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URINARY 3-METHOXY-4-HYDROXYPHENYLGLYCOL DETERMINATION USING REVERSED-PHASE CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

A reversed-phase liquid chromatographic method with amperometric detection has been developed for the determination of urinary 3-methoxy-4-hydroxyphenylglycol. Before and after enzymatic deconjugation, it was purified by an extraction procedure and rapidly quantified under isocratic conditions. The 24-h excretion profile in normal human subjects (eight males and seven females) was determined; our results are consistent with those arrived at in a number of other studies. The present method is highly sensitive and selective; in addition, a good degree of precision is assured by use of 4-methoxy-3-hydroxyphenylglycol as internal standard.

INTRODUCTION

The determination of urinary 3-methoxy-4-hydroxyphenylglycol (MHPG) levels offers a valuable aid for the diagnosis of catecholamine-secreting tumors such as pheochromocytoma, neuroblastoma and ganglioneuroma [1—4].

In addition, the activity of the brain noradrenergic system has been related to a variety of pathological states such as depression, obesity, hypertension and mania [5—7]. A number of studies have determined that 20—50% of the MHPG excreted in urine originates in the central nervous system, while Maas et al. [8] recently estimated the average contribution by the brain to the total body production of MHPG to be 63% [8]. Hence, the measurement of MHPG concentrations in the urine may provide an index of the cerebral norepinephrine (NE) turnover and may prove helpful in the diagnosis of mental disease and in selection of an appropriate therapy.

MHPG is present in urine as a free metabolite (MHPG-free) or, more

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frequently, as the conjugate of sulfuric acid (MHPG-SO_4), which has been reported to be derived from the central NE metabolism [9], and as β-conjugate of glucuronic acid (MHPG-Glu); this latter seems to reflect the metabolism of systemic NE [10].

Various methods have been reported for the determination of this metabolite in urine. At the present time, the most commonly used techniques are gas chromatography with flame ionization detection [11] or electron-capture detection [12, 13], and gas chromatography—mass spectrometry [14]. Unfortunately these procedures require a pretreatment of the sample (e.g. derivatization) and call for equipment too sophisticated and too expensive for routine evaluation.

Reversed-phase liquid chromatography (RPLC) with electrochemical detection (ElCD) has recently been introduced and is an excellent analytical tool for the measurement of MHPG in urine. However, to ensure elimination of interferences, the most reliable procedures call for additional analytical steps, e.g. chemical conversion of the metabolite [15] or thin-layer chromatography for its purification [16].

This report presents a specific RPLC-ElCD method for determining free and conjugated urinary MHPG; it is based exclusively on extraction steps before chromatographic analysis. High precision was achieved by use of 4-methoxy-3-hydroxyphenylglycol (iso-MHPG) as internal standard.

MATERIALS AND METHODS

Apparatus and liquid chromatographic conditions

An LC-50 liquid chromatograph (Bioanalytical Systems, West Lafayette, IN, U.S.A.) equipped with a detector Model TL-3 packed with CP-O carbon paste (Bioanalytical Systems) was made use of for all determinations; the electrode potential was set at +0.80 V versus an Ag/AgCl reference electrode. The chromatographic column was a prepacked μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm average particle size) obtained from Waters Assoc. (Milford, MA, U.S.A.).

The mobile phase, 0.009 *M* citric acid and 0.089 *M* sodium acetate buffered to pH 5.1 containing 10% methanol, was degassed by filtration under vacuum through a Millipore 0.2-μm membrane and delivered at a flow-rate of 1.0 ml/min.

Reagents

All reagents used were of the highest purity (A.C.S. certified grade). Arylsulfatase (type VI from *Aerobacter aerogenes*, 31.2 units/ml), β-glucuronidase/arylsulfatase (glusulase, from *Helix pomatia*, containing 19,500 units/g sulfatase and 420,000 Fishman units/g β-glucuronidase) and MHPG piperazine salt were obtained from Sigma (St. Louis, MO, U.S.A.); iso-MHPG was from Paesel (Frankfurt, G.F.R.)

