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ANALYSIS OF ACIDIC METABOLITES OF BIOGENIC AMINES IN THE NERVOUS TISSUES OF THE *PERIPLANETA AMERICANA* BY GAS CHROMATOGRAPHY/NEGATIVE ION CHEMICAL IONISATION-MASS SPECTROMETRY. (GC/NICI-MS)

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Acidic metabolites of biogenic amines in the brain tissues of *Periplaneta americana* have been identified and quantified by the extraction-derivatisation method. This involves differential acetylation of alcoholic groups in the presence of phenolic hydroxyls, using pentafluorobenzyl bromide. The method establishes unambiguously the presence of *p*-hydroxybenzoic acid, 3,4-dihydroxyphenyl acetic acid, *p*-hydroxymandelic acid and 3,4-dihydroxymandelic acid in the brains of individual insects in sub-nanogram concentrations. This has been confirmed by the novel extraction-derivatisation method using dichlorotetrafluoro acetone.

Key words:Mono-amine oxidative de-amination, Acidic metabolites, Biogenic amines.

Introduction

Oxidative deamination of catecholamines and 5-hydroxytryptamine [1,2] by monoamine oxidase (MAO) has been reported to occur at very low levels in some insect tissues. Metabolites of MAO induced oxidative deamination have been reported to occur in the brain, haemolymph tissues and in the malpighian tubules of *Periplaneta americana* [3-7]. More recently, Czapla *et al.* [8] have identified the MAO metabolites in its haemolymph and in nerve cord by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) [9]. However, Sloley and Downer *et al.* [10-13] failed to detect any metabolites of MAO induced activity in *Periplaneta americana* by the above method. In addition, attempts to demonstrate MAO activity in a number of other insect species, including *Periplaneta americana*, by manometric measurements, histochemical methods or by radioenzymatic assays have also been unsuccessful [14-17]. With a view to establishing unequivocally the monoamine oxidative deamination activity in *P. americana*, a technique involving extraction- derivatisation of acidic metabolites of biogenic amines and their quantitation by gas chromatography/negative ion chemical ionisation-mass spectrometry (GC/NICI-MS) has been developed. The technique is specific and allows for the first time the unambiguous measurement of such metabolites in sub-nanogram concentrations.

Experimental

Gas chromatography-mass spectrometry (GC-MS). 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°, 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 12.5m x 0.2mm, i.d. or 25m 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 follows: injector temperature 250°, transferline temperature 280°, the oven temperature was maintained either at 100° for 1 min. or at 60° for 1 min. and then programmed at 10° min^{-1} to 300°. Injections were made using a Grob splitless injection system.

Nuclear magnetic resonance (n.m.r.) Spectra were recorded using either a BRUKER WM 250 (250 MHz) or a Perkin Elmer R 32 (90 MHz) spectrophotometer with tetramethylsilane as an internal standard and using ds-DMSO as the solvent, unless otherwise stated. The following abbreviations have been used. s : singlet, d : doublet, t : triplet, q : quartet, m : multiplet and br : broad.

Materials and reagents. All solvents used in extraction and derivatisation were HPLC grade (Rathburn chemicals, Peebleshire, U.K.); while the Chemicals DCTFA:1,3-Dichlorotetrafluoroacetone,PFB-Br: Pentafluorobenzylbromide, TEA : Triethylamine, DHMA : 3,4-Dihydroxymandelic acid, PHMA : *p*-Hydroxybenzoic acid, DOPAC:3,4-Dihydroxyphenylacetic acid, HVA : Homovanillic acid, ($^2H_1$) Cl : Deuterium chloride, ($^2H_1$) Br : Deuterium bromide, ($^2H_2$)

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O:Deuterium oxide, C($^2H_3$) COO ($^2H_1$): Deuterioacetic acid, were obtained from Fluorochem. Ltd. and the Aldrich Chemical Co. Ltd. U.K.

