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DETERMINATION OF TOTAL 3-METHOXY-4-HYDROXYPHENYLGLYCOL IN PLASMA USING REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION*

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SUMMARY

A method is described for determination of total (free plus conjugated) 3-methoxy-4-hydroxyphenylglycol (MHPG) in plasma using high-performance liquid chromatography with electrochemical detection. After enzymatic hydrolysis of conjugates and deproteinization with zinc sulfate and sodium hydroxide, free MHPG is isolated by extraction prior to isocratic chromatographic separation. The absolute detection limit is about 30 pg which corresponds to a plasma concentration of 2—3 ng/ml when 0.5 ml of plasma is analyzed. Average within-analysis and between-analysis coefficients of variation (6.5 and 8.9%, respectively) are suitable for routine clinical analyses. Severe icterus is without effect, but even mild hemolysis causes detectable losses of MHPG from plasma. The mean ± S.D. plasma MHPG concentration observed in sixteen healthy individuals (14.5 ± 3.9 ng/ml) compares favorably with values reported using more complex reference methods.

INTRODUCTION

3-Methoxy-4-hydroxyphenylglycol (MHPG) is the principal metabolite of norepinephrine in human brain [1]. Studies in animals and man suggest that

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the concentration of MHPG in plasma can be used as an index of central nervous system noradrenergic activity [1]. There is evidence that certain psychopathological states and hypertension may be associated with abnormalities of central noradrenergic neuronal function [2, 3]. A method for measuring MHPG in plasma should therefore have considerable clinical utility. Because of its low concentration, MHPG has most often been measured in plasma using gas chromatography (GC) with either electron-capture detection (ECD) or mass spectrometric (MS) detection [4—8]. Due to their technical complexity and the high cost of the required equipment, these methods are not usually available to the average clinical laboratory. Recently, several methods using high-performance liquid chromatography (LC) with electrochemical detection (ED) to measure MHPG in plasma have been described [9—15]. With one exception [9], these methods have measured only free MHPG. Because most of the MHPG in plasma is conjugated [4, 7, 8], we desired a procedure for measuring total (free plus conjugated) MHPG. Described here is a relatively simple and inexpensive method for measuring total MHPG in plasma using LC—ED. The method has proved to be reliable in routine use in our laboratory during the past eighteen months.

EXPERIMENTAL

Apparatus

The LC—ED system used consisted of a Model M-45 pump, a Model U6K injector and a C_{18} Guard-Pak precolumn from Waters Assoc. (Milford, MA, U.S.A.) and a Model LC-4B electrochemical detector from Bioanalytical Systems (West Lafayette, IN, U.S.A.). The reversed-phase analytical columns (30 cm × 3.9 mm, 10 μm particle size) were either prepacked with μBondapak C_{18} (Waters Assoc.) or repacked with ASI C_{18} (Analytical Sciences, Santa Clara, CA, U.S.A.). The TL-5 glassy carbon electrode was set at a potential of +0.75 V versus a Ag/AgCl reference electrode. The mobile phase was 0.05 *M* disodium hydrogen phosphate containing 1.34 m*M* Na_2EDTA, which had been adjusted to pH 3.0 with perchloric acid (a strong acid which does not corrode stainless-steel pump parts), filtered, mixed with methanol (980 ml of buffer per 20 ml of methanol) and then degassed. Elution was isocratic at a flow-rate of 1.0 ml/min.

Chemicals

Ethyl acetate was distilled in glass from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Florisil (60—100 mesh), HPLC-grade hexane and HPLC-grade methanol were from Fisher Scientific (Springfield, NJ, U.S.A.). Sulfatase Type H-2 (a mixture of about 10 000 U/ml of arylsulfatase and 100 000 U/ml of β-glucuronidase) and bovine albumin (Fraction V powder) were from Sigma (St. Louis, MO, U.S.A.). MHPG, piperazine salt was from Regis (Morton Grove, IL, U.S.A.). MHPG sulfate, potassium salt was from Research Biochemicals (Wayland, MA, U.S.A.). All other chemicals were ACS reagent grade. Water was deionized and then distilled in a glass Mega-Pure still from Corning Glass Works (Corning, NY, U.S.A.).

