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Dichlorotetrafluoroacetone as a derivatisation reagent in the analysis of mandelic acids in human urine

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SUMMARY

Dichlorotetrafluoroacetone has been used to prepare 4-substituted 2-bis(chlorodifluoromethyl)-1,3-dioxolan-5-one derivatives of mandelic acids which were found to be suitable for the analysis of these compounds by gas chromatography–negative-ion chemical ionisation mass spectrometry (GC–NICIMS). The high specificity of the derivatising agent facilitates the identification and quantitation of small amounts of mandelic acids in complex biological matrices. The derivatisation procedure was used to determine the concentrations of *m*- and *p*-hydroxymandelic acids and vanillylmandelic acid in to the urine of normal subjects. The method may also have application in the determination of isomeric phenylethylene glycols, the corresponding products of reductive metabolism of biogenic amines.

INTRODUCTION

In earlier investigations we measured the concentrations of *o*-, *m*- and *p*-hydroxymandelic acids in the urine of normal subjects [1] and that of sub-

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jects suffering from phenylketonuria [2]. In this work O-pentafluoropropionyl methyl ester derivatives of the acids were used for the gas chromatographic–mass spectrometric (GC–MS) analysis which was carried out in the electron-impact (EI) mode. We have found that, under negative-ion chemical ionisation (NICI) conditions, these derivatives of the acids fragment so that most of the ion current in their mass spectra is carried by reagent-specific ions; this renders these derivatives of limited use for the quantitation of mandelic acids under NICI conditions. Dichlorotetrafluoroacetone (DCTFA) has been used to prepare oxazolidinones from amino acids [3,4]; these derivatives could be prepared under mild conditions and were suitable for the analysis of these compounds by GC and GC–MS in the EI mode. The advantages of DCTFA as a reagent are that: (i) it reacts specifically with appropriate groups on adjacent carbon atoms (and probably also with functionalities separated by one carbon atom) and therefore should produce a smaller number of interfering peaks in comparison with less specific reagents such as fluoracylating reagents; (ii) the reagent is very volatile and the excess is easily removed following derivatisation; (iii) the cyclic derivatives formed by the reaction of DCTFA with two functionalities are very stable [5] and amenable to further manipulation (e.g. to chromatographic clean-up) after derivatisation; (iv) the resultant derivatives contain several halogen atoms and therefore should be suitable for analysis by GC–NICIMS. Thus we report the formation of cyclic 1,3-dioxolan-5-one derivatives of mandelic acids via their reaction with DCTFA and the application of these derivatives to the analysis of mandelic acids in human urine.

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: *p*-hydroxymandelic acid (PHMA), vanillylmandelic acid (VMA), 3,4-dihydroxymandelic acid (DHMA) and 4-hydroxyphenylglyoxylic acid (Aldrich, Gillingham, U.K.); *m*-hydroxymandelic acid (MHMA) (Sigma, Poole, U.K.); *o*-hydroxymandelic acid (OHMA) was synthesised as described previously [1]; dichlorotetrafluoroacetone (Fluorochem, Glossop, U.K.); solvents, glass-distilled grade (Rathburn, Walkerburn, U.K.). Pyridine was redistilled from and stored over potassium hydroxide.

Deuterated internal standards

2,4,6-[$^2H_3$]MHMA was available from earlier work [2]. In our earlier work [1] 3,5-[$^2H_2$]PHMA was synthesised by acidic exchange of the activated protons by deuterium atoms using ^2HCl; this gave a red tar containing ca. 10% of the required material. This product, although adequate as an internal standard, had to be standardised before use and a better internal standard was sought. Briefly, the synthesis of the 3,5,α-[$^2H_3$]PHMA was carried out as fol-

lows: 4-hydroxyphenylglyoxylic acid was brominated at positions 3 and 5 and this was followed by reduction of the keto group and removal of the bromine atoms by reaction with sodium borodeuteride in the presence of $Pd^{II}Cl_2$ [6]. The resultant pink powder contained ca. 20% of the desired product and was used as an internal standard without further purification. This procedure will be reported more fully when we have optimised the final step.

Derivatisation procedure

Standard samples were prepared by dissolving the mandelic acid (50 μg) in acetonitrile (30 μl) to which pyridine (5 μl) was added followed by DCTFA (10 μl). The mixture was allowed to stand at room temperature (15 min) and the solvent and reagents were removed under a stream of nitrogen at 80°C. Acetic anhydride (15 μl) and pyridine (5 μl) were then added and the mixture was heated (70°C, 15 min); the reagents were removed under a stream of nitrogen and the residue was dissolved in ethyl acetate (0.5 ml) for GC–MS analysis.

