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DETERMINATION OF URINARY 3-METHOXY-4-HYDROXYPHENYLETHYLENE GLYCOL AND ITS CONJUGATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL AND ULTRAVIOLET ABSORBANCE DETECTION

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SUMMARY

A simple method for the determination in urine of the norepinephrine metabolite 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) and its conjugated derivatives is described. After an extraction procedure similar to that described by Gaertner and Wiatr [J. Clin. Chem. Clin. Biochem., 18 (1980) 579] isocratic high-performance liquid chromatographic separation is performed. MHPG can be detected by either electrochemical or absorbance detection (278 nm). Free MHPG is determined directly, whereas MHPG sulphate and MHPG glucuronide are determined after enzymic hydrolysis. Since total MHPG, which represents the sum of these substances, is determined separately, a comparison of the results allows the effectiveness of the hydrolysis of the MHPG derivatives for each urine sample to be determined. For quantitation, the method of adding standard amounts of MHPG is used.

INTRODUCTION

Determination of the norepinephrine metabolite 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) as a marker for possible disturbances in noradrenergic neurons of the central nervous system (CNS) has been used in

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biological psychiatry for years. However, Kopin and co-workers [2,3] have demonstrated that only 20% of total urinary MHPG is of brain origin. The same holds true for the total MHPG in plasma [4]. Based on these results, determination of total MHPG in peripheral body fluids as a marker for the central norepinephrine metabolism is of limited value.

Free MHPG represents only a small fraction of total urinary MHPG. Most MHPG is excreted conjugated as the sulphate or glucuronide. Some authors have suggested that urinary MHPG glucuronide originates primarily from peripheral metabolism of norepinephrine. In contrast, MHPG sulphate is considered to derive mainly from metabolism of CNS norepinephrine [5,6]. Experimental findings of Peyrin and Pequignot [7] and our previous results [8,9] confirm this theory. Therefore, separate measurements of total MHPG and its conjugated derivatives are of considerable importance. We describe in the present paper a simple and accurate procedure to measure these fractions in urine.

EXPERIMENTAL

Chemicals and enzymes

Ethylenediaminetetraacetic acid disodium salt (EDTA), sodium acetate, sodium disulphite, sodium chloride, potassium hydrogencarbonate, potassium dihydrogenphosphate, phosphoric acid (85%), chloroform, and ethyl acetate, all of analytical-reagent grade, were obtained from Merck (Darmstadt, F.R.G.). Before use, ethyl acetate was distilled over potassium carbonate. Methanol and water, analysed HPLC reagents, were purchased from Baker (Gross-Gerau, F.R.G.). Acetonitrile, HPLC grade S, was from Zinsser (Dreieich, F.R.G.). MHPG hemipiperazine salt was obtained from Sigma (Munich, F.R.G.).

β-Glucuronidase from *Helix pomatia* (EC 3.2.1.31) and β-glucuronidase/arylsulphatase from *Helix pomatia* (EC 3.2.1.31/3.1.6.1) were from Boehringer (Mannheim, F.R.G.). The specific activity of β-glucuronidase was 3 U/mg of protein (25°C; *p*-nitrophenyl-β-D-glucuronide as substrate). The contamination by arylsulphatase was less than 2%. The activity of the β-glucuronidase of β-glucuronidase/arylsulphatase was 5.2 U/ml (38°C; phenolphthalein monoglucuronide as substrate) and the activity of arylsulphatase was 2.6 U/ml (38°C; phenolphthalein disulphate as substrate). Arylsulphatase type VI, EC 3.1.6.1, from *Aerobacter aerogenes*, containing 2–5 U/mg of protein (37°C; *p*-nitrophenylsulphate as substrate) was from Sigma. It had no detectable β-glucuronidase activity at pH 7.0.

MHPG stock solution

A stock solution of MHPG (16 μg/ml) was prepared by dissolving MHPG hemipiperazine salt in a sodium acetate buffer (1 mol/l) and was stored at 4°C. Under these conditions MHPG remained stable for more than six months.

Filters

The all-glass filtration device Pyrex XX1504700 (Millipore, Eschborn, F.R.G.) was used. It was equipped with a Millipore AW0304700 prefilter to filter aqueous phases and with a Millipore FHUP04700 fluoropore membrane for degassing acetonitrile. Samples were clarified using Millex HV4 filters (Millipore) with a nominal pore size of 0.45 μm.

