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Assay of urinary free and conjugated 3-methoxy-4-hydroxyphenylethyleneglycol by high-performance liquid chromatography with amperometric detection

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Abstract

The aim of this study was to develop an analytical method for free and conjugated 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) in urine. After hydrolysis of the conjugated forms, the urinary MHPG was purified by solid-phase extraction on anion exchanger and eluted with a water–methanol (1:1, v/v) mixture. After addition of ethyl acetate to the eluate and back-extraction into acetic acid, the aqueous phase was separated on a C_{18} column by HPLC and detected amperometrically. The results obtained from forty healthy human subjects were compared with the literature values. The precision and accuracy of the assay were studied using 4-methoxy-3-hydroxyphenylethyleneglycol (iso-MHPG) as internal standard.

1. Introduction

3 - Methoxy - 4 - hydroxyphenylethyleneglycol (MHPG), the main metabolite of brain norepinephrine is preferably measured in the urine [1]. It can also be evaluated in the cerebrospinal fluid and the plasma. In biological fluids, MHPG is present in the free and conjugated form. The sulfate conjugate is derived from the brain, whereas the glucuronide conjugate originates from pools of norepinephrine outside the central nervous system [2–5].

Several reports concern mainly the urinary MHPG evaluation in patients with bipolar and unipolar affective disorders. Most of the data suggests that noradrenergic and serotonergic systems in the brain may be involved in the pathophysiology of depression. Reduction in neurotransmitter concentrations was postulated to be a determining factor in the ethiology of depression. Furthermore these neurotransmitter systems have been implicated in the therapeutic actions of antidepressant drugs [6–8]. Many individual antidepressants block the reuptake of both norepinephrine and serotonin, thereby in-

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creasing the concentrations of these transmitters. The main objective of our study was to prove that from the quantities of urinary MHPG the response to antidepressant drugs could be predicted. In many reports, only the total MHPG (MHPG-T) was evaluated, whereas the sulfate MHPG (MHPG-S) seems to be a better indicator of the metabolism of brain norepinephrine. It was therefore essential to measure the free MHPG, the MHPG-S, the glucuronide MHPG (MHPG-G) and the MHPG-T. MHPG-G is obtained by subtraction of the free and MHPG-S from the MHPG-T.

Methods using only ethyl acetate for extraction are tedious because they require several washing steps [9–15]. Furthermore they are not very selective. Liquid–solid extraction methods are very easy to perform and require only small sample volumes. Therefore we developed a method for measuring the free and conjugated MHPG by HPLC and amperometric detection after solid-phase extraction on AG 1×4 (Biorad) resin and liquid–liquid extraction with ethyl acetate.

2. Experimental

2.1. Reagents

3 - Methoxy - 4 - hydroxyphenylethyleneglycol-(MHPG), 4-methoxy-3-hydroxyphenylethyleneglycol (iso-MHPG), 3-hydroxy-4-methoxy-mandelic acid, lithium aluminum hydride, purified glucuronidase type VIII-A, β-glucuronidase type H-2S (glusulase) from *Helix pomatia*, arylsulfatase type VI from *Aerobacter aerogenes* were obtained from Sigma (St. Quentin Fallavier, France). All other reagents used were analytical grade. AG 1×4 resin (100–200 mesh) and the chromatography Econocolumn were purchased from BioRad (Ivry sur Seine, France).

2.2. Instrumentation

The liquid chromatographic-electrochemical detection system consisted of a Waters Model 510 pump (St. Quentin, France), a Superspher 100 RP18 reversed-phase column (4 μm particle size, 250×4 mm I.D.) with a LiChrospher 100 RP18 (7 μm particle size, 4×4 mm I.D.) precolumn (Merck, Nogent sur Marne, France), and an amperometric LC 4B detector with glassy carbon electrodes (Biochrom, Angouleme, France). The electrochemical detector was operated at +0.85 V versus a Ag/AgCl reference electrode.

A 0.05 *M* potassium dihydrogenophosphate buffer (pH 3.0) containing 2% acetonitrile was used as isocratic mobile phase. Elution of the compounds from the column was carried out at ambient temperature with a flow-rate of 1.0 ml/min. The resultant pressure was about 15.1 MPa.

