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Note

High-performance liquid chromatographic method for determining plasma and urine 3-methoxy-4-hydroxyphenylglycol by amperometric detection

PHILIP A. SHEA* and J. BARRY HOWELL

Department of Psychiatry, Institute of Psychiatric Research, Indiana University School of Medicine, 791 Union Drive, Indianapolis, IN 46223 (U.S.A.)

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The levels of urinary, plasma and/or cerebral spinal fluid (CSF) 3-methoxy-4-hydroxyphenylglycol (MHPG) have been used as a biochemical index of noradrenergic function in man and have been extensively studied in their relationship to the major psychiatric disorders. Based on measurements of MHPG there have been attempts to biochemically subtype the depressive disorder [1], differentiate other mental disorders [2] and to select appropriate drug therapy or associate drug response [3]. The analytical methods for determining MHPG content have principally been based on the use of gas chromatography (GC) either with electron-capture detection (ECD) [4] or mass spectrometry (MS) [5]. Recently new procedures have been introduced which use high-performance liquid chromatography (HPLC) with electrochemical detection [6, 7]. These methods in general require extensive pre-purification [i.e., multiple extractions, derivatization, thin-layer chromatography (TLC), etc.] before chromatography which often results in low sample recovery and time-consuming procedures. Furthermore these procedures are often not sensitive enough to detect the low levels of MHPG found in blood. None of the HPLC methods have been used for both plasma and urinary MHPG determinations.

This article presents a HPLC method coupled with electrochemical detection (ED) for determining both free plasma and total urinary MHPG. The procedure is simple, rapid, and highly sensitive. Chromatographic separation involves pre-purification of either hydrolyzed urine or deproteinized plasma by passage through a short mixed-bed anion—cation exchange column. This is followed by extraction of MHPG from the eluate into an organic phase which is then evaporated. Residues are resuspended in the HPLC buffer and run on an

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isocratic HPLC system using a reversed-phase column. The eluted MHPG is then detected electrochemically.

MATERIALS AND METHODS

Chemicals

3-Methoxy-4-hydroxyphenylglycol, piperazine salt, 99% was obtained from Aldrich (Milwaukee, WI, U.S.A.). β-Glucuronidase sulfatase from Sigma (St. Louis, MO, U.S.A.), a crude preparation from *Helix pomatia* containing 100,000 units per ml of β-glucuronidase and 10,000 units sulfatase activity and was used as supplied. All other reagents used were analytical grade, unless otherwise indicated. All water used was double-distilled in glass.

Urine collection

Urine was collected from human subjects over a period of 24 h. To the collection, 0.5 mg/ml sodium metabisulfite was added after total volume was recorded. A 50-ml aliquot was stored in a −20° C freezer prior to analysis. Urine collections were checked for 24-h completeness by determination of creatinine.

Plasma collection

Whole blood was collected from human subjects into evacuated tubes containing heparin. The samples were spun (900 *g* for 15 min) to separate the plasma which was drawn off and stored in a −20° C freezer prior to analysis.

Preparation of anion—cation column

The cation-exchange resin (Bio-Rad AG-50W-X4, 200—400 mesh), in the H^+ form, was converted to the Na^+ form by washing with 2 vol. of 1 *M* sodium hydroxide and rinsing with 4 vol. of double-distilled water. The resin was further washed with 2 vol. of 0.01 *M* sodium phosphate buffer, pH 6.0, and stored at 4° C in this buffer until use. The anion-exchange (Bio-Rad resin AG-1-X4, 200—400 mesh, Cl^-) was conditioned by adding 2 vol. of the same buffer and storing at 4° C. Chromaflex column tubes (Kontes, Martin, IL, U.S.A.), 6 cm × 0.5 cm I.D. were fitted with column tube tips that had a small amount of silanized glass wool placed in the tip to hold the packing. Onto the silanized glass wool was placed 1.0-cm height (volume, 0.19 cm^3) of anion resin. This was followed by the same amount of cation resin. The columns were then washed with 4 vol. of 0.01 *M* sodium phosphate buffer, pH 6.0.

