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COMPARATIVE STUDIES OF BIOGENIC AMINES AND THEIR METABOLITES IN *PERIPLANETA AMERICANA* BY GAS CHROMATOGRAPHY/NEGATIVE ION CHEMICAL IONISATION-MASS SPECTROMETRY (GC.NICI-MS)

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Biogenic amines and their metabolites in the brain tissue of the American Cockroach have been identified and quantified by different methods of extraction and derivatisation. The molecular ion of these derivatives carried most of the ion current under NICI conditions, with potential limits of detection low to the picogram level.

Key words: Biogenic amines, *Periplaneta americana*, GC-NICIMS.

Introduction

The comparative studies of the metabolism of amines in insects have suffered from the lack of a sensitive and specific method for measuring the very small quantities of amines and their metabolites in biological systems. Possible pathways for the biosynthesis and degradation of biogenic amines have been summarized in Schemes 1-3. *P*-Octopamine has been extensively studied in the central nervous system (CNS) of insects, and measurements have been made almost exclusively using modifications of the radioenzymatic method first introduced by Molinoff *et al.* [1] which is sensitive but lacks specificity [2,3]. High performance liquid chromatography (HPLC) with electrochemical detection (ECD) is a relatively new procedure for estimation of catecholamines [4] and biogenic amines [5-8] in the central nervous system of insects. The combination of gas chromatography with mass spectrometry (GC-MS) has resulted in a technique of considerable applications in that compounds may be identified by high resolution capillary GC combined with monitoring of significant ions in the mass spectrum of a compound, and their m/z values may be changed in a predictable manner, to provide additional proof of identity, by the preparation of different derivatives of the same chemicals class.

Experimental

Gas chromatography-mass spectrometry. GC-MS in the NICI mode was carried out using a Hewlett Packard 5988A gas chromatograph-mass spectrometer interfaced with a HP RTE-6/VM data system. The following mass spectrometric conditions were used:

The instrument was tuned in the NICI mode to the ions at m/z 452, 595 and 633 from the perfluorotributylamine (PFTBA) calibrant, source temperature was 140°C, electron energy 200 ev and methane reagent gas was introduced to give a source pressure ~0.9 Torr. The gas chromatograph was fitted with a HP-1 fused silica column (either 12.5 m x 0.2 mm i.d., or 25 m x 0.2 mm i.d.); helium carrier gas was used with a head pressure of 8 p.s.i. for the 12.5 m column or 25 p.s.i. when the 25 m column was installed.

The GC conditions. Were as, injector temperature 250°C, transfer line temperature 280°C, the oven temperature was maintained at 100°C for 1 min., then programmed for phenylethanolamines, catecholamines and indolealkylamines at 10°C min^{-1} to 300°C. Injections were made using a Grob splitless injection system.

Materials and reagents. Chemicals were obtained from the following sources: DTFMBCl: 3,5-ditrifluoromethylbenzoyl chloride, ECF or MCF: Ethyl or Methylchloroformate, MTBSTFA:*N*-Methyl-*N*-(tertiarybutyldimethylsilyl) trifluoroacetamide,MTFA: Methyltrifluoroacetamide, DCTFA:1,3-Dichlorotetrafluoroacetone,PFB-Br: Pentafluorobenzylbromide, PFPA Pentafluoropropionicanhydride, were obtained from Fluorochem Ltd; BSA: Bistrimethylsilylacetamide, TBDSCl: Tertiarybutyldimethylsilylchloride imidazole, IPDMS: Isopropyldimethylsilylchloride. TA: Tyramine, DA: Dopamine hydrochloride, OA: Octopamine hydrochloride, Ad: Adrenaline, NorAd: Noradrenaline hydrochloride, Syn: Synephrine hydrochloride, 5-HT: 5-Hydroxytryptamine hydrochloride, 5-HTP: 5-Hydroxytryptophanethylester hydrochloride, were obtained from the Aldrich Chemical Co. Ltd.

Extraction and derivatisation. Adult cockroaches *(Periplaneta americana)* were left undisturbed at room tem perature for 1-2 hr. after removal from the main colony. The insects were anaesthetized by inserting them in a freezer (-20°C, 30 min.) and then stored in crushed ice until dissection. The brain was removed directly from the anaesthetised insect and either processed immediately or frozen by placing it on a piece of dry ice followed by storage at -20°C until required.