Liquid chromatography (LC) apparatus

A Beckman Model 110 LC pump (Beckman, Frankfurt, F.R.G.) and a Model 210 injector from Beckman with a 20-μl loop were used. The absorbance detector, Model BT 3030 (Biotronik, Maintal, F.R.G.), was operated at 278 nm. The electrochemical detector from Metrohm (Herisau, Switzerland) consisted of the controller unit "VA-Detektor 641" and the detector cell "Electrochemischer Detektor 656" (glassy carbon electrode). The electrode potential was set at +0.80 V versus an Ag/AgCl reference electrode. The sensitivity of the detector was set at 0.1 or 1.0 μA. The chromatographic column was an Ultrasphere ODS column (250 mm×4.6 mm I.D., 5 μm particle size) obtained from Beckman. For solvent switching a motor-driven six-way valve ("TMV-6 Teflon-6-Wege-Drehventil 1,5 D mit Motorantrieb", Latek, Heidelberg, F.R.G.) was used. It was controlled by the process-timer PT810S from Latek. Chromatograms were recorded using the pen recorder BD 41 (Kipp & Zonen, Kronberg/Taunus, F.R.G.).

Sample preparation for analysis of free MHPG, total MHPG, MHPG glucuronide and MHPG sulphate

Three consecutive 24-h urine samples were collected and the volumes recorded. After the addition of sodium disulphite (0.5 g/l), urine samples were filtered through Millipore AW0304700 prefilters. Then they were stored at −25°C until analysis. The extraction procedures for the urine samples are outlined in Table I.

The residue obtained was dissolved in 200 μl of a potassium dihydrogenphosphate buffer (2 mmol/l, pH 2.5) and passed through a Millex HV4 filter. A 20-μl sample of the resulting solution (total volume 150 μl) was used for the chromatographic analysis.

Liquid chromatographic conditions

Isocratic LC conditions were chosen. The solvent was 2 mmol/l potassium dihydrogenphosphate buffer (pH 2.5) containing 10% methanol. Before use it was degassed by filtration under vacuum through a Millipore AW0304700 prefilter. Acetronitrile, which was necessary to wash the late-eluting peaks off the column, was subjected to the same procedure using a Millipore FHUP04700 fluoropore membrane.

The flow-rate was 1.0 ml/min. For LC, 20 μl were placed onto the column

TABLE I

INCUBATION AND EXTRACTION PROCEDURE TO PREPARE URINE SAMPLES FOR HPLC

	MHPG derivative			
	Free	Sulphate	Glucuronide	Total
Preparation				
Urine sample	1000 μl	500 μl	500 μl	500 μl
Add MHPG stock solution[a]	0, 15, 30 and 45 μl respectively			
Add EDTA (20 g/l)	100 μl	100 μl	100 μl	100 μl
Add sodium acetate buffer (1 mol/l, pH 6)	-	150 μl	400 μl	100 μl
Add enzyme	-	250 μl (Sulphatase)	10 μl (Glucuronidase)	300 μl (Glucuronidase/ sulphatase)
Incubation (37°C,18 h)	-	+	+	+
Extraction				
Bring to room temperature	+	+	+	+
Take sample	1100–1145 μl	1000–1045 μl	1010–1055 μl	1000–1045 μl
Add chloroform	4000 μl			
Shake (1 min), centrifuge (4 min, 3000 g), discard organic layer				
Take from aqueous phase	900 μl			
Add sodium chloride	400 mg			
Add potassium dihydrogen-phosphate (2 mmol/l, pH 2.5)	2000 μl			
Add ethyl acetate	4000 μl			
Shake (1 min), centrifuge (4 min, 3000 g), collect organic layer				
Take aqueous phase				
Add ethyl acetate	4000 μl			
Shake (1 min), centrifuge (4 min, 3000 g), collect organic layer, discard aqueous layer				
Take from pooled organic phase	7800 μl			
Add sodium chloride	400 mg			
Add potassium hydrogen-carbonate (1 mol/l, pH 9.2)	1000 μl			
Shake (1 min), centrifuge (4 min, 3000 g)				
Take from organic phase	7200 μl			
Dry under a stream of nitrogen				

[a]Stock solution, 16 μg of MHPG per ml of sodium acetate buffer (1 mol/l, pH 6).

via a loop injector. When a UV detector was used, the buffer was automatically switched to pure acetonitrile by a motor-driven valve 10 min after injection; after further 30 min, the solvent was switched back to the initial methanolic buffer and the next sample could be injected 10 min later. Following this procedure, up to ten samples could be assayed within 8 h.

