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Improved high-performance liquid chromatographic method for the routine determination of unconjugated 3-methoxy-4-hydroxyphenylethyleneglycol in human plasma using solid-phase extraction and electrochemical detection

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ABSTRACT

An improved semi-automated high-performance liquid chromatographic method is described for the routine determination of unconjugated 3-methoxy-4-hydroxyphenylethyleneglycol in plasma. The 3-ethoxy analogue of the compound is used as an internal standard. The method is based on purification of 0.5-ml plasma samples with phenyl-type reversed-phase extraction columns, reversed-phase separation with an acetate–citrate–methanol mobile phase with an octadecyl-bonded column, and dual-electrode coulometric detection with oxidation at +0.44 V and reduction at −0.25 V. The precision and accuracy of the assay are satisfactory: the lower limit of reliable detection corresponds to a plasma concentration of 1.5 n*M*. The validity of the determination is demonstrated by an 18% mean increase in plasma levels of 3-methoxy-4-hydroxyphenylethyleneglycol during physical exercise (duration 16 min, $n = 13$) and a 50% mean reduction in plasma levels induced by a single dose of the monoamine oxidase inhibitor, moclobemide ($n = 8$). The method is suitable for routine use in pharmacological and physiological experiments.

INTRODUCTION

3-Methoxy-4-hydroxyphenylethyleneglycol (MHPG) is a major metabolite of the neurotransmitter norepinephrine (NE); its formation from NE is dependent on three enzymes, monoamine oxidase, catechol-O-methyltransferase and an aldehyde reductase [1,2]. Measurements of MHPG concentrations in blood plasma are widely used to monitor NE turnover and metabolism in human subjects. Such information is important in many pharmacological and physiological studies, as well as in clinical studies of various illnesses suspected of being related to disorders of noradrenergic neurotransmission in the central nervous system and/or the peripheral sympathetic nerves [2].

Most previous high-performance liquid chromatographic (HPLC) methods for unconjugated MHPG in human plasma have utilized either extraction with organic solvents [3–5] or complex column separation procedures [6,7] for puri-

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fication of the samples prior to HPLC analysis based on reversed-phase separation and electrochemical detection (ED).

The objective of the present study was to develop and validate a sensitive, reproducible, rapid and less tedious method for the routine quantitation of unconjugated MHPG in human plasma, for use in pharmacological and physiological studies. For this purpose, the method previously used in this laboratory [3] was modified according to the currently available extraction and HPLC technology.

EXPERIMENTAL

Materials

Bond Elut® extraction columns with 1-ml phenyl (PH) bonded phase were from Analytichem International (Harbor City, CA, U.S.A.). Methanol, sodium acetate and citric acid (analytical grade) were purchased from E. Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris, 99.0–99.5%) and MHPG (hemipiperazinium salt) were from Sigma (St. Louis, MO, U.S.A.). The internal standard, 3-ethoxy-4-hydroxyphenylethyleneglycol (EHPG), was synthesized by Dr. Kenneth Kirk (NIH, Bethesda, MD, U.S.A.), as previously described [8]. Type HV filters (0.45 μm) were from Millipore (Yonezawa, Japan). Water was first distilled and then deionized in a Milli Q apparatus (Millipore, Bedford, MA, U.S.A.).

Instrumentation

A vacuum-operated sample processing station designed for use with the solid-phase extraction columns (Vac Elut SPS 24™, Analytichem International) was used in sample preparation. The HPLC system consisted of an LKB Model 2150 HPLC pump (Bromma, Sweden), a pressure filter (Model LP-21, Scientific Systems, State College, PA, U.S.A.), a Model 460 HPLC autosampler (Kontron Instruments, Zürich, Switzerland), a pre-column filter (0.5 μm × 3 mm, Rheodyne, Berkeley, CA, U.S.A.), and an Ultrasphere ODS column (5 μm particle size, 250 mm × 4.6 mm I.D., Beckman Instruments, San Ramon, CA, U.S.A.).