Analysis of urine samples

Samples were obtained from normal volunteers (the age range of the subjects was 21–60 years) who had fasted for 12 h prior to providing a specimen. They were not taking medication which was likely to affect the results. Samples were stored at −20°C after the addition of ca. 50 mg of ascorbic acid and, prior to analysis, the creatinine concentrations were determined [7]. 2,4,6-[$^2H_3$]MHMA (100 ng) and 3,5,α-[$^2H_3$]PHMA (20 μg) were added to a volume of urine (ca. 1 ml) equivalent to 2 mg of creatinine. The pH of the sample was then adjusted to 1 with hydrochloric acid (6 *M*, 100 μl) and it was extracted twice with an equal volume of ethyl acetate. The combined organic extracts were then dried (anhydrous sodium sulphate) and the residue was derivatised as described above; the solution in ethyl acetate (0.5 ml) was passed through a plug of silica gel (ca. 1 cm, 40–60 mesh, in a Pasteur pipette) and the final solution (1 μl) was injected into the GC–MS system. Quantitation was carried out by measuring the intensity of the ion at *m/z* 192 (for MHMA and PHMA) and that of the ion at *m/z* 222 (for VMA) and relating these to those of the ions obtained for the derivatised added deuteriated internal standards: [$^2H_3$]PHMA (*m/z* 194, 195) and [$^2H_3$]MHMA (*m/z* 195). VMA was quantitated against [$^2H_3$]PHMA using the ions at *m/z* 194 and 195.

Instrumentation

GC–MS in the NICI mode was carried out using a Hewlett-Packard 5988A GC–MS instrument with an RTE 6V/M data system. Methane was used as the reagent gas with a source pressure of ca. 1.2 Torr and a temperature of 140°C. The Hewlett-Packard 5890 gas chromatograph was fitted with a cross-linked methyl silicone column (12 m×0.25 mm I.D., SGE BP-1, Burke Elec-

tronics, Glasgow, U.K.) and programmed at 10 °C/min from 60 °C; the injector temperature was 250 °C and interface temperature 280 °C. Injections were made in the splitless mode.

RESULTS AND DISCUSSION

Mass spectra of DCTFA-acetate derivatives of mandelic acids

Table I shows retention indices and mass spectral data for the DCTFA-acetate derivatives of a number of mandelic acids. The mass spectra under NICI conditions contain two major ions; in each case the base peak (*m/z* 198) due to DCTFA itself is eliminated from the derivative. There is a corresponding peak (in most cases ca. 50% of the intensity of the base peak) due to that fragment of the derivatised acid which remains after DCTFA has been eliminated from the molecular ion. Fig. 1 shows the mass spectrum for the DCTFA-acetate derivative of PHMA. The DCTFA-acetate derivative of OHMA gives a mass spectrum where the ion current is carried almost entirely by the reagent fragment (of *m/z* 198) which makes it less satisfactory for the analysis of this compound. However, the specificity of the derivatising agent makes it possible to monitor the reagent ion at *m/z* 198, even when low nanogram amounts of

TABLE I

RETENTION INDICES AND MASS SPECTRAL DATE FOR DCTFA-ACETATE DERIVATIVES OF MANDELIC ACIDS

Mandelic acid	*I* value[a]	Base peak	Other major ion[b] (%)
PHMA	1772	198	192 (43)
MHMA	1739	198	192 (53)
OHMA	1655	198	192 (3)
VMA	1910	198	222 (43)
DHMA	2021	198	250 (81)

[a] *I* values were determined on an HP-1 column (80 °C for 1 min then 10 °C/min to 300 °C).
[b] M^- – DCTFA.

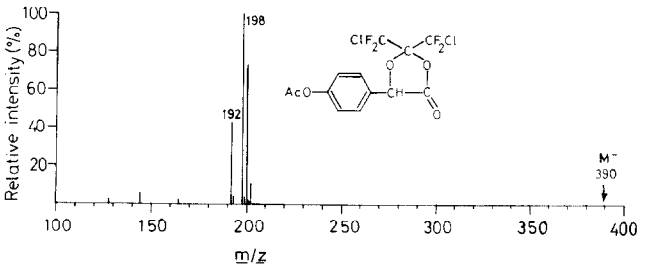

Fig. 1. NICI mass spectrum of the DCTFA-acetate derivative of *p*-hydroxymandelic acid.

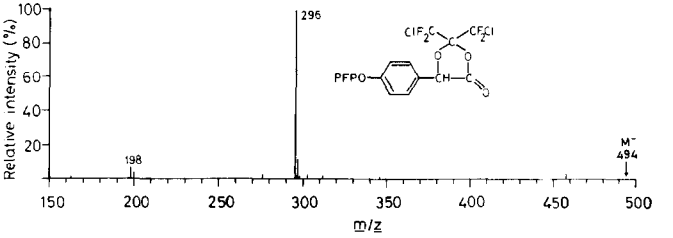

Fig. 2. NICI mass spectrum of the DCTFA-pentafluoropropionyl derivative of *p*-hydroxymandelic acid.

compound are being analysed, without the chromatogram being swamped by reagent peak; presumably the volatility and ease of removal of DCTFA also contribute substantially to this. These factors, combined with the stability of the derivatives to chromatographic clean-up (50-ng amounts of DCTFA–acetate derivatives were satisfactorily recovered from a short column of silica gel), make these derivatives useful in electron-capture analysis of mandelic (and possibly other α- and β-hydroxy) acids. The phenolic hydroxyl group(s) of the mandelic acids may be reacted with agents other than acetic anhydride (e.g. the reaction of PHMA with DCTFA followed by pentafluoropropionic anhydride results in the mass spectrum shown in Fig. 2, where the ion current is now carried largely by the fragment of the derivative remaining after the elimination of DCTFA). We are currently investigating such potentially useful variations in the derivatisation procedure.