Solutions of standards were prepared in redistilled deionized water and kept frozen until used.

Procedure

Twenty-four-hour urine samples from laboratory personnel or student volunteers (eight men and seven women) were collected in glass bottles which contained 0.5 mg of sodium metabisulfite per ml of urine and maintained at 4°C. After collection, urine volume was recorded and creatinine was measured colorimetrically using a modification of Jaffe's method [17].

An aliquot of 20 ml was stored at −20°C until the time of assay. For determining MHPG, three samples were prepared as follows. Sample 1 (MHPG-free): 0.5 ml of urine plus 0.2 ml of 1.0 *M* sodium acetate buffer, pH 6.5 plus 0.1 ml of 2% EDTA solution. Sample 2 (MHPG-free + MHPG-SO_4): the same as sample 1 plus 25 μl of arylsulfatase type VI. The pH was adjusted to 7.1. Sample 3 (MHPG total): the same as sample 1 plus 10 mg of glusulase. The pH was adjusted to 5.2. These enzyme amounts have been used because further addition of glusulase or sulfatase did not increase the estimates of MHPG. To each sample 100 μl of a 20 ng/μl iso-MHPG solution were added and samples were incubated at 37°C for 20 h [15].

After cooling and addition of 0.7 ml of 1.0 *M* sodium acetate buffer, the pH was adjusted to 6.5 and each sample, saturated with sodium chloride, was extracted three times with 2 ml of ethyl acetate. The ethyl acetate pool was re-extracted two times with 0.5 ml of 0.1 *M* H_3BO_3. The boric acid pool was washed twice with 2 ml of diethyl ether, its pH adjusted to 8.7, and then extracted twice with 2 ml of ethyl acetate.

The ethyl acetate extracts were evaporated to dryness under reduced pressure using a rotavapor; 20 μl of residue, reconstituted with 0.5 ml of glass-distilled water, were injected into the RPLC system.

To determine the MHPG content of the original samples, a urine pool was made from the samples to be analysed. Different amounts of pure MHPG (125, 250, 500, 1000 and 2000 ng per 100 μl) and 100 μl of a 20 ng/μl iso-MHPG solution were added to each 0.5-ml sample of the pool; these were hydrolysed with arylsulfatase and subjected to the whole procedure. A standard curve was prepared by plotting the MHPG/iso-MHPG peak height ratio versus the MHPG added. A linear regression analysis was performed to determine the best linear graph. The equation for the standard curve was $Y = 0.45X + 0.85$ ($r = 0.994$). The slope of the linear regression line was found to be not significantly different when the standard curve was prepared from samples of urine, whether these were untreated or hydrolysed with glusulase.

The concentration of MHPG in the unknown samples was calculated according to the following equation:

$$\text{MHPG } (\mu\text{g/ml}) = \frac{\text{MHPG peak height}}{\text{Iso-MHPG peak height}} \times \frac{1}{\text{Slope of standard curve}}$$

RESULTS AND DISCUSSION

The major difficulty in the determination of MHPG in urine is its separation from several substances usually extracted by organic solvents at neutral pH; nor has the problem been fully solved by HPLC with ElCD, a technique increa-

singly applied to the evaluation of catecholamines and their metabolites in biological fluids. Despite the high resolving power of HPLC and of the selectivity of the electrochemical detector, MHPG still requires at least partial purification before chromatographic analysis. To achieve this end, in two recently published methods, after extraction into ethyl acetate, MHPG was oxidized to vanillin with periodate and then reduced to vanillyl alcohol [15] or isolated by thin-layer chromatography [16].

We obtained a satisfactory degree of purification by an extraction procedure where final washing with diethyl ether and MHPG reextraction from the borate buffer at pH 8.7 by ethyl acetate were important steps towards obtaining chromatograms with fewer interfering peaks and a straight baseline.

The precision of this method was increased by use of iso-MHPG as internal standard, iso-MHPG being a compound with extraction, chromatographic and detection characteristics similar to those of MHPG.

