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Analysis of Acidic Monoamine Metabolites by Gas Chromatography–Mass Spectrometry

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Derivatization of vanillylmandelic acid and 5-hydroxyindoleacetic acid using fluorinated and nonfluorinated alcohols and pentafluoropropionic anhydride produced a number of products. The structures of these products were determined by gas chromatography–mass spectrometry. Reaction conditions are described to substantially reduce the formation of undesirable products, thus making the procedures more suitable for the quantitative determination of these acidic metabolites in biological samples.

Gas chromatography–mass spectrometry (GC-MS) has recently been applied to the quantitative determination of aromatic monoamines and their metabolites in biological samples (*1–3*). Volatile monoamine derivatives for GC-MS analysis have been prepared through acylation with halogenated anhydrides such as trifluoroacetic, pentafluoropropionic, and heptafluorobutyric anhydride (*4*). For acidic monoamine metabolites, prior methylation of the carboxyl moiety with ethereal diazomethane followed by acylation with pentafluoropropionic or heptafluorobutyric anhydride has provided suitable volatile derivatives for analysis (*3, 5*). However, esterification of these metabolites using the above reagents may result in multiple products depending on the number of potential molecular sites of esterification and the reaction conditions employed (*6, 7*). These multiple products may give reduced sensitivity and also may result in loss of specificity in the case of selective ion monitoring GC-MS or gas–chromatography (GC) with electron capture detection.

Recently, less cumbersome procedures for determination of acidic monoamine metabolites have been reported (*8–10*). These methods employ halogenated alcohols such as hexafluoroisopropanol or pentafluoro-*n*-propanol for carboxyl esterification. Mass spectral verification of the derivatives formed has been reported (*9*) but little information was provided about potential by-products or products of incomplete reactions. In this paper we report a systematic investigation of the multiple esterification products of 5-hydroxyindoleacetic acid and vanillylmandelic acid using the principle of esterification described by Watson et al. (*9*) and describe alternative reaction conditions to obtain single derivatives by this chemical approach.

EXPERIMENTAL

5-Hydroxyindoleacetic acid (5-HIAA) and vanillylmandelic acid (VMA) were obtained from Sigma Chemical Co., St. Louis, Mo. Pentafluoropropionic anhydride, pentafluoro-*n*-propanol and trifluoroethanol were obtained from PCR Chemical Co., Gainesville, Fla. The latter reagents were redistilled prior to their use. For determination of mass spectra, 5 μg of each compound in 0.01 N HCl was evaporated to dryness under N_2 in a 1.0-mL reaction vial at room temperature. 5-HIAA and VMA were then derivatized with a mixture of pentafluoropropionic anhydride and pentafluoro-*n*-propanol (4:1) by the procedure of Watson et al. (*9*). The final reaction was terminated by evaporation of the reagents under N_2 and the products were reconstituted in 100 μL of redistilled dry ethyl acetate. Two-μL aliquots were injected for GC-MS analysis. For selective ion monitoring, 50 ng of each compound was treated as above and 2-μL aliquots were analyzed. Esterification of VMA was also examined using a mixture of pentafluoropropionic anhydride and trifluoroethanol in the same ratio as above.

Samples were analyzed using a Finnigan 3200 GC-MS system equipped with a programmable multiple ion monitor (Promim). Chromatographic separation was performed on a 1.5 m × 2 mm i.d. glass column, packed with 3% OV-17 on Gas Chrom Q, 100-120 mesh (Applied Science Laboratories, State College, Pa.). The injector port was maintained at 230 °C and the carrier gas (helium) flow rate was 20 mL/min. The separation of the products of VMA treated with pentafluoropropionic anhydride and pentafluoro-*n*-propanol was achieved at an oven temperature of 115 °C; for the products of VMA reacted with pentafluoropropionic anhydride and trifluoroethanol, an oven temperature of 130 °C was used. The products obtained following reaction of 5-HIAA with pentafluoropropionic anhydride and pentafluoro-*n*-propanol were separated by temperature programming from 145 to 200 °C at a rate of 10 °C/min after running isothermally at 145 °C for 60 s. Mass spectrometer conditions were as follows: separator temperature 230 °C; ion source temperature 70 °C; electron energy 70 eV; and emission current 1 mA. The solvent vehicle was vented through a diverter valve that was maintained open for 30 s following injection of the sample.