2.3. Standard solutions

The internal standard 4-methoxy-3-hydroxyphenylethyleneglycol (iso-MHPG) was prepared from the 3-hydroxy-4-methoxy-mandelic acid by reduction of the acid function with lithium aluminum hydride according to the previously described method [12–16]. All standard solutions were prepared in a 0.05 *M* HCl solution containing sodium metabisulfite (0.5 g/l). From the stock solutions of MHPG (100 mg/l) and MHPG-S (100 mg/l), dilutions to 5 mg/l and 25 mg/l were prepared for the evaluation of free and conjugated MHPG respectively. The standard solutions were stable for more than six months at 4°C.

2.4. Urine samples

Before analysis, the 24-h urine samples, non acidified, were stored at −20°C after addition of a solution containing 1 g/l sodium metabisulfite in order to prevent oxidation of the catechol compounds. The creatinine concentration was evaluated in each urine sample by Jaffé's method without deproteinisation, using an Hitachi 704 analyser [17].

2.5. Extraction

The extraction procedures for the urine samples are shown in Fig. 1. For each extraction

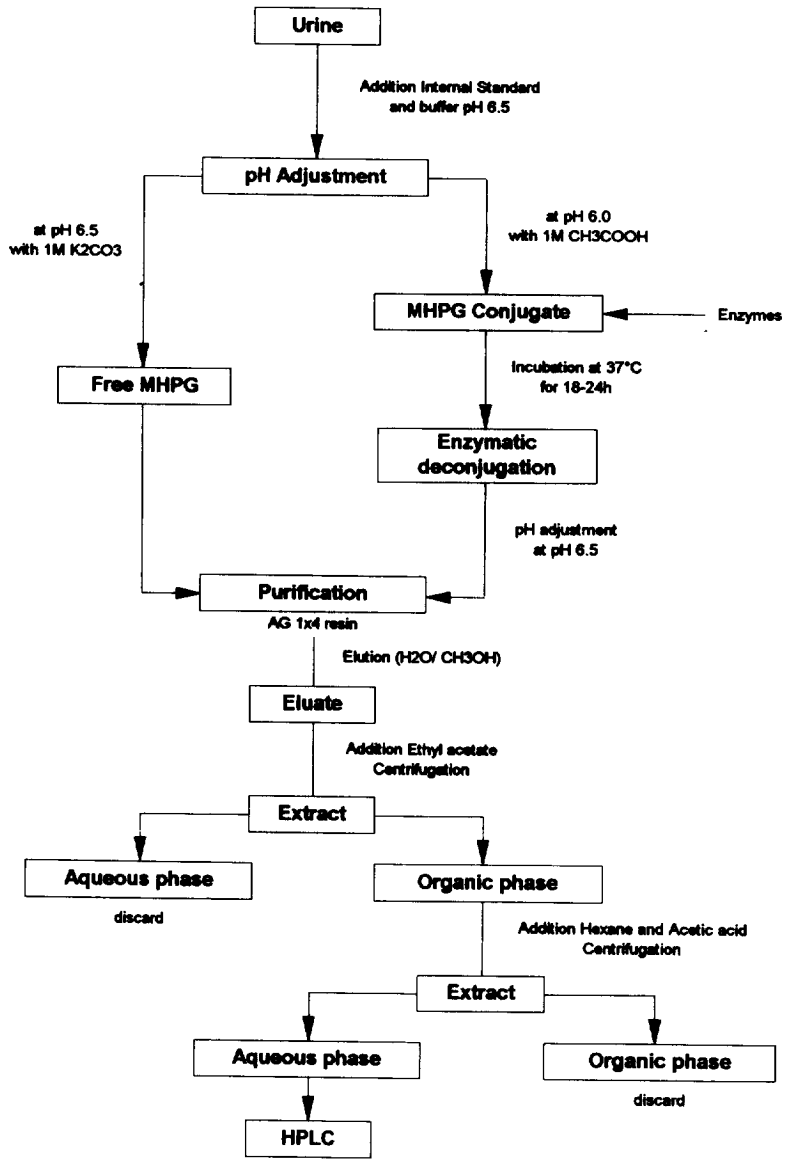

Fig. 1. Schematic outline of the extraction procedures.