The tissue was homogenised in 1 ml of 0.1 M hydrochloric acid. The extract was centrifuged for 30 min. (2500 g). The supernatant liquid was transferred to a sample tube (3.5 ml)

and the PH adjusted to 7.2 with an equal volume of 1M potassium phosphate buffer (pH 7.2). The solution was then shaken with 3,5-ditrifluoromethylbenzoyl chloride (DTFMBCI) (2 µl) for 10 min, extracted with ethyl acetate (2 X 1.5 ml) and the organic Layer Shaken with aqueous ammonia (0.5 ml, 10 M) on a wrist-action Shaker for 10 min. to hydrolyse phenolic ester groups. The solvent removed to dryness and the residue then derivatised separately. (i) The residue was reacted (10 min., 70°C) with bistrimethylsilylascetamide (BSA) (30 µl). The reagents were removed under a stream of nitrogen and the residue redissolved in ethyl acetate. Alternatively, in order to form (ii) t-butyldimethyl silyl ether (TBDMS) derivatives, the residue was reacted (15 min., 70°C) with TBDMSCl and imidazole (each 1M in dimethylformamide, 30 µl). (iii) For *N*-methyl-*N* (t-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) derivatives, the residue was reacted with MTBSTFA (20 µl, 3 hr. 65°C) and TMCS (2%, 20 µl). (iv) The *N*-3, 5-ditrifluoromethylbenzyl amidex isopropyldimethylsilyl ether (DTFMB-xIPDMS) derivatives, were prepared in a manner similar to that described above except that, following hydrolysis of the phenolic ester groups with 10 M aqueous ammonium hydroxide, the residue was then reacted (60 min., 65°C) with IPDMS-MTFA) (20µl). Most of the reagent was removed by evaporation and the derivatised compound was dissolved in ethyl acetate for GC-MS analysis.

All of the deuteriated internal standards (20 ng each) were subjected to the extraction procedures, which did not change their isotopic composition appreciably. Standard mixtures (20 ng) of deuteriated and undeuteriated amines (1:1) were derivatised and analysed when each batch of samples was processed and blanks were also performed on the reagents on each occasion.

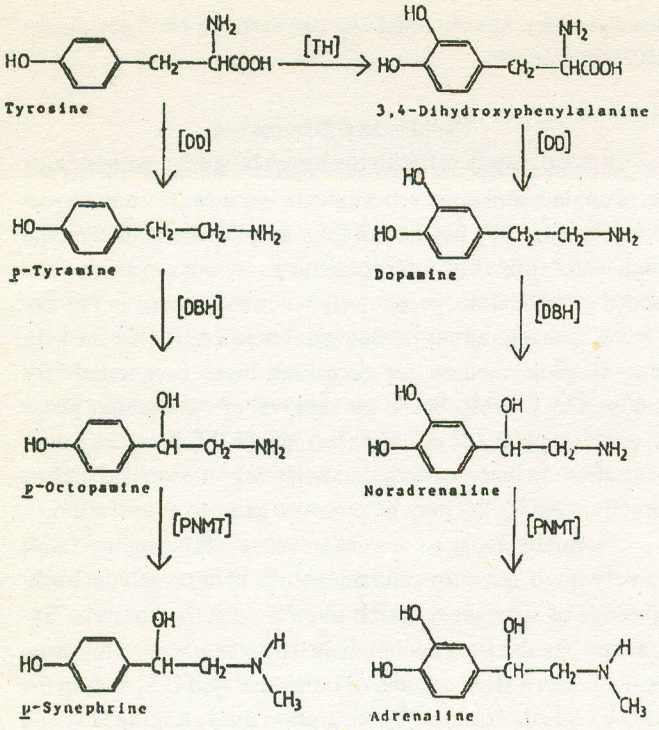
O, N-Diacyl-N-Pentafluoropropionyl (Pr-PFP) Spirocyclic derivatives of Indolealkylamines. Separate Stock Solutions (1 µg/µl) of each indolealkylamine and the corresponding isotopomer were prepared. 5ng - 20 µg of the Stock Solution was added to 0.3 M perchloric acid (0.5 ml). Acylation was accomplished by the addition of saturated aqueous Sodium carbonate (0.2 ml), pyridine (30 µl) and propionic anhydride (0.2 ml.), followed by vigorous vortexing (2 min.,) on Shaker. The *O*, *N*-dipropionylated product was extracted into ethyl acetate and solvent removed to dryness. The residue was then reacted (15 min., 60°C) with pentafluoropropionic anhydride (PFPA) (200 µl). The excess of the reagent was removed by evaporation with a Stream of nitrogen. The residue was dissolved in benzene (0.5 ml) and the resultant solution was thoroughly vortexed with 0.05 M aqueous potassium phosphate buffer (0.3 ml) until it was clear. The organic layer was separated and then removed to dryness. The derivatised product thus obtained was dissolved in ethyl acetate for GC-MS analysis.

