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A NON-ENZYMIC PROCEDURE FOR THE QUANTITATIVE ANALYSIS OF (3-METHOXY-4-SULPHOXYPHENYL)ETHYLENE GLYCOL (MHPG SULPHATE) IN HUMAN URINE USING STABLE ISOTOPE DILUTION AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

STEPHEN MURRAY, THOMAS A. BAILLIE and DONALD S. DAVIES

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Ducane Road, London (Great Britain)

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SUMMARY

A method is described for the quantitative analysis of (3-methoxy-4-sulphoxyphenyl)-ethylene glycol (MHPG sulphate) in human urine, based on selected ion monitoring gas chromatography—mass spectrometry and using a specifically deuterium-labelled analogue of MHPG sulphate as internal standard. The procedure involves extraction of the urine sample on Amberlite XAD-2, followed by isolation of MHPG sulphate by column chromatography on Sephadex LH-20. Cleavage of the sulphate conjugate and formation of the MHPG tris(trifluoroacetate) derivative are carried out in a one-step reaction, without recourse to enzymic hydrolysis.

INTRODUCTION

Studies on the metabolic fate of noradrenaline in man have shown that (3-methoxy-4-hydroxyphenyl)ethylene glycol (MHPG) is a major metabolite [1,2] and that this compound is excreted in urine mainly as its sulphate and glucuronide conjugates [1,3]. Whereas MHPG appears to be conjugated mainly with glucuronic acid in peripheral tissues, it has been suggested that sulpho-conjugation of this compound takes place predominantly in the brain [4]. If this is the case, measurement of urinary MHPG sulphate should provide a convenient, non-invasive technique for the assessment of central noradrenergic function in man [4].

A number of methods have been reported for the analysis of free and conjugated MHPG in urine, based on spectrophotometry [5—8], gas—liquid chromatography [4, 9—15] and, more recently, on gas chromatography—mass spectrometry (GC—MS) [16—19]. Of these techniques, the latter combines highest specificity and sensitivity and permits the use of stable isotope-labelled analo-

gues of the compounds of interest as internal standards [20].

MHPG sulphate may be hydrolysed by the action of acid [16], but since free MHPG is itself unstable under these conditions [5, 7, 9], methods for the measurement of the sulphate conjugate rely on the "selective" action of sulphatase enzymes; unconjugated MHPG in urine is determined before and after hydrolysis and the levels of MHPG sulphate are obtained by difference. Although enzymic hydrolysis is a powerful technique for the qualitative study of conjugated metabolites, the use of enzymes for quantitative determinations on a routine basis suffers from a number of disadvantages. First, most sulphatase preparations contain low, and often variable, amounts of β-glucuronidase and, if steps are not taken to selectively inhibit β-glucuronidase activity [21], erroneously high values for MHPG sulphate will be obtained. This problem may, however, be overcome if MHPG sulphate is separated from the corresponding glucuronide prior to enzymic hydrolysis. Chromatographic systems based on thin-layer [22], paper [3] and ion-exchange chromatography [22] have been described for the separation of MHPG and its conjugates, but these have not been adopted for routine use.

A second disadvantage associated with the use of enzymic hydrolysis is that hydrolytic activity may be diminished by the presence in urine samples of endogenous inhibitors of enzyme action [3] or by the use of a large excess of the enzyme in the incubation [4]. Problems of this nature can be overcome satisfactorily only through the use of an appropriate, sulpho-conjugated internal standard. Finally, certain enzyme preparations have been shown to contain traces of MHPG itself and of other interfering substances [23], thus necessitating the use of a "blank" incubation with each batch.

In view of the above disadvantages, we have investigated alternative approaches to the analysis of MHPG sulphate in human urine. In the present communication, we report the development of an improved stable isotope dilution assay for urinary MHPG sulphate, of which a specifically deuterium-labelled analogue has been synthesized for use as internal standard. Following extraction of MHPG and its conjugates from urine, MHPG sulphate is isolated by liquid–gel chromatography and is converted to a derivative suitable for GC–MS analysis by a novel procedure which does not entail hydrolysis by an aryl sulphatase.