RESULTS

Using the reaction conditions described by Watson et al. (*9*) two distinct 5-HIAA esterification products were obtained. The first product was the pentafluoropropyl ester of 1,5-

Table I. Fragmentation Profiles of VMA and 5HIAA Reaction Products

Product	M^+	$M^+ - A^a$	$M^+ - A - B$	$M^+ - A - 2B$	$M^+ - A - D$	$M^+ - A - D - B$	RRT
Ia	615 (17.8)	438 (100)	291 (25)	144 (78.6)			1.00
Ib	469 (10)	292 (100)	145 (31.5)				6.60
IIa	622 (10.6)	445 (90.9)	298 (30.3)	151 (100)			0.17
IIb		431 (100)				151 (44)	0.24
IIc	572 (12.9)	445 (93.5)	298 (29)	151 (100)			0.24
IId	508 (14.2)	381 (100)			298 (5.2)	151 (50)	0.40
IIe	504 (7.0)	445 (35.3)	298 (29.4)	151 (100)			0.60
IIf		313 (100)		151 (71.4)			1.68
	M^+	$M^+ - C$	$M^+ - A$	$M^+ - C - A$	$M^+ - C - A - B$	$M^+ - C - A - D$	
Ic	511 (1.7)	469 (40)	334 (13.3)	292 (100)	146 (76.7)		5.60
Id	365 (3)	323 (10)	188 (6)	146 (100)			8.21
IIIa	468 (6)	426 (69)		299 (100)	152 (32)		1.44
IIIb		362 (5)		235 (100)		152 (28)	2.30

[a] A = $COOCH_2C_2F_5$, $COOCH_2CF_3$, or $COOCH_3$ for the respective esters of VMA or 5HIAA; B = COC_2F_5; C = $COCH_2$; and D = $CH_2C_2F_5$ or CH_2CF_3 for the respective ether products of VMA. Fragments indicated are those most useful for structure determination. The normalized intensities of the fragments are indicated in parentheses. RRT = relative retention time.

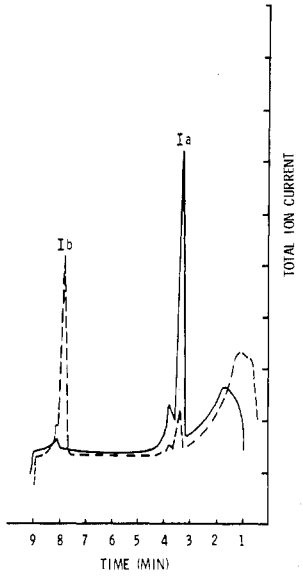


Figure 1. Total ion chromatograms of the products Ia and Ib of 5-HIAA reacted with pentafluoropropionic anhydride–pentafluoro-*n*-propanol for 10 min (- - -) and 3 h (—)

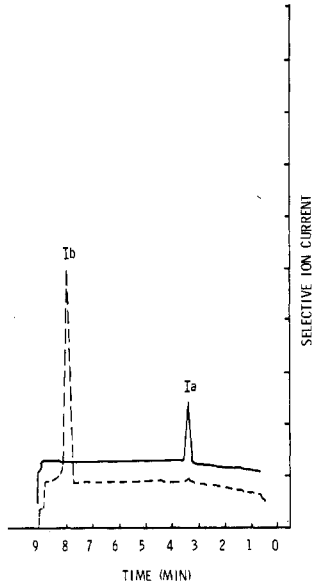
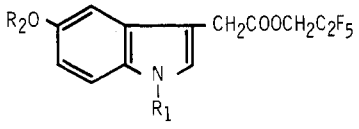


Figure 2. Selective ion monitoring of the products of 5-HIAA reacted with pentafluoropropionic anhydride–pentafluoro-*n*-propanol for 10 min. (—) 438 amu (for product Ia), (- - -) 469 amu (for product Ib)

dipentafluoropropionyl-5-hydroxyindole-3-acetic acid (Ia). As seen in the total ion chromatogram (Figure 1), this product was a minor one (14% of Total Ion Current [TIC]) and its mass spectrum (Table I) corresponded with the one reported by Watson et al. (*9*). The second and major product (86% of TIC) was the partially reacted monopentafluoropropionyl derivative (Ib). The mass spectrum of this product (Table I) revealed a molecular ion at 469 amu and a base peak at 292 amu ($M^+ - COC_2F_5$). We assigned structure Ib to the latter product (see below). These two products were also found



