Journal of Chromatography, 233 (1982) 97—105
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1462

SIMULTANEOUS DETERMINATION OF FREE 3-METHOXY-4-HYDROXY-MANDELIC ACID AND FREE 3-METHOXY-4-HYDROXYPHENYL-ETHYLENEGLYCOL IN PLASMA BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(First received April 29th, 1982; revised manuscript received July 29th, 1982)

SUMMARY

A new assay method is described for the simultaneous determination of free 3-methoxy-4-hydroxymandelic acid and 3-methoxy-4-hydroxyphenylethyleneglycol in plasma utilizing separation and purification by Bio-Gel P-10 followed by high-performance liquid chromatography with electrochemical detection. This technique is sensitive and reliable, and offers an inexpensive and practical alternative to gas chromatographic—mass fragmentographic methods for the monitoring of plasma levels of these catecholamine metabolites in the study of selective metabolic pathways of endogenous norepinephrine originating in the peripheral and the central nervous systems.

INTRODUCTION

Determination of the changes in the turnover of the monoamine transmitters and their metabolites has indicated their importance in the normal and pathological functioning of the brain. For instance, variations of urinary 3-methoxy-4-hydroxymandelic acid (VMA) and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) have been associated with depressive disorders [1—3] or states of stress and anxiety [4, 5].

The determination of the monoamine metabolites in physiological samples has mostly been performed by gas chromatography [6, 7] or by a gas chromatographic—mass fragmentographic procedure [8—11]. These latter techniques, while offering excellent selectivity and high sensitivity are either slow or very expensive to employ on a routine basis. Moreover, they require a derivatization step before chromatography can be carried out.

Reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection has been shown to provide high selectivity and sensitivity for the analysis of monoamine metabolites in urine [12—14], in cerebrospinal

0378-4347/82/0000—0000/$02.75

fluid [15] and brain tissue [16—18]. These procedures cannot however be applied to plasma samples since a low plasma concentration of these metabolites and the presence of electroactive compounds carried by proteins and lipoproteins generate peaks which hamper greatly the resolution of the chromatogram.

The aim of this article is to describe a simple, fast and sensitive method for the plasma determination of catecholamine metabolites using molecular sieve chromatography as a clean-up procedure followed by HPLC analysis with electrochemical detection.

MATERIALS

Solvents and chemicals

Methanol, HPLC grade, was purchased from Fisher Scientific (Montreal, Canada). All other reagent grade chemicals were obtained from Baker (Canlab, Montreal, Canada). The standards, *dl*-VMA and MHPG piperazine salt, were purchased from Sigma (St. Louis, MO, U.S.A.); normetanephrine (NMN), 3,4-dihydroxyphenylethyleneglycol (DOPEG), 3,4-dihydroxy-*d*-mandelic acid (DOMA) and 3,4-dihydroxyphenylacetic acid (DOPAC) from Calbiochem (La Jolla, CA, U.S.A.). The Bio-Gel P-10 (100—200 mesh) was obtained from Bio-Rad (Mississauga, Canada). The water used throughout the experiment was deionized and glass double-distilled.

Instrumentation for extraction and evaporation

The equipment used in the extraction procedure was the Evapo Mix and the Vortex evaporator (all from Buchler, Fort Lee, NJ, U.S.A.), connected to a vacuum pump for the organic solvent evaporation.

METHODS

Preparation of the gel column

Dry Bio-Gel P-10 powder (1 g) was suspended in 50 ml of distilled water by gentle stirring and allowed to swell for at least 30 min. It was rinsed and decanted twice with distilled water, then equilibrated by replacing the water with the eluting buffer, decanting and renewing this buffer four times. The eluting buffer was 0.05 *M* sodium phosphate, pH 6, containing $2.7 \cdot 10^{-3}$ *M* EDTA and $6.3 \cdot 10^{-5}$ *M* ascorbic acid.