Synthesis of Deuterium Labelled Internal Standards. (2,5,6- $^2H_3$) Homovanillic acid was synthesised by the established procedures by adjusting the variables [18,19] briefly the product was obtained by heating the unlabelled compound with aqueous ($^2H_1$) Cl in a sealed tube at 105-110° for 17 hrs. The procedure was repeated with ($^2H_1$) Cl for 6 hrs. to remove exchangeable protons as completely as possible. T.l.C system (benzene : acetic acid : water 2 : 3 : 1) showed that it was a mixture of the corresponding deuteriated demethylated and *o.* methylated products. This mixture was applied to precoated silica gel plates and pure compound was then extracted from the band with ethyl acetate in colourless microcrystalline needles in 40% yield (m.p. 140 - 142°; Lit. [20] m.p. 143°)[a*]. DMSO : 2.29 (S, ^2H, - CH_2 COOH); 3.8 (S, ^3H, CH_3 O-Ar); 8.78 ppm (br S, ^1H, Phenolic OH exchangeable with D_2O).

The ^{1}HNMR spectrum showed that signals due to the aromatic protons attached to C_2 , C_5 and C_8 were absent and GC/NICI-MS with selected ion monitoring of the *o*-acetyl pentafluorobenzyl ester (Ac-PFB) derivative gave the isotope composition as : ($^2H_3$) 88.4% and ($^2H_0$) 0.03%. [2,5,6- $^2H_3$] 3,4-Dihydroxyphenylacetic acid ([$^2H_3$] DOPAC) was prepared in a manner similar to that described by Shimamura *et al.* [21] in 15% yield. (m.p. 125-127°; Lit. 22 m.p. 131-32°)[a*].

δDMSO : 2.29 ppm (S, 2H, - CH_2 COOH). The ^{1}HNMR spectrum showed that the signals due to the aromatic protons attached to C_2, C_5 and C_8 were absent. GC/NICI-MS with SIM of the Ac-PFB ester derivative gave the isotopic composition as ($^2H_3$) 69.9% and ($^2H_0$) 0.00%

Extraction and Derivatisation. Adult *Periplaneta americana*, of both sexes were obtained from the Department of Zoology, University of Glasgow, U.K. The insects were left undisturbed at room temperature for 1-2 hrs and then anaesthetized by inserting them in a freezer (-20°, 30-60 mins). The brain was removed directly from the anaesthetized insect and either processed immediately or frozen at -20° until required. The tissues were homogenized manually in acetonitrile (1 ml) and the suspension was then centrifuged at 20° (4500 rpm, 30 mins.). The supernatant liquid was removed to dryness and the residue converted into the derivatised compounds using Ac-PFB ester and 1,3-dichlorotetrafluoroacetone acetate (DCTFA-Ac) derivatization methods. In the former method, the residue was treated with acetic anhydride and dry pyridine at 60° for 30-35 mins. The acetylated compound thus obtained was dissolved in acetonitrile, PFB-Br and TEA was added and left for 15 mins at room temperature. The excess of reagents were removed and the residue was dissolved in a mixture of ethyl acetate and hexane and was then chromatographed on Sephadex LH 20. The column was eluted with hexane and the solvent evaporated to dryness with a stream of nitrogen. The derivatised compound thus obtained was dissolved in ethyl acetate for GC-MS analysis. In the later method, the residue (in acetonitrile) was reacted with DCTFA and dry pyridine at room temperature for 10 mins. The reaction mixture was then heated at 85° for 5 mins. The excess of solvent and reagents were removed and the residue redissolved in acetic anhydride and allowed to stand at room temperature for 20 mins. The excess of reagent was removed by evaporation with a stream of nitrogen and the derivatised compound thus obtained was dissolved in ethyl acetate for GC-MS analysis. In all cases, internal standards were added to the extraction medium prior to homogenisation. Standard mixture (20 ng) of deuteriated and undeuteriated acids (1:1) were derivatised and analysed when each batch of samples was processed, with almost quantitative rate of recovery of each compound, and blanks were also performed on the reagents on each occasion.