Ia $R_1 = R_2 = COC_2F_5$

Ib $R_1 = H$, $R_2 = COC_2F_5$

Ic $R_1 = COC_2F_5$, $R_2 = COCH_3$

Id $R_1 = H$, $R_2 = COCH_3$

when the reactions were performed using 50-ng quantities. In this case the products were detected by double ion monitoring at 438 amu ($M^+ - COOCH_2C_2F_5$) for Ia and 469

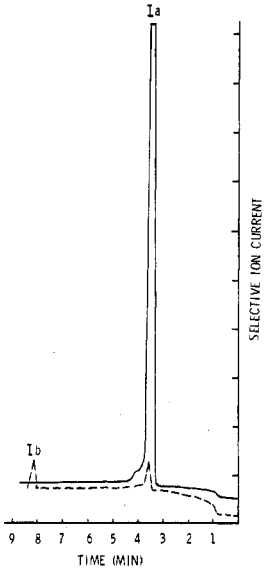


Figure 3. Selective ion monitoring of the products of 5-HIAA reacted with pentafluoropropionic anhydride–pentafluoro-*n*-propanol for 3 h. (—) 438 amu (for product Ia), (- - -) 469 amu (for product Ib)

(M^+) for Ib (Figure 2). When the heating time of the second reaction with pentafluoropropionic anhydride was increased

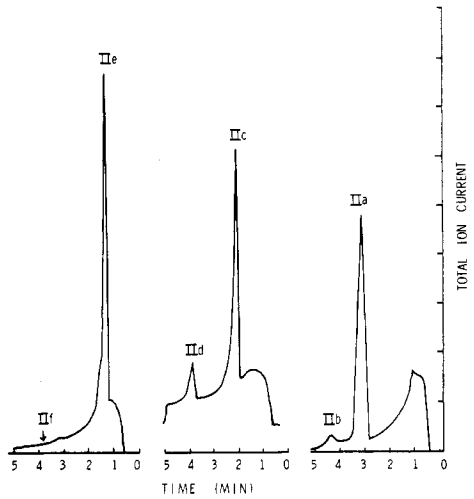
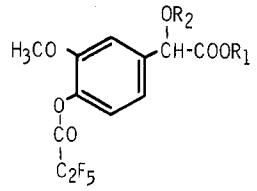


Figure 4. Total ion chromatograms of the products of VMA reacted with pentafluoropropionic anhydride–pentafluoro-*n*-propanol (IIa and IIb), pentafluoropropionic anhydride–trifluoroethanol (IIc and IId) and methanol–HCl followed by pentafluoropropionic anhydride (IIe and IIf)

to 3 h, as described by Gelpi et al. (*6*), product Ia was formed almost quantitatively (Figure 3).

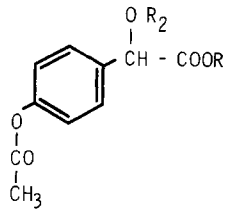
Since there were two possible alternative structures for Ib (i.e., where R_1 = H and R_2 = COC_2F_5 or R_1 = COC_2F_5 and R_2 = H) (Gelpi et al. (*6*)), it was necessary to verify the proposed structure of Ib as shown. The alternative structure for Ib would have an unsubstituted 5-hydroxyphenolic group. Thus we attempted to acetylate the reaction product by a modification of the procedure of Warsh (*11*) described for 5-hydroxyindoles. The mixture of derivatives was dissolved in diethyl ether containing 1% acetic anhydride and shaken for 5 min with 1 M bicarbonate buffer (pH 10.3). The ethereal extract was separated and evaporated to dryness. GC-MS analysis revealed that product Ib was recovered unchanged, suggesting that the 5-hydroxyl group was already acylated. Alternatively, when the 5-hydroxyl group of 5-HIAA was first selectively acetylated as above and then treated with the pentafluoropropionic anhydride and pentafluoro-*n*-propanol mixture, as described by Watson et al. (*9*), products Ic and Id were obtained (Table I). These compounds were separated at a column temperature of 175 °C. The mass spectrum of product Ic showed the molecular ion at 511 amu with other prominent ions at 469 ($M^+ - COCH_2$) and 292 ($M^+ - COCH_2 - COOCH_2C_2F_5$) amu. GC-MS analysis of product Id showed the molecular ion at 365 with other ions at 323 ($M^+ - COCH_2$) and 188 ($M^+ - COOCH_2C_2F_5$) amu. These observations confirm the structure of Ib.