The barrel of a 10-ml disposable syringe (Beckton-Dickinson, Mississauga, Canada) was used as a column. For this purpose, a cotton-wool plug was placed at the bottom. In order to standardize the chromatographic procedure, a two-channel peristaltic pump (Buchler) was used. The outlet of the column was linked to the fraction collector by a catheter and a tubing connector passing through one of the channels of the pump. An identical tubing system going through the other pump channel provided the connection between the buffer reservoir and the upper part of the column. These instrumental conditions ensured reproducibility of the flow-rate and flexibility in the operation. The entire system can, in effect, be shut off and reopened at any time and still retain the same characteristics.

The syringe was partly filled with buffer and the gel slurry was added, stirring constantly, then allowed to pack under gravity. The final volume of the gel bed was 5 ml. This and the following operations were all carried out at room temperature.

The characteristic parameters of the elution diagram were established using a 2 mg/ml solution of Dextran Blue (Pharmacia, Uppsala, Sweden) containing 100 ng/ml each of VMA and MHPG. An LKB (Bromma, Sweden) Model 7000 fraction collector with disposable styrene tubes (12 × 75 mm) was used for collecting the eluate.

Plasma sample preparation

Clean-up step by gel filtration. The liquid above the gel bed was drained. When the surface of the gel bed was almost dry, the flow was stopped and 1 ml of plasma mixed with 10 μl of internal standard solution containing 8 ng of hydroquinone (HQ) was carefully applied along the walls in order to avoid disturbances in the gel bed. As soon as the sample was applied, the flow was resumed and the sample allowed to penetrate the gel until the surface was again almost dry. Then, 1 ml of eluting buffer was applied, as described above, in order to rinse the walls of the column and the gel surface and the draining procedure was repeated. A final aliquot of 1 ml of buffer was applied and the column connected to the reservoir of eluting buffer. The elution was carried out at a flow-rate of 30 ml/cm^2/h. Fractions of 0.5 ml were collected from the moment that the plasma entered the gel bed. The first eight tubes, corresponding to a volume of 4 ml, containing proteins and high-molecular-weight substances were discarded. The following seven tubes, for a total of 3.5 ml, were collected and their contents pooled for the subsequent analytical step. The gel column was rinsed with 2 ml of buffer before the application of the next sample.

Extraction. The 3.5 ml of pooled eluate were put into a 20-ml screw-cap test tube and 2 g of sodium chloride were added. The pH was adjusted to 3 with phosphoric acid, 30% (v/v). A volume of 10 ml of ethyl acetate was then added. The tube was shaken for 5 min on the Evapo Mix and centrifuged for 5 min at 2000 *g*. The organic upper layer was collected and the aqueous phase was then reextracted twice with 5 ml of ethyl acetate. The three ethyl acetate extracts were pooled and evaporated to dryness under vacuum at 30°C on the Vortex evaporator. The tube was then rinsed with 1.5 ml of methanol, which were evaporated to dryness. The residue was redissolved in 150 μl of the mobile phase and 100 μl were injected onto the HPLC column.

HPLC instrumentation

The HPLC system consisted of a Model 6000 A solvent delivery pump with a U6K injector (all from Waters Assoc., Milford, MA, U.S.A.), and an ODS reversed stationary phase column (Spherisorb 5 μm, 250 × 3 mm, Brownlee, Santa Clara, CA, U.S.A.) protected by a MPLC guard column (Brownlee). The detection system consisted of a thin-layer flow-through electrochemical cell with glassy carbon as the working electrode, a silver/silver chloride reference electrode (Bioanalytical Systems, W. Lafayette, IN, U.S.A., Models TL 5 and LC 4A), and a recorder–integrator (Data Module 730, Waters Assoc.).

The glassy carbon electrode was repolished once a week or as required, using the polishing kit supplied with the detector.

HPLC conditions

The solvent system consisted of a 97:3 (v/v) mixture of 0.07 *M* sodium phosphate buffer and methanol containing sodium EDTA (0.01%). The pH was adjusted to 2.5 with phosphoric acid. This mobile phase was filtered through 0.45-μm type HA Millipore filters and degassed under vacuum. The flow-rate was set at 1.2 ml/min. The effluent was passed through the detector cell and monitored at a potential of +0.8 V versus the silver/silver chloride reference electrode. The chart speed of the recorder was set at 0.25 cm/min.

