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Note

Assay of 3-methoxy-4-hydroxyphenylglycol in human plasma using high-performance liquid chromatography with amperometric detection

SERGIO SCHINELLI*, GIUSEPPE SANTAGOSTINO, PIETRO FRATTINI, MARIA LAURA CUCCHI and GIAN LUIGI CORONA

Institute of Pharmacology, University of Pavia, Via Taramelli 14, 27100 Pavia (Italy)

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The measurement in plasma of 3-methoxy-4-hydroxyphenylglycol (MHPG), a major metabolite of norepinephrine, may provide a useful tool for assessing central and peripheral noradrenergic activity in humans. Since the half-life of the metabolite is 45—75 min, its plasma levels reflect the average norepinephrine metabolism over an interval of several hours, providing a more integrated measure of presynaptic neuron activity than does the parent amine [1]. MHPG levels in plasma have thus been examined in various disorders in which the noradrenergic system appears to be involved, such as anxiety, hyperactivity and some neuropsychiatric diseases [2—4].

The only method with a sufficient degree of sensitivity and specificity employed for determination of MHPG in plasma has been gas chromatography—mass spectrometry (GC—MS) [5—7]. Unfortunately, not many clinical and research laboratories have the financial and technical means required for this technique.

Recently, reversed-phase high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ED) gave good results when applied to urinary MHPG measurement [8—11]; in plasma the determination of MHPG is more difficult because of the low levels of the metabolite in this fluid.

In this note we describe a method for the determination of unconjugated MHPG in human plasma based on protein precipitation, solvent extractions and isocratic HPLC with ED. High precision was achieved using 3-hydroxy-4-methoxyphenylglycol (iso-MHPG) as internal standard.

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EXPERIMENTAL

Apparatus and liquid chromatographic conditions

A Knauer HPLC pump, Model 64 (Knauer, Berlin, F.R.G.) and a Model 7125 injector from Rheodyne (Cotati, CA, U.S.A.) were used. The electrochemical detector from Bioanalytical Systems (West Lafayette, IN, U.S.A.) consisted of an LC 4B controller and a TL-3 cell packed with CP-0 carbon paste; the electrode potential was set at +0.75 V versus an Ag/AgCl reference electrode. The chromatographic column was a prepacked Ultrasphere ODS column (250 × 4.6 mm, 5 μm particle size) with a self-packed ODS precolumn (Altex, Berkeley, CA, U.S.A.).

The mobile phase was a mixture of 0.09 mol/l sodium acetate with 0.009 mol/l citric acid buffered to pH 5.0 and containing 10% methanol, degassed under vacuum by filtration through a Millipore 0.2-μm membrane and delivered at a flow-rate of 1.0 ml/min.

Chemicals and reagents

All reagents used were of the highest purity (A.C.S. certified grade). MHPG hemipiperazine salt was obtained from Sigma (St. Louis, MO, U.S.A.); iso-MHPG was from Paesel (Frankfurt, F.R.G.).

Solutions of standards were prepared in distilled deionized water and kept frozen until use.

Procedure

Plasma samples (2 ml), obtained from heparinized blood, were transferred into polycarbonate tubes each containing 100 μl of iso-MHPG in water (0.2 ng/μl). After brief mixing and incubation on ice (10 min), 200 μl of cold 4 mol/l perchloric acid were added to each tube. The samples were vigorously mixed and centrifuged at 30 000 *g* for 10 min at 4°C. The supernatants were removed and adjusted to pH 6.5 with cold 5 mol/l potassium hydroxide in 0.5 mol/l phosphate buffer and then, after saturation with sodium chloride, extracted twice with 3 ml of ethyl acetate for 1 min in a vortex mixer. In order to facilitate the separation between the phases, the test tubes were placed in glycol antifreeze solution at −25°C for 3 min and the separated ethyl acetate pools were re-extracted twice with 1 ml of 0.1 mol/l phosphate buffer, pH 5.3. Each aqueous phase was washed with 4 ml of diethyl ether, its pH adjusted to 9.0 with 5 mol/l sodium hydroxide and then extracted twice with 2 ml of ethyl acetate. The ethyl acetate extracts were evaporated to dryness under reduced pressure using a rotavapor; each residue was reconstituted with 250 μl of mobile phase and 100 μl were injected into the HPLC column.

Quantitative analysis

Quantitative analysis was performed using the internal standard addition method. A plasma pool was made from ten different subjects and increasing amounts of pure MHPG (2, 4, 8, 16 ng) were added to each 2 ml sample of the pool; these, after addition of internal standard (100 μl of a 0.2 ng/μl iso-MHPG solution), were subjected to the whole procedure.

After chromatography, ratios of responses of MHPG to those of iso-MHPG

were calculated for each sample and were plotted versus the amount of metabolite added. Linear regression analysis was performed to determine the best linear graph. The equation for the standard curve was $y = 0.146x + 0.492$ ($r = 0.999$). The concentrations of MHPG in the unknown samples were calculated according to the equation

$$\text{Concentration of MHPG (ng/ml)} = \frac{\text{MHPG peak height}}{\text{iso-MHPG peak height}} \times \frac{1}{\text{slope of standard curve}}$$

RESULTS AND DISCUSSION

Despite the high resolving power of HPLC and the selectivity of the electrochemical detector, the determination of MHPG in plasma requires at least partial purification before chromatographic analysis.