EXPERIMENTAL

General

Amberlite XAD-2 (BDH, Poole, Great Britain) was washed exhaustively with acid, alkali, water and solvents [24]. The resin was stored in water and was used as described by Bradlow [25]. Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was washed in turn with 5% acetic acid in ethanol, water, ethanol, chloroform and finally methanol. Trifluoroacetic anhydride was purchased from Sigma (London, Great Britain), as were aryl sulphatase (Type H-1, containing 18,600 units/g sulphatase and approx. 300,000 Fishman units/g β-glucuronidase) and β-glucuronidase (Type II, approx. 40,000 units/g). All solvents employed in the analytical procedure were of analytical grade and were redistilled before use. Ethyl acetate and dimethylformamide were dried over calcium hydride and redistilled.

Acetovanillone, benzyl chloride and [O-^{2}H]ethanol were purchased from Koch-Light Labs. (Colnbrook, Great Britain). Sodium borodeuteride and sodium deutero-oxide in deuterium oxide (40%, w/w) were obtained from Merck Sharp and Dohme (Montreal, Canada) and dicyclohexylcarbodiimide from BDH. A sample of the potassium salt of MHPG sulphate was kindly donated by Hoffmann-La Roche (Basle, Switzerland) while further quantities were purchased from Fluka (Buchs, Switzerland). The piperazine salt of unconjugated MHPG was obtained from Sigma. Thin-layer chromatography (TLC) was carried out using glass plates (5 × 20 cm), precoated with 0.25-mm layers of silica gel G_{F254} (Merck, Darmstadt, G.F.R.).

Infrared (IR) spectrometry. IR spectrometry was carried out on Nujol mulls using a Perkin-Elmer 157G instrument.

Nuclear magnetic resonance (NMR) spectrometry. NMR was performed at 60 MHz on a Hitachi Perkin-Elmer R-24 instrument. Spectra were obtained on solutions in deuterochloroform, except where otherwise stated.

Gas chromatography—mass spectrometry. A Finnigan Model 3200 instrument was used, equipped with a 5 ft. × 2 mm I.D. glass column packed with 3% OV-17 on Gas-Chrom Q (100—120 mesh), and operated at 115° with helium (30 ml/min) as carrier gas. Under these conditions, the retention time of the MHPG tris(trifluoroacetate)(tris-TFA) derivative was approximately 5 min. The mass spectrometer was operated in the electron impact mode with an electron energy of 25 eV, emission current of 400 μA and continuous dynode electron multiplier voltage of 1.3 kV. The pre-amp range was 10^{-7} A · V^{-1} for recording of reference spectra and 10^{-8} A · V^{-1} for analysis of urinary extracts. Data acquisition and reduction was performed by a Finnigan Model 6000 interactive data system, using revision H software.

Synthesis

3-Methoxy-4-benzyloxy-ω-bromoacetophenone (III). This compound was prepared from acetovanillone (I; 10.0 g, 60.2 mmole) by the method of Hegedüs [26]. The crude product was recrystallised from ethanol to give III as fine crystals (yield = 8.0 g, 40%). TLC (chloroform): single spot, R_F = 0.57. MS: *m/e* 334 (M^+), 255, 241, 214 and 91 (base peak). IR: 3065 (w), 1760 (s), 1580 (s), 1510 (s), 1260 (s) and 1155 cm^{-1} (s). NMR: 3.93 (3H, s), 4.36 (2H, s), 5.23 (2H, s), 7.15 (3H, m) and 7.36δ (5H, broad s).

1-(3-Methoxy-4-benzyloxyphenyl) [1,2,2-$^2H_3$] ethylene glycol (V). To a solution of the bromide III (7.5 g, 22.4 mmole) in [O-^{2}H]ethanol (100 g) was added anhydrous potassium acetate (4.39 g) and the mixture was heated under reflux for 19 h. The reaction product was allowed to cool to room temperature, when sodium borodeuteride (1 g, 23.8 mmole) was added in portions with stirring. After 3 h at room temperature, the mixture was treated with a solution of sodium deutero-oxide in deuterium oxide (40% w/w; 5 ml), followed by a further 10 ml of deuterium oxide and allowed to equilibrate for 1 h. The reaction mixture was then poured into water (200 ml) and extracted into diethyl ether (three 150-ml portions). The combined ether extracts were washed with water (two 200-ml portions), dried (sodium sulphate), filtered and evaporated under reduced pressure to give V as an oil. Crystallisation from benzene—

hexane afforded 4.94 g of product, containing benzene of crystallisation [26]. TLC (ethyl acetate): single spot, R_F = 0.29. MS: *m/e* 277 (M^+), 259, 244 and 91 (base peak). NMR: 2.8 (2H, very broad s, —OH), 3.97 (3H, s), 5.27 (2H, s), 7.0—7.8δ (14H, m, includes one molecule of benzene of crystallisation).