Standard solutions of HQ, VMA and MHPG

Stock solutions of HQ, VMA and MHPG were prepared at concentrations of 100 μg/ml in methanol and stored at −40°C. Working standard solutions were obtained by appropriate dilution of the stock solutions with 0.05 *M* citric acid.

Preparation of spiked plasma samples

One volume of pooled human plasma was dialysed for 24 h against 200 volumes of an 0.15 *M* sodium phosphate buffer pH 7.4 for elimination of endogenous VMA and MHPG. The dialysed plasma was then spiked with known concentrations of VMA and MHPG ranging from 2 to 32 ng/ml and with HQ (8 ng/ml) as internal standard.

Calculations

All measurements were done by estimation of peak heights. Four calibration standards were used with each set of unknown samples to determine the concentrations of VMA and MHPG. The calibration standards were prepared from the pooled dialysed plasma spiked with a known quantity of VMA and MHPG (4—8 ng/ml) and internal standard. The calibration standards were run through the entire procedure along with other samples to be analysed. The ratio of the peak height (PHR) for each metabolite to that of the internal standard was used to calculate the concentration according to the formula:

$$\text{Concentration sample} = \frac{\text{PHR}_{\text{sample}}}{\text{PHR}_{\text{plasma standard}}} \times \text{concentration}_{\text{plasma standard}}$$

RESULTS AND DISCUSSION

Clean-up procedure

A molecular sieve was selected for the elimination of proteins from plasma prior to chromatography; the principle was identical to the desalting of a protein solution. The current methods for plasma deproteinization by perchloric acid, ethanol or by ultrafiltration did not give satisfactory results due to the presence of interfering peaks on the chromatograms, thus resulting in poor resolution. The experimental conditions were chosen based on several

criteria: a satisfactory separation of proteins from the small molecular size compounds enabling a chromatogram, devoid of interfering peaks, to be obtained together with recovery of the metabolites in a volume of eluate suitable for extraction with small volumes of solvent. These objectives were met by using a short and wide gel column providing a high flow-rate. The size of the sample, which was fairly large compared to the total bed volume, nevertheless remained compatible with the characteristics of the column. Indeed, the parameters, as determined for our system on the elution diagram (see Fig. 1) gave a separation volume of 3 ml: thus, theoretically, a 3-ml sample could be applied. Although sample volumes as high as 30% of the total bed volume which would represent in our case 1.5 ml [19], could be used, we have deliberately kept the volume of the applied sample to 1 ml in order to minimize the risk of cross contamination by overlapping peaks. The polyacrylamide matrix, Bio-Gel P-10, proved to be satisfactory for the deproteinization procedure, as it is easy to handle and re-usable for months without deterioration once the volume characteristics of the column have been established.

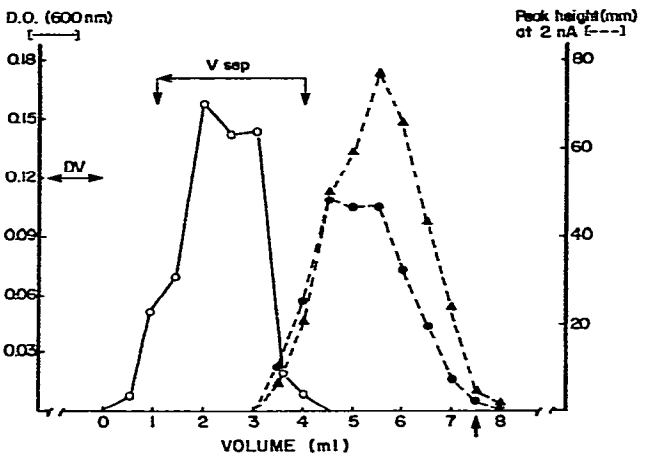

Fig. 1. Elution profile of a solution of Dextran Blue, VMA and MHPG. The sample volume applied is 1 ml. The gel bed dimensions are 3 × 1.46 cm. V sep = V_e (MHPG or VMA) — V_e (Dextran Blue). V_e is the volume eluted from the start of the sample application on the column to the inflexion point (↓) of the elution peak. Dead volume (DV) refers to the volume of the tubing system. ○, Dextran Blue; ●, VMA; ▲, MHPG.

