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SELECTIVE GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC METHODS FOR THE QUANTITATION OF NORMETANEPHRINE, METANEPHRINE AND VANILLYLMANDELIC ACID

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SUMMARY

The partial derivatization of normetanephrine, metanephrine and vanillylmandelic acid (VMA) with alcoholic hydrochloric acid yields β- or α-O-ethyl ethers suitable for selective quantitation by gas chromatography–mass spectrometry (GC–MS). The isomeric 3-methoxy-4-hydroxy and 4-methoxy-3-hydroxy derivatives can be identified and quantitated in biological samples by this method. The GC–MS characteristics of the partial O-ethyl ethers of normetanephrine isothiocyanate, metanephrine and VMA ethyl ester are described.

INTRODUCTION

Normetanephrine (NM), 3-methoxy-4-hydroxyphenylglycol (MHPG) and 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA) are key metabolites in catecholamine metabolism. Several methods involving column, ion-exchange, paper or thin-layer chromatography (TLC) followed by colorimetry or fluorimetry have been used to determine the levels of these compounds in body fluids and tissues[1] (for earlier publications on methodology, see the bibliography in ref. 1). Gas chromatography (GC) of the trimethylsilyl (TMS) ethers or of the trifluoro- or heptafluorobutyryl derivatives of the methyl esters has also been used for their quantitation[2,3]. Although the *p*-methylation of catecholamines by catechol-O-methyltransferase (COMT) has been established[4], no attempt has been made to separate or to take into account such isomeric compounds.

In a recent study, we showed that the ethyl or methyl esters of homovanillic acid (HVA) and isohomovanillic acid (iso-HVA) could be separated on an OV-225 GC column[5]. We also reported the identification of these isomers by gas chromatography–mass spectrometry (GC–MS) as well as their ratios in urine and cerebrospinal fluid[6]. It was pointed out that the complete derivatization of the phenolic hydroxyl groups as TMS ethers renders the isomers inseparable.

A method for the partial derivatization of the β-hydroxy compounds to ethyl esters (alcoholic) so that the isomeric 3- and 4-methoxy compounds can be separated and quantitated is now reported.

EXPERIMENTAL

Materials and methods

NM, metanephrine, MHPG, VMA and *p*-methoxymandelic acid were obtained from commercial sources. Freshly distilled reagent-grade carbon disulphide was used in the preparation of isothiocyanate (NCS) derivatives[7]. A 3 *N* solution of ethanolic hydrochloric acid was prepared by passing hydrogen chloride gas through absolute ethanol.

Two millilitres of ethanolic hydrochloric acid and 3 mg of the compound were heated in a stoppered tube for 2 h at 90–100°. The solution was then evaporated to dryness under vacuum with more ethanol added for complete dehydration. The residues of the ethyl esters were dissolved in ethyl acetate to give a solution containing 1 mg/ml. The residues of NM and metanephrine were shaken for 15 min with 10 ml of ethyl acetate and 1 ml of 10% ammonia solution. After centrifugation, the aqueous layer was discarded and the ethyl acetate extract was dried over sodium sulphate and evaporated to dryness under vacuum. The residue of NM was redissolved in ethyl acetate and shaken for 30 min with 0.5 ml of carbon disulphide to form the NCS derivative.

Gas–liquid chromatography

The acid esters, the NCS derivatives and the β-O-ethyl ether of metanephrine were run on a 6-ft. column of 3% OV-225 on a Varian Model 2740 gas chromatograph at 190° (isothermal). Helium was used as the carrier gas at a constant flow-rate of 30 ml/min.

Gas chromatography–mass spectrometry

The mass spectra were recorded on a Varian CH 7 mass spectrometer interfaced with a Varian Model 2740 gas chromatograph and a Watson–Biemann separator. The GC conditions were as described above. The temperatures of the separator and the ion source were maintained at 280° and the ionization potential was 70 eV.

