SYNTHESIS OF CD_3-LABELLED 3-METHOXYTYRAMINE, NORMETANEPHRINE AND METANEPHRINE FOR USE AS INTERNAL STANDARDS IN MASS FRAGMENTOGRAPHIC ANALYSES

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SUMMARY

The preparation of CD_3-labelled 3-methoxytyramine, normetanephrine and metanephrine from their parent catecholamines is described. The syntheses include N-maleylation, O-methylation by dimethylsulfate-D_6, removal of the protecting group by acid hydrolysis and partial purification of the reaction product. Examples of the use of the deuterium labelled O-methylated catecholamine metabolites as internal standards for the ammonia chemical ionisation mass fragmentographic determination of their naturally occurring analogs in urine, are given.

Key Words: CD_3-labelled-3-O-methylated catecholamine metabolites; mass fragmentography.

INTRODUCTION

The determination of catecholamines and their metabolites in biological fluids is particularly important for the diagnosis and follow-up of patients with neural crest tumours (neuroblastoma and phaeochromocytoma).

The main pathway in the degradation of catecholamines include aerobic deamination by monoamine oxidase (MAO, EC 1.4.3.4), finally leading to acidic- and alcoholic metabolites; and 3-O-methylation by catechol-O-methyltransferase (COMT, EC 2.1.1.6.), resulting in the formation of 3-methoxytyramine (3MT) from dopamine, normetanephrine (NM) from norepinephrine and metanephrine (M) from epinephrine. Both classes of 'primary' metabolites can subsequently be metabolised by MAO and COMT, giving rise to the main urinary catecholamine metabolites homovanillic acid, vanilmandelic acid and 3-methoxy-4-hydroxyphenylethylene glycol (for review see reference 1).

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Due to the difference in the distribution of MAO and COMT in the body, there are differences in the relative contributions of the various catecholamine producing compartments to the three mentioned metabolite classes in urine (2). In this respect the excretion of the 3-O-methylated catecholamine metabolites can largely be attributed to the action of COMT on catecholamines secreted into the bloodstream, making their determination of considerable diagnostic importance particularly for patients with phaeochromocytoma.

Numerous methods have been described for the determination of 3-O-methylated catecholamines in urine including advanced methods such as high performance liquid chromatography with fluorimetric (3), ultraviolet (4) and electrochemical detection (5) and gas chromatography with flame ionisation (6), electron capture (7) and mass spectrometric (8-10) detection. Until recently mass spectrometric methods have been dependent on the synthesis of commercially unavailable deuterated 3-O-methylated catecholamine metabolites as internal standards. However, besides the hazard of exchange of the label under severe conditions, the yield of the synthesis (11) of ring deuterated NM and M (9,10) is low, whereas the use of nowadays available side-chain trideuterated NM and M (8) is expensive.

In this paper we describe the synthesis of unexchangeable CD_3-labelled 3MT, NM and M, that can alternatively be used as internal standards for the mass fragmentographic measurements of their naturally occurring analogs.

MATERIALS AND METHODS

Standards, reagents and equipment

Metanephrine, normetanephrine, 3-methoxytyramine, dopamine, epinephrine and norepinephrine were purchased from Sigma Chemical Co; 4-O-methyldopamine (iso-3MT) from Janssen; Affi-Gel 601 (boronate gel) and AG 1-X10 anion exchange resin were from BioRad; pentafluoropropionic anhydride (PFPA) from Pierce Chemical Co, and all other reagents were from Merck.

Gas chromatography with flame ionisation detection was performed on a Varian 3700 gas chromatograph equipped with a 25m x 0.26mm (i.d.) OV-1701 coated (0.07µm film thickness) fused silica column from Chrompack.

Gas flow was 0.8 ml He/min; split ratio 1:7.5; detector temperature was 280°C, injector temperature 240°C. Oven temperature was programmed from 120°C to 240°C at 5°C/min.

Gas chromatography - mass spectrometry was performed using a Varian 3700 gas chromatograph interfaced to a Varian MAT 44S mass spectrometer by an open-split coupling. Data were collected on a Finnigan MAT SS 200 data system. The gas chromatograph contained a 25m x 0.23 mm (i.d.) SE-54 coated (0.2µm film thickness) fused silica column from Chrompack. Injector and interface temperatures were 250°C, source temperature was 200°C. Ionisation energy for ammonia chemical ionisation was 120eV. Oven temperature program was from 140-210°C at 10°C/min.