The column effluent was monitored with a three-electrode coulometric detector (Model 5100A, equipped with a Model 5011 high-sensitivity analytical cell and a Model 5021 conditioning cell; ESA, Bedford, MA, U.S.A.). The conditioning cell potential was set to +0.44 V, and the potentials of the analytical electrodes to ±0.00 V (detector 1) and −0.25 V (detector 2); these settings were found to provide optimal detection conditions. The chromatography was carried out at room temperature (20–25°C). The output from detector 2 was connected to a data system, which consisted of an Osborne AT microcomputer, a Model 4100-4541 PC integration pack program (Kontron Instruments) and a dot matrix printer (Epson EX-800, Nagano, Japan). The autosampler was cooled with an Endocal RTE-110 refrigeration system (Neslab Instruments, Newington, NH,

U.S.A.). The cooling fluid was water–ethylene glycol (80:20, v/v, at 1°C). Alternatively, a syringe-loading sample injector with a 50-μl loop (Model 7125, Rheodyne) and a linear strip chart recorder (Kipp & Zonen BD41, Delft, The Netherlands) could be used. The chart speed was 5 mm/min.

Mobile phase preparation

The mobile phase consisted of a mixture of 0.1 *M* sodium acetate with 0.09 *M* citric acid and methanol (94:6, v/v). The final pH was adjusted to 5.25 ± 0.02 with a few drops of 10% (w/v) citric acid. The mixture was filtered with a 0.45-μm Millipore HA filter and degassed under reduced pressure. The flow-rate was 1.5 ml/min (pressure 21 MPa); the mobile phase was allowed to recirculate.

Sample preparation

Venous blood samples were collected into pre-chilled polypropylene tubes with K_2EDTA as anticoagulant, chilled in ice and centrifuged (10 min, 3000 *g*, +4°C) within 30 min to separate the plasma. Frozen human plasma samples kept at −70°C were thawed and then centrifuged at 3000 *g* for 10 min at +4°C. Aliquots of 500 μl were supplemented with the internal standard, EHPG (15 μl of a 1.25 μM solution in 0.05 *M* Tris–HCl, pH 7.2, to yield a final concentration of 36 n*M* in the sample), and subsequently used for the analysis.

The Bond-Elut extraction columns were activated in the Vac Elut manifold by passing first 1 ml of methanol, then 1 ml of water and finally 1 ml of 0.05 *M* Tris–HCl buffer (pH 7.2) through the columns. The columns were not allowed to run completely dry during the procedure.

With the vacuum off, 450-μl aliquots of the samples and standard solutions were loaded on the columns. After passage of the samples through the columns under reduced pressure, 200 μl of water were used to wash the columns. MHPG and EHPG were then eluted with 200 μl of 75% (v/v) methanol in water. The eluates were filtered with Millipore HV filters. At this point the samples were ready for HPLC analysis. They were either kept in the refrigerated autosampler or in ice (no longer than 24 h), or were stored frozen at −70°C overnight. The injection volume was 25 μl.

Standard solutions of 0–25 n*M* MHPG were made daily in 0.05 *M* Tris–HCl buffer (pH 7.2) from an aqueous stock solution (500 n*M*). They were supplemented with the internal standard (EHPG) and extracted analogously to the samples.

Precision and accuracy

The precision of the assay was tested using pooled plasma samples. The within-assay variability was assessed with five to nine independent replicates of seven different plasma samples, with MHPG concentrations in the physiological range. Values of peak heights obtained by manual measurement were compared with those obtained by computer integration. The between-assay variability was tested with two control samples, one analysed in nine and the other in seventeen different assays within a period of several weeks.

The accuracy and linearity were tested by spiking plasma and Tris–HCl buffer with different concentrations of MHPG (0, 12.5, 25, and 50 n*M* in one experiment; 0, 3, 6, and 12 n*M* in another) and analysing the resulting samples in the normal manner. The extraction recovery of the analytes was assessed analogously with plasma spiked with 10 and 25 n*M* MHPG.

