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Note

Microassay of free 3-methoxy-4-hydroxyphenylglycol in plasma using high-performance liquid chromatography with electrochemical detection

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The central noradrenergic neurons are considered to play an important role in the pathogenesis of various cardiovascular and psychiatric disorders [1–3]. 3-Methoxy-4-hydroxyphenylglycol (MHPG) has been shown to be a major norepinephrine metabolite in the mammalian brain [4,5]. Since there is a significant correlation between MHPG concentrations in plasma and those in the brain in animals treated with drugs that alter the central norepinephrine metabolism [6], MHPG in the systemic circulation may reflect a functional activity of the central noradrenergic neurons. Although routine measurement of plasma MHPG remains controversial [7], several clinical studies have suggested that plasma MHPG concentrations may be related not only to the therapeutic response in patient with certain psychiatric disorders [8,9] but also to the prognosis in patients with subarachnoid hemorrhage [10,11]. A method for measuring plasma MHPG concentrations in humans and experimental animals, therefore, may have a considerable clinical and research utility.

Although the specific measurement of MHPG in biological fluids has been achieved by gas chromatography with mass spectrometric detection (GC–MS) [12–14], this method requires expensive equipment and laborious sample preparation. Recent studies [15–19] have demonstrated that high-performance liquid chromatography with electrochemical detection (HPLC–ED) is useful for determining MHPG in human plasma. However, in these reports, except for one

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from Minegishi and Ishizaki [17], the methods employed for sample preparation were tedious and often suffered from low recoveries. In addition, these HPLC methods seemed to have some drawback in terms of their sensitivity. Since at least 1.0 ml of plasma was required to perform the assay, it was difficult to carry out the assay in a small amount of plasma (e.g. 200 μl) obtained from paediatric patients (i.e. neonates and infants) or small experimental animals. The present report describes a sensitive HPLC–ED method for determining free MHPG in a 200-μl plasma sample using a newly developed, highly efficient carbon graphite electrode coupled with a simple solid-phase extraction method. Using the present method, serial determinations of plasma MHPG concentrations can easily be carried out in paediatric patients and in small experimental animals.

EXPERIMENTAL

Reagents

MHPG hemipiperazine salt and (±)-isoprenaline hydrochloride were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium 1-octanesulphonic acid, used as an ion-pairing agent, was purchased from Eastman Kodak (Rochester, NY, U.S.A.). All other reagents were of analytical-reagent grade and purchased from Wako (Osaka, Japan).

Instrumentation and chromatographic conditions

The analyses were performed with an HPLC system consisting of an Altex Model 110A pump (Altex Scientific, Berkeley, CA, U.S.A.), an Eicopack ODS reversed-phase column (250 mm × 4.6 mm I.D., 5-μm particle size) from Eicom (Kyoto, Japan), a Rheodyne 7125 sample injector (Rheodyne, Berkeley, CA, U.S.A.) and a Model 100 electrochemical detector with a newly developed carbon graphite electrode (Eicom). The column temperature was maintained at 12°C using a water-bath connected to a Coolnics circulator (Model CTE-22, Yamato, Tokyo, Japan).

The detector potential was set at +0.7 V versus the Ag/AgCl reference electrode. The mobile phase consisted of 0.05 *M* sodium citrate buffer (pH 4.5), 10 μM Na_2EDTA and 100 μM sodium 1-octanesulphonic acid. This mobile phase solution was mixed with methanol (92:8, v/v) and delivered at a flow-rate of 1.0 ml/min. All chromatograms were recorded on a pen recorder (Model 561, Hitachi, Tokyo, Japan).

Sample preparation

Blood was collected in a chilled tube containing 0.1% Na_2EDTA and 0.1% sodium metabisulphite, and put on ice. Plasma was separated by centrifugation at 600 *g* for 5 min at 4°C and then stored at −70°C until analysed.

Extraction was carried out using Bond-Elut columns pre-packed with 100 mg of C_{18}-bonded silica (40 μm) in a 1-ml disposable syringe (Analytichem International, Harbor City, CA, U.S.A.). The columns were rinsed with 100% methanol (1 ml) and then with double-distilled water (1 ml) before the samples were applied.

A 200-μl plasma sample mixed with the internal standard (I.S.), 20 μl of an isoprenaline solution (equivalent to 2 ng) and 200 μl of 500 μM sodium 1-octanesulphonic acid was applied to the Bond-Elut column. The column was rinsed with 400 μl of a 500 μM ion-pairing agent solution to remove the easily eluted hydrophilic plasma constituents. The elution of MHPG and the I.S. from the column was carried out with 400 μl of 0.1 M hydrochloric acid mixed with methanol (70:30, v/v). The final eluate was passed through a 4.5-μm membrane filter (Gelman Science, Tokyo, Japan) and 40 μl of this solution were injected onto the HPLC column.