Special reagents and standards

The following reagents were used: 0.5 *M* acetate buffer, pH 5.0, containing 5.4 m*M* Na_2EDTA; 0.5 *M* borate buffer, pH 10.5; 0.5 *M* sodium hydroxide, balanced with the zinc sulfate solution so that 10 ml of the zinc sulfate solution diluted to 50—70 ml with water required 10.8—11.2 ml of the sodium hydroxide solution to obtain a pink color with phenolphthalein; 0.348 *M* zinc sulfate; 0.05 *M* phsophate buffer, pH 7.0, containing 15 g/l bovine albumin.

The MHPG stock standard solution (1 mg/ml MHPG free base) was prepared by dissolving 12.3 mg of MHPG piperazine salt in 10 ml of 0.01 *M* hydrochloric acid. Stored at 4°C, this solution is stable for at least six months. On the day of analysis, 0.01 ml of this stock was diluted to 10 ml in 0.01 *M* hydrochloric acid to give stock solution B (1 mg/l). Then, 0.2 ml of stock solution B was diluted to 1.0 ml in 0.01 *M* hydrochloric acid to give stock solution C (0.2 mg/l). For the standards carried through the assay, 0.02 ml (20 ng) of stock solution B was used. For the external standard, 0.02 ml of stock solution C was mixed with 0.18 ml of mobile phase, and 100 μl (2 ng) was injected onto the HPLC column.

The MHPG sulfate stock standard solution (1 mg/ml MHPG free base) was prepared by dissolving 8.2 mg of the potassium salt in 5 ml of 0.01 *M* hydrochloric acid. Stored at 4°C, this solution is stable for at least twelve months. On the day of analysis, 0.01 ml of this stock solution was diluted to 10 ml in 0.01 *M* hydrochloric acid, and 0.02 ml (20 ng of MHPG free base) was used in the analysis.

Plasma specimens

Plasma samples were obtained from sixteen healthy volunteers (eight men and eight women) ranging in age from 21 to 52 years. Plasma remaining after analysis of a large number of specimens from normal subjects and depressed patients was mixed to obtain pooled plasma for our validation studies. Venous blood was collected in heparinized or EDTA tubes, centrifuged at 4°C and the separated plasma was stored frozen at —30°C until analyzed.

Procedure

In 100 × 17 mm polypropylene tubes, were mixed 0.06 ml of ascorbic acid solution (28.4 m*M*), 0.02 ml of standard (20 ng) or vehicle, 1.0 ml of phosphate buffer or a mixture of 0.5 ml of buffer with 0.5 ml of plasma, 1.0 ml of acetate buffer and 0.1 ml of sulfatase solution. The tubes were capped and placed in a 37°C water bath overnight (16—18 h). The next morning, the tubes were removed and chilled on ice. Then 1.0 ml of the zinc sulfate solution was added, followed by 1.0 ml of the sodium hydroxide solution, the tubes were mixed vigorously and centrifuged for 10 min at 5900 *g* and 4°C. An aliquot (3.0 ml) of clear supernatant was transferred to a 50-ml plug-seal screw-cap polypropylene tube (Corning 25330) containing 1 g of dry sodium chloride and 0.2 g of florisil, and 10 ml of ethyl acetate was added. The tubes were capped and shaken mechanically for 15 min, then centrifuged for 10 min at 1086 *g*. An aliquot (8.0 ml) of the clear organic layer was transferred to a fresh 50-ml polypropylene tube containing 8.0 ml of hexane and 4.0 ml of 0.1 *M* boric acid solution. The tubes were capped and shaken mechanically for

20 min, then centrifuged. The top organic layer was aspirated off and discarded. The pH of the lower aqueous layer was adjusted to 8.7 with pH 10.5 borate buffer (about ten to twelve drops), 8 ml of ethyl acetate was added and the tubes were vortexed for 30 s, centrifuged to separate the layers, and then the upper organic layer was transferred to a 100-ml round-bottom flask. The extractions were repeated with a second 8-ml aliquot of ethyl acetate which was combined with the first extract. The combined ethyl acetate extracts were evaporated to dryness on a rotating evaporator at reduced pressure without heat. The residue was transferred to an acid-washed glass tube (100 × 13 mm) using three 0.5-ml portions of methanol. The methanol extracts were evaporated to dryness under a stream of nitrogen. The residue was dissolved in 200 μl of mobile phase, and 100 μl was injected onto the HPLC column. A reagent blank and two or three standards were analyzed along with each batch of plasma samples. Quantification was based on comparison of peak heights with the mean height of three external standards injected at the start, middle and end of each day's analysis. Plasma concentrations are corrected for the recovery of the simultaneously analyzed standards.