Physiological and pharmacological experiments

The usefulness of the assay for human studies was tested with plasma samples collected in the course of four different experiments. The subjects were healthy male medical students, 22–28 years old, and had given their informed consent. All study protocols had approval from the local Ethics committee.

The within-subject variability was tested with frequent plasma samples obtained over 6 h from six supine, resting subjects who had fasted overnight. A low-monoamine meal was served between 4 and 5 h. The effect of submaximal physical exercise was investigated in a bicycle exercise test with stepwise increments in work-load, performed by thirteen subjects. They had abstained from coffee, smoking and monoamine-containing foodstuffs on the test day. The duration of exercise was 16 min, and the final work-load was adjusted to result in heart rates of *ca.* 170 beats per min. Blood samples were collected immediately before and after the test, with the subjects sitting on the bicycle ergometer [9].

Two sets of samples from pharmacological experiments were also investigated. One consisted of plasma samples taken for eight subjects before and 4 h after intake of a single 300-mg dose of moclobemide, an inhibitor of the enzyme monoamine oxidase, which is assumed to cause marked reductions in the plasma MHPG concentration [10]. Another set of samples was derived from an experiment in which a 50-μg dose of dexmedetomidine, a selective α_2-adrenoceptor agonist, was administered intravenously to five subjects; plasma NE concentrations were reduced by 80%, on the average, 30 min after the injection [11], and we predicted that this reduction in neuronal NE release would be reflected in the MHPG levels in plasma.

Statistical methods

Linear regression analysis was used in the assessment of the accuracy of the method. Student's *t*-test was used to compare two means. One-way analysis of variance (ANOVA) for repeated measurements was used to evaluate the changes in plasma MHPG levels over time.

RESULTS

Chromatographic conditions

Fig. 1 shows typical chromatograms obtained by injecting extracts of a standard mixture (A) and human plasma (B and C). The resolution of the analytes from other plasma components was sufficient in all the samples studied. The

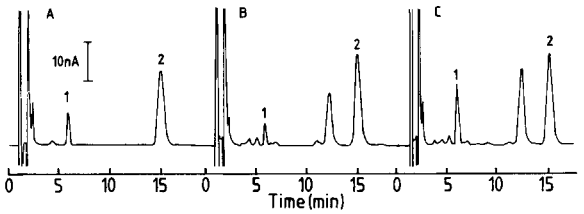

Fig. 1. Typical chromatograms of (A) extracted 25 n*M* MHPG standard in Tris buffer, (B) plasma with 12.6 n*M* endogenous MHPG, and (C) the same plasma with 25 n*M* added MHPG. Peaks 1 = MHPG; 2 = EHPG. Sample preparation and HPLC conditions are given in the text. The identity of the peak appearing at 12 min has not been confirmed.

retention times of MHPG and EHPG were typically *ca.* 6 and 15 min, and were not markedly altered by small changes in the pH of the mobile phase. The detector potentials used, +0.44 V for oxidation and −0.25 V for reduction, were chosen based on hydrodynamic voltammograms for both analytes (data not shown); these settings provided a maximal signal-to-noise ratio and could be maintained unchanged over a period of two months.

The detector response was proportional to the MHPG concentration from the lower limit of detection to at least 25 pmol MHPG injected (25 μl of 1000 n*M* solution). The lower limit of reliable detection (signal-to-noise ratio greater than 3) in actual plasma samples was *ca.* 0.08 pmol MHPG injected, corresponding to a plasma concentration of 1.5 n*M*.

Precision and accuracy

Assessment of the within-asay reproducibility with seven plasma pools containing physiological MHPG concentrations resulted in coefficients of variation (C.V.) in the range 3.6–8.0% (Table I). Measurement of peak heights manually and by computer resulted in almost identical concentrations and C.V. estimates (Table I, sample 5); in nine replicate plasma samples quantitated in parallel with both methods the difference between the two results ranged between 0.2 and 4.4%. Similar C.V. were obtained with nine repeat injections of a 50 n*M* standard solution: 2.5% by hand and 4.4% by computer. In contrast, computer integration based on peak-area measurements resulted in more variability (10%, $n = 9$).