1-(3-Methoxy-4-benzyloxyphenyl) [1,2,2-$^2H_3$] ethylene glycol diacetate (VI). A portion of the labelled glycol V (150 mg) was acetylated in acetic anhydride—pyridine (4:1, v/v; 2.5 ml) for 16 h at room temperature. The mixture was then poured into ice-water (20 ml) and the product was extracted with diethyl ether (twice 15 ml). The combined ether extracts were washed with 1 *N* hydrochloric acid (twice 10 ml), saturated sodium hydrogen carbonate solution (20 ml), dried (sodium sulphate) and filtered. Evaporation of the solvent gave VI as a pale yellow oil (yield: 170 mg). TLC (chloroform): single spot with R_F = 0.48. MS: *m/e* 361 (M^+), 300, 258, 211, 209, 159, 157, and 91 (base peak). NMR: 2.03 (3H, s), 2.08 (3H, s), 3.87 (3H, s), 5.12 (2H, s), 6.82 (3H, broad s) and 7.33δ (5H, broad s).

1-(3-Methoxy-4-hydroxyphenyl) [1,2,2-$^2H_3$] ethylene glycol diacetate (VII). The diacetate VI (170 mg, 0.471 mmole) was dissolved in methanol (30 ml) and 5% palladium on charcoal (100 mg) was added. This mixture was hydrogenated for 2.5 h in a Parr shaker, using a hydrogen pressure of 30 p.s.i. The resulting mixture was filtered and evaporated under reduced pressure to yield VII as a colourless oil. TLC (chloroform): single spot, R_F = 0.30. MS: *m/e* 271 (M^+), 210, 196, 168, 154, 94 and 43 (base peak). NMR: 1.99 (3H, s), 2.03 (3H, s), 3.87 (3H, s), 5.55 (1H, very broad s, —OH) and 6.80δ (3H, s).

1-(3-Methoxy-4-sulphoxyphenyl) [1,2,2-$^2H_3$] ethylene glycol potassium salt (IX). The product from the above reaction was dissolved in anhydrous dimethylformamide (5 ml), dicyclohexylcarbodiimide (700 mg) was added and the resulting solution was cooled to 0°. To this solution was added 1.5 ml of a mixture of concentrated sulphuric acid (0.45 ml) and anhydrous dimethylformamide (7.7 ml), which had previously been cooled to 0°. The reaction was allowed to proceed for 2 h at 0° and was quenched by the addition of 75% aqueous ethanol (20 ml). The pH of the mixture was adjusted to 7.5 by the addition of a few drops of 2 *N* potassium hydroxide solution. The mixture was centrifuged and the supernatant was removed. The residue was extracted with 75% aqueous ethanol (thrice 20 ml), and the combined supernatant and extracts were taken to dryness in vacuo.

The residue was redissolved in 5 ml chloroform—methanol (1:1, v/v, 0.01 *M* with respect to potassium chloride) and applied to a column of Sephadex LH-20 (20 g), packed in and eluted with the same solvent system. The effluent between 210 and 270 ml was collected and evaporated to dryness to give VIII as a white solid (103 mg), which was hydrolysed for 3 h at 80° in 1 *M* aqueous potassium hydroxide (3 ml). The resulting solution was neutralized by the careful addition of hydrochloric acid and was lyophilized to yield 306 mg of solid material, of which approximately 10% by weight corresponded to the labelled sulphate conjugate IX. NMR (^2H_2O): 3.90 (3H, s) and 7.20δ (3H, m).

Treatment of a portion of the above product with trifluoroacetic anhydride—ethyl acetate (1:1, v/v) for 90 min at room temperature and subsequent GC—MS analysis showed the formation of a single derivative whose

mass spectrum was almost identical to that previously published for the tris-TFA derivative of [$^2H_3$]MHPG [18]. Deuterium content of this derivative, as measured from the molecular ion cluster, was found to be as follows (atoms % excess): 0.03% $^2H_0$, 0.37% $^2H_1$, 10.60% $^2H_2$ and 88.99% $^2H_3$.

The deuterated sulphate IX was stored at −20° and stock solutions in distilled water (concentration approx. 250 μg/ml) were prepared as required. To aliquots of these solutions was added a fixed amount of unlabelled MHPG sulphate to serve as internal standard and the mixtures were derivatised with trifluoroacetic anhydride in ethyl acetate. The resulting MHPG tris-TFA derivatives were analysed by selected ion monitoring GC—MS, when the ratios of peak heights in the recordings for *m/e* 358 and 360 were used to calculate the concentration of [$^2H_3$]-MHPG sulphate in the original stock solutions.