Synthesis of CD_3-labelled 3-O-methylated catecholamine metabolites

N-maleylation

N-maleylation of catecholamines was performed according to Miwa et al. (12). One millimole of dopamine.HCl, norepinephrine.HCl or epinephrine. HCl was suspended in 5 ml of methanol. Two millimoles of pulverised maleic anhydride and eight drops of triethylamine were added to each tube. After standing at room temperature for 15 min., the solvents were removed under reduced pressure. The yellow residues were washed with 2x20 ml of diethylether.

O-methylation

N-maleylated catecholamines were dissolved in 4 ml of cold water and 16 ml of cold 2 mol/l NaOH solution, containing 0.53 mol/l sodium metabisulfite, were added. Methylation was performed by adding 1.2 ml of dimethylsulphate-D_6 in several portions. The tubes were cooled in an ice bath and occasionally mixed until the dimethylsulphate-D_6 was completely dissolved.

Hydrolysis of the maleyl group

The pH was adjusted to 1-2 with a concentrated HCl solution and the tubes heated at 60°C for 16 hours in a heating block.

Partial purification of the products

The pH was adjusted to 8 with a concentrated solution of ammonia and the reaction mixtures passed over 0.8 x 10 cm columns of AG 1-X10 (100-200 mesh, CL^- form). The columns were washed with 10 ml of water

and the combined eluates were passed through 0.8 x 10 cm Affi-gel 601 columns. The columns were washed with 10 ml of 0.1 mol/l of NH_4HCO_3 solution pH 8.2. The then combined eluates were adjusted to pH 1 with a concentrated HCl solution. Aliquots were evaporated to dryness at 40°C under a stream of nitrogen and derivatised with pentafluoropropionic anhydride as previously described (9).

RESULTS AND DISCUSSION

The described syntheses of CD_3-labelled 3-O-methylated catecholamines from their respective parent catecholamines include four major steps viz. protection of the nitrogen group by N-maleylation with maleic anhydride, O-methylation with $(CD_3)_2SO_4$, removal of the protecting group by acid hydrolysis and purification by means of anion exchange chromatography (removal of maleic acid) and boronate affinity chromatography (removal of unreacted catecholamines). Analyses of the reaction products as their PFP derivatives by gas chromatography and gas chromatography - mass spectrometry revealed relatively pure O-methylated products, containing 3-O and 4-O-methylated (iso-) compounds in about equal amounts. Their ammonia chemical ionisation mass spectra together with those of the naturally occurring 3-O-methylated catecholamine metabolites are shown in figures 1-3. In case of CD_3-labelled 3MT we were unable to provide direct evidence for the co-synthesis of CD_3-labelled iso-3MT, as these two isomers could not be separated under the gas chromatographic conditions used. The total yields of 3-O and 4-O-methylated products amounted to about 50%.

Figure 4 shows examples of the use of the synthesized internal standards for the quantitative mass spectrometric measurements of 3-O-methylated catecholamines in urine by a modification of our previously described method (9). Besides the positions of the labels, the main modifications comprised the use of capillary columns instead of packed columns and ammonia chemical ionisation instead of electron impact. Mass fragmentograms were prepared by simultaneously monitoring the respective $M+NH_4^+$-ions of PFPA derivatised urinary ethyl acetate extracts of a normal healthy adult (A), a patient with phaeochromocytoma (B) and a patient with neuroblastoma (C). Table 1 summarizes the excretion values calculated from these analyses, indicating increased urinary excretion of NM and M in the patient with phaeochromocytoma and increased excretion of 3MT in

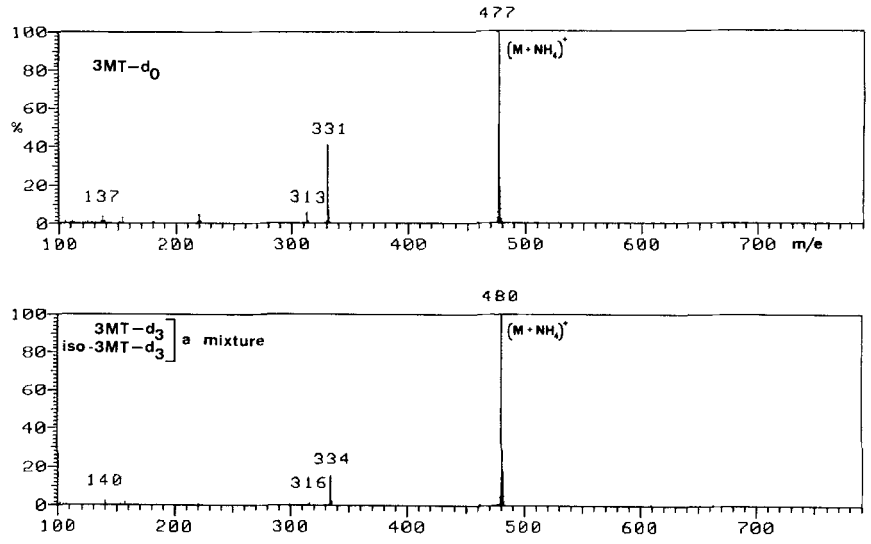

FIGURE 1: Ammonia chemical ionisation mass spectra of pentafluoropropionated 3MT (top) and a mixture of CD_3-labelled 3MT and iso-3MT (bottom).