RESULTS

Chromatographic examples

Typical chromatograms are shown in Fig. 1. The MHPG peak appeared to be well separated from other electroactive constituents of plasma. Because small but detectable amounts of MHPG were present in some lots of sulfatase (Fig. 1A), we always ran a blank with each batch of plasma samples. If there were co-eluting peaks in this blank, we subtracted them from the plasma peaks. We did not purify the enzyme preparations we used because others [8, 9] have demonstrated that purification of crude sulfatase causes a significant loss of hydrolysis efficacy. Although elution was usually complete within 25—30 min, occasional plasma specimens contained slow eluting peaks which appeared at about 45 min. We tried using a simple step gradient, increasing

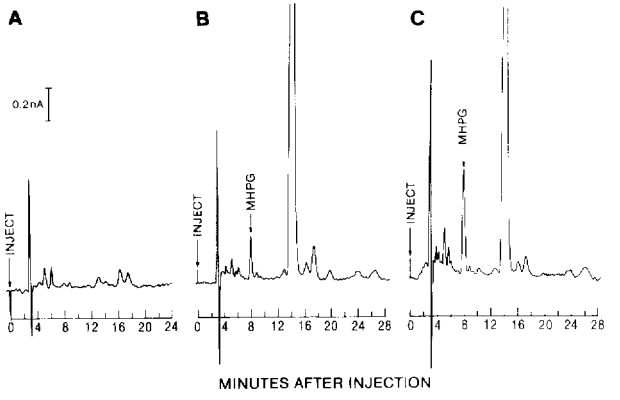

Fig. 1. Typical chromatograms of (A) a buffer blank, (B) a 0.5-ml sample of pooled human plasma and (C) a 0.5-ml sample of pooled plasma to which 20 ng of MHPG sulfate had been added before analysis. The absolute detection limit is about 30 pg which corresponds to a plasma concentration of 2—3 ng/ml when 0.5 ml of plasma is analyzed.

the methanol concentration briefly, to speed elution of these peaks but the additional time required to reequilibrate the system made this approach impractical. The extract volume was large enough so that it was possible to make a second injection in those rare instances where late eluting peaks from a preceding sample interfered with an analysis. We chose not to use an internal standard because the substances we tried either lacked the stability characteristics of MHPG, thus giving us a false sense of security, or they co-eluted with other plasma constituents and thus gave inaccurate values.

Linearity and sensitivity

We verified the linearity of the assay by adding known amounts of MHPG sulfate to aliquots of a pooled plasma specimen and carrying these samples through the assay. The curve was linear over the range tested ($y = 0.0366x + 0.291$; $r = 0.999$). The detection limit, defined as the amount of MHPG which gave a detector response equal to twice the background noise, was about 30 pg. This corresponded to a plasma concentration of 2—3 ng/ml when 0.5 ml of plasma was analyzed.

Precision

We evaluated with-analysis precision by analyzing sequentially eight replicates of a pooled plasma sample and nineteen duplicate plasma samples over a period of one month. To assess between-analysis precision, we analyzed one pooled and three individual plasma samples separately over a period of one month. We used two lots of sulfatase and several lots of ethyl acetate during these studies. Within-analysis and between-analysis coefficients of variation averaged 6.5 and 8.9%, respectively (Table I).

TABLE I

ASSAY PRECISION

Sample	*n*	Mean MHPG concentration (ng/ml)	Coefficient of variation (%)
Within-analysis			
Replicates	8	11.9	5.0
Paired samples	19	15.0	8.0
Between-analysis			
Pooled plasma	8	14.0	9.3
Sample 1	4	17.8	4.5
Sample 2	4	17.0	15.9
Sample 3	4	15.4	5.8

Analytic recovery

The mean recovery of 20 ng of free MHPG carried through the entire assay was 25.6 ± 3.9% (n=76); that of free MHPG from an equimolar amount of MHPG sulfate was 26.7 ± 4.6% (n=76). The difference was not significant, indicating that hydrolysis was complete. There was no detectable difference between recovery of MHPG from plasma (28.4 ± 4.6%; n=4) or from buffer (28.9 ± 3.0%; n=4). These recoveries were calculated from data obtained during

our routine plasma analyses over a period of nine months. The greater variance in the recovery data than in the precision data is due to use of occasional lots of ethyl acetate which contained impurities that destroyed some of the MHPG during the evaporation step. We found that we could remove these impurities by redistilling the ethyl acetate. Analytical reagent grade ethyl acetate always had to be purified. Since most lots of glass-distilled ethyl acetate from Burdick & Jackson Labs. were satisfactory, we chose to use it and to purify the ethyl acetate only when the recovery data indicated that impurities were causing problems.