Results and Discussion

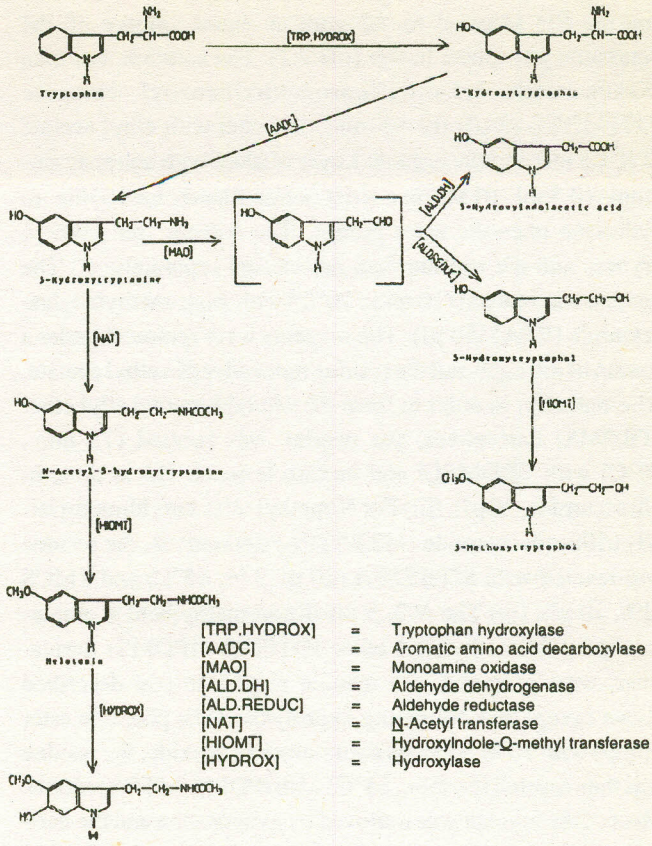
It is extremely difficult to extract biogenic amines (such as phenylethanolamines and catecholamines) from the aqueous phase directly because of their amphoteric character and high water solubility, their sensitivity towards oxidation is an added complication, particularly when they occur in Physiological Concentrations in biological matrices (Schemes 1-3). The simplest method for preparing these compounds for analysis by CG-MS lies in the removal of the aqueous phase evaporation [10, 11] or lyophilization [12] followed by derivatisation. In these techniques interfering substances (such as proteins and lipids) may be removed prior to evaporation.

Normally, biogenic amines are present in biological fluids in very small quantities and this results in high residual backgrounds of substances which interfer with the analysis. Extraction has also been accomplished by adsorption of biogenic amines onto a short column of boric acid gell [13] and, in the case of catecholamines, by adsorption onto alumina [14] or a short column of an appropriate ion exchange resin [15-18]. Substances which might interfer with the analysis pass through the column and the biogenic amines are then eluted by adjusting the pH of the eluting solvent.