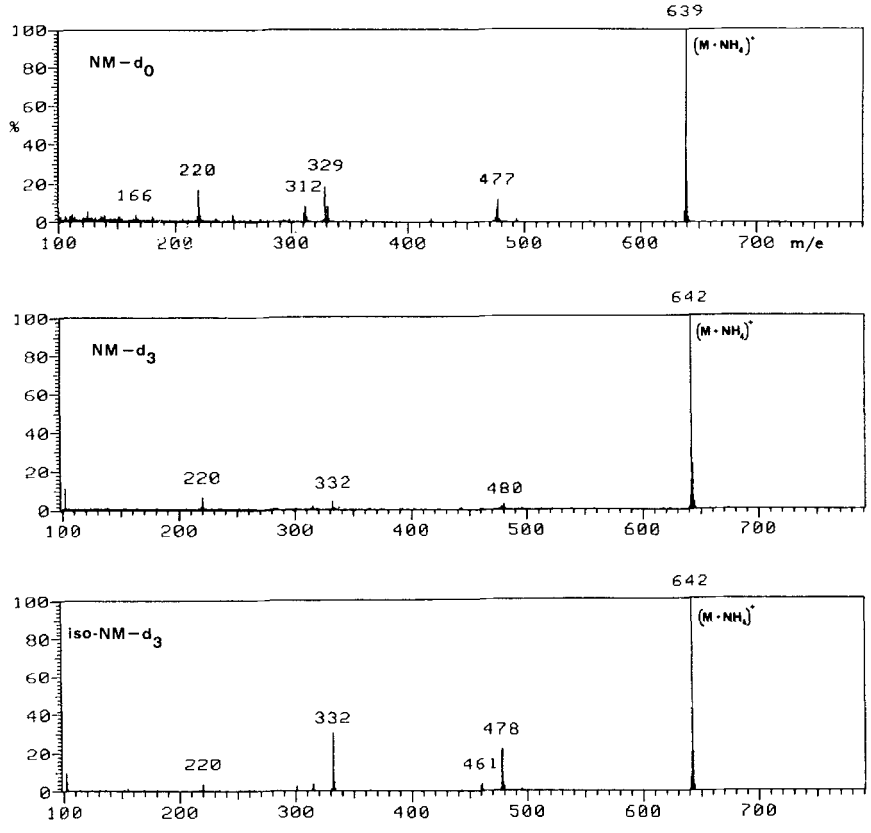

FIGURE 2: Ammonia chemical ionisation mass spectra of pentafluoropropionated NM (top), CD_3-labelled NM (middle) and CD_3-labelled iso-NM (bottom).