Freeing of urine MHPG conjugates

Duplicate urine samples (2.0 ml) were mixed with 0.1 ml β-glucuronidase sulfatase and 1.0 μl 2% (w/v) ethylenediamine tetraacetate (EDTA) in 1.0 *M* sodium acetate in 15-ml glass centrifuge tubes. Stoppered tubes were incubated 24 h at 37° C with agitation. The crude β-glucuronidase sulfatase preparation gave no peaks on the chromatogram when saline reagent blanks were run through the entire assay.

Deproteinization of plasma

Duplicate plasma samples (1.0 ml) were deprotienized by adding 0.5 ml of formic acid, diluted to 2% (w/v) concentration in water—acetone (1:3), followed by 0.25 ml 10% (w/v) sodium tungstate. After mixing, the samples sat at room temperature for 15 min, then were centrifuged 10 min at 1000 *g*. The supernatants were transferred to 15-ml glass-stoppered centrifuge tubes, the pellets were then resuspended in the formic acid solution and re-centrifuged. The pooled supernatants were extracted in the centrifuge tubes with heptane–chloroform (8:1), using 30 sec vigorous mixing followed by 5 min centrifugation at 300 *g* to separate phases. The organic phase, containing lipophilic material, was discarded. The aqueous phase, much reduced in volume, was adjusted to pH 6.0 by addition of 2 *M* disodium phosphate.

Preparation of standard curves

Standard curves were prepared by replicate additions of MHPG to samples from a single pool of plasma or urine following which the samples containing added MHPG were treated identically with other samples.

For urine, the standard curve consisted of duplicate 2.0-ml samples from a single pool which received 0, 0.2, 1.0, 1.5 or 2.5 mg MHPG per sample. For plasma, the standard curve comprised duplicate 1.0-ml samples from a single pool which received 1, 5, 10, 25 ng MHPG.

Pre-column purification of urine and plasma

Urine and plasma samples were partially purified by eluting 0.5 ml of the hydrolyzed urine or the total aqueous phase of the deproteinized plasma extract, through the anion—cation column with 1.2 ml of 50% methanol. Eluents were collected and reduced to about 0.6 ml volume in a Buchler vortex-evaporator (about 10 min). Each sample was extracted with ethyl acetate (2.5 ml), then samples spun at 300 *g* for 2 min to separate phases. The organic phase was transferred to 5.0-ml conical glass centrifuge tubes, and the ethyl acetate extraction of the remaining aqueous phase was repeated. The pooled ethyl acetate extracts were then evaporated to dryness (under vacuum). Urine samples were resuspended in 1.0 ml and plasma samples in 50 μl of the HPLC buffer.

HPLC

The HPLC apparatus consisted of an Altex pump Model 110-A, a Rheodyne Model 7000 loop injector with 20-μl loop, and a 3-μm Chromantics Spherisorb ODS-2 (100 × 4.6 mm) column (J & S Scientific, Crystal Lake, IL, U.S.A.). Samples were eluted isocratically at 1.5 ml/min using a mobile phase buffered at pH 4.0 with 0.01 *M* sodium acetate—acetic acid, and containing 1 m*M* EDTA.

Detection of MHPG was by an amperometric system (Bioanalytical Systems, West Lafayette, IN, U.S.A.) using a glassy carbon working electrode, a silver—silver chloride reference electrode, and a Model LC4A detector-potentiometer.

The working electrode was maintained at a potential of 0.9 V vs. the silver—silver chloride reference electrode, and the amperometric detector was operated using a 0.1-sec response time constant. Detector response was displayed on a chart recorder (Omniscribe, Houston Instruments).

RESULTS AND DISCUSSION

Resolution

Typical chromatograms of the separation of MHPG in urine and plasma are shown in Figs. 1 and 2, respectively. The left profile of each figure represents endogenous amounts and the right, a duplicate of the same sample containing added MHPG. The MHPG peak is free of obvious interferences.

Precision

Within-assay variation, evaluated by independent replicate determinations of the apparent MHPG content of single samples, was small. For urine, the coefficient of variation (C.V.) for six replicates of a single sample carried through the entire assay (including the hydrolysis step) was only 0.09, while for plasma, eight replicate determinations of free MHPG showed a C.V. of 0.10. This high precision was judged to render the use of recovery standards unnecessary.