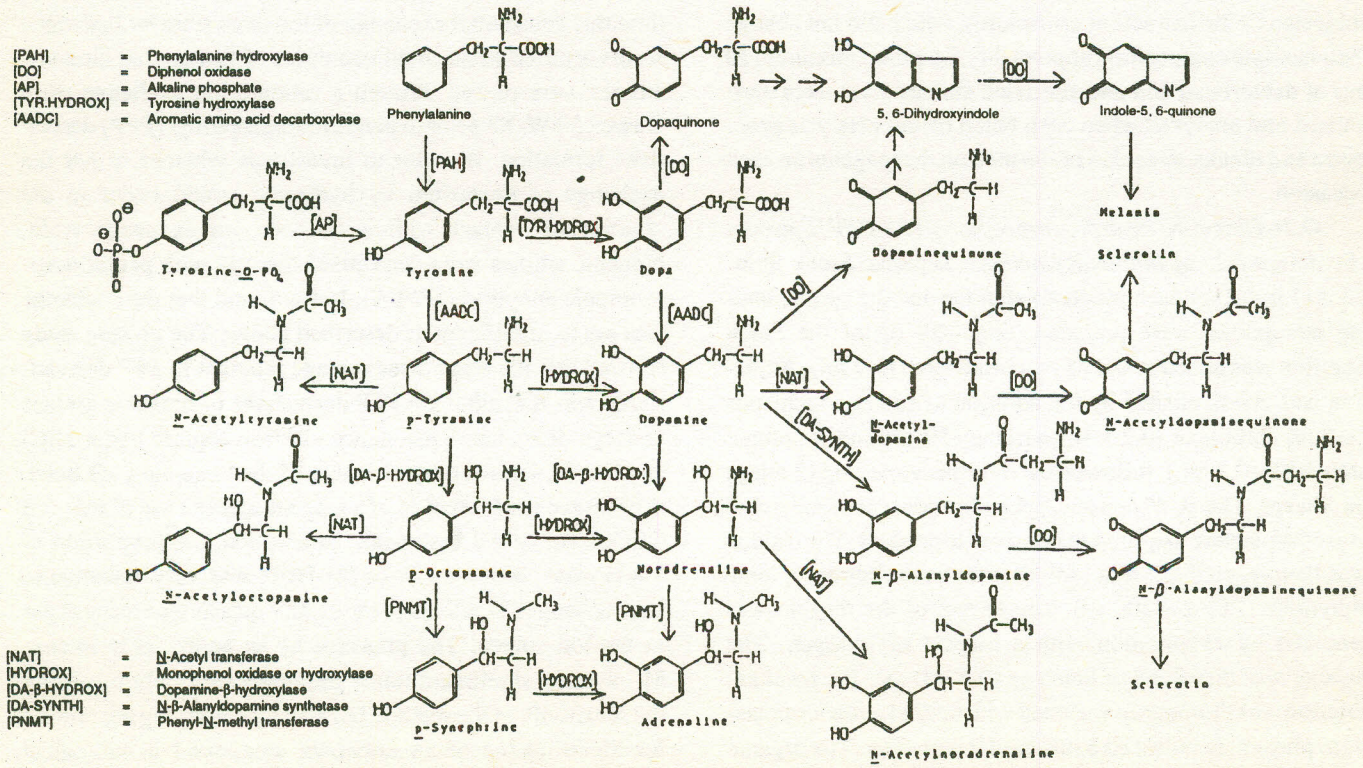
When biogenic amines were processed by the method described by earlier workers [17], it was found for the first time that substantial exchange of the deuterium by hydrogen atoms occured when small quantities of deuteriated biogenic amines were passed through a cationic ion exchange resin Dowex 50 W-X8 prior to pentafluoropropionyl (PFP) derivatives formation. In order to investigate whether or not the exchange of deuterium by hydrogen would occur in the absence of contact with a cationic ion exchange resin, biogenic amines were derivatised directly with pentafluoropropionic anhydride (PFPA). It was found that the exchange was not as significant as described above. The present study showed that the other disadvantage inherent in PFP derivatisation was that all of the PFP derivatives of biogenic amines undergo dissociative resonance electron capture under NICI conditions, followed by cleavage of the benzylic C-O bond, resulting in the formation of a reagent specific ion of m/z 163 ($-CO_2C_2F_5$) as the base peak. In addition, the proportion of ion current carried by M-or $(M-HF)^-$ was very sensitive to slight changes in the temperature and pressure of reagent gas in the ion source. The presence of an appreciably intense M- or other significant ion is important, not only regarding the sensitivity of the method but also for satisfying the criteria for identification of an unknown compound in biological samples by gas chromatogrphy-mass spectrometry, with single



Scheme 1. Biosynthesis of catecholamines and phenolamines.



Scheme 2. Possible pathways for the biosynthesis and degradation of indolalkylamines.



Scheme 3. Pathways in the biosynthesis and degradation of monophenolamines and catecholamines.

ion monitoring (GC-MS, SIM).

An alternate method for the extraction of biogenic amines and their metabolites relies on the rapid reaction of phenolic and amino functions with acid anhydrides or alkyl chloroformates in aqueous solution [18,20]. The resultant esters/amides extracted readily into an organic solvent and then reacted subsequently with tertiary-butyldimethylsilyl chloride (TBDMSCI) or, bis trimethylsilylacetamide (BSA), followed by analysis by GC-MS in the electron impact (EI) mode [18].

Thus, preliminary work carried out by the author leading to the development of direct extraction procedure for biogenic amines from the brain tissue of the cockroach encompassed different methods of extraction. Homogenisation of the brain tissues in boiling water was compared to the more usual extraction of biogenic amines with perchloric acid. Latter, extraction with hydrochloric acid has been adopted as the general method of extraction of the nanogram concentrations of biogenic amines from single brains of the American cockroach.

