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Note

Routine determination of unconjugated 3-methoxy-4-hydroxyphenylglycol in plasma using high-performance liquid chromatography with electrochemical detection

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3-Methoxy-4-hydroxyphenylglycol (MHPG) is the major metabolite of noradrenaline [1] and it has been reported that unconjugated (free) MHPG in plasma may closely reflect central noradrenaline turnover [2]. Free plasma MHPG levels have been reported to be altered in both depression [3—5] and anxiety [6], thus suggesting that MHPG may be used as a clinical marker.

Plasma MHPG is generally determined by gas chromatography with either electron-capture detection [7, 8] or mass spectrometry [9, 10]. These techniques require derivatization of MHPG prior to analysis and although they are very selective, they are either too expensive or too slow to be used routinely. High-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been used to measure MHPG in urine [11, 12] and cerebrospinal fluid [13, 14].

The rather complex nature of plasma has made the determination of free MHPG potentially more difficult. A search of the relevant literature revealed three methods using HPLC with amperometric detection. Prior to extraction, the first method [15] involves deproteination of the plasma and subsequent pH adjustment with two buffers whilst the other two [16, 17] require lengthy and complex column separation and purification steps.

The recent improvement in the coulometric detector as opposed to the traditional amperometric detector has given the chromatographer increased versatility. The coulometric detector oxidises 100% of the analyte passing through the cell while an amperometric detector cell oxidises only 1—5% [18]. By incorporating a guard cell between the delivery system and the injector the

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expected increase in background noise owing to the solvent can be eliminated. In addition the detector cell itself contains two electrodes and some potentially interfering compounds may be eliminated by their complete oxidation or reduction at the first electrode. In this instance the necessity for a tedious purification and extraction procedure has been avoided. In this paper we describe a fast and simple method for the routine determination of free MHPG in plasma.

EXPERIMENTAL

Reagents and standards

All the standards apart from the internal standard 4-methoxy-3-hydroxyphenylglycol (iso-MHPG) were supplied by Sigma (Poole, U.K.). Iso-MHPG was kindly donated by Dr. H.J. Gaertner, although it can be easily prepared [11]. Methanol and sodium acetate (HPLC grade) were purchased from Fisons (Loughborough, U.K.). The remaining reagents (AnalaR grade) were obtained from BDH (Poole, U.K.).

All water was deionised and glass-distilled prior to use.

HPLC instrumentation

The HPLC system comprised of a Model 302 pump fitted with an 802 Manometric module (Gilson SA, Villiers Le Bel, France), a Kontron Model MSS1 660 autosampler with a 20-μl loop (Kontron Instruments, St Albans, U.K.,), an ODS reversed stationary phase column (Rainin, 3 μm particle size, 100 × 4.6 mm, Anachem) protected by a 5-μm ODS HPLC guard column (Brownlee, Anachem). The detection system consisted of a Model 5100A Coulochem^{TM} detector and a Model 5020 guard cell (ESA, Bedford, MA, U.S.A.) protected by a 5-μm ODS HPLC guard column (Brownlee). The detector was linked to an LDC CI-10 integrator with an NEC printer—plotter (LDC, Stone, U.K.).

HPLC conditions

The mobile phase consisted of a mixture of 0.1 *M* sodium acetate—methanol (90:10). The pH was adjusted to 5.0 with glacial acetic acid and the solvent degassed under vacuum prior to use. The flow-rate was set at 1.0 ml/min. The potentials for detectors 1 and 2 were selected after injection of fixed amounts of MHPG and the internal standard over the range 0.1—0.6 V for each detector (Fig. 1). The potentials for the guard cell and detectors 1 and 2 were +0.5, +0.1 and +0.4 V, respectively. The response time was 2 sec.

Collection and storage of blood for analysis

There have been several reports of sample deterioration under different storage conditions [15, 19]. To date samples have been stored in the manner described below for up to three months without any evidence of decay.

Whole blood (10 ml) was added to a 10-ml lithium heparin tube containing 100 μl of 10 μM iso-MHPG and 0.2 *M* sodium metabisulphite. This was gently mixed and then centrifuged at 1500 *g* for 10 min at 4°C. The plasma was removed and stored at −20°C until analysis. Prepared samples were found to be