Interference studies

Relative retention times of monoamines and related substances which might be present in plasma samples are shown in Table II. Only epinine, carbidopa and metanephrine eluted near MHPG. These compounds were removed during the preliminary extractions and did not interfere. To simulate severe icterus, we added bilirubin to plasma to produce a final concentration of 0.2 g/l. No interference was detected (Table III). To simulate mild and severe hemolysis, we added 10 or 30 μl of lysed washed red blood cells (RBCs) to plasma. Mild hemolysis caused a 27% decrease and severe hemolysis a 41% decrease in the measured MHPG concentration, indicating that hemolyzed plasma is unsuitable for MHPG analysis (Table III).

TABLE II

RELATIVE RETENTION TIMES OF MONOAMINES AND RELATED SUBSTANCES TESTED FOR INTERFERENCE IN THE ASSAY

Relative retention time (RRT) = retention time compound/retention time MHPG.

Compound	RRT	Compound	RRT
Norepinephrine (NE)	0.28	3-Methoxy-4-hydroxyphenylglycol (MHPG)	1.00
3,4-Dihydroxymandelic acid (DOMA)	0.30	Carbidopa	1.02
Epinephrine (E)	0.40	Metanephrine (M)	1.08
3,4-Dihydroxyphenylglycol (DHPG)	0.40	3-Methoxytyrosine	1.14
3,4-Dihydroxyphenylalanine (DOPA)	0.42	5-Hydroxytryptophan (5-HTP)	1.44
3,4-Dihydroxybenzylamine (DHBA)	0.42	3,4-Dihydroxyphenylethanol (DHPE)	1.52
Uric acid	0.48	3,4-Dihydroxyphenylacetic acid (DOPAC)	1.97
Normetanephrine (NM)	0.51	5-Hydroxytryptamine (serotonin, 5-HT)	2.15
Vanilmandelic acid (VMA)	0.57	3-Methoxytyramine (3-MT)	2.36
Dopamine (DA)	0.64	5-Hydroxyindole-3-acetic acid (5-HIAA)	5.10
α-Methyldopa (ALDOMET)	0.79	3-Methoxy-4-hydroxyphenylethanol (MHPE)	5.82
Epinine	0.94	Homovanillic acid (HVA)	7.51

TABLE III

EFFECT OF BILIRUBIN AND HEMOGLOBIN ON MHPG IN PLASMA

Sample	MHPG concentration (ng/ml)		
	1	2	Mean
Plasma alone	13.4	12.2	12.8
Plasma + bilirubin (0.2 g/l)	13.9	11.9	12.9
Plasma + 10 μl lysed RBCs*	9.2	9.6	9.4
Plasma + 30 μl lysed RBCs*	7.7	7.4	7.6

*Packed red blood cells (RBCs) were washed with saline three times, diluted with an equal volume of water, refrigerated overnight, and then frozen and thawed once. Aliquots of the supernatant obtained after centrifugation were added to plasma before hydrolysis.

MHPG in plasma from normal subjects

The mean concentration of MHPG in plasma from sixteen healthy subjects was 14.5 ± 3.9 ng/ml (range: 9.0—22.5 ng/ml). Men had a slightly higher mean concentration (16.3 ± 3.9 ng/ml) than women (12.6 ± 3.2 ng/ml), but the difference did not quite reach statistical significance ($0.05 < P < 0.1$). Our data compare favorably with values reported by others using more complex reference methods (Table IV).

TABLE IV

TOTAL MHPG CONCENTRATIONS IN PLASMA OF HEALTHY ADULTS OBTAINED BY VARIOUS METHODS

The values between parentheses represent the number of subjects.