* a = m.p. of the non deuteriated compound

Results and Discussion

O-Acetyl-pentafluorobenzyl (Ac-PFB) ester derivative. Preliminary work to develop a procedure for the identification and quantitation of acidic metabolites of biogenic amines was carried out in a manner described by De Jong *et al.* [23]. Here phenolic hydroxy groups were acetylated in buffered aqueous solution followed by the formation of the pentafluorobenzyl (PFB) ester and then acetylation of the aliphatic hydroxy groups under anhydrous conditions. The mass spectra of the resulting per-*o*-acetyl-pentafluorobenzyl (Ac-PFB) esters, determined under negative ion conditions, exhibited intense peaks due to diagnostically useful anions. However, under NICI SIM conditions, the background, due to interfering residues from the extraction-derivatisation procedure in the region of the chromatogram of interest, was very high.

Consequently, several efforts were made to improve the signal-to-noise ratio. The acidic metabolites were isolated directly by extraction of the biological tissue with acetonitrile and no further purification was required. The solvent was removed and the hydroxyl groups were acetylated in almost quantitative yield by reacting the compound with acetic anhydride, using dry pyridine. After esterification of the product with PFB-Br, the presence of residual traces of the reagent (or its decomposition products) were removed by

precipitating them with ethyl acetate, followed by hexane. The resultant suspension was passed through a column of Sephadex LH20. This contrasts with an earlier report by De Jong *et al.* [23] where the reagents and by-products formed were removed by washing the organic solution with 0.1 M HCl. The Ac-PFB ester derivatives of nine acidic metabolites of biogenic amines showed good chromatographic characteristics in sub-nanogram concentrations. The retention times and base peaks of the mass spectra determined under NICI conditions are summarized in Table-1.

In each case the principal ion in the mass spectrum was due to α-cleavage resulting in the loss of (C_6F_5-CH_2), from the molecular ion. This situation is typified by Fig. 1 which shows the NICI mass spectrum of Ac-PFB ester derivative of p-hydroxymandelic acid (PHMA). Under NICI SIM conditions, the per-O-acetyl-PFB derivatives of acidic metabolites gave abundant structurally diagnostic anions, which allowed the quantitative determination of these compounds in subnanogram concentrations from an extract of single brain. Generally, the limits of detection of these compounds using selected ion monitoring (SIM) was less than 10 picogram on column.

The selected ion trace containing the [M-$CH_2C_6F_5$]- ions (*m/z* values respectively 179, 226, 253, 254, 237 and 309) of the Ac-PFB ester derivatives of 4-hydroxybenzoic acid (PHBA), [$^2H_3$] homovanillic acid (HVA), [$^2H_2$] *p*-hydroxymandelic acid (PHMA), [$^2H_3$] 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxybenzoic acid (DHBA) and 3,4-dihydroxymandelic acid (DHMA) and that containing the [M-$CH_2C_6F_5$]- ions (*m/z* 179, 251, 251, 237 and 309 respectively) of the same derivatives of endogenous

TABLE 1. RETENTION TIMES AND BASE PEAKS [M-$CH_2C_6F_5$]- IN THE NICI MASS SPECTRA OF AC-PFB DERIVATIVES OF ACIDIC METABOLITES OF BIOGENIC AMINES AND THEIR CORRESPONDING ISOTOPOMERS.

Sample No.	Compound	tR (Min)	Base peak *(m/z)*
1.	[$^2H_0$] PHBA	11.38	179
2.	[$^2H_0$] HVA	12.73	223
3.	[$^2H_3$] HVA	12.72	226
4.	[$^2H_0$] PHMA	13.18	251
5.	[$^2H_2$] PHMA	13.17	253
6.	[$^2H_0$] DOPAC	13.60	251
7.	[$^2H_3$] DOPAC	13.59	254
8.	[$^2H_0$] DHBA	13.71	237
9.	[$^2H_0$] DHMA	14.98	309

where PHBA = *p*-Hydroxybenzoic acid. HVA = Homovanillic acid. PHMA = *p*-Hydroxymandelic acid. DOPAC = 3,4-Dihydroxyphenylacetic acid. DHMA = 3,4-Dihydroxymandelic acid. DHBA = 3,4-Dihydroxybenzoic acid.