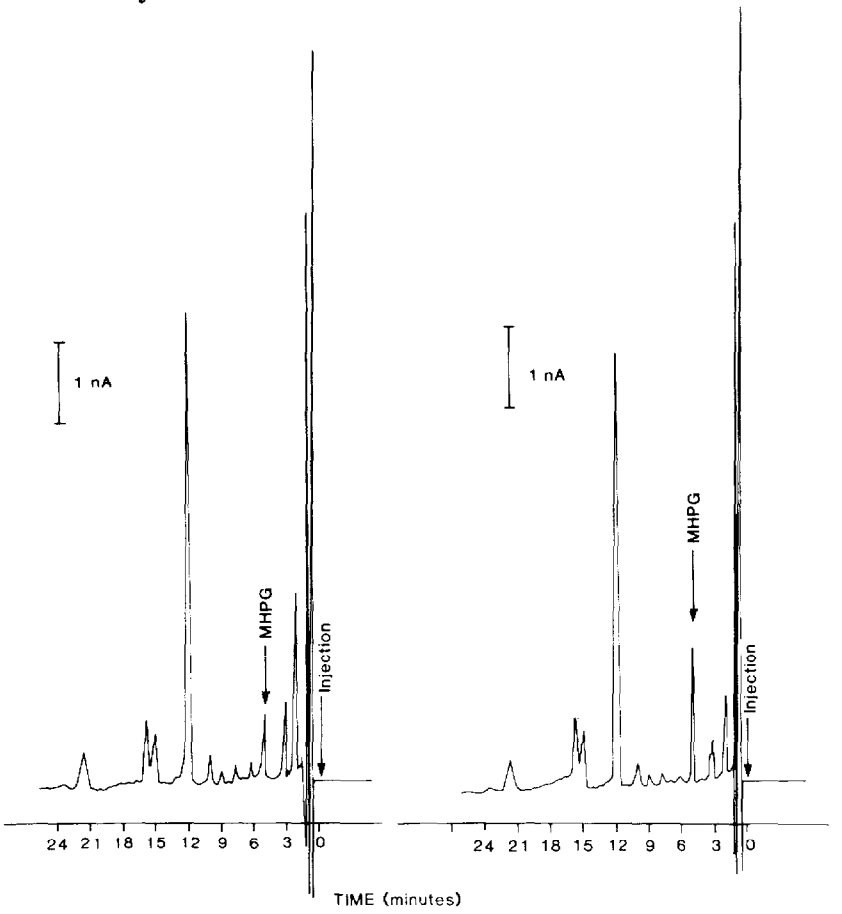

Fig. 1. Shown are typical chromatographic profiles for total urinary MHPG using a 3-μm, C18 reversed-phase column under isocratic conditions and amperometric detection. The left profile is endogenous MHPG and the right an addition of MHPG to the same split sample. From an original 2.0-ml urine extract 20 μl were injected. Sensitivity of the detection system was set at 1 nA with a working electrode potential of 0.90 V.