When the electrochemical detector was used (external flow through pulse dampener, ambient temperature), we assayed eight samples with a separation time of 8 min each before washing the column by switching from the methanol-containing phosphate buffer to pure acetonitrile. After an acetonitrile flush of 1.5 h, the original buffer was used for 2 h until the next eight samples were measured. By this method we assayed sixteen samples within 8 h.

Peak assignment was achieved by comparison of retention time of reference MHPG with that of the corresponding peak in the urine samples.

Quantitation

From the stock solution of MHPG (16 μg/ml), 20 μl (equivalent to 320 ng of MHPG) were injected onto the LC column via the loop. The absorbance maximum of the resulting peak at 278 nm after 6.5 min was determined to be 0.0238. The corresponding current intensity at +0.80 V was 415 nA. Linear standard curves were obtained using the stock solution of MHPG and dilutions thereof. The UV absorbance and the current intensity, respectively, for 1 ng MHPG dissolved in 20 μl of buffer were calculated. The values obtained were used in eqns. 1–4 (see below), symbolized by c.

To evaluate the exact amounts of MHPG in the urine aliquots the following method of adding standard amounts was applied (Table I). Four aliquots of each urine sample were used for the determination of one MHPG derivative. From the stock solution, different amounts (15, 30 and 45 μl, equivalent to 240, 480 and 720 ng of MHPG) were added to three of these aliquots. Then the further procedure as described in Table I was carried out followed by LC. For each MHPG derivative (free, sulphate+free, glucuronide+free and total MHPG) four determinations were made by measuring the peak heights. By means of linear regression analysis four linear curves were obtained (peak height versus the amount of MHPG). The regression lines allowed the quantification of the amounts of MHPG originally present in the samples without additional MHPG and the loss of substance due to the analytical procedure (see below). From the intersections with the abscissa the amount of the MHPG derivative in 1.0 ml of urine was obtained as follows: the value for free MHPG represented the amount directly; the MHPG values of the other three intersections had to be doubled to obtain total MHPG, glucuronide+free and sulphate+free (see Table I).

Recovery

In order to calculate the recovery (%) (R) from the regression line, an equa-

tion was developed from the following considerations.

If the recovery was 100%, the absorbance $A^*_{(de)}$ of the 20 μl injected into the column, resulting from the sum of MHPG originally present in the urine sample and one of the three amounts added before the procedure was started (see Table I), could be calculated as follows:

$$A^*_{(de)} = (d+e)bc/a \qquad (1)$$

where a is the amount of buffer (200 μl) used to dissolve the residue dried under nitrogen, b is the volume (20 μl) injected into the column, c is the absorbance (peak height) resulting from 1 ng of MHPG in 20 μl of buffer [reference value c (ng^{-1}), see above], d is the amount of MHPG (ng) originally present in the urine sample (1 ml and 0.5 ml, respectively, see Table I) and e is the amount of MHPG (ng) added to the urine sample before the start of the procedure (see Table I).

If the recovery is less than 100%, then instead of $A^*_{(de)}$, $A_{(de)}$ is obtained from the regression line. Therefore the recovery (R) is given by:

$$R = 100A_{(de)}a/[(d+e)bc] \qquad (2)$$

In this equation the value of d is unknown. The intercept of the regression line with the ordinate (where $e=0$) gives an absorbance $A_{(d)}$ which has to be proportional to the amount d. By solving eqn. 2 for d, we obtain:

$$d = 100A_{(d)}a/(bcR) \qquad (3)$$

Now, from eqn. 3 and eqn. 2, R can be calculated from

$$R = 100a[A_{(de)} - A_{(d)}]/(ebc) \qquad (4)$$

When the electrochemical detector was used, the corresponding calculations were made by replacing the absorbance values in eqn. 4 by the current values (in nA).

Precision and detection limit

To evaluate the reproducibility of the assays for total MHPG, free MHPG, MHPG sulphate and MHPG glucuronide, duplicate analyses of six urine samples each obtained from a different depressed patient were performed. The standard deviations and the prognostic ranges ($P=95\%$) were calculated by means of eqn. 21a in ref. 10.

The detection limit was defined as the lowest concentration giving a peak height three times higher than the baseline noise.