Method	Reference	MHPG concentration (mean ± S.D.) (ng/ml)		
		Male	Female	Unspecified or both sexes
LC—ED	This method	16.3 ± 3.9 (8)	12.6 ± 3.2 (8)	14.5 ± 3.9 (16)
LC—ED	Karege [9]	18.8 ± 4.5 (—)	15.6 ± 3.1 (—)	—
GC—ECD	Dekirmenjian and Maas [4]	—	—	15.7 ± 2.0 (5)
GC—ECD	Markianos and Beckmann [16]	10.9 ± 3.4 (11)	—	—
GC—ECD	Halaris et al. [6]	7.6 ± 4.9 (4)	8.4 ± 1.9 (2)	7.9 ± 3.9 (6)
GC—MS	Sjöquist et al. [5]	—	—	10.3 ± 0.6 (18)
GC—MS	Takahashi et al. [7]	16.5 ± 4.4 (10)	—	—
GC—MS	Karoum et al. [17]	—	—	22.0 ± 5.5 (10)
GC—MS	Jimerson et al. [18]	—	—	10.7 ± 2.5 (25)
GC—MS	Hjemdahl et al. [19]	—	—	18.0 ± 2.0 (13)

DISCUSSION

Due to its low concentration and neutral, hydrophilic character, MHPG is a very difficult compound to measure in a complex biological fluid such as plasma. Some selectivity can be achieved by using a reversed-phase LC separation coupled with ED. MHPG, however, requires a rather high potential (+0.75 to 0.80 V) for optimal detector response. At this potential many other constituents of plasma are also electroactive and they appear in the chromatograms. The retention of MHPG is not easily manipulated. MHPG is poorly retained on a reversed-phase column and, because it is not ionized, its retention is insensitive to changes in mobile phase pH or to the presence of ion-pairing agents. Thus, extensive pre-purification is required to ensure adequate separation of MHPG from potentially interfering components of plasma during the chromatographic analysis. In the present method, we have adapted strategies from three previously described LC—ED procedures for determination of MHPG in urine [20—22] to permit assay of MHPG in plasma. It was necessary to insert a preliminary step to remove plasma proteins and to increase the sensitivity by concentrating the final extract into a small volume so that a greater proportion of the initial sample could be injected onto the column.

We chose a mixture of zinc sulfate and sodium hydroxide for protein precipitation and removed the precipitated protein by centrifugation. This technique has the advantage of producing a clear protein-free centrifugate with a pH of about 7.0. Although extraction of MHPG into ethyl acetate is somewhat more

efficient at acid than at neutral pH [23], it is less selective. At pH 7.0, weakly acidic components of plasma are ionized and therefore poorly extracted into organic solvents. Also, some potentially interfering electroactive constituents of plasma, notably uric acid, are removed with the protein. Others [10] have used gel filtration for removal of proteins. We tested both Sephadex G-10 and Bio-Gel P-10 columns. In our hands, gel filtration was not only time-consuming but also it increased the sample volume three- to five-fold without effecting a clean separation of MHPG from plasma proteins.

In our initial work, we extracted deproteinized plasma with ethyl acetate (3 × 6 ml). The combined organic layers were evaporated to dryness, the residue was reconstituted in 0.2 ml of mobile phase and 0.1 ml was injected onto the column. This technique was simpler than the method we describe here and the mean recovery of MHPG sulfate from plasma (61.9 ± 7.6%; n=18) was better, but there were many interfering peaks in the chromatograms. We used a step gradient, increasing the methanol content of the mobile phase to 50% for 10 min, to remove highly retained compounds from the column, but each analysis required 36 min and retention times tended to vary. We therefore decided to add some additional steps to our preliminary purification procedure. Washing the ethyl acetate extracts with sodium bicarbonate solution decreased recovery of MHPG and did not remove any late eluting peaks from our chromatograms. Recovery of MHPG from ion-exchange columns (Dowex 1×4 or a mixed-bed anion/cation-exchange column [11]) was good but, in our experience, the columns failed to remove interfering peaks. Back-extraction into acetic acid, as described by Moleman and Borstrok [20], gave a good recovery of MHPG (60%) but the 2-ml acid extract volume diluted the MHPG too much to permit assay of the small amounts of MHPG present in plasma. We therefore extracted MHPG back into ethyl acetate and reduced the volume by evaporating the ethyl acetate. These modifications decreased the recovery of MHPG but they produced chromatograms free of interfering peaks. The absence of late peaks allows injection every 25—30 min under isocratic conditions. An added bonus is that the column lifetime is prolonged and the electrode rarely has to be resurfaced.

In conclusion, the method we described is reproducible and specific for MHPG. Although we were interested in measuring total MHPG in plasma, it is possible to use our method to measure free MHPG by simply omitting the enzymatic hydrolysis and increasing the plasma volume to 1.0 ml. The method is simple enough to be used routinely. We have measured total MHPG concentrations in more than 1000 plasma samples without encountering problems. The method should be of particular value to clinical and research laboratories which lack expensive GC—MS equipment.

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