HPLC analysis

Under the conditions described, HQ (internal standard), VMA and MHPG gave sharp, well separated peaks with retention times of 9, 10.3 and 14.8 min, respectively. The chromatograms of aqueous standards, dialysed plasma blanks, dialysed plasma spiked with HQ, VMA and MHPG, and plasma sample from one subject are illustrated in Fig. 2A, B, C and D. Under these chromatographic conditions, no interference with the other naturally occurring acidic and neutral electroactive endogenous compounds were observed, as shown in Fig. 2E.

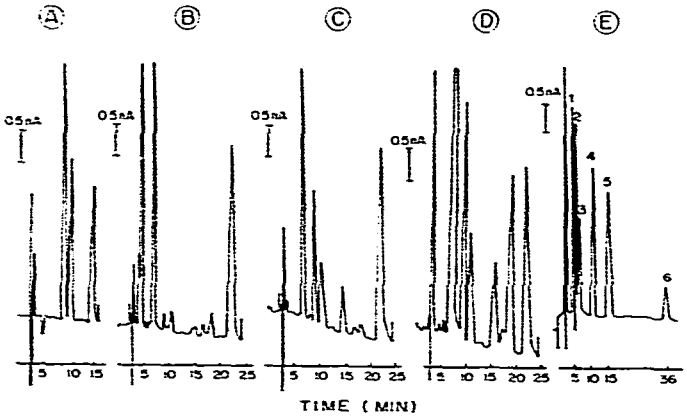

Fig. 2. Chromatography of VMA and MHPG. (A) Chromatogram of aqueous standards (5 ng of HQ, VMA, MHPG, in their order of elution); (B) plasma blank (dialysed plasma); (C) plasma blank spiked with 8 ng of HQ (internal standard) and 4 ng each of VMA and MHPG; (D) plasma sample from one subject (the estimated concentrations for VMA and MHPG are 4.12 and 4.07 ng, respectively); (E) separation of a synthetic mixture containing 5 ng each of the following: (1) 3,4-dihydroxymandelic acid; (2) 3,4-dihydroxyphenylglycol; (3) normetanephrine; (4) VMA; (5) MHPG; (6) 3,4-dihydroxyphenylacetic acid. Chromatographic conditions: 250 × 3 mm column of Spherisorb-ODS, 5 μm; mobile phase, 0.07 *M* phosphate buffer—methanol (97:3, v/v) containing 0.01% sodium EDTA; flow-rate 1.2 ml/min; detector, 0.8 V vs. the Ag/AgCl reference electrode.

Recovery

Known amounts of VMA and MHPG were added to the dialysed plasma samples and were put through the whole analytical procedure. The peak heights of VMA and MHPG from these samples were compared to those of aqueous standard solutions of these metabolites, at known concentrations, to determine the absolute percent recovery. The data are given in Table I. The average percent recovery is 59.3% and 52.7% for VMA and MHPG, respectively.

To investigate the influence of the pH of the eluting buffer with which the ethyl acetate extraction was performed, aliquots of 3.5 ml were spiked

TABLE I

ABSOLUTE RECOVERY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF VMA AND MHPG IN PLASMA

Concentration spiked into plasma* (ng/ml)	VMA		MHPG	
	Absolute recovery (%)	Relative standard deviation (%) (n = 3)	Absolute recovery (%)	Relative standard deviation (%) (n = 3)
2	63.1	6.0	57.4	5.2
4	59.5	5.1	49.1	11.4
8	51.6	7.5	55.8	7.7
16	59.6	6.7	51.7	5.2
32	62.8	8.5	49.5	6.3

*Concentrations were identical for VMA and MHPG.