assay, the following samples were prepared: a standard range (free MHPG: 0.1, 0.25 and 0.5 mg/l; MHPG-S: 0.5, 1, 2, and 3 mg/l), a urinary pool from several subjects as control and the urine samples of the patients. Aliquots of 25 μl and 100 μl of internal standard were added to each tube for the assay of free and conjugated MHPG (MHPG-S and MHPG-T) respectively. The extracts were buffered by addition of 500 μl of 1 *M* sodium acetate buffer (pH 6.5) and adjusted to pH 6.0 ± 0.05 with 1 *M* CH_3COOH or 1 *M* $KHCO_3$ when necessary.

After addition to each extract of the corresponding enzyme (100 μl of arylsulfatase for MHPG-S and 200 μl of glusulase for MHPG-T), all the tubes were incubated between for a 18–24-h period at 37°C. After incubation, the pH was adjusted in all tubes to 6.5 ± 0.05 with 1 *M*

$KHCO_3$. In the arylsulfatase type VI (50 U) preparation, no β-glucuronidase activity could be detected. In the glusulase preparation, the activities of β-glucuronidase and arylsulfatase were about 100 000 U/ml (at pH 5.0) and 1000–5000 U/ml, respectively.

Each extract (standards, control and urine samples) was applied to a BioRad Econocolumn containing AG 1 × 4 resin (100–200 mesh) suspended in water, then washed twice with 1 ml of water and finally eluted with 2 ml of a water–methanol mixture (1:1, v/v) containing 0.5 g/l EDTA and 0.5 g/l sodium metabisulfite [18]. Then 1 g of dry NaCl and 8 ml of ethyl acetate were added to each eluate. The tubes were shaken for 15 min and centrifuged at 3000 *g* for 5 min. From the organic layer 5 ml were withdrawn and added to 5 ml of hexane and 2 ml of 0.1 *M* acetic acid (pH 3.0), containing 0.5 g/l EDTA and 0.5 g/l sodium metabisulfite. The extracts were then shaken for 15 min and centrifuged at 3000 *g* for 5 min. The organic layer was aspirated and 1 ml of the aqueous phase (pH 3.0) was transferred into an haemolysis tube. The traces of organic solvent were removed under nitrogen at 40°C for 5 min. A 50-μl volume of each extract was injected.

3. Results and discussion

The assay of MHPG conjugate requires the use of a hydrolysing agent. After an exhaustive review of the commercially available hydrolytic enzymes, the following were selected: arylsulfatase type VI and β-glucuronidase type H-2S (glusulase) for the deconjugation of MHPG-S and MHPG-T, respectively. After subtraction of the free MHPG and MHPG-S from MHPG-T, we obtained the MHPG-G value. However, several times during the adjustment of the extraction method, 40 μl of glucuronidase type VIII-A were added in order to verify complete glycolysis and to evaluate the MHPG-G. The results obtained from four urine samples (Table 1) showed no significant difference between the measured and calculated values of the MHPG-T.

Unlike catecholamines, MHPG does not have a functional group that allows the specific extraction of MHPG from a complex matrix such as urine. In some previously reported procedures, MHPG was extracted with ethyl acetate [9–15]. In some cases, MHPG was purified or prepared by solid-phase extraction before treatment with ethyl acetate [9,19–22]. Our extraction procedure is essentially the same as that of Julien et al. [21] with some modifications to increase sensitivity and precision. We have purified the urine with the strong anion-exchange resin Dowex 1 × 4 before extraction with ethyl acetate. We have improved the specificity of the MHPG extraction by back-extracting the ethyl acetate extract into acetic acid. The addition of hexane makes the organic phase extraction easier by decreasing its polarity. With the liquid chromatographic technique of Julien et al. the eluate injected onto the chromatographic system

Table 1
Correlation between measured and calculated total MHPG values

Urine sample	Concentration (mg/l)				
	Free MHPG	MHPG-S	MHPG-G	MHPG-T measured[a]	MHPG-T calculated[b]
1	0.09	1.00	1.57	2.65	2.66
2	0.04	0.47	0.57	1.12	1.08
3	0.11	1.76	2.12	4.05	3.99
4	0.08	0.80	1.20	2.15	2.08

