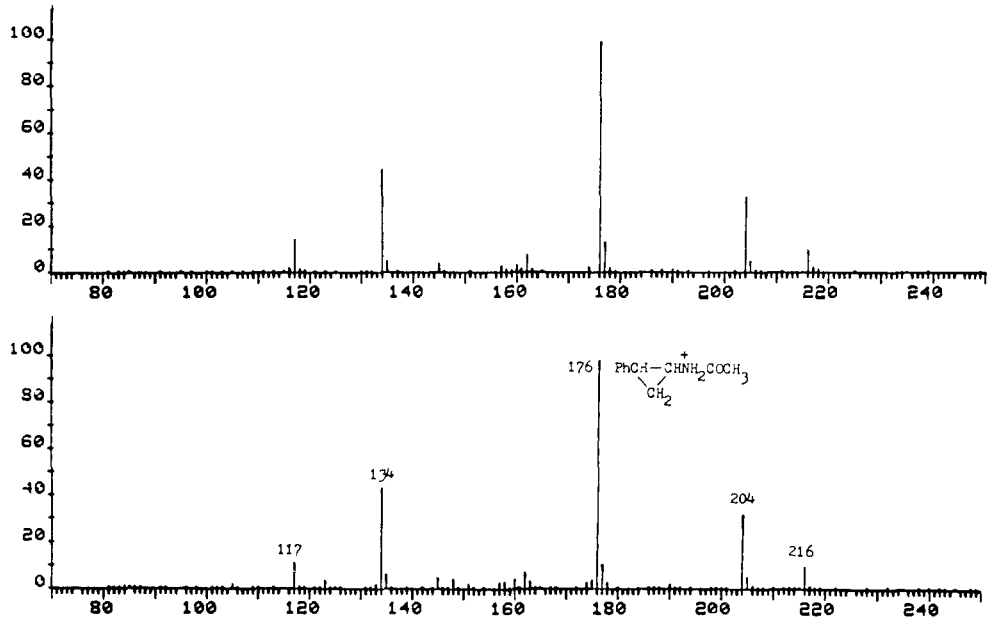


Fig. 3. Chemical ionization mass spectra of authentic *N*-acetyl-tranylcypromine (top) and ethyl acetate extract of brain homogenate from a rat treated with tranylcypromine, 118 μmoles/kg, 1.5 hr prior to death (bottom). *m/e* (identify): 216 $(M + C_3H_5)^+$; 204 $(M + C_2H_5)^+$; 176 $(MH)^+$; 134 (*m/e* 176—$CH_2{=}C{=}O$); 117 (*m/e* 176—$CH_2CONH_2)^+$ or (*m/e* 134—$NH_3)^+$.

Third, the ethyl acetate solution was evaporated to dryness and the residue was taken up in 20 μl ethyl acetate. The chemical ionization mass spectrum obtained on an HP 5985A GC–MS from this ethyl acetate extract of rat brain tissue was virtually identical (Fig. 3) to that obtained from authentic *N*-acetyl-TCP. Methane, at a flow rate of 4.0 ml/min, was used as g.c. carrier gas and reactant gas. The ion source pressure was 0.8 Torr and the ionization potential was 175 eV. A glass column (2 ft × 2 mm i.d.) packed with 3% SP-2250 on Supelcoport, 1001/120 mesh, was employed at an oven temperature of 180°.

In two different TCP-treated animals (118 μmoles/kg, 1.5 hr) 15 and 17 per cent of the apparent TCP measured using the shortened analytical method was due to *N*-acetyl-TCP. These percentages were determined using aliquots of brain tissue homogenate composed of the four areas of interest in this paper, combined together. We have not determined, therefore, whether the brain distribution of this compound is differential. Its presence, however, arouses interest regarding its site of production (not necessarily within the brain) and possible activity within the brain. An arylamine *N*-acetyl transferase has been identified in both peripheral and CNS tissues of the rat [19]. *In vitro*, this enzyme catalyzes the acetylation of a variety of arylamines including tryptamine, 5-methoxytryptamine, 5-hydroxytryptamine, histamine, phenylethylamine, amphetamine, tyramine, octopamine, normetanephrine and mescaline [19–21]. It is conceivable that such an enzyme would also catalyze the *N*-acetylation of TCP which is similar in structure to both phenylethylamine and amphetamine.

Advantages of the technique

The technique described is relatively inexpensive. It is rapid and allows for the simultaneous extraction and reaction of a large number of samples. The derivatives formed, in particular the PFP derivatives, are quite stable when kept refrigerated or frozen, and results are very reproducible. The standard curve is linear over a very wide range of TCP concentrations (150 ng to 10 μg in the original tissue sample). The technique has the added advantage of eliminating any possible interference by endogenous *N*-acetyl-TCP, which may not be the case for other techniques currently available for measurement of TCP.

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