Analysis of samples of urine from normal subjects using DCTFA-acetate derivatives

The concentrations of PHMA, MHMA and VMA in urine were determined by our method and the results are presented in Table II. Calibration curves of deuteriated against undeuteriated PHMA and MHMA were linear over the concentration range under investigation, as was the calibration curve for VMA against deuteriated PHMA. Coefficients of variation obtained from triplicate analyses of ten samples were as follows: PHMA, 6.6%; MHMA, 8.7%; VMA, 10.6%; the latter (i.e. least reproducible analysis) may be due to the fact that the quantitation was not carried out in relation to the deuteriated isotopomer of VMA.

The amounts of MHMA and PHMA detected are within the range (11–71 and 1300–4280 ng/mg of creatinine, respectively) found in our previous work [1].

Fig. 3 shows a typical selected-ion trace of a derivatised extract obtained from urine containing 2 mg of creatinine; the ions of *m/z* 192 for MHMA and PHMA and of *m/z* 222 for VMA were monitored. However, some additional clean-up steps may be needed before the method is applicable to the quanti-

TABLE II

CONCENTRATIONS OF MANDELIC ACIDS IN URINE FROM NORMAL SUBJECTS

Subject	Concentration (ng/mg of creatinine)		
	PHMA	MHMA	VMA
1	1701	15.9	3480
2	1038	15.9	616
3	1175	9.6	2790
4	2341	5.9	3857
5	666	14.0	2883
6	830	23.7	3633
7	1216	13.0	4230
8	873	11.5	3435
9	1054	28.3	6767
10	1123	13.5	4063
Mean	1202	15.1	3575
Range	666-2341	5.9-28.3	616-6767

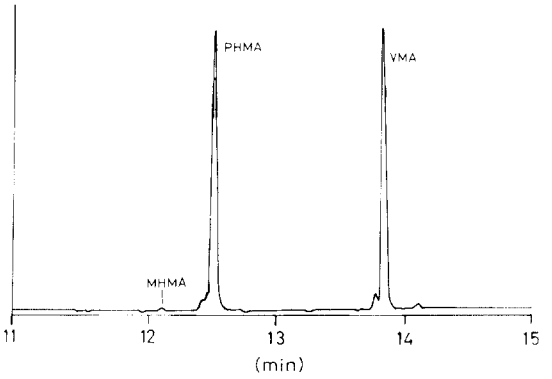

Fig. 3. Selected-ion trace of mandelic acids from human urine after treatment to form DCTFA-acetate derivatives.

tation of DHMA, where there were a number of large interfering peaks in the chromatogram.

Application of DCTFA in the analysis of phenylethylene glycols

DCTFA shows promise in the derivatisation of α-glycols to yield 1,3-dioxolanes which are suitable for analysis by GC–NICIMS; we have found that although perfluoroacyl derivatives of phenylethylene glycols are highly electron-capturing, they yield mass spectra under NICI conditions which are dominated by reagent-specific ions. DCTFA rapidly forms stable hemiketal intermediates

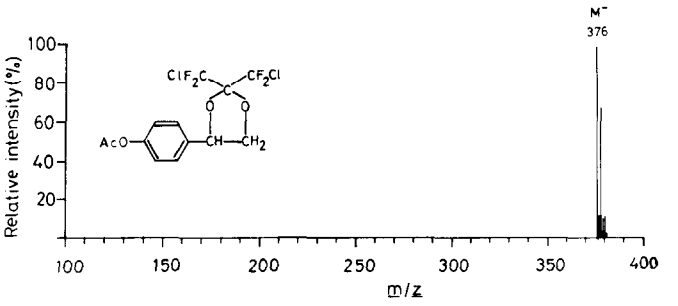

Fig. 4. NICI mass spectrum of the DCTFA-acetate derivative of *p*-hydroxyphenylethylene glycol.

with glycols [7,8] but a cyclised product is not formed under normal circumstances. Using acid catalysis we have been able to produce cyclic products of the type shown in Fig. 4 but the reaction still requires optimisation before the yields of products are reliable enough to allow the analysis of such substances by this method in biological matrices. However, this method of derivatisation is particularly attractive since, as is shown in Fig. 4 for *p*-hydroxyphenylethylene glycol, the mass spectra of DCTFA derivatives of the glycols are dominated by a molecular ion cluster with very little of the reagent-specific ion (which is found as the base peak in the mass spectra of the DCTFA derivatives of the mandelic acids).

Thus we have established that DCTFA is a useful selective derivatisation reagent agent for the analysis of mandelic acids; we are using it successfully for this purpose in invertebrate nervous tissue and in human biological fluids in order to determine the role of monophenolic phenylethanolamines (such the isomeric octopamines) in nervous systems.

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