Urine samples

Twenty-four-hour urine samples were collected from healthy laboratory personnel who were not receiving any medication, and were stored at −20° until analysed.

Analytical procedures

Urinary MHPG sulphate. To an aliquot (5 ml) of a 24-h urine collection is added [$^2H_3$]MHPG sulphate (approx. 25 μg) in distilled water (100 μl). The sample is mixed thoroughly on a Vortex mixer and is passed through a column (10 × 2 cm) of Amberlite XAD-2. The column is washed in turn with water (10 ml) and hexane (10 ml) and the washings are discarded. MHPG sulphate is then eluted with methanol (30 ml) and the solvent is evaporated under reduced pressure. The residue is taken up in 2 ml chloroform—methanol (1:1, v/v, 0.01 *M* with respect to sodium chloride) and is applied to a column (28 × 1 cm) of Sephadex LH-20 (5 g), prepared in and eluted with the same solvent system. The fraction corresponding to 70—100 ml of effluent is taken to dryness under reduced pressure and is transferred with methanol (two 0.5-ml portions) to a screw-capped Reactivial®. The solvent is evaporated under a stream of nitrogen and the residue is further dried for 2 h in a vacuum desiccator in the presence of phosphorous pentoxide.

Anhydrous ethyl acetate (250 μl) and trifluoroacetic anhydride (350 μl) are added to the sample and the vial is securely capped and shaken for 2 h at room temperature. Residual sodium chloride from the LH-20 chromatography solvent system is removed by brief centrifugation and the clear supernatant is transferred to a second Reactivial and evaporated to dryness under a stream of dry nitrogen. The residue is taken up in anhydrous ethyl acetate—trifluoroacetic anhydride (95:5, v/v, 20 μl) and aliquots (2 μl) are injected into the GC—MS instrument, which is focused to monitor the ions at *m/e* 358 and 360.

The concentration of endogenous MHPG sulphate in the urine sample is determined by reference to a standard curve of peak height ratio (*m/e* 358:360) versus quantity of unlabelled MHPG sulphate. Standard curves, which are prepared with each batch of samples, are linear over the range of MHPG sulphate concentrations encountered in normal subjects and are constructed from known mixtures of the reference (unlabelled) and deuterated conjugate.

Free and "total" MHPG in urine. Measurement of free and "total" urinary MHPG is also carried out by GC—MS using a modification [27] of the procedure described by Bertilsson [28] for cerebrospinal fluid samples. [$^2H_2$]MHPG (prepared by reduction of vanillylmandelic acid with lithium aluminium deuteride) serves as the internal standard in both assays. The volume of urine required for estimation of free MHPG is 200 μl, while that for "total" MHPG is 10 μl. Cleavage of MHPG conjugates is carried out using Sigma Type H-1 enzyme, a preparation containing both aryl sulphatase and β-glucuronidase activity.

RESULTS AND DISCUSSION

Synthesis of [$^2H_3$]MHPG sulphate (Fig. 1)

Preparation of the internal standard, [$^2H_3$]MHPG sulphate, was carried out by modification of a published procedure for the synthesis of the corresponding unlabelled conjugate [26]. Acetovanillone (I) was converted to the bromo derivative III, which was then heated under reflux with potassium acetate in monodeuteroethanol. This treatment gave the acetoxy ketone IV with simultaneous incorporation of two atoms of deuterium in the side-chain. Reduction of IV in situ with sodium borodeuteride, followed by alkaline hydrolysis, afforded the trideuterated glycol derivative V. Acetylation of V and subsequent removal of the benzyl protecting group yielded [$^2H_3$]MHPG diacetate (VII), which was converted to the corresponding sulphate conjugate VIII as described by Mumma [29]. This compound was purified as its potassium salt by chromatography on Sephadex LH-20 [30] and finally hydrolysed under alkaline conditions to give [$^2H_3$]MHPG sulphate (IX).