Extraction - derivatisation procedures. Ethylcarbamate - x tertiary-butyldimethylsilyl ether derivative. The anitial experiments in the development of a direct extraction procedure for biogenic amines were carried out by reacting the amines with ethylchloroformate in phosphate buffer, followed by reaction of the product with TBDMSCl/imidazole. The relative sensitivity for the detection of biogenic amines in nanogram concentration by this method of derivatisation was too low.

N-3, 5-Ditrifluoromethylbenzyl amide - x terbutyldimethyl silyl ether (DTFMB-XTBDMS) Derivative. In order to overcome the shortcomings of the ethylcarbamate- XTBDMS derivative and to improve the signal to noise ratio in biological motrices, further aqueous phase derivatisation reactions were carried out using 3,5-ditrifluoromethyl benzoyl chloride (DTFMBCl) [21]. Under negative ion chemical ionisation (NICI) conditions the ion current of each of the N-DTFMB-XTBDM derivatives of a number of biogenic amines was almost completely carried by the molecular ion [22] (Fig IA and Fig IB). The characteristics of the derivative ensured that it is highly suitable for quantitation of biogenic amines in biological matrices.

TABLE 1. CONCENTRATIONS OF BIOGENIC AMINES IN INDIVIDUAL BRAINS OF THE COCKROACH.

Biogenic amine	Concentration (ng/Brain) (a)	(b)	(c)
p-Tyramine	2.0 ± 0.6 (n=4)	3.5 ± 1.2 (n=14)	3.4 ± 1.6 (n=34)
p-Octopamine	2.2 ± 0.4 (n=4)	2.4 ± 0.6 (n=12)	3.7 ± 1.9 (n=43)
p-Synephrine	0.26 ± 0.4 (n=4)	0.08 ± 0.06 (n=12)	-
Dopamine	2.1 ± 0.7 (n=4)	2.7 ± 0.5 (n=14)	2.7 ± 0.6 (n=12)
Norarenaline	0.85 ± 0.15 (n=2)	0.7 ± 0.3 (n=13)	0.8 ± 0.3 (n=11)
5-Hydroxytryptamine	7.2 ± 3.9 (n=4)	2.9 ± 2.3 (n=14)	3.2 ± 2.3 (n=12)
Adrenaline	-	0.09 ± 0.07 (n=10)	-

n= Number of estimates on individual insects.

The figures are the average obtained by different methods of derivatisations: (a) Concentration of biogenic amines (ng, as DTFMB-x IPDMS derivatives.), (b) concentration of biogenic amines (ng, as DTFMB-xTMS derivatives), (c) concentration of biogenic amines (ng, as DTFMB-xTBDMS derivatives) extracted with HCl (0.1 N).

DTFMB-x trimethylsilyl ether (DTFMB-XTMS) derivative. Following the initial work with DTFMB-XTBDMS derivatives it was found that the corresponding DTFMB-XTMS derivatives were more satisfactory for the following reasons. The presence of residual traces of bistrimethylsilylacetamide (BSA) assisted the removal of residual traces of DTFMBCl or its decomposition products from the gas chromatograph column and the TMS derivatives gave better chromatographic resolution of mixtures of amines and their isomers than did the TBDMS derivatives.

Mass spectral data were obtained for 22 DTFMB-XTMS derivatives of biogenic amines and corresponing isotopomers from the extract of a single brain tissue (Fig. 2A and Fig 2B). In each case the principal ion in the mass spectrum was the molecular ion, which carried > 60% of the ion current under NICI conditions. The average quantities (ng/brain) of each biogenic amine determined by these means were in good agreement with the quantities obtained previously by different methods of analysis [7, 23]. Each peak was identified by comparing the retention time and the m/z value of the molecular ion with those of the standard samples processed immediately before each batch of biological samples was as analysed. In view of the very low concentrations of both *p*- synephrine and adrenaline it is not too surprising that they have not been reported previously in any cockroach tissue. Although it has been reported (using HPLC with ECD) that *p*- synephrine and adrenaline, together with the products of metabolism by monoamine oxidase (MAO) occurred in the central nervous system of the Cockroach [24]. There are accounts that adrenaline occurred in some other insect species [25 -27].

DTFMB-xN-methyl-N-(T-butyldimethylsilyl) trifluoroacetamide (DTFMB-xMTBSTFA) derivatives. The method of slective formation of DTFMB amides of biogenic amines has the additional advantage that the silylating reagent may be changed in order to shift both the m/z value of the molecular ion and the retention time of the derivative to ensure that its indentification is unequivocal. Thus, the silylating reagent was changed and the DTFMB amides were reacted with *N*-methyl *N*- (t-butyldimethylsilyl) tri-fluoroacetamide (MTBSTFA) in the presence of trimethylchlorosilane (TMCS). After analysis by GC-MS, the chromatogram showed a relatively low signal to noise ratio for each ion.