[a] Results obtained after hydrolysis with 100 μl of aryl-sulfatase for MHPG-S, 40 μl of glucuronidase for MHPG-G and 200 μl of glusulase for MHPG-T.
[b] MHPG-T = free MHPG + MHPG-S + MHPG-G.

caused erratic recovery of the internal standard (vanillyl alcohol). Our attempts to use vanillyl alcohol in ethyl acetate extractions were unsuccessful. The chromatographic conditions used in Ref. [21] were insufficiently specific and the ODS column (10 μm particle size) showed neither satisfactory efficiency nor good resolution. In most hydrolyzed samples, the peak of the internal standard was partly obscured by an endogenous compound, and some late-eluting analytes were occasionally observed in the chromatogram. Consequently Julien et al. performed a rapid cleaning of the column between two consecutive analyses to only evaluate free MHPG and MHPG-T. In our assay, the precision of the method was increased by using iso-MHPG as internal standard. The extraction, chromatographic and detection characteristics of iso-MHPG are similar to those of MHPG. This compound, which has not been commercialized yet, was prepared in situ using the method of Muskiet et al. [12].

Typical chromatograms obtained after extraction are shown in Fig. 2. Two unknown compounds (A and B) were extracted along with MHPG and the internal standard. These compounds did not originate from the enzyme preparation but most likely from deconjugation of

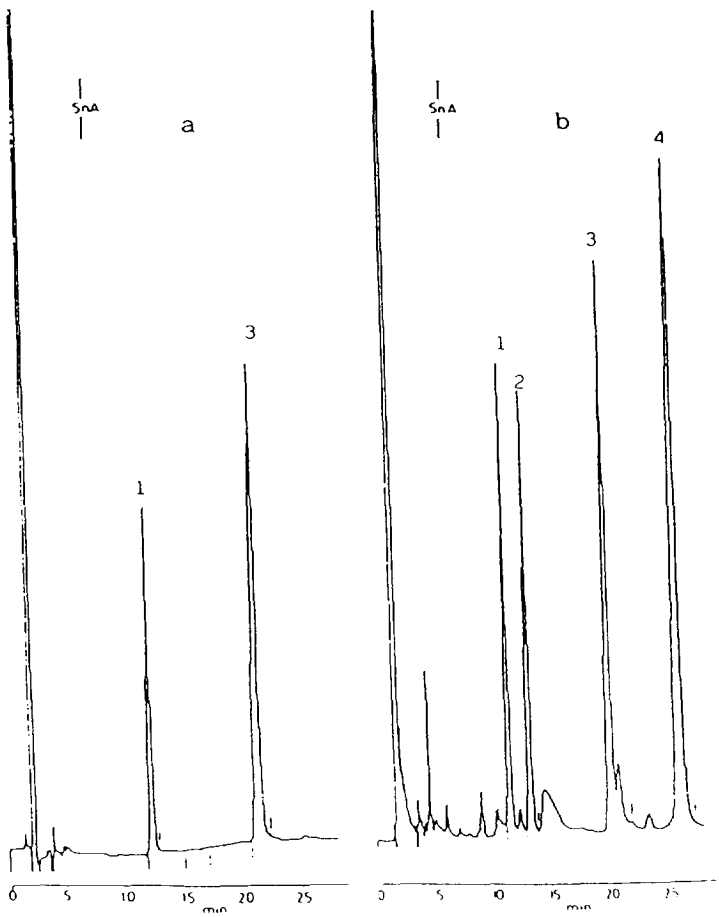

Fig. 2. Typical chromatograms obtained after extraction of (a) 1 mg/l MHPG-S standard solution, (b) urine containing 1.2 mg/l MHPG-S after hydrolysis. Peaks: 1 = MHPG, 2 = compound A, 3 = iso-MHPG, 4 = compound B. Volume injected: 20 μl; detection: amperometric, +0.85 V; range: 20 nA; integrator attenuation: 4.

endogenous compounds. The chromatographic separation of the four compounds (MHPG, iso-MHPG, A and B) was studied as a function of temperature (Fig. 3). The best separation was obtained at ambient temperature with a Superspher 100 RP18 column (4 μm particle size, 250 × 4 mm I.D.). The peaks of the unknown compounds A (13 min) and B (27.6 min) did not interfere with those of MHPG and iso-MHPG (11.8 min and 20.9 min), respectively.