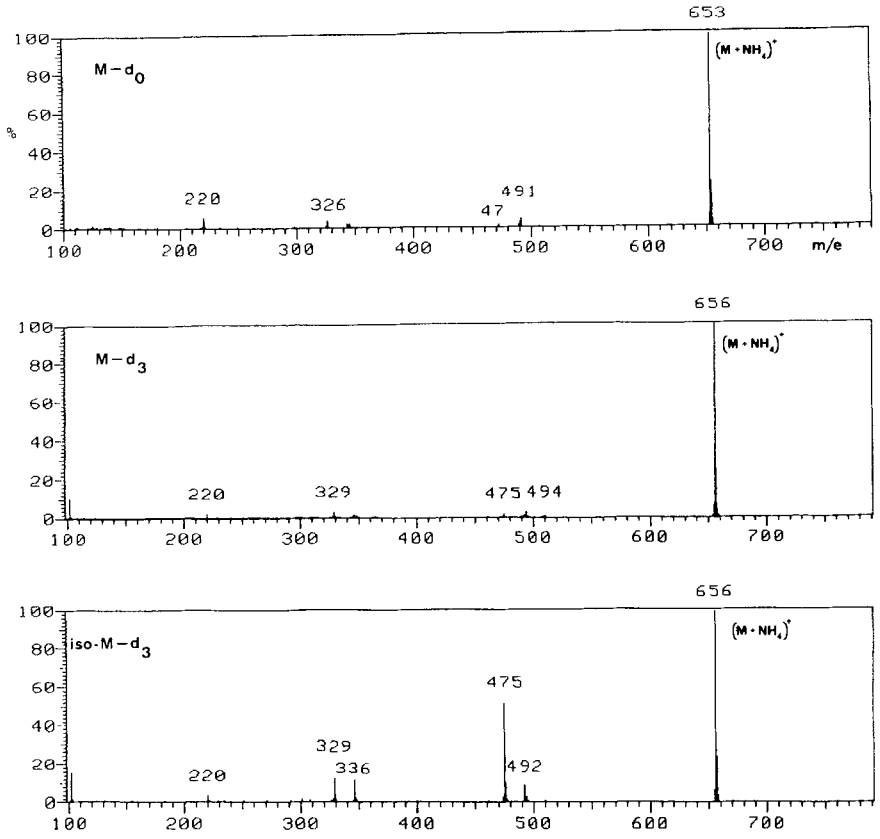

FIGURE 3: Ammonia chemical ionisation mass spectra of pentafluoropropionated M (top), CD_3-labelled M (middle) and CD_3-labelled iso-M (bottom).

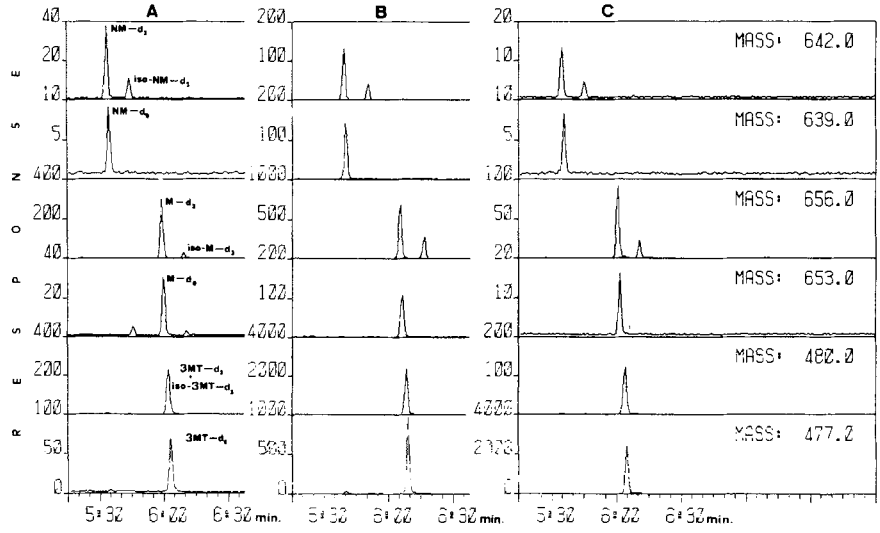

FIGURE 4: Typical ammonia chemical ionisation mass fragmentograms obtained from the simultaneous determination of 3MT, NM and M in derivatised urinary extracts of a normal healthy adult (A), an adult patient with phaeochromocytoma (B) and a pediatric patient with neuroblastoma (C).

Table 1: *Urinary excretion values of 3MT, NM and M calculated from the mass fragmentograms shown in figure 4.*

	age	3MT[2]	NM	M
normal healthy person	*28y*	*52*	*133*	*55*
patient with phaeochromocytoma	*52y*	*142*	*780*	*120*
reference value [1]	*13-65y*	*170*	*260*	*70*
patient with neuroblastoma	*2y 11m*	*1270*	*586*	*151*
reference value [1]	*3y*	*300*	*600*	*175*

Urinary excretion values were calculated by comparing peaks area ratios of the endogenous compound and its internal standard to those of known amounts of standard compounds, using linear regression analysis. Values are expressed in µmol/mol of creatinine.1, cut-off values according to reference 9; 2, corrected for recovery (see text); y, years; m, months.

case of the patient with neuroblastoma. Day-to-day spiking experiments (n=5) with standard 3-O-methylated catecholamines revealed mean extraction recoveries relative to the internal standards of 102 and 110% for NM and M, respectively, and 165% for 3MT. The too high recovery of 3MT was further investigated by means of spiking experiments with 3MT and iso-3MT, using ring-deuterated 3MT-D_3 as an internal standard. These experiments revealed a much lower relative recovery of iso-3MT (12%) than 3MT (100%), indicating a need for further optimalisation of the method by (1) gas chromatographic separation of CD_3-labelled 3MT and CD_3-labelled iso-3MT; (2) purification of the CD_3-labelled mixture prior to its use as an internal standard , or (3) the combined use of CD_3-labelled NM and M and the easily prepared ring deuterated 3MT-D_3 as internal standards.

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