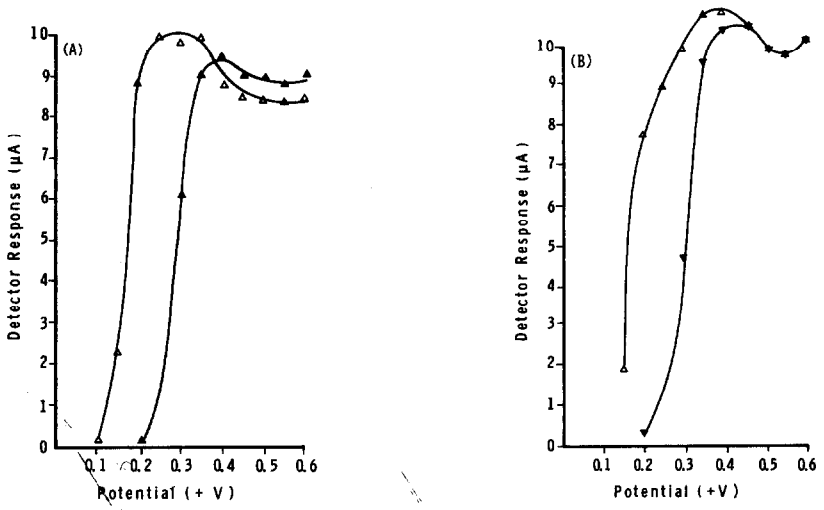

Fig. 1. Voltagrams of MHPG and iso-MHPG from both detectors at different electrode potentials. Each voltagram was determined whilst the other electrode was set at zero potential. (A) Voltagram of MHPG from detector 1 (△) and detector 2 (▲). (B) Voltagram of iso-MHPG from detector 1 (△) and detector 2 (▼).

stable for more than 24 h at room temperature. This also allowed the system to be automated.

Extraction procedure

To 1 ml of plasma spiked with internal standard contained in a 15-ml glass stoppered centrifuge tube, were added 5 ml of ethyl acetate. The tube was vortex-mixed for 1 min and then centrifuged for 5 min at 1500 *g* at 4°C. The organic phase was aspirated into a 15-ml glass centrifuge tube containing 1 ml of 0.1 *M* potassium bicarbonate. This was vortex-mixed, centrifuged and separated as above. The organic phase was transferred to a third glass centrifuge tube, and vortex-evaporated to dryness at 30°C under vacuum in a Buchler Vortex Evaporator (Fortlee, NJ, U.S.A.). The residue was re-dissolved in 100 μl of mobile phase and 20 μl were injected onto the column. Standards and blanks were extracted in the same manner.

RESULTS

Resolution and sensitivity were determined by an injection of an extracted plasma standard (Fig. 2b). The retention times of MHPG and the internal standard, iso-MHPG, were 4.1 and 7.6 min, respectively. The linearity of both the extraction procedure and detector response (determined from the peak height) was verified over the anticipated range of the assay (1—200 nmol/l). The linearity was determined by assaying pooled plasma that had been dialysed for 24 h against 200 vols. of water and then spiked with known amounts of MHPG. A calibration curve was calculated for MHPG and a linear relationship was observed between MHPG concentration and the peak height ratio over the concentration range studied. The equation for the calibration curve was $y = 0.01833x + 0.00037$; $r = 0.9999$. Each point on the calibration curve was

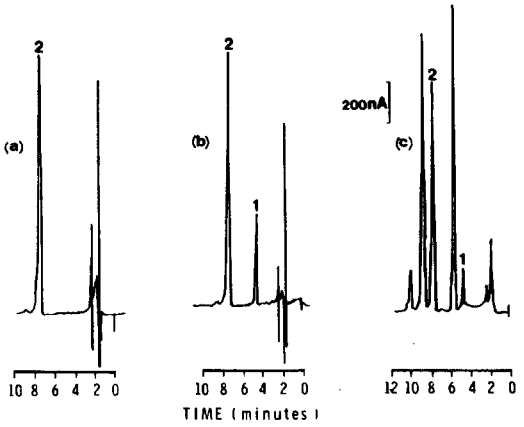

Fig. 2. Chromatography of MHPG and iso-MHPG, internal standard. (a) Chromatogram of a dialysed plasma extract spiked with 100 nmol/l iso-MHPG. (b) Dialysed plasma extract spiked with 100 nmol/l iso-MHPG and 20 nmol/l MHPG. (c) Plasma extract from a normal male subject 30 min after insertion of a catheter (12.8 nmol/l MHPG). Peaks: 1 = MHPG and 2 = iso-MHPG.

TABLE I

INTRA- AND INTER-DAY COEFFICIENTS OF VARIATION (C.V.) (n = 5)

MHPG Concentration (nmol/l)	Intra-day C.V. (%)	Inter-day C.V. (%)
1	9.0	22.5
5	6.5	22.5
10	2.8	11.0
20	2.0	13.3
50	2.0	9.6
100	1.8	11.1
150	2.4	11.5
200	1.2	12.3

calculated from the means of the inter-day assay variation data (Table I).