In order to improve the assay, several columns were tested: Hypersil ODS (5 μm particle size, 250 × 4 mm I.D.), Nucleosil CN (5 μm particle size, 250 × 4.6 mm I.D.), Brownlee RP18 (5 μm particle size, 220 × 4.6 mm I.D.). The resolution of these columns did not prove successful after optimization of the chromatographic conditions. The use of acetonitrile rather than methanol in the mobile phase and the Superspher C_{18} column rather than other manufacturer's columns was crucial for the separation of the unidentified peaks. Special care was taken to prevent the presence of ethyl acetate in the injected extracts, because peak shapes were dramatically affected in the presence of this solvent.

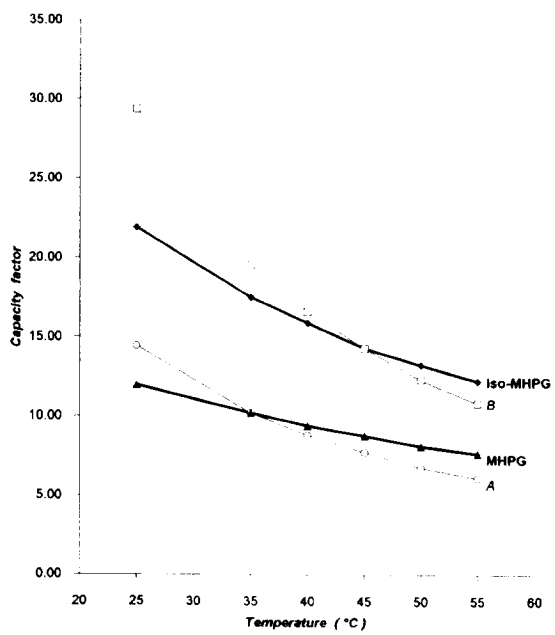

Fig. 3. Effect of temperature on the chromatographic separation of the four compounds: MHPG, iso-MHPG, A, and B.

Standard curves were obtained with aqueous solutions of free MHPG and MHPG-S. When the free MHPG concentration was plotted versus the ratio free MHPG peak height/iso-MHPG peak height, the response was linear between 0.075 and 1 mg/l (regression equation: $y = 2.189x - 0.002$; $r = 0.998$). When the MHPG-S concentration was plotted versus the ratio MHPG-S peak height/iso-MHPG peak height, the response was linear between 0.25 and 7 mg/l (regression equation: $y = 0.582x - 0.022$; $r = 0.999$). The detection limit for both compounds, based on a signal-to-noise ratio of 2, was found to be 0.05 mg/l for free MHPG and 0.125 mg/l for MHPG-S. The precision of the assay was tested using two control urine pools containing 0.115 and 0.09 mg/l of free MHPG and 2.10 and 0.93 mg/l of MHPG-S. The within-run ($n = 10$) precision of quantitative results gave coefficients of variation (C.V.) of 3.4% and 5.5% for free MHPG, and 1.9% and 3.2% for MHPG-S. The higher C.V. for free MHPG compared to MHPG-S is probably due to the lower concentrations and hence quite understandable. For each assay, the standard range and two control urinary pools were used to determine the day-to-day variability with twelve different assays on different days; accurate C.V.s were observed.

The urine samples from forty healthy human subjects (twenty males and twenty females) were collected to determine the normal concentrations of the free and conjugated forms of MHPG. The free MHPG (0.12 ± 0.05 mg/l) and MHPG-S (1.20 ± 0.34 mg/l) values did not differ significantly between male and female subjects. The present results are consistent with the values obtained by other authors [2–5,10,23,24]. However, the MHPG-G (1.89 ± 0.86 mg/l) and MHPG-T (3.20 ± 1.07 mg/l) values are much higher in our study but no sex effect was detected. Some investigators reported that muscular exercise or sleep could influence the peripheral MHPG-G values. Only some authors [5,10] have observed that the MHPG values differ according to the sex.

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