RESULTS

Under the conditions of the reaction with ethanolic hydrochloric acid, the β-hydroxyl group is quantitatively converted into the O-ethyl derivative. In a series of experiments to determine the optimal conditions for the reaction, it was found that a 3 *N* solution of hydrogen chloride gas in absolute ethanol gave the best results when the sample was heated in a stoppered tube at 90–100°. When the reaction with VMA was monitored by TLC and GLC, it was found that VMA ethyl ester and VMA ethyl ester-β-O-ethyl ether were distinctly separated and that underivatized β-hydroxy compound was present at levels of 5–10% of the mixture. Similar results were obtained with *p*-methoxymandelic acid. However, with NM and metanephrine, the reaction was quantitative.

The GC data for the various compounds are presented in Table I.

TABLE I

GC RESULTS FOR SOME CATECHOLAMINE METABOLITES

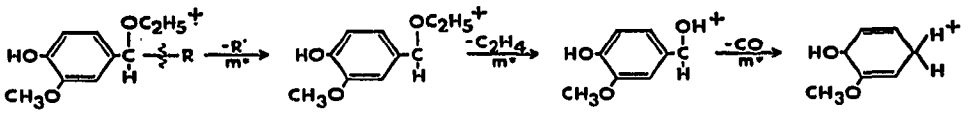
Column: 6 ft., 3% OV-225, 190° (isothermal), helium flow-rate 30 ml/min.

Metabolite	*Retention time (min)*
4-Methoxymandelic acid ethyl ester-α-O-ethyl ether	3.66
4-Methoxymandelic acid ethyl ester	4.65
VMA-ethyl ester-α-O-ethyl ether	9.62
VMA-ethyl ester	13.7
Metanephrine-β-O-ethyl ether	4.88
Normetanephrine-NCS-β-O-ethyl ether *	3.8

* Column temperature 200°.

Mass spectral data

The mass spectra of all the derivatives agreed with those required for the α- or β-O-ethyl ethers. In all instances the molecular ion was noticeable and the base peak due to the β-fission in the NCS derivatives[7] was at *m/e* $(M-72)^+$, at *m/e* $(M-73)^+$ for the acid esters and $(M-44)^+$ for metanephrine-β-O-ethyl ether. Thus NM-NCS-β-O-ethyl ether, metanephrine-β-O-ethyl ether and VMA-ethyl ester-α-O-ethyl ether had the base peak at *m/e* 181 and the other fragment ions derived from these were identical in all instances. The fragmentation is represented schematically below. The fragmentation mechanisms are confirmed by metastable transitions.



The structures of the β-O-ethyl ethers were further confirmed by the preparation of O-TMS ethers, where mono-TMS derivatives were obtained. The mass spectra of the derivatives agreed with the required structures. As a typical example, the mass spectra of VMA-ethyl ester-β-O-ethyl ether and of the free ester, which is a minor component in the reaction, are given in Fig. 1. The mass spectral data of all the compounds studied are presented in Table II.

Under these experimental conditions of derivatization, MHPG loses water and forms an epoxide. The mass spectrum of the epoxide is shown in Fig. 2. The molecular ion at *m/e* 166 and the base peak at *m/e* 137 by loss of ·CHO are the characteristic features of the spectrum.

DISCUSSION

This method for the partial derivatization of the β-hydroxyl group of NM and metanephrine and the α-hydroxyl group of VMA renders the compounds volatile enough for GC analysis. The free phenolic hydroxyl group permits the