Analysis of the reaction products of VMA using pentafluoropropionic anhydride and pentafluoro-*n*-propanol revealed the formation of two products which were separated at 115 °C (Figure 4). The first product IIa (94% of TIC) had a mass spectrum similar to the one published by Watson et al. (*9*) while the spectrum (Table I) of the minor product (6% of TIC) had a base peak at 431 amu and another major peak at 151 amu. The latter ion was also present in IIa. We tentatively assigned structure IIb to this product which is formed by acid catalyzed alkylation of the side chain hydroxyl group. The expected molecular ion for this substance was calculated to occur at 608 amu but was not detectable. The base peak was formed by removal of $COOCH_2C_2F_5$ ($M^+ - 177$). The products obtained by reaction of VMA with pentafluoropropionic anhydride and trifluoroethanol were separable at 130 °C (Figure 4) and were similar to the ones obtained with pentafluoropropionic anhydride and pentafluoro-*n*-propanol. Their structures, as verified by their mass spectra, were assigned as IIc and IId (Table I). Product IId (16% of



Product	R_1	R_2
IIa	$CH_2C_2F_5$	COC_2F_5
IIb	$CH_2C_2F_5$	$CH_2C_2F_5$
IIc	CH_2CF_3	COC_2F_5
IId	CH_2CF_3	CH_2CF_3
IIe	CH_3	COC_2F_5
IIf	CH_3	CH_3

TIC) did have the expected molecular ion at 508 amu. The by-products IIb and IId were also obtained by first reacting VMA with trifluoroacetic acid and pentafluoro-*n*-propanol or trifluoroethanol and subsequently with pentafluoropropionic anhydride after evaporating the volatiles with N_2. Since products IIb and IId should be formed through the benzyl carbonium ion, similar reactions were carried out with mandelic acid or *m*-methoxy mandelic acid. In both cases no by-product was obtained. This suggested that the carbonium ion stabilizing effect of the para-substituted oxygen is necessary for the formation of the by-product. To test this hypothesis, the phenolic group of VMA was selectively acetylated in a similar way as described for 5-HIAA. The acetylated product was extracted into ethyl acetate after adjusting the pH of the aqueous phase to 1.0 using 1 N HCl. The organic phase was evaporated to dryness and the residue was treated with pentafluoropropionic anhydride and trifluoroethanol. The two products IIIa and IIIb were separated at a column temperature of 160 °C. IIIa was the major



Product	R_1	R_2
IIIa	CH_2CF_3	COC_2F_5
IIIb	CH_2CF_3	CH_2CF_3

product (96% of TIC) with fragments at 426 ($M^+ - COCH_2$) and 299 ($M^+ - COOCH_2CF_3$) amu (Table I). IIIb accounted for only 4% of the TIC. Its mass spectrum (Table I) showed a base peak at 235 amu ($M^+ - COCH_2 - COOCH_2CF_3$) with the heaviest ion occurring at 362 amu ($M^+ - COCH_2$). The molecular ion was not detectable.

The foregoing evidence indicates that the acid catalyzed alkylation of the benzyl group is facilitated by the electron withdrawing nature of the fluorine, thus making the pentafluoro-*n*-propanol or trifluoroethanol nucleophilic. This suggested that the esterification of VMA with methanol and HCl should give minimum alkylation. The procedure of Karoum et al. (*12*) was used to esterify VMA followed by derivatization with pentafluoropropionic anhydride. Total ion detection showed a single peak (IIe) at a column temperature of 140 °C (Figure 4). However, a very small amount (0.1% of TIC) of by-product (IIf) eluted later. Product IIf

had fragments at 313 ($M^+ - COOCH_3$) and 166 ($M^+ - COOCH_3 - COC_2F_5$) amu but had no detectable molecular ion which was calculated to occur at 372 amu (Table I).