The between-assay reproducibility was estimated with the use of two control samples. The resulting C.V. were 8.0% (MHPG 11.9 n*M*, $n = 9$) and 9.2% (MHPG 14.8 n*M*, $n = 17$).

The accuracy of the determination was tested with known amounts of MHPG added to plasma and Tris–HCl buffer. Linear regression analysis yielded similar slopes (0.015–0.016) for both types of sample, *i.e.* standard curves prepared in plasma and buffer, both in the low (3–12 n*M*) and high (12.5–50 n*M*) concentration ranges of added MHPG. The *y*-intercepts were not significantly different from zero for the buffer, and corresponded to 6.2 n*M* in the plasma pool with low

TABLE I

ESTIMATION OF INTRA-ASSAY PRECISION USING POOLED PLASMA SAMPLES

Sample No.	Mean concentration (n*M*)	C.V. (%)	*n*
1	9.7	8.0	6
2	9.9	5.6	6
3	11.6	3.6	9
4	11.7	3.9	4
5	17.1	5.6	9
5[a]	16.8	5.2	9
6	18.1	5.6	8
7	36.4	7.2	5

[a] Manual peak-height measurement.

endogenous MHPG content (measured: 6.4 n*M*) and to 13.4 n*M* in the plasma pool with higher MHPG content (measured: 13.4 n*M*). The correlation coefficients of the equations ranged between 0.981 and 1.000 (Pearson's *r*).

Recovery

The extraction recovery of MHPG added to pooled plasma was 89 ± 7% at 10 n*M* added ($n = 6$) and 93 ± 9% at 25 n*M* added ($n = 6$). The recovery of EHPG from these plasma samples was 95 ± 4% ($n = 12$). The completeness of the elution of the analytes from the extraction columns was investigated by passing another 200 μl of 75% (v/v) methanol through the column; less than 2% of the applied MHPG and less than 5% of the applied EHPG were recovered in this fraction. The extraction recovery was not dependent on the MHPG concentration applied on the columns in the range studied (3–50 n*M*), as shown by the linear regression analysis.

Physiological and pharmacological experiments

MHPG concentrations in plasma were relatively stable over the 6-h sampling period in resting human volunteers (Table II, group A). A low-monoamine meal was served after the 4-h sample; this did not result in marked changes in the MHPG levels. The within-subject variability (C.V.) ranged from 11 to 18% over this sampling period. In another experiment, a graded bicycle exercise test lasting 16 min and elevating the heart rate to *ca.* 170 beats per min resulted in an 18% increase in the mean plasma MHPG concentration (from 16.0 ± 3.9 to 18.9 ± 3.5 n*M*, mean ± S.D., $p < 0.001$, paired *t*-test, $n = 13$).

Administration of the monoamine oxidase inhibitor moclobemide to human volunteer subjects was followed by 52 ± 8% reduction in plasma MHPG levels 4 h after oral drug intake (from 18.1 ± 2.6 to 8.5 ± 1.3 n*M*, $p < 0.001$, $n = 8$). In

TABLE II

MHPG CONCENTRATIONS IN PLASMA IN RESTING MALE VOLUNTEERS (GROUP A) AND AFTER INTRAVENOUS ADMINISTRATION OF DEXMEDETOMIDINE (GROUP B)

ANOVA: no significant changes from baseline.