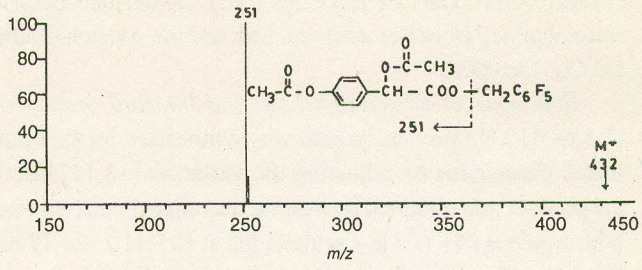

Fig. 1. NICI mass spectrum of Ac-PFB derivative of PHMA.

PHBA, PHMA, DOPAC, DHBA and DHMA extracted from brain tissues are shown in Fig. 2(A) and Fig. 2(B) respectively. There is a possibility that these undeuteriated acids may be produced, wholly or in part, from the deuteriated internal standards by the exchange of deuterium with hydrogen under the conditions of extraction and/or derivatisation. However, the analysis of a mixture (5 ng each; 0.5 ng on column) of the Ac-PFB esters of [$^2H_2$] HVA, [$^2H_3$] DOPAC and [$^2H_2$] PHMA showed that replacement of deuterium with hydrogen did not occur to a measureable extent under the conditions of extraction and derivatisation. In addition, later, biological samples were analysed without the addition of [$^2H_2$] PHMA and [$^2H_3$] DOPAC as internal standards; all of the acids were quantified against [$^2H_3$] HVA (20 ng, added as the internal standard, Fig.2B. The results did not differ significantly from those obtained in the presence of deuteriated internal standards.

By these means, the acidic metabolites of biogenic amines were identified unequivocally and quantified in the central nervous system of the *Periplaneta americana*. The average amounts/brain of most of the acids (identified as their corresponding Ac-PFB ester derivatives) were in the sub-nanogram range as follows: PHBA, 1.3 ± 0.4 ng (n=3); DHBA, 0.7 ± 0.2 ng (n=5); PHMA, 0.7 ± 0.3 ng (n=5); DHMA, 0.5 ± 0.2 ng (n=4) and DOPAC, 0.44 ± 0.37 ng (n=4). (The figures are the averages of the number of estimates on individual insects shown in the parentheses).

1,3-Dichlorotetrafluoroacetone acetate (DCTFA-Ac) derivatives of mandelic acids. The presence of the products of the MAO activity in the central nervous system of the insect was confirmed by changing the method of derivatisation.

Here dichlorotetrafluoroacetone (DCTFA) was used to prepare 2-bis (chlorodifluoromethyl) -4-substituted- 1,3-dioxolan-5-one derivatives of mandelic acids. The high specificity of the bifunctional derivatising agent facilitated the detection of small amounts of mandelic acids in complex biological matrices. The advantage of DCTFA as a reagent is that it reacts specifically with appropriate groups on adjacent carbons (and probably also with functionalities separated by one carbon atom). In addition, the resultant