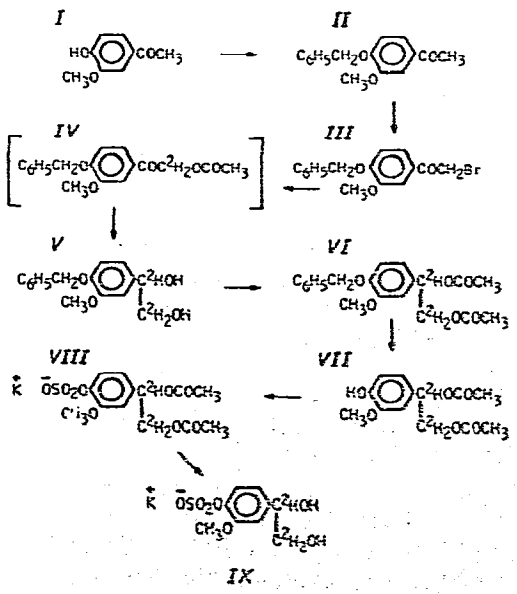

Fig. 1. Synthesis of [$^2H_3$]MHPG sulphate.

MS analysis of compounds V and IX (the latter following trifluoroacetylation) indicated that the deuterium atoms had been completely retained during the latter stages of the synthesis.

Analysis of urinary MHPG sulphate

In the first stage of the assay procedure, a known amount of the labelled internal standard, [$^2H_3$]MHPG sulphate, is added to a 5 ml aliquot of a 24-h urine collection. Free MHPG and its conjugates are then extracted from urine by chromatography on a small column of Amberlite XAD-2. MHPG sulphate is only partially retained on the resin, and, although overall recoveries from the column are poor (yield approximately 30%), this has not proved to be a serious drawback in the analysis.

MHPG sulphate is next separated from its free and glucuronide-conjugated forms by liquid—gel partition chromatography on a column of Sephadex LH-20. The use of this lipophilic gel with methanol—chloroform (1:1, v/v) as the mobile phase has been widely employed for the isolation of steroid sulphates from lipid extracts of biological material [30]. In this system, the presence or absence of a sulphate group determines chromatographic mobility and enables group separations to be carried out, based on conjugate class. Calibration of the LH-20 column with a sample of authentic MHPG sulphate indicated that this conjugate is strongly retained on the gel and is eluted slightly later than the majority of steroid monosulphates, which confirms the relative insensitivity of the LH-20 system towards structural modifications in the lipophilic region of the molecule. In an experiment designed to establish the chromatographic behaviour of MHPG glucuronide on LH-20, an XAD-2 extract of human urine was applied to a column containing 5 g of the gel and the column was eluted with methanol—chloroform (1:1, v/v, 0.01 *M* with respect to sodium chloride). Fractions (10 ml) of the effluent were collected and each fraction was split into three equal parts and taken to dryness. One aliquot was derivatized directly with trifluoroacetic anhydride, while the other two aliquots were incubated with β-glucuronidase and arylsulphatase, respectively, prior to derivatization. The amount of MHPG tris-TFA formed in each case was determined by GC—MS and the results are summarized in Fig. 2. This shows that MHPG glucuronide has an elution volume intermediate between those of free MHPG and its sulphate conjugate and that the LH-20 column fraction (between 70 and 100 ml of effluent) collected in the assay of MHPG sulphate will not contain any of the glucuronide.

In the final stage of the analytical procedure, MHPG sulphate is converted to a derivative suitable for GC—MS analysis by reaction at room temperature with a mixture of trifluoroacetic anhydride and ethyl acetate. This mild treatment has been found to result in quantitative conversion of MHPG sulphate to the MHPG tris-TFA derivative in a single step. A similar type of reaction was observed when isoetharine sulphate [31] and isomeric dopamine sulphates [32] were treated with trimethylsilylating reagents, and when a number of steroid sulphates were reacted with heptafluorobutyric anhydride in benzene [33]; in each case, the sulphate ester is displaced and a derivative of the resulting alco-

TABLE I

VALUES FOR THE EXCRETION OF FREE MHPG, MHPG SULPHATE AND TOTAL MHPG (FREE + CONJUGATED) IN 24-H URINE COLLECTIONS FROM HEALTHY VOLUNTEERS

Subjects	Age (years)	mg MHPG/24 h			mg MHPG/g creatinine		
		Free	Sulphate*	Total*	Free	Sulphate*	Total*
Males							
ARB	28	0.10	1.57	3.11	0.06	0.89	1.76
TB	28	0.04	0.55	1.54	0.03	0.43	1.20
CD	31	0.06	1.05	2.51	0.04	0.74	1.77
HJ	29	0.07	0.89	1.82	0.04	0.50	1.03
SJ	23	0.09	1.19	2.36	0.05	0.68	1.34
SM	28	0.14	1.55	4.21	0.07	0.81	2.20
JR	32	0.09	1.35	2.83	0.04	0.66	1.38
Females							
CH	29	0.11	1.12	2.42	0.09	0.94	2.03
BN	23	0.05	0.55	1.25	0.05	0.52	1.18
PT	33	0.06	0.65	2.25	0.05	0.49	1.71
Mean		0.08	1.05	2.43	0.05	0.67	1.56
± S.D.		± 0.03	± 0.38	± 0.84	± 0.02	± 0.18	± 0.39

* Values expressed in terms of free MHPG present in conjugated form.