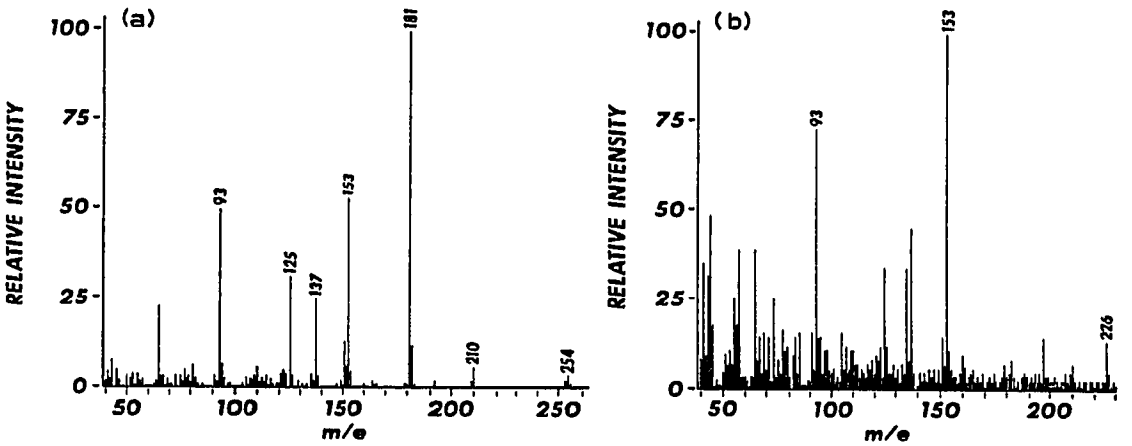


Fig. 1. Mass spectra of (a) VMA ethyl ester-β-O-ethyl ether and (b) VMA ethyl ester.

TABLE II
MASS SPECTRAL DATA FOR SOME CATECHOLAMINE METABOLITES

Metabolite	*m/e* (%)
VMA ethyl ester-α-O-ethyl ether	254 (7.5), 181 (100), 153 (42), 136 (6), 125 (18), 93 (36)
VMA ethyl ester-α-O-ethyl-4-O-TMS ether	326 (9), 311 (8), 253 (100), 225 (14), 197 (6), 73 (34)
Normetanephrine-NCS-β-O-ethyl ether	253 (13), 181 (100), 153 (60), 137 (14), 125 (40), 110 (10), 93 (87)
Metanephrine-β-O-ethyl ether	225 (5), 181 (100), 153 (52), 137 (9), 125 (22), 93 (31)
4-Methoxymandelic acid ethyl ester-α-O-ethyl ether	238 (1.2), 193 (3.5), 165 (100), 137 (82), 109 (30), 94 (13)
MHPG with HCl	166 (25), 137 (100), 122 (14), 94 (16), 77 (22)

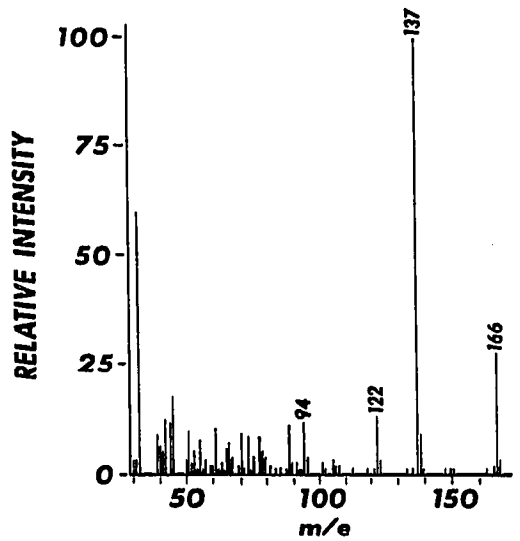


Fig. 2. Mass spectrum of the product formed from 3-methoxy-4-hydroxyphenylglycol (MHPG) with ethanolic hydrochloric acid.

separation of the isomeric 3-methoxy-4-hydroxy and 4-methoxy-3-hydroxy compounds on GC for quantitation by GC–MS.

Even if the isomeric compounds are obtained by standard techniques of liquid chromatography, ion-exchange column chromatography or TLC, when the isomers are not separated, they can subsequently be derivatized by this method and identified if they should occur in biological systems.

As overlapping of other peaks has been reported to occur with the use of the TMS derivatives of the methyl ethers[8], we reinvestigated by the present method the levels of VMA in urine samples of Parkinson patients receiving L-DOPA. When the levels were low and other peaks interfered, we performed preliminary preparative TLC. The ester fraction from the urine and standard VMA-ethyl ester-β-O-ethyl ester were spotted on a silica gel G thin-layer plate which was developed with chloroform–acetic acid (100:2). The reference standard was rendered visible by spraying with diazotized *o*-tolidine and the sample fraction corresponding to the reference standard was eluted with ethanol–ethyl acetate (1:1). This fraction was then quantitated by GC on an OV-225 column and also by monitoring the base peak at *m/e* 181 during GC–MS. The homogeneity of the peak was confirmed by its mass spectrum. The results obtained by the two methods agreed closely, and were further confirmed when the recovery was checked by using the same procedure with adequate controls with added standards. The values obtained by this specific method were lower than those in the literature[8]. Details of these results will be published elsewhere.

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