DISCUSSION

GC-MS methods are capable of providing sensitive and highly specific determinations of aromatic monoamines and their metabolites, provided that suitable volatile derivatives of these compounds can be formed. The present data demonstrate potential problems of reduced sensitivity that may arise in derivatization of monoamine metabolites using pentafluoropropionic anhydride and halogenated alcohols. Such reduced sensitivity is attributed to multiple derivative formation.

Although mass fragmentographic methods are known to be specific at high sensitivities, there is always a possibility of detecting ghost peaks from unknown impurities or from multiple products such as those demonstrated. The smaller and simpler the fragment monitored, the more likely is the chance of occurrence of a ghost peak, since such a fragment will be present in many other molecules in a complex biological sample. In the case of 5-HIAA, the completely derivatized product (Ia) showed a fragment at 291 amu while the partially derivatized product (Ib) showed a fragment at 292 amu. If 5-HIAA-d_2 is used as an internal standard, the corresponding fragments are found at 293 and 294 amu. The product Ib migrates very slowly with a relative retention time of 6.6 and therefore its presence will produce a peak at 292 amu and to a lesser extent at 293 amu due to the isotopic contribution of the elements in that fragment. Hence, for each injection of the 5-HIAA derivatives (Ia and Ib) a ghost peak will be produced at 293 amu which may interfere with the quantitation procedure. Double ion monitoring for both the metabolite and the internal standard will detect such an effect.

It is obvious from the structures of these products that they will also be detected by GC using a electron capture detector. In the work of Watson et al. (*9*) a number of GC-electron capture detection peaks are shown for 5-HIAA. The main product was shown to have a retention time of 10 min with a carrier gas flow rate of 45 mL/min. Comparing with our data, it seems that this product should have the structure Ib instead of Ia, as assigned by these authors. One of the earlier and minor peaks in the chromatogram will be Ia. With respect to 5-HIAA, the formation of the partially derivatized monopentafluoropropionyl product Ib was substantially reduced by increasing the heating interval of the reaction mixture to 3 h. In the case of VMA, extending the duration of the first reaction up to 3 h or running the reaction between room temperature and 75 °C did not reduce the by-product formation in the presence of pentafluoro-*n*-propanol or trifluoroethanol.

Our results are in agreement with Gelpi et al. (*6*) who found that longer heating times are required for complete derivatization of the indole compounds. They also found that when 3,4-dihydroxymandelic acid was esterified with methanol and HCl, a methylated by-product was formed which matched the Kovats Index of IIe but its mass spectrum indicated methylation of the β-hydroxyl group. In the present work we have confirmed unequivocally the structures of such by-products of VMA. Pentafluoro-*n*-propanol and trifluoroethanol used for esterification of *p*-hydroxy substituted mandelic acids such as VMA or dihydroxymandelic acid partially alkylate the side chain hydroxyl group and, therefore, are not preferable for this purpose. The more suitable esterification reagent is methanol and HCl which is simple to prepare and inexpensive.

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Determination of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Environmental Samples by High-Resolution Gas Chromatography and Low Resolution Mass Spectrometry

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High-resolution gas chromatography in combination with mass spectrometric detection (mass fragmentography) was demonstrated to be a powerful means for detecting trace amounts of 2,3,7,8-TCDD in environmental samples. The separation of different TCDD isomers was studied on OV-101 and OV-17 glass capillary columns and some differences in the mass spectra of these isomers were pointed out. TCDD in environmental samples from Seveso, Italy, had the same retention times on these columns and the same mass spectrum as standard 2,3,7,8-TCDD.

The accidental release of a chemical cloud containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) and 2,4,5-trichlorophenol (2,4,5-TCP) into the atmosphere and over the surrounding area at Seveso on July 10, 1976, caused a most serious environmental contamination. 2,3,7,8-TCDD (for structure, see Figure 1) has been recognized as an extremely toxic (*1*), teratogenic (*2*), mutagenic (*3*), and possibly carcinogenic compound stable in biological systems. It therefore presents a threat to life and environment.

The high toxicity of 2,3,7,8-TCDD requires very sensitive