Recently, Scheinin et al. [12] adopted a preliminary extraction of the compound in ethyl acetate. However, this step alone was not sufficient to obtain a good separation of both MHPG and EHPG (3-ethoxy-4-hydroxyphenylglycol, internal standard) from interfering substances, unless rigorously optimized chromatographic conditions (mainly pH and temperature) were adopted. Injections of methanol at the end of the chromatographic analysis were also necessary to reduce the analysis time.

In the present procedure we introduced a purification step based on re-

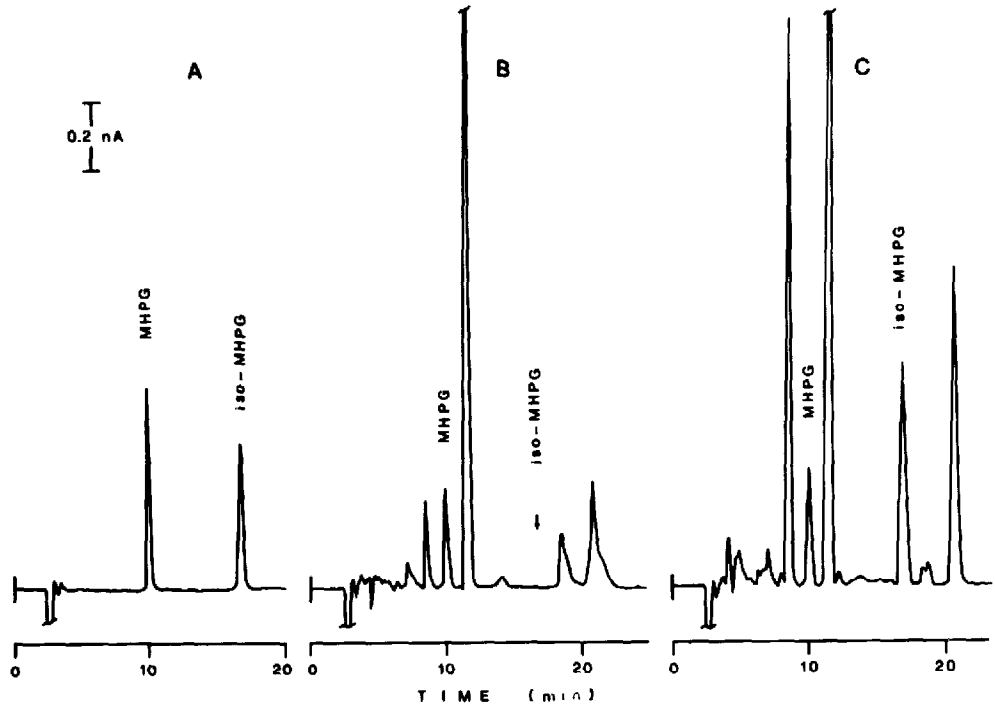

Fig. 1. Typical chromatograms of human plasma from healthy subjects: (A) MHPG and iso-MHPG standards; (B) pooled plasma sample; (C) plasma sample (3.66 ± 0.055 ng/ml, mean ± S.D. of five observations) with iso-MHPG added. For chromatographic conditions, see text.

extraction of the metabolite with phosphate buffer and subsequent washing with diethyl ether to eliminate any trace of ethyl acetate from the aqueous phase. Of considerable importance also is the final extraction into ethyl acetate at pH 9.0. The result was that, despite a lower recovery of MHPG, we obtained chromatograms free of interfering peaks and with a straight baseline, with the possibility of using higher sensitivity levels.

The usefulness of iso-MHPG as internal standard for the determination of MHPG and its possible presence in man, had been previously discussed [10]. Even if in some chromatograms a very small peak was detected with a retention time corresponding to that of iso-MHPG, nevertheless the height of this peak was negligible compared with the height of the internal standard (always less than 2%), so that correction was unnecessary.

Although an increase in the oxidation potential above 0.75 V results in an increase in the electrochemical response of compounds, this potential was adopted in our procedure because it allows a highly sensitive analysis of MHPG and iso-MHPG without loss of selectivity.

Typical chromatograms of a mixture of MHPG and iso-MHPG and endogenous free MHPG in plasma without and with added iso-MHPG are shown in Fig. 1.

The absence of late peaks in this assay allows injection of samples every 25 min with isocratic elution. The chromatographic peak was identified as MHPG since the retention time and voltage curve were similar to those of authentic MHPG.

The recovery of MHPG added to plasma was 35.1 ± 1.7% (mean ± S.D. of five observations). The sensitivity of the assay is suitable for clinical studies, with a detection limit of 0.18 ng/ml, based on a signal-to-noise ratio of 2. The precision of the assay was evaluated by analysing samples of a plasma pool. The intra-assay coefficient of variation was 4.1% while the inter-assay coefficient of variation was 5.6% (n = 10).

The concentrations of free MHPG in plasma samples of fifteen apparently healthy subjects (volunteers or laboratory personnel, aged between 20 and 65 years) are reported in Table I: these results agree well with recently published data obtained by GC—MS and by HPLC—ED [5, 6, 12].

TABLE I

FREE MHPG CONCENTRATIONS IN PLASMA OF HEALTHY SUBJECTS

Values are given in ng/ml.

n	Males	n	Females
1	3.66	9	3.53
2	2.66	10	3.55
3	3.67	11	3.83
4	2.83	12	3.70
5	4.14	13	3.85
6	4.71	14	2.51
7	3.11	15	3.01
8	4.08		
Mean	3.60		3.42

In conclusion, this method provides a sensitive and precise means for routine determination of the metabolite 3-methoxy-4-hydroxyphenylglycol in laboratories where GC—MS is not available.

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