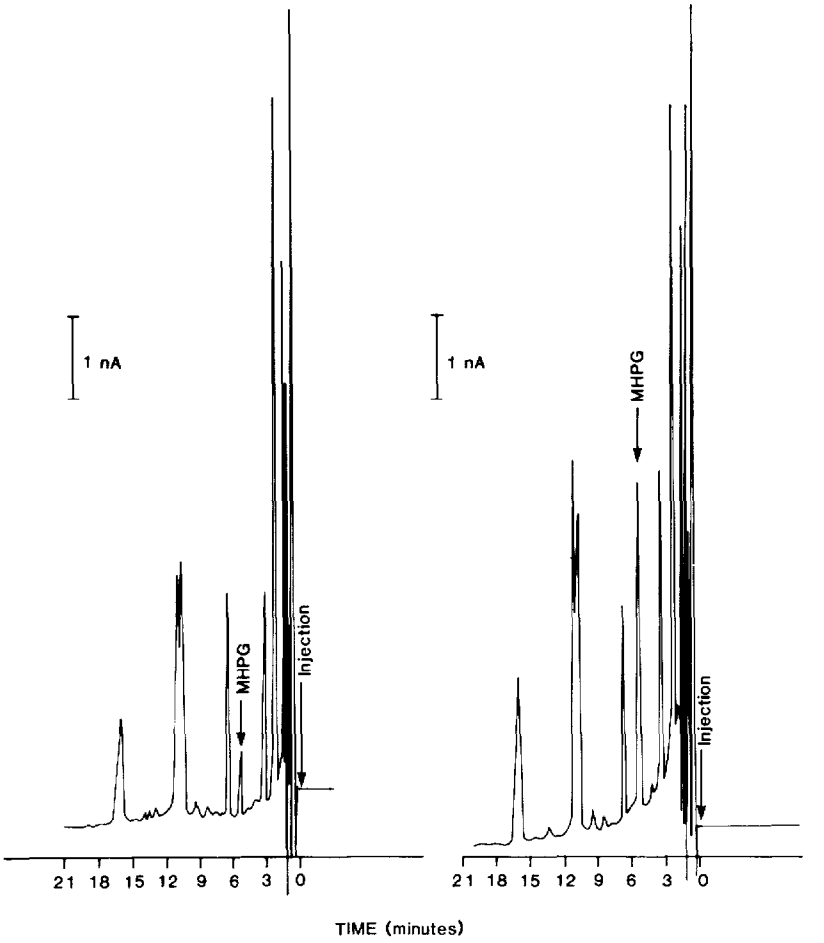

Fig. 2. A typical chromatographic profile for free plasma MHPG using the conditions as in Fig. 1. The left profile is endogenous MHPG and the right an addition of MHPG to the same split sample. From an original 1.0-ml plasma extract 20 μl were injected.

Recovery and accuracy

Recovery, estimated by addition of known amounts of MHPG to samples, was 50% for plasma and 70% for urine. The two steps which gave significant losses were at the anion—cation column step and during the ethyl acetate extraction. Using a greater volume to elute off the column and repeating the ethyl acetate extraction more than twice on the eluents would improve recoveries by 20% or more. Plasma recoveries are lower than urine due to the initial extraction step. However, with the detection limit of the assay at 0.1 ng MHPG per injection, it was felt the extra time involved to improve recoveries was unnecessary. A linear detector response from the lower limit of sensitivity (0.1 ng MHPG per injection) up to 40 mg per injection was verified.

The values for total urinary MHPG in ten subjects and free MHPG in plasma from seven subjects, as determined by this HPLC method, were 1.26 ± 0.58 (S.D.) mg/24 h and 3.34 ± 1.45 ng/ml, respectively. These means compare closely with other procedures using similar subjects populations (Table I).

TABLE I

COMPARISON OF FREE PLASMA AND TOTAL URINARY MHPG AS DETERMINED BY VARIOUS METHODS

All results are taken from the literature and are expressed as the means ± standard deviations with the number of subjects (n) given in each case. All measurements include normal male and female total urinary MHPG determinations.

Method	Reference	Total urinary MHPG (mg/24 h)	n	Free plasma MHPG (ng/ml)	n
HPLC—ED	This method	1.26 ± 0.58	10	3.34 ± 1.45	7
HPLC—ED	[6], [7]	2.65 ± 0.20	12	4.62 ± 1.11	3
GC—ECD	[8], [9]	1.92 ± 0.71	17	5.4 ± 1.5	15
GC—MS	[5], [10]	2.62 ± 0.34	7	4.6 ± 1.0	10

Advantages of this method

The main advantage of the HPLC method described is the ability to assay both urine and plasma samples using the same system. The 3-μm column gives good separation, with MHPG peaks starting from and returning to baseline. Equipment used in this procedure is less expensive than GC equipment and samples do not require derivatization. Other HPLC methods have been used only for urinary MHPG [6, 11] with the exception of one method that requires a more difficult pre-column purification procedure for plasma MHPG [7].

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