Time	Concentration (mean ± S.D.) (n*M*)	
	Group A (n = 6)	Group B (n = 5)
Baseline	11.5 ± 2.6	13.3 ± 1.6
15 min	9.8 ± 2.2	11.1 ± 1.9
30 min	10.2 ± 2.8	11.5 ± 2.8
60 min	10.3 ± 2.5	12.5 ± 2.0
90 min	9.8 ± 2.5	13.8 ± 2.7
2 h	10.8 ± 2.8	13.3 ± 2.0
3 h	11.0 ± 1.5	14.2 ± 2.3
4 h	12.0 ± 3.5	13.8 ± 3.0
5 h	10.3 ± 2.7	
6 h	10.3 ± 2.1	

contrast, the plasma MHPG concentration remained unchanged after intravenous injection of the α_2-adrenoceptor agonist dexmedetomidine (Table II, group B).

DISCUSSION

The present HPLC separation system coupled with selective dual-electrode ED provided more than sufficient separation of MHPG and EHPG from each other, from the solvent front and from all impurities present in the plasma extracts (Fig. 1). This is a clear improvement over our previous MHPG assay [3], where resolution of the two analytes from interfering plasma components was critical and temperature-dependent. Only a few of the compounds recognized by oxidizing HPLC electrochemical electrodes undergo reversible electrochemical reactions, which provided the basis for the improved selectivity for MHPG and EHPG at the reducing analytical electrode. This principle has been widely used in the measurement of catecholamines in plasma, but has less often been applied to catecholamine metabolites [1].

The improved selectivity of detection allowed us to use a simplified plasma extraction procedure. Bond-Elut PH columns have previously been used by Karege [12] for the purification of MHPG after enzymic hydrolysis of MHPG conjugates from brain tissue, urine and protein-free ultrafiltrate of plasma. MHPG and EHPG are not, however, very well retained on reversed-phase extraction columns, and we found it necessary to adhere strictly to all details of the procedure

outlined above in order to achieve optimal results. Direct injection of plasma ultrafiltrate has also been used in the assay of unconjugated MHPG in plasma; this, however, resulted in gradual inactivation of the detector electrodes [13].

The reproducibility of the extraction of MHPG was improved by the use of a close chemical analogue as an internal standard. The MHPG homologue used by us, EHPG, fulfils most of the criteria for a suitable internal standard, but is not an ideal compound owing to its commercial unavailability. The same applies to the MHPG isomer 3-hydroxy-4-methoxyphenylethyleneglycol used by Molyneux and Franklin [4] for this purpose, but both compounds can be readily synthesized [4,8].

The present system allows one well trained person to process up to 60 samples within a working day, making the method useful for routine measurement of plasma MHPG concentrations. This increase in sample throughput compared with our previous method [3] results from three factors: a simplified extraction procedure, automated sample injection and automated data processing. The number of samples could probably be further increased by speeding up the chromatographic separation; the resolution is now more than sufficient and would allow this. This was, however, considered unnecessary in our setting and would require more than one person to run the assay. Manual injection and peak-height measurement are also feasible, but result in a smaller throughput. The reproducibility of the assay is equally satisfactory using either manual or automated operation.

The preliminary physiological and pharmacological experiments reported here demonstrate the usefulness of the present method for clinical studies. Plasma MHPG levels were found to be relatively stable over a few hours in resting subjects, and to be modestly increased by a short bout of submaximal physical exercise, known to increase NE release from sympathetic nerves. Inhibition of the enzyme monoamine oxidase, essential in the metabolism of NE to MHPG, resulted in *ca.* 50% reduction in the plasma MHPG concentration; this is in line with previous observations [14]. In contrast, administration of an α_2-adrenoceptor agonist drug, known to inhibit NE release [11], was not followed by a decrease in the plasma MHPG level. Previous studies have reported conflicting results in this respect [14]; it may be that NE release, as opposed to NE metabolism, is not accurately reflected by metabolite concentrations in plasma, or that the duration of the action of the applied drug was too short, or that the drug also impeded the clearance of MHPG from plasma, thereby masking a reduction in its rate of appearance [2]. This question will be addressed in future studies.

CONCLUSIONS

This HPLC method for the determination of unconjugated MHPG concentrations in human plasma is precise, accurate and rapid, and has proved to be suitable for routine use in pharmacological and physiological experiments.

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