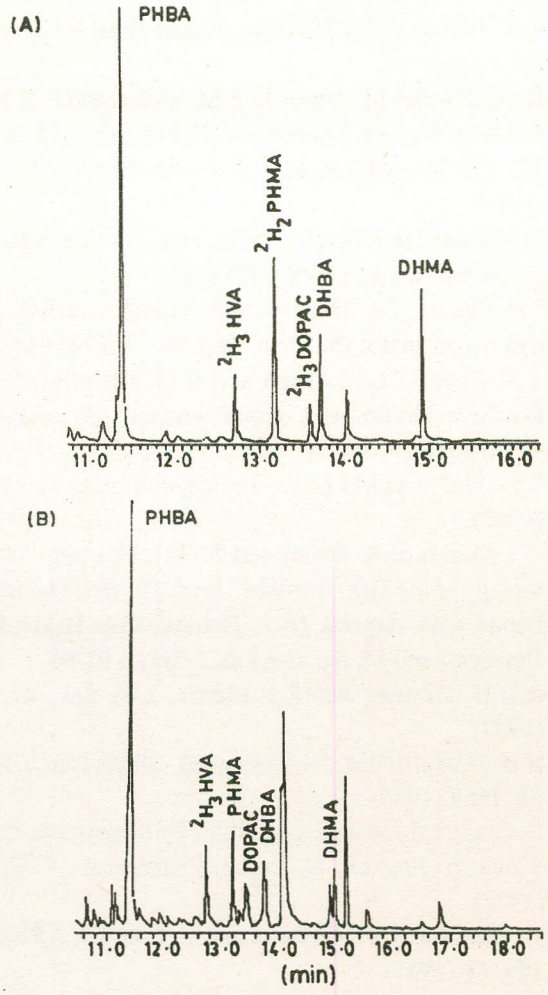

Fig. 2. (A) NICI SIM trace of Ac-PFB derivatives of deuteriated and undeuteriated carboxylic acids (each 20 ng) and (B) the corresponding endogenous acidic metabolites of biogenic amines from a single brain of American cockroach after addition of deuteriated internal standards (20 ng).

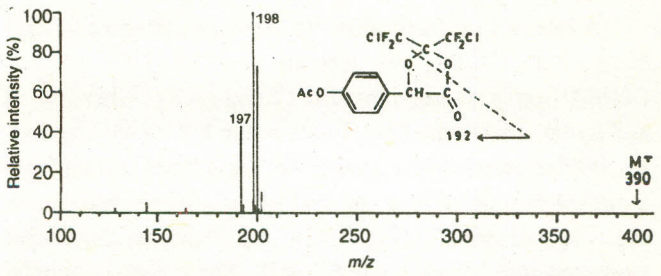

Fig. 3. NICI mass spectrum of DCTFA-Ac derivative of PHMA.

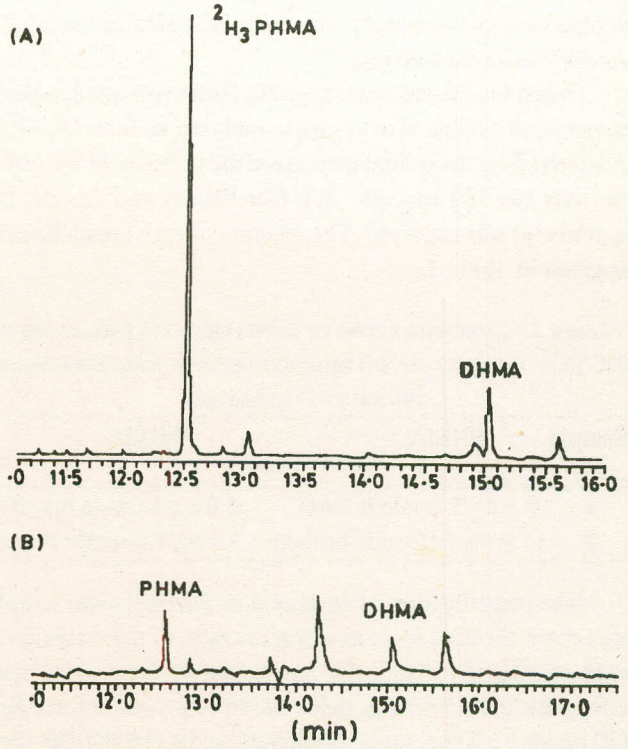

Fig. 4. (A) NICI SIM trace of DCTFA-Ac derivatives of deuteriated and undeuteriated mandelic acids (each 20 ng) and (B) the corresponding endogenous mandelic acids from pooled brain of 5 male American cockroach after addition of deuteriated internal standards (20 ng).

cyclic derivatives formed by the reaction between DCTFA and compounds with two appropriate functionalities were quite stable [24] and would be amenable to chromatographic clean-up after derivatisation. Retention times and mass spectral data for the DCTFA-Ac derivatives of mandelic acids are shown in Table-2.