TABLE II

INFLUENCE OF pH ON THE RECOVERY OF VMA, MHPG AND HQ FOLLOWING THE ETHYL ACETATE EXTRACTION

Results represent the absolute percent recovery by comparing the peak heights of VMA, MHPG and HQ obtained from spiked samples [2] to that of aqueous standard solutions.

pH of buffer	VMA	MHPG	HQ
1.5	80.7%	38.9%	50.5%
3	59.5%	58.5%	43.5%
5	37.9%	54.6%	9.0%

with 5 ng each of VMA, MHPG and HQ and the pH varied. The results are shown in Table II. The VMA recovery is lower at pH 3 and pH 5 where this acidic metabolite is partially ionized. As expected, the recovery of the neutral metabolite MHPG is not affected between pH 3 and pH 5. Nevertheless, a lower recovery has been noted at pH 1.5. The recovery of the internal standard is 43.5%. The use of an antioxidant such as ascorbic acid at $6.3 \cdot 10^{-5}$ *M* in the eluting buffer is necessary for constant recovery of VMA, MHPG and HQ. Other antioxidants, dithiothreitol and sodium bisulfite were tested. In our experimental conditions, dithiothreitol is not useful, since it produces a peak interfering with MHPG. Important variations in the results have been noted when using sodium bisulfite at $5.3 \cdot 10^{-3}$ *M* in the eluting buffer.

Linearity of response and detection limits

For calibration purposes, the standard curves for VMA and MHPG, added to dialysed plasma in concentrations ranging from 2 to 32 ng/ml, were prepared by plotting concentration against peak height ratio. Least squares linear regression analysis was used to determine the slope, y intercept and correlation coefficient. For VMA, $y = 0.024x + 0.003$ ($r = 0.996$); for MHPG, $y = 0.028x + 0.016$ ($r = 0.998$) where y is peak height ratio and x is the concentration in ng. Under these conditions, good linearity is obtained for both metabolites. In other experiments, a linear relationship was also obtained for these substances at concentrations up to 100 ng/ml.

The detection limit for both metabolites in spiked plasma samples, based on a signal-to-noise ratio of 2, was found to be 2 ng/ml for VMA and 1.2 ng/ml for MHPG.

Reliability and accuracy

The reliability of the assay was tested with three plasma samples for each concentration of 2, 4, 8, 16 and 32 ng/ml of both metabolites. The relative standard deviations are given in Table I.

In order to check the accuracy of the method, a technician to whom the exact concentration was unknown, did triplicate determinations of plasma samples spiked with VMA, MHPG (3 ng/ml) and with HQ (8 ng/ml) as an internal standard. The values obtained were 2.8 ± 0.23 ng/ml (mean ± S.D.) for VMA and 2.8 ± 0.28 ng/ml for MHPG.

Method validation

Using vacuum blood collection tubes containing EDTA as an anticoagulant, blood samples (7—8 ml) were collected from the antecubital vein of three volunteers after an overnight fast. Plasma was removed immediately after centrifugation and stored at —40°C. The results of triplicate determinations of free VMA and MHPG present in these samples are given in Table III. Notable individual differences are observed in the plasma level of MHPG. Our findings confirm the results reported by Dekirmenjian and Maas [7]. The MHPG average concentration in our study is almost identical to that reported by Takahashi et al. [10] with the gas chromatographic—mass fragmentographic procedure. Nevertheless, plasma levels of free VMA from our determinations are lower [9, 10].

TABLE III

MEAN PLASMA FREE VMA AND MHPG IN VOLUNTEERS

Experiments were done in triplicate.

Subject	VMA (ng/ml)	MHPG (ng/ml)
1	4.77 ± 0.14	4.87 ± 0.32
2	4.27 ± 0.007	5.85 ± 0.7
3	4.58 ± 0.225	3.15 ± 0.25

In conclusion, liquid chromatographic analysis coupled with sensitive electrochemical detection as described in this paper, combined with a simple clean-up procedure using a polyacrylamide gel provides an inexpensive method to determine the profile of endogenous catecholamine metabolites. This method also offers an attractive possibility for monitoring plasma levels of VMA and MHPG in patients with psychiatric and metabolic disorders.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. G. Deziel for her skilled technical assistance. This work was supported by a grant from le Comité d'attribution de fonds interne de recherche de l'Université de Montréal and in part, by a development grant from the Medical Research Council of Canada (MRC). D. Lamontagne was recipient of a summer undergraduate research scholarship from MRC.

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