To determine if this assay method could reliably measure plasma MHPG concentrations in 50-μl plasma samples, the sample preparation was performed as above, but adding a smaller amount of the I.S. (equivalent to 1 ng of isoprenaline for each sample). The final eluate was freeze-dried. The residue was reconstituted in 50 μl of the mobile phase, and 10–20 μl of this solution were injected onto the HPLC column.

Quantitation

The ratio of the peak height of MHPG to that of the I.S. was used to calculate the concentration in each sample. The calibration standards to which the known amounts of MHPG (equivalent to the final concentrations of 0, 5 and 10 ng/ml) were added were run through the entire procedures along with other samples to be analysed.

RESULTS AND DISCUSSION

Selection of an appropriate I.S. for assay of plasma MHPG using HPLC–ED is difficult, and internal standards were not used in some of the recently reported HPLC–ED assay methods [18,19]. The maximum detector responses to MHPG and structural analogues, which were evaluated as candidate internal standards and included 4-hydroxybenzyl alcohol, 2-hydroxybenzyl alcohol, 3-hydroxy-4-methoxybenzyl alcohol and 3-hydroxyphenyl-1-propanol, were obtained at applied potentials higher than +0.75 V versus Ag/AgCl reference electrode. At such high potential differences there was a substantial increase in interfering peaks and the background current, which was attributable to oxidation of various plasma constituents and detracted from the assay selectivity and sensitivity. In the present study, isoprenaline, which is oxidized at considerably lower applied potentials than MHPG (Fig. 1), was selected as the I.S. An additional advantage of using isoprenaline as the I.S. was that its retention time on the chromatogram can be manipulated independently of MHPG by using an ion-pairing agent.

Typical chromatograms obtained from plasma samples extracted from a pooled plasma with and without adding authentic MHPG and isoprenaline (I.S.) are shown in Fig. 2. Under the selected chromatographic conditions, MHPG and the I.S. are well separated from their adjacent, unidentified peaks. Since MHPG is a neutral and hydrophilic compound, it is poorly retained on a reversed-phase column, and its retention on the column is insensitive not only to changes in pH but also to the presence of ion-pairing agents in the mobile phase. We found that

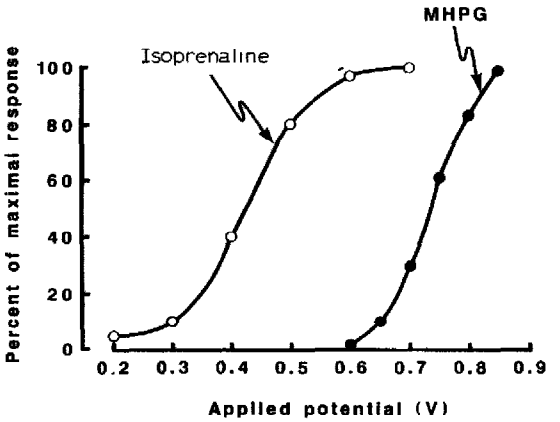

Fig. 1. Detector responses to the authentic MHPG and isoprenaline standards (1 ng each) with respect to the applied potentials (+0.20 to +0.85 V) versus Ag/AgCl reference electrode. Data were obtained under the chromatographic conditions described in the text and are expressed as percentage values relative to the maximum responses obtained for the respective compounds.

among the chromatographic conditions employed the column temperature was particularly important in ensuring the complete resolution of MHPG from its interfering peaks. The column temperature was, therefore, maintained at 12°C despite somewhat prolonged total assay time. Although we attempted to reduce the total assay time by using a simple, step-gradient technique by briefly increasing the methanol concentration in the mobile phase, the additional time required to re-equilibrate the system made this approach impractical. The total assay time might have been reduced by using a continuous gradient or column-switching

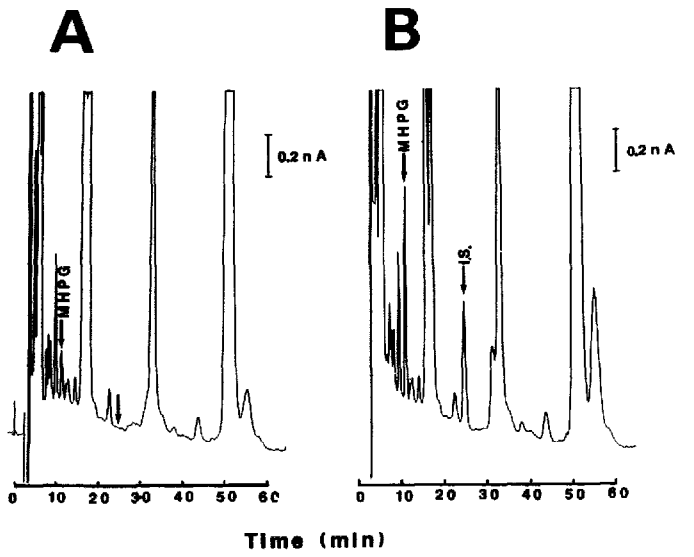

Fig. 2. Typical chromatograms obtained from (A) a blank plasma and (B) a plasma spiked with 2 ng each of MHPG and the internal standard (I.S.).