TABLE 2. RETENTION TIMES AND MASS SPECTRAL DATA FOR DCTFA-AC DERIVATIVES OF MANDELIC ACIDS AND CORRESPONDING ISOTOPOMERS.

Sample No.	Compound	tR (min)	Base peak (*m/z*)	Other major ion (*m/z*)	(%)
1.	[$^2H_0$] PHMA	12.54	198	192	(43)
2.	[$^2H_3$] PHMA	12.52	198	195	(42)
3.	[$^2H_0$] VMA	13.88	198	222	(43)
4.	[$^2H_0$] DHMA	15.05	198	250	(81)

The mass spectra produced under NICI conditions exhibited two ions of high intensity; the base peak (*m/z* 198; due to DCTFA itself) which is eliminated from the derivative and a corresponding ion (in most cases, *ca.* 50% of the intensity of the base peak) due to the fragment of the derivatized acid which remains after DCTFA has been eliminated. The mass spectrum of the DCTFA-Ac derivative of PHMA is shown in Fig. 3. The specificity of the derivatizing agent made it possible to monitor the reagent ion (*m/z* 198) when amounts of compound in the 0-1 nanogram range were being analysed without the chromatogram being swamped by the reagent peak. Thus the method was not only sensitive and specific but convenient also, since, these results were obtained without a clean-up step, either before or after derivatisation.

A selected ion (*m/z* values $^2H_3$ 195 and 250 respectively) trace of the DCTFA-Ac derivatives of [$^2H_3$] PHMA and DHMA from a standard mixture (20 ng each) of deuteriated and undeuteriated acids is shown in the Fig. 4(A). The corresponding selected ion trace of the derivatised extract from a pooled sample of five cerebral ganglia where the ions at *m/z* 192 and *m/z* 250 (for PHMA and DHMA respectively) were monitored, is shown in Fig.4(B). The average amounts/brain of PHMA and DHMA determined by this method were 0.88±0.07 ng (n=3) and 0.39±0.1 (n=3), respectively. This compares very favourably with the results obtained using the Ac-PFB ester derivatives.

When the pooled brain samples (from five adult male or seven adult female insects) were analysed as their DCTFA-Ac derivatives, a ten-fold increase in the intensity of the peaks for ions *m/z* 192 and *m/z* 250 (for PHMA and DHMA respectively) was observed. The quantity of each mandelic acid is given in Table 3.

TABLE 3. CONCENTRATIONS OF MANDELIC ACIDS (NG, AS THEIR DCTFA-AC DERIVATIVES) FROM EXTRACTS OF POOLED BRAINS OF THE ADULT *P. AMERICANA*

Sample no.	PHMA	DHMA
1.	9.5 ng/5 (male brains)	6.0 ng/5 (male brains)
2.	11.9 ng/7 (female brains)	5.6 ng/7 (female brains)

The identification of each acid in the biological sample was cross checked by comparing the ratio of the intensity of each structurally diagnostic ion to that of the reagent specific peak (*m/z*198) with those ratios in a standard mixture (20 ng each). Thus, identification of acidic metabolites confirmed, for the first time, the unequivocal presence of MAO activity in the nervous system of *P. americana.* Although the activity is at a lower rate than that of *N*-acetyl-transferase, the high sensitivity and specificity of GC-MS made it possible to analyse acidic metabolites in sub-nanogram concentrations in the cerebral ganglion of the insect.

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