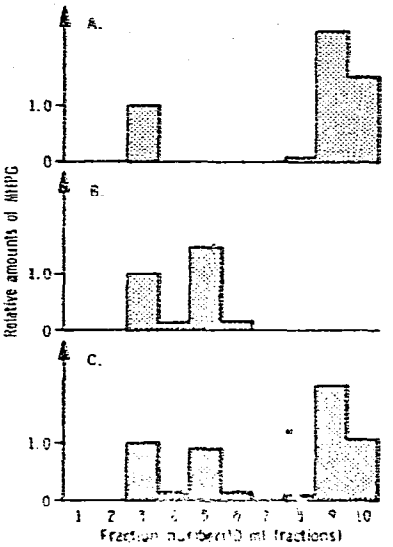

Fig. 2. Chromatographic separation of free MHPG (fr. 3), MHPG glucuronide (fr. 5) and MHPG sulphate (fr. 8—10) on Sephadex LH-20 (mobile phase: methanol—chloroform, 1:1, v/v; 0.01 *M* in NaCl) from an extract of urine. The amount of MHPG in each fraction was determined by GC—MS, (A) following direct derivatization with trifluoroacetic anhydride—ethyl acetate, (B) after incubation with β-glucuronidase, extraction into ethyl acetate and derivatization and (C) after incubation with aryl sulphatase (containing β-glucuronidase activity), extraction into ethyl acetate and derivatization.

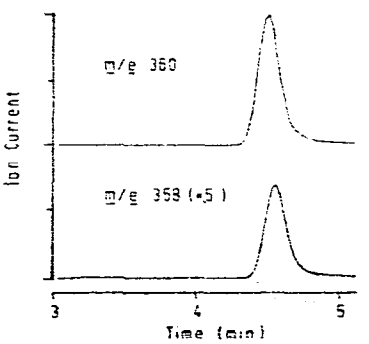

Fig. 3. Selected ion recordings obtained from a urinary extract of MHPG sulphate; *m/e* 358, MHPG tris-TFA derivative; *m/e* 360, internal standard. The concentration of MHPG sulphate in this sample was 0.48 μg (as free MHPG)/ml urine.

hol is formed. Although we have not investigated the general applicability of this reaction, it would appear to provide an attractive alternative to the use of sulphatases, particularly in cases where the conditions employed for acid hydrolysis result in destruction of the liberated alcohol. As is evident from Fig. 2, the glucuronide conjugate of MHPG is stable towards trifluoroacetic anhydride and does not give rise to MHPG tris-TFA under the derivatization conditions employed.

Quantification of endogenous MHPG sulphate is based on ratios of peak heights in the selected ion records for *m/e* 358 (unlabelled MHPG derivative) and *m/e* 360 (internal standard) (Fig. 3). These fragments correspond to loss from the molecular ions of the elements of trifluoroacetic acid and monodeutero-trifluoroacetic acid, respectively [28] Monitoring of the molecular ions, at *m/e* 472 and 475, may also be used for quantitative measurements, although monitoring of these masses gives a lower response per unit mass of sample injected.

In a study of the precision of the analytical procedure, the concentration of MHPG sulphate was determined in five 5-ml aliquots of a 24-h urine collection, when each sample was analysed twice by GC—MS. The mean value obtained was 0.70 μg/ml, with a standard deviation of ±2%.

Levels of MHPG sulphate in 24-h urine collections from ten healthy volunteers are given in Table I. For comparison, corresponding values for free and "total" MHPG, the latter obtained using a conventional enzymic hydrolysis, are also shown. Mean figures for the excretion of MHPG sulphate and of free and "total" MHPG in these subjects were found to fall within the range reported by other authors [3, 4, 16], although it should be noted that literature values for the excretion of these compounds vary widely.

Application of the present method to studies of the effect of antihypertensive drugs on the turnover of noradrenaline in the central and peripheral nervous system is currently in progress.

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