The presence in man of this compound, a possible metabolite of 4-O-methylation of norepinephrine, has been investigated by Mathieu et al. [18] and Muskiet et al. [19] by means of gas chromatography with electron-capture detection and mass fragmentography.

For normal subjects iso-MHPG was found to be present, though at an extremely low level (mean percentage of iso-MHPG relative to MHPG = 0.77), only by Muskiet et al., whereas Mathieu et al. did not detect it at all. Furthermore, the percentage of iso-MHPG relative to MHPG was in the range determined for normal subjects even in specimens of urine taken from patients with neural crest tumors (neuroblastoma and pheochromocytoma) or from patients treated with dopamine or catecholamine precursors (L-DOPA). Hence iso-MHPG should be regarded as generally not produced, or as produced only in negligible quantities; as a result it has been used as internal standard.

The potential at which the maximum oxidation current is generated was established for iso-MHPG at between + 0.7 and + 1.0 V. Given that the potential of + 0.8 V allows a highly sensitive analysis of MHPG without loss of selectivity, this potential was adopted for the protocol procedure.

Some authors [16] report the possible presence of contaminating peaks in ethyl acetate and small amounts of MHPG in commercially available enzyme preparations. In order to check this a reagent blank was run, and no measurable peaks were detected in the chromatograms.

In Fig. 1 typical chromatograms of a urine sample hydrolyzed with glusulase are shown. A peak whose retention time corresponds to that of MHPG was observed, while no peaks were detected which would correspond to that of iso-MHPG. The identity of this peak was further confirmed by the addition of authentic MHPG, and also on the basis of ratios of responses at several oxidation potentials against the reference standard.

In these preliminary experiments a mobile phase without methanol was used; to shorten the chromatographic analysis time, and in view of the very clear separation of MHPG and iso-MHPG from interfering peaks, in the protocol procedure 10% methanol was added. The retention times of two compounds were then decreased to 7 min and 10 min without loss of resolution (Fig. 2).

The precision of the assay was evaluated by analysing samples of a urine

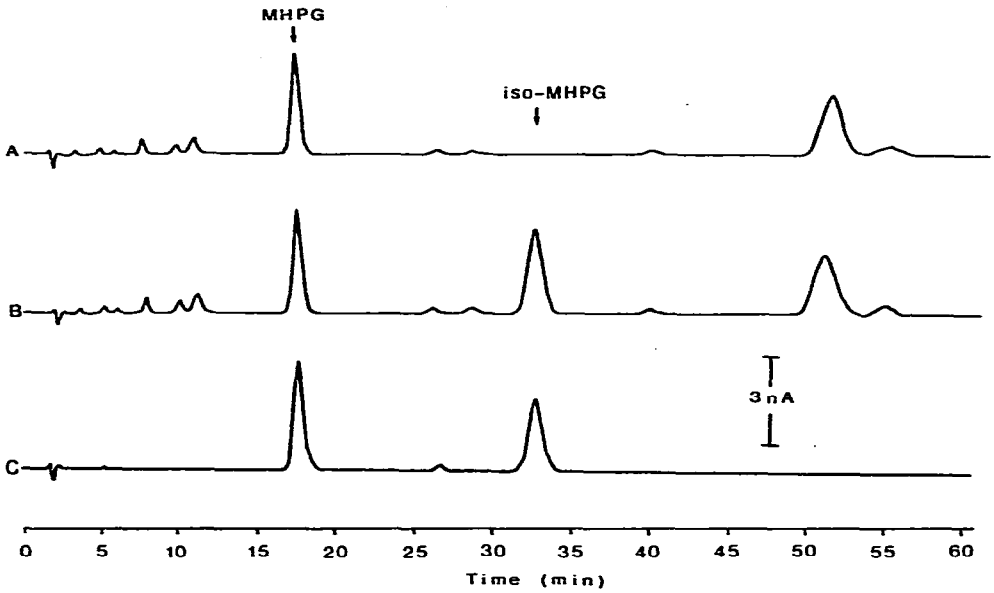

Fig. 1. Chromatogram of human urine. Aliquots of a urine sample were hydrolysed with glusulase and processed as described in the text. (A) Urine. (B) Urine with iso-MHPG added. (C) MHPG and iso-MHPG standards. Chromatographic conditions: C_{18} reversed-phase column; 0.009 *M* citric acid and 0.089 *M* sodium acetate buffer (pH 5.1); flow-rate 1.0 ml/min; electrochemical detector +0.8 V; room temperature.