TABLE I

RETENTION TIMES OF MONOAMINES, THEIR PRECURSORS AND THEIR METABOLITES

Compound	Retention time (min)
L-DOPA	4.4
Norepinephrine	4.6
3,4-Dihydroxyphenylglycol	5.4
Epinephrine	6.0
Tyrosine	6.2
5-Hydroxytryptophan	9.5
Tryptophan	10.4
MHPG	11.5
Dopamine	13.2
3,4-Dihydroxyphenylacetic acid	18.4
Isoprenaline (I.S.)*	24.0
5-Hydroxyindoleacetic acid	46.0
Serotonin	49.4
Homovanillic acid	60.0

*I.S. = internal standard in the present assay.

technique, although these would have required additional expensive equipment (e.g. a gradient controller, a pump plus a column).

Effective and rapid extraction of MHPG from plasma is difficult owing to its neutral and hydrophilic chemical character. Previously reported extraction methods for plasma MHPG using various kinds of organic solvent were tedious and often suffered from low recoveries [15,16,18,19]. A novel solid-phase extraction method using a small C_{18} column for plasma MHPG and other monoamine metabolites, which was first introduced by Minegishi and Ishizaki [17,20], has attracted wide interest as an alternative strategy offering speed and high recovery. We adopted this approach with some minor modifications in the present study. Because fairly hydrophilic compounds, such as MHPG, are weakly adsorbed on the C_{18} sorbent, the volume of rinsing solution prior to the elution is critical to obtain a high recovery. When the rinsing volume was set at 400 μl, the loss of MHPG was minimized and the most of the hydrophilic plasma constituents, which might have interfered with the assay, were promptly and successfully removed. Volume (400 μl), methanol content (30%) and pH (ca. 1.0) of the eluent were also critical to ensure the maximum recoveries for MHPG and the I.S. Using the present method, the entire extraction procedure for a batch of ten samples was performed within a few minutes and the mean ($\pm$S.D.) recovery values for MHPG and the I.S. were 89 ± 4.9 and 103 ± 3.2%, respectively.

We also investigated whether or not basic monoamines (i.e. norepinephrine, dopamine, serotonin and epinephrine), their precursors (i.e. tyrosine, tryptophan, L-DOPA and 5-hydroxytryptophan) and their acidic and neutral metabolites (3,4-dihydroxyphenylglycol, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid and homovanillic acid) interfere with the detection of

TABLE II

ANALYTICAL PRECISION IN THE DETERMINATION OF MHPG FROM A POOLED PLASMA

	n	Concentration measured (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Within-day variation	6	3.52 ± 0.10	2.9
Day-to-day variation	6	3.53 ± 0.23	6.7

MHPG and the I.S. under the assay conditions. None of the compounds examined were found to interfere with the detection of MHPG and the I.S. (Table I).

The linearity of the assay was examined by adding known amounts of MHPG to aliquots of a pooled plasma and by analysing these samples through the entire assay procedure. There was a good linearity ($y=0.582x+0.003$, $r=0.998$) over the plasma MHPG concentration range examined (1–15 ng/ml).

We evaluated the analytical precision of the present method analysing aliquots of a pooled sample sequentially on the same day (within-day variation, $n=6$) and over the period of one month (day-to-day variation, $n=6$). The mean (± S.D.) values for plasma MHPG concentrations measured in the within-day and day-to-day precision studies were almost identical and the coefficients of variation (C.V.) obtained were small (Table II).

The mean (± S.D.) plasma concentration of MHPG measured in plasma obtained from six healthy subjects, 3.24 ± 0.54 ng/ml (range 2.74–4.18 ng/ml), showed a good agreement with those reported by several investigators using GC–MS [12–14] or HPLC–ED [15–19].

A newly developed carbon graphite electrode was demonstrated to be sensitive enough to allow the determination of MHPG in a 50-μl plasma sample, provided that the final eluate (400 μl) from the small C_{18} column was condensed before injection. In our preliminary studies an excellent linearity ($y=0.0925$, $x+0.0068$, $r=0.998$) for the calibration curve was obtained using 50-μl plasma samples spiked with MHPG to make final concentrations equivalent to 1–15 ng/ml. In addition, the mean within-day and day-to-day coefficients of variation at 5.7 ng/ml were 2.4 and 11.0%, respectively. These data indicate that with some minor modifications the present assay method could be used to perform serial determinations of plasma MHPG concentrations in small-volume blood samples, obtained from paediatric patients (neonates and infants) and small experimental animals.

In conclusion, the method described here is simple, sensitive and precise for determining plasma MHPG using 200-μl samples. Its high sensitivity is considered particularly valuable in studies involving serial determinations of plasma MHPG concentrations in a small amount of plasma sample. A simple and rapid extraction procedure will significantly reduce the time required for sample preparation.

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