DTFMB-Xisopropyldimethylsilyl ether (DTFMB-XIPDMS) Derivative. In view of these results, DTFMB-amides of a variety of biogenic amines were reacted with N-isopropyldimethylsilyl-*N*-methyltrifluoroacetamide (IPDMS-MTFA). In each case the principal ion in the mass spectrum was the molecular ion which carried almost all of the ion current under NICI conditions and the sensitivity of this method of derivatisation for a given biogenic amine was comparable to that of the corresponding DTFMB-XTMS ether derivative, i.e. <1 pg on column (Fig 3A an Fig 3B).

Thus, the results of the study using different methods of derivatisation showed that *p*-tyramine, *p*-octopamine, dopamine, and 5-hydroxytryptamine were widely distributed and occurred in higher amounts, while noradrenaline was also present in the central nervous system of each insect but in half the amount of that of dopamine (Table 1). Both adrenaline and *p*-synephrine also occured together with other amines but in very low concentrations.

The identification of subnanogram concentrations of the *N*-methyl derivatives of both *p*-octopamine and noradrenaline suggested that it was not possible to rule out the possibility that *N*-methyltransferase activity was present in the central nervous system of the insect as a minor route of biosynthesis.

O, N-Diacyl-N-pentafluoropropionyl spirocyclic (Pr-PFP) derivatives of indolealkylamines. It was possible to quantify the 5- hydroxytryptamine (5-HT) together with the other biogenic amines satisfactorily, from extracts of single brains by both method of derivatisation (i.e., DTFMB-XTMS and DTFMB-XIPDMS). However, the coefficients of variance of these determinations were generally higher for those

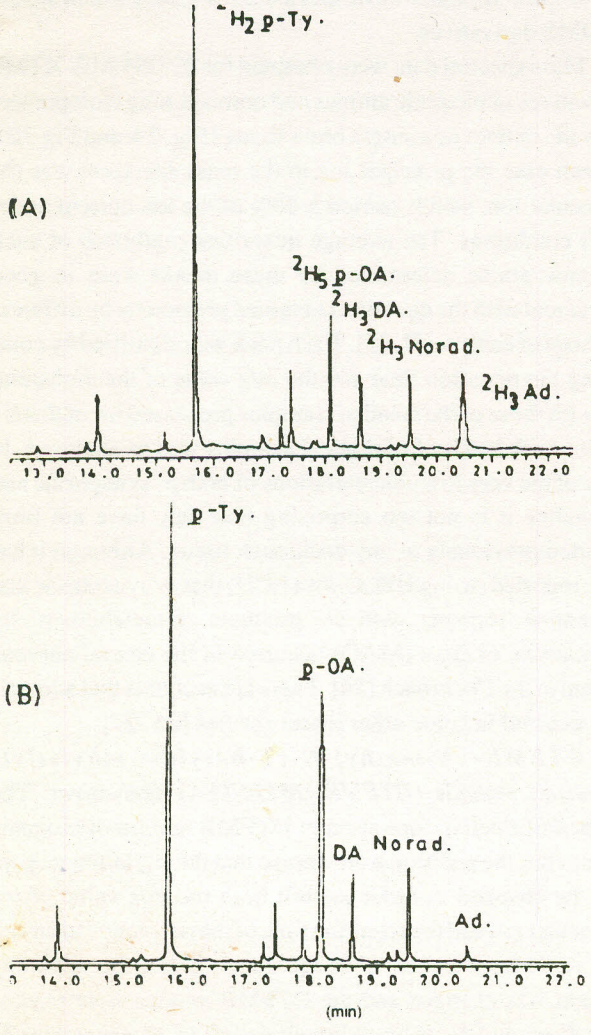


Fig. 1. (A) NICI SIM trace of DTFMB-TBDMS derivatives of deuteriated and undeuteriated biogenic amines (each 20 ng) and (B) the corresponding endogenous biogenic amines from a single brain of American cockroach after addition of deuteriated internal standards (20 ng).