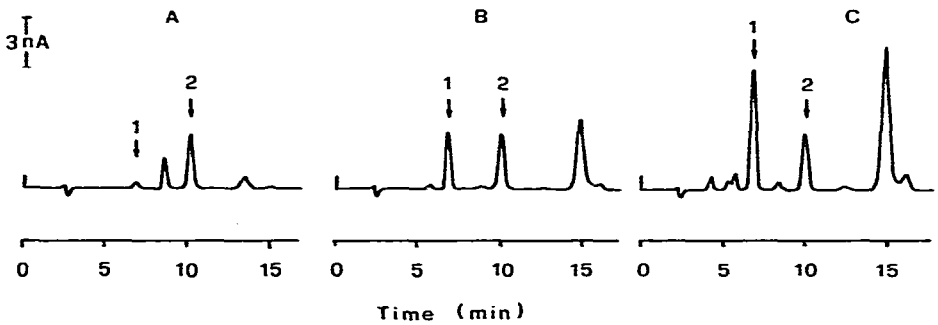

Fig. 2. Typical chromatograms of MHPG extracted from: (A) untreated urine (MHPG-free, 0.19 ng/μl); (B) urine hydrolyzed with arylsulfatase (MHPG-SO_4 + MHPG-free, 1.61 ng/μl); and (C) urine hydrolyzed with glusulase (MHPG-total, 3.46 ng/μl). Chromatographic conditions: as in Fig. 1 with a mobile phase containing 10% (v/v) methanol. Peaks: 1 = MHPG, 2 = iso-MHPG.

specimen either untreated or hydrolysed with glusulase. The within-run precision of quantitative results gave coefficients of variation of 1.7% and 1.9%, respectively; day-to-day precision evaluations yielded coefficients of variation of 2.6% and 3.0% (n=10).

The values of 24-h urinary MHPG-free, MHPG-SO_4, MHPG-Glu and MHPG total excretion are shown in Table I and are consistent with recently published data [15, 16]. Although the excretion of total and conjugated MHPG was lower

TABLE I

24 h URINARY MHPG EXCRETION IN NORMAL HUMAN SUBJECTS

Data are given as mean ± S.D. M = males (n=8), F = females (n=7).

		mg MHPG per 24 h	μg MHPG per mg creatinine
MHPG-free	M	0.112 ± 0.03	0.058 ± 0.02
	F	0.140 ± 0.12*	0.120 ± 0.09*
MHPG-SO_4	M	1.340 ± 0.30	0.700 ± 0.22
	F	0.809 ± 0.31	0.696 ± 0.28
MHPG-Glu**	M	1.470 ± 0.43	0.783 ± 0.34
	F	0.751 ± 0.31	0.625 ± 0.27
MHPG total	M	2.930 ± 0.73	1.540 ± 0.57
	F	1.702 ± 0.50	1.440 ± 0.40

*F significant at 1% level (F vs. M).
**As the difference of the MHPG total − (MHPG-free + MHPG-SO_4).

in women than in men, no significant difference was found when the values were expressed as mg MHPG excreted per g creatinine; free MHPG excretion alone was significantly higher in women. In addition, our results confirm that MHPG-SO_4 is less than, or equal to, MHPG-Glu in 24-h human urine samples [6, 14, 15].

In conclusion, the present method is very reproducible, and selective and sensitive enough for the determination of low concentrations of urinary free MHPG; in addition, it appears to be simpler than the existing HPLC procedures.

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