RESULTS

Fig. 1 shows four typical chromatograms obtained with absorbance detection at 278 nm. They represent the determination of the MHPG derivatives in four

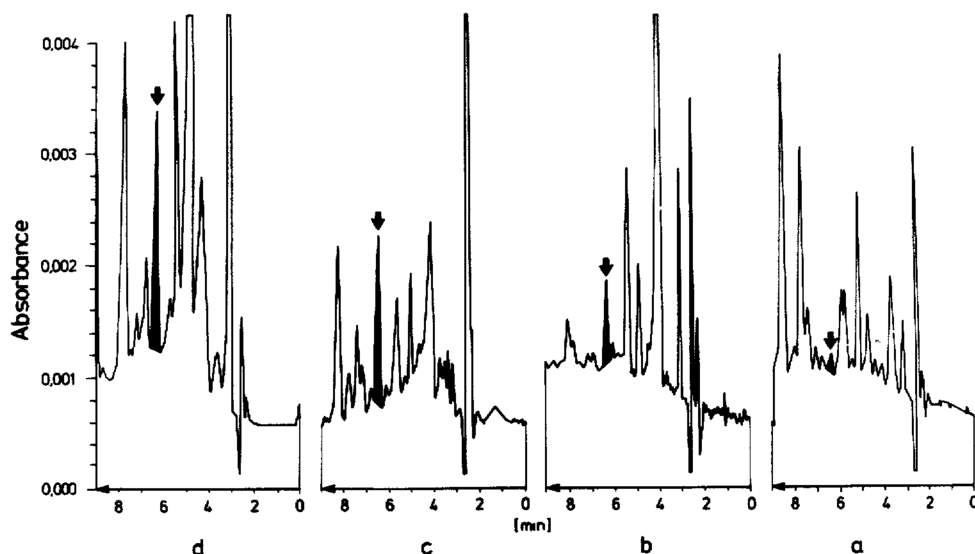

Fig. 1. Chromatograms of MHPG detected with absorbance detection at 278 nm after extraction of urine as described in Table I. For chromatographic conditions see text. Marked peaks represent MHPG. (a) Determination of free MHPG after extraction of 1000 µl. (b) Determination of MHPG after treatment of 500 µl of urine with sulphatase and subsequent extraction. (c) Determination of MHPG after treatment of 500 µl of urine with glucuronidase and subsequent extraction. (d) Determination of MHPG after treatment of 500 µl of urine with glucuronidase/sulphatase and subsequent extraction.

aliquots of one urine sample (no standard added) after extraction, as described in Table I. The sensitivity at a signal-to-noise ratio of 3 was ca. 1.5 ng per 20 µl injected solution. This is equivalent to 40 ng MHPG per ml urine when 1 ml of urine was extracted, as it was for the determination of free MHPG (recovery of free MHPG was 38%, see below). As has been confirmed by analysis of an enzyme blank, no peak arose from the enzymes added to hydrolyse the MHPG derivatives.

When the electrochemical detector was used fewer peaks were detected and, compared with the UV detector, the sensitivity (signal-to-noise ratio of 3) was 4.5 times higher. Fig. 2 shows the chromatograms of four aliquots of a single urine sample. No MHPG was added to the first aliquot, but 240, 480 and 720 ng were added to each of the following aliquots of 1000 µl. Before LC, all aliquots were subjected to the procedure described in Table I.

The recoveries were calculated as described above. They were (means ± relative standard deviations, $n=12$): total MHPG, 52 ± 10%; MHPG sulphate + free MHPG, 50 ± 5.2%; MHPG glucuronide + free MHPG, 52 ± 11%; free MHPG, 38 ± 3.2%. The recoveries were independent of the detector chosen. The calibration curves were linear for both detection methods between 1.6