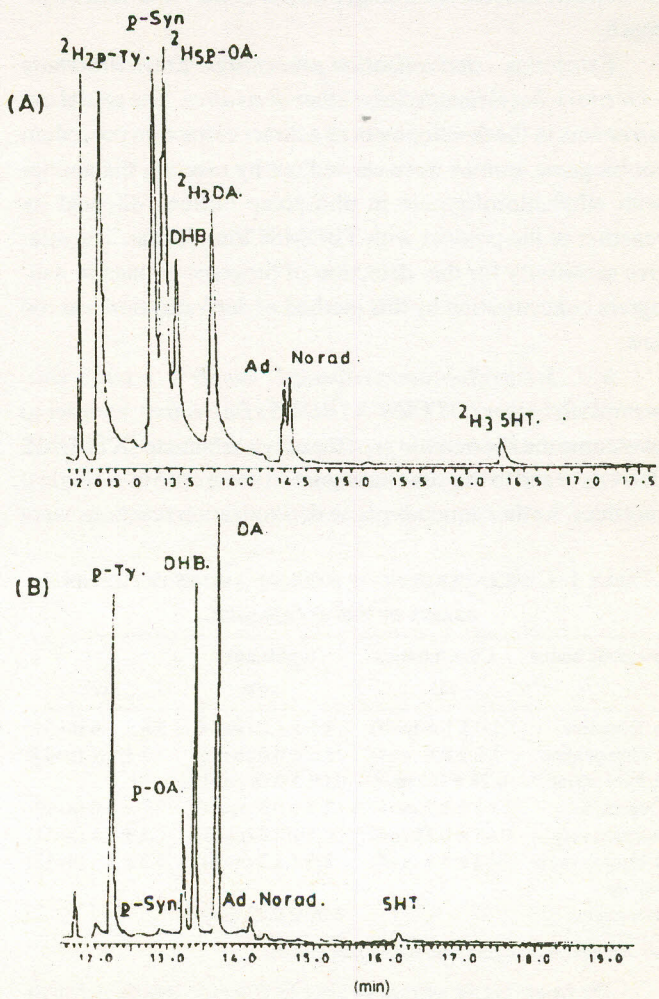


Fig. 2. (A) NICI SIM trace of DTFMB-TMS derivatives of deuteriated and undeuteriated biogenic amines (each 20 ng) and (B) the corresponding endogenous biogenic amines from a single brain of American cockroach after addition of deuteriated internal standards (20 ng).

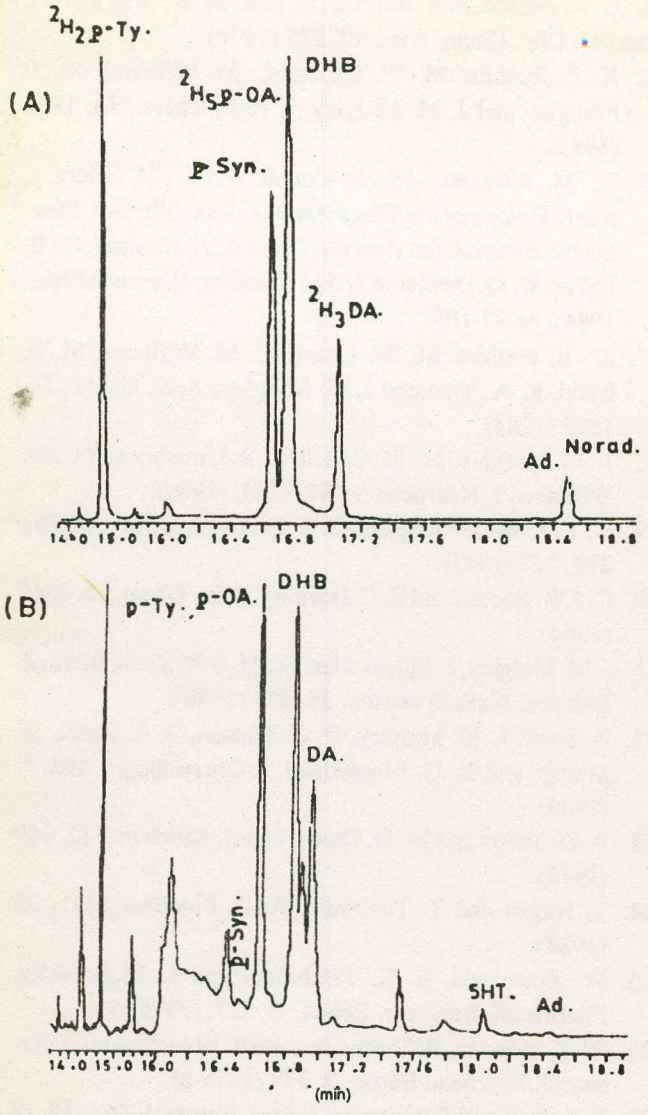


Fig. 3. (A) NICI SIM trace of DTFMB-IPDMS derivatives of deuteriated and undeuteriated biogenic amines (each 20 ng) and (B) the corresponding endogenous biogenic amines from a single brain of American cockroach after addition of deuteriated internal standards (20 ng).