The actual recovery of MHPG over the anticipated concentration range was 35% (this value is not corrected for volume losses), which is lower than that reported elsewhere [7, 8, 15, 17]. Further study has shown that this can be improved to 55% with a second ethyl acetate extraction, but this was thought to be unnecessary as the minimum quantifiable concentration calculated from a peak height of twice the baseline noise was 0.5 nmol/l (2 pg of MHPG injected).

Several catecholamines, indoleamines, their metabolites and precursors have been examined for possible interference with the assay; their retention times are given in Table II. MHPG values form normal volunteers are given in Table III and are within the range reported by others [15—17, 19].

The methodology has been used in a recent research study. In this study normal healthy male volunteers received diazepam for a period of three weeks, the dose being increased over the first three days to a maximum of 25 mg per day. Subjects were sampled the morning prior to the investigation and during the third week.

TABLE II

CHROMATOGRAPHIC CHARACTERISTICS OF CATECHOLAMINES, INDOLEAMINES, THEIR PRECURSORS AND METABOLITES

Each value is for a single extraction from water of a 1 μmol/l solution prepared on the day of analysis.

Compound	Capacity factor, k
3-Methoxy-4-hydroxyphenylglycol	0.46
4-Methoxy-3-hydroxyphenylglycol	1.00
5-Hydroxytryptamine	N.D.*
4-Hydroxy-3-methoxyphenylacetic acid	N.D.
4-Hydroxy-3-methoxyphenethanol	1.84
Tryptophan	N.D.
4-Hydroxy-3-methoxymandelic acid	N.D.
3,4-Dihydroxyphenylglycol	N.D.
Noradrenaline	N.D.
Adrenaline	N.D.
Dopamine	N.D.
Normetanephrine	N.D.
Metanephrine	N.D.
5-Hydroxyindoleacetic acid	N.D.
3,4-Dihydroxymandelic acid	N.D.
Tryptamine	N.D.
Tryptophol	N.D.
5-Hydroxytryptophol	N.D.

*N.D. = Not detected.

TABLE III

FREE MHPG CONCENTRATIONS IN PLASMA OF NORMAL MALE VOLUNTEERS

All the subjects were sampled in a supine position, 30 min after insertion of a cannula.

Subject	Free MHPG (nmol/l)
1	17.4
2	15.4
3	12.8
4	20.6
5	8.4
6	17.5
7	26.5
8	19.8
Mean	17.3 ± 5.41

Subjects, in a supine position, were sampled three times at 15-min intervals via a butterfly cannula. Table IV shows that there was a significant increase ($p > 0.001$) in baseline MHPG levels during the third week of diazepam treatment as compared to normals.

TABLE IV

BASELINE MHPG LEVELS BEFORE AND DURING DIAZEPAM TREATMENT (25 mg PER DAY)

Subject	MHPG concentration (mean ± S.D., nmol/l)	
	Before diazepam treatment	After diazepam treatment
1	21.4 ± 2.0	28.5 ± 0.4
2	7.9 ± 0.5	25.5 ± 2.4
3	14.9 ± 0.9	16.7 ± 0.5
4	11.5 ± 1.2	16.5 ± 1.1

DISCUSSION

The use of the coulometric detector as opposed to the amperometric detector has several advantages, which enable it to be performed in the manner described. A full and comprehensive treatise of the potential use of coulometric detectors is given by Matson et al. [20]. The detector cell which oxidises 100% of the eluent, compared with 1—5% oxidised by the amperometric cell [18, 20] produces a substantial increase in sensitivity. Fortunately, the sensitivity is not matched by a corresponding increase in noise for two reasons. Firstly, the guard cell oxidation potential is set higher than that of the detecting cell, which removes most of the background noise associated with the solvent system. Secondly, by setting the potential of the first detector cell at 0.1 V, it reduces the response of the second detector cell (the analytical cell) to the solvent front and thus enables it to attain baseline stability earlier.

The 3-μm 10-cm column was found to decrease the assay time whilst giving good resolution of the extracted compounds. One possible disadvantage of this system is that the pore sizes of the cells are very small and could block. However, this has not yet been a problem.

It is recognized that the recovery of MHPG is relatively low, but it is felt that this is more than adequately compensated for by the greater sensitivity of the coulometric detector. The possibility of sample deterioration has been reduced by adding the internal standard to the sample prior to storage at −20° C. This approach is justified because samples have been stored for three months without any evidence of breakdown. Free plasma MHPG concentrations found in our normal volunteers compare well with those reported elsewhere [15—17, 19].

CONCLUSIONS

A novel technique using HPLC with ED has been described. It is a reliable, quick and inexpensive alternative to gas chromatography—mass spectrometry and other HPLC methods available, as a method for the determination of free MHPG in plasma. These factors render the method suitable for routine clinical analysis and neurochemical research.

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