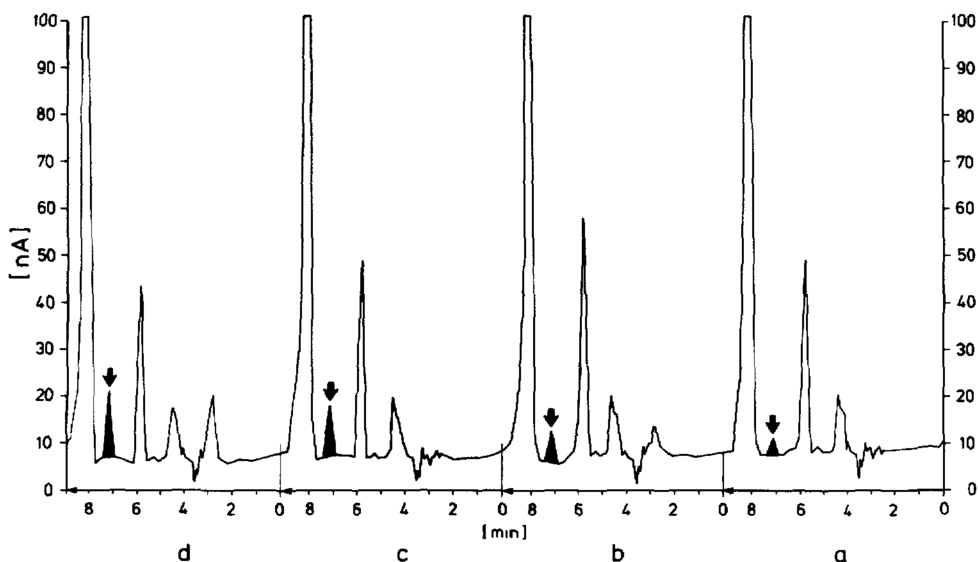

Fig. 2. Chromatograms of free MHPG detected with electrochemical detection at +0.80 V after extraction of 1000 μl of urine as described in Table I. For chromatographic conditions see text. Marked peaks represent MHPG. (a) No MHPG was added to urine. (b) 240 ng of MHPG were added to 1000 μl of urine (see Table I). (c) 480 ng of MHPG were added to 1000 μl of urine (see Table I). (d) 720 ng of MHPG were added to 1000 μl of urine (see Table I).

ng (absorbance detector) and 0.3 ng (electrochemical detector), respectively, and 320 ng per 20 μl injected solution. The reference value c (see eqns. 1–4) was calculated to be 0.0000744 ng^{-1} (absorbance detector) and 1.297 nA/ng (electrochemical detector).

The assay precisions for the MHPG derivatives investigated were determined from duplicate analyses of urine samples of six different depressed patients. For both detection methods, the coefficients of variation (C.V.) and the calculated prognostic ranges ($P=95\%$) for the individual derivatives are listed in Table II. The former were less than 10% when total MHPG, MHPG sulphate + free MHPG and MHPG glucuronide + free MHPG were determined. They were 20% (absorbance detector) and 18% (electrochemical detector) in determinations of free MHPG.

The specificity for MHPG of both detection methods was similar (Fig. 3). When the total MHPG was calculated as the sum of the measured MHPG derivatives it was $102 \pm 10.0\%$ (mean ± S.D.; $n=12$) of total MHPG determined directly by means of the absorbance detector and $101 \pm 9.3\%$ (mean ± S.D.; $n=12$) of total MHPG measured directly using the electrochemical detector.

TABLE II

ASSAY PRECISION

Determined from duplicate analyses of six different human urine samples independently for free MHPG, MHPG sulphate + free MHPG, MHPG glucuronide + free MHPG and total MHPG (directly measured).

MHPG derivative	Absorbance detector		Electrochemical detector	
	C.V. (%)	U^a (%)	C.V. (%)	U^a (%)
Free MHPG	20	49	18	44
MHPG sulphate + free MHPG	8	19	6	15
MHPG glucuronide + free MHPG	10	24	8	20
Total MHPG	4	9.1	3	7.4

[a] U = prognostic range (P = 95%).

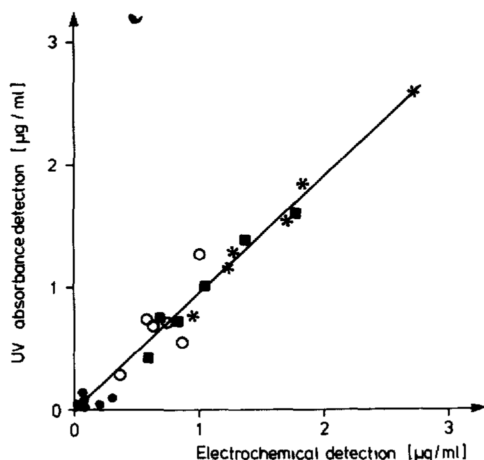

Fig. 3. Comparison of MHPG and its derivatives in six different human urine samples determined by absorbance detection and by electrochemical detection, respectively. Values are means of duplicate analyses. $y = 0.9524x$ ($r = 0.9869$). (●) free MHPG; (○) MHPG sulphate + free MHPG; (■) MHPG glucuronide + free MHPG; (∗) total MHPG (directly measured).