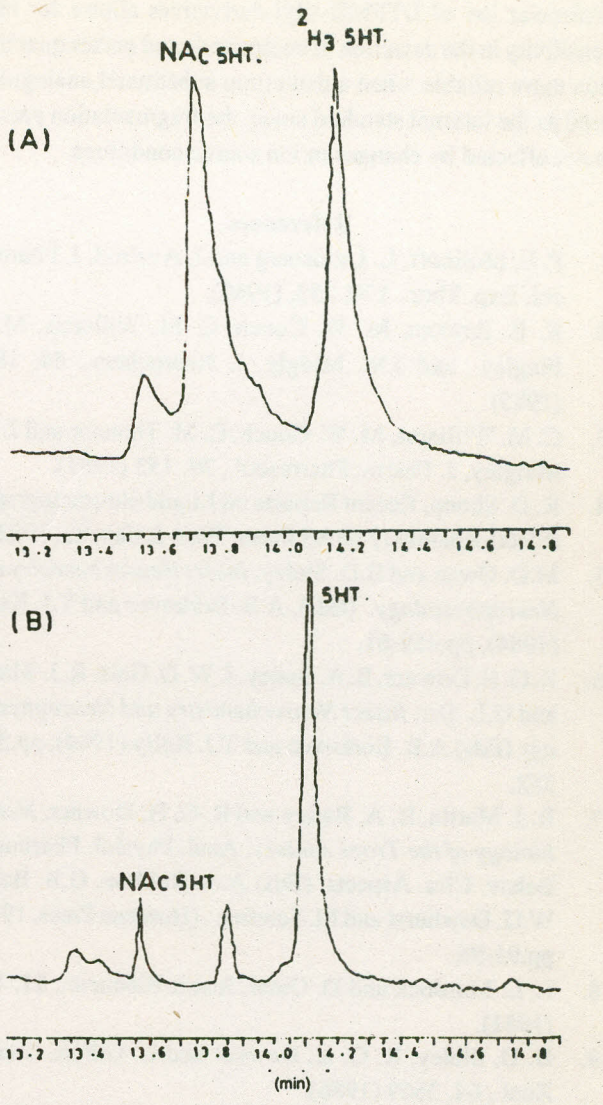


Fig. 4. (A) NICI SIM trace of PR-PFP derivatives of deuteriated and undeuteriated biogenic amines (each 20 ng) and (B) the corresponding endogenous biogenic amines from a single brain of American cockroach after addition of deuteriated internal standards (20 ng).

of other biogenic amines. Ttherefore, 5-HT and *N*-acetyl-5-HT were quantitated using the modified and highly sensitive method of markey et al. [28] where perfluoroacylanhydrides further reacted with),*O* acylated 5-HT and *O*, *N*-diacylated 5-HT to produce stable spirocyclic products. The base peak was due to the loss of HF or DF from the molecular ion (M-) with a relative abundance of 90%. The limit of detection of these indolealkylamines on-column was less than 15 pg (Fig 4A and Fig 4B. The average amounts/brain of 5-HT and *N*-AC-5HT were 3.8± 2.4 (n=8) and 0.4± 0.1 (n=7), respectively. The concentrations of these compounds in the cerebral ganglia of the insect by this method of derivatisation were similar to those reported previously [28,30].

All the solvents used in extraction and derivatisation were of HPLC grade (Rathburn chemicals, Peebleshire, U.K.) Deuterium labelled internal standards were synthesised, for the first time, as their crystalline deuteriochloride salts [22] and were used as reference standards throughout this work.

Conclusions

The author has developed highly sensitive and specific methods for the identification and quantitation of biogenic amines in biological systems where variation in the derivatisation reagent at the silylation stage allowed a shift in the m/z values of characteristic ions and retention time of a given compound. These derivatives have another advantage that the molecular ion carried most of the ion current and there were negligible amounts of reagentspecific ions. The stability of the

molecular ion of DTFMB-silyl derivatives allows for high sensitivity in the detection of compounds and makes quantitation more reliable when a deuterium substituted analogue is used as the internal standard since, the fragmentation pattern is not effected by changes in ion source conditions.

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