DISCUSSION

Since MHPG was shown [11] to be the major metabolite of norepinephrine in the CNS, a variety of methods has been developed for its quantitative analysis in urine. At first, methods based on gas chromatography (GC), including

mass spectrometric (MS) determinations (e.g. ref. 12) were used (summarized in refs. 13 and 14). However, compared with LC techniques they are time-consuming because a derivatization step is needed to form a volatile derivative. Furthermore, the most widely used technique in GC using electron-capture detection seems to correlate only modestly with a GC–MS reference method [14]. To overcome this problem, LC methods have been increasingly applied [13,15–21], preferentially using electrochemical detection [13,16–21].

Our method offers several advantages over the previously published LC procedures. The incubation procedure to detect the MHPG derivatives is similar to that described in ref. 13, but our extraction procedure is less laborious. It combines the methods of ref. 1 and ref. 18: one extraction using chloroform followed by an extraction at acidic pH and by one at alkaline pH.

We chose isocratic LC conditions using a methanol-containing phosphate buffer similar to ref. 16. After elution of MHPG, the system was automatically switched to 100% acetonitrile in order to wash the column, as in the method described by Moleman and Borstrok [17]. The use of an electrochemical detector was not indispensable, though the chromatograms obtained showed fewer peaks and a straighter baseline. The absorbance detector, which is easier to handle, was operated at 278 nm thus permitting detection even of free MHPG. No difference was observed between the specificities of the two detection methods.

In contrast to others [1,18,21], we did not use 4-methoxy-3-hydroxyphenylethylene glycol (iso-MHPG) as internal standard for two reasons: first, iso-MHPG is not available commercially; secondly, it was found to be present in human urine [22] and in plasma [23], though at very low concentrations. The use of other compounds as internal standard does not seem to be reasonable because of their different extraction behaviour [17]. Furthermore, an internal standard does not guarantee an improvement in the assay precision [17]. Therefore we used the method of standard addition, which allowed the calculation not only of the MHPG derivatives originally present in the urine but also of the recovery. As far as we know, the mathematical method for calculation of the latter has not yet been published.

Except for the free MHPG, the recovery of our method, which allows the use of an absorbance detector, is in the same range as the recoveries determined by others using electrochemical detectors (e.g. ref. 16, 57%; ref. 17, 57%). The recovery of free MHPG is lower (38%). This might be the reason for the high C.V. in the determination of free MHPG. As suggested in ref. 24, the precision was determined from duplicate analyses of different urine samples and not by repeated analyses of only one urine sample. The C.V. is thus higher because it reflects the actual differences in the composition of the samples.

The 24-h urinary MHPG values obtained by our method in seventeen controls (ten males, seven females) are published in ref. 9. They were (means $\pm$ S.D.): free MHPG, 158 ± 86 μg, range 50–346 μg (males) and 116 ± 63

μg, range 30–200 μg (females); MHPG sulphate, 1033 ± 454 μg, range 490–1740 μg (males) and 654 ± 340 μg, range 330–1300 μg (females); MHPG glucuronide, 1746 ± 645 μg, range 660–2770 μg (males) and 1055 ± 559 μg, range 480–2090 μg (females); total MHPG, 2937 ± 980 μg, range 1320–4090 μg (males) and 1855 ± 630 μg, range 920–3570 μg (females).

These results are consistent with data obtained by LC methods [13,18] and with those obtained by GC–electron-capture detection [25]. Free MHPG was 0.16–0.47 mg ($n=2$) [13], 0.112 mg (S.D. = 0.03 mg, eight males) [18] and 0.140 mg (S.D. = 0.12 mg, seven females) [18]. MHPG sulphate was 0.60–1.01 mg ($n=2$) [13], 1.34 mg (S.D. = 0.30 mg, eight males) [18], 0.809 mg (S.D. = 0.31 mg, seven females) [18] and 1.02 mg (S.D. = 0.39 mg, $n=13$) [25]. MHPG glucuronide was 1.42–1.52 mg ($n=2$) [13], 1.47 mg (S.D. = 0.43 mg, eight males) [18], 0.751 mg (S.D. = 0.31 mg, seven females) [18] and 1.49 mg (S.D. = 0.34 mg, $n=13$) [25]. Total MHPG was 2.69–3.60 mg ($n=2$) [13], 2.93 mg (S.D. = 0.73 mg, eight males) [18], 1.70 mg (S.D. = 0.50 mg, seven females) [18] and 2.74 mg (S.D. = 0.55 mg, $n=13$) [25]. These results are comparable with those obtained by others [7,26–28], which indicate that the level of MHPG sulphate is less than or equal to that of MHPG glucuronide in 24-h human urine samples.

In conclusion, we have developed a method that does not require complex and expensive equipment. It is sensitive and precise for routine determination of urinary MHPG and its derivatives.

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