Journal of Chromatography, 424 (1988) 255–262
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3976

AUTOMATIC DETERMINATION OF URINARY 4-HYDROXY-3-METHOXYMANDELIC (VANILLYLMANDELIC) ACID BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

P. NI, F. GUYON*, M. CAUDE and R. ROSSET

Laboratoire de Chimie Analytique de l'Ecole Supérieure de Physique et de Chimie de Paris, 10 Rue Vauquelin, 75005 Paris (France)

(First received June 11th, 1987; revised manuscript received September 28th, 1987)

SUMMARY

An automated liquid chromatographic method for the determination of urinary concentrations of 4-hydroxy-3-methoxymandelic acid (VMA) is described. Urine samples are purified by solid-phase extraction on an anion-exchange cartridge and automated on-line chromatographic elution is carried out using a Varian AASP (advanced automated sample processor) system. The column effluent is monitored with an electrochemical detector using a glassy carbon working electrode. The method allows the determination of VMA in 0.05 ml of normal urine with a relative standard deviation of less than 3%. The analysis time can be shortened by use of back-flushing technique, and the correlation with a classical (but non-automated) VMA analysis method is excellent.

INTRODUCTION

Vanillylmandelic acid (4-hydroxy-3-methoxymandelic acid, VMA) is the major metabolite of epinephrine (E) and norepinephrine (NE) in urine. A knowledge of the excretion patterns of VMA is an effective aid in the diagnosis of catecholamine-secreting tumours such as pheochromocytoma, neuroblastoma and ganglioneuroma [1].

Many procedures for the determination of VMA have been described, including spectrophotometry [2], paper chromatography [3], thin-layer chromatography [4], electrophoresis [5] and gas chromatography with flame ionization detection [6], electron-capture detection [7] or coupled with mass spectrometry [8]. Today, high-performance liquid chromatography (HPLC) is more frequently used, with UV, fluorimetric, post-column reaction and electrochemical detection, and the last method may be considered to be the best approach for routine investigations [9].

0378-4347/88/$03.50

The direct determination of VMA in urine after its separation by HPLC is complicated because many other urinary compounds are electrochemically oxidizable. Hence purification of urine samples before HPLC is required. Various methods for isolating urinary organic acids have been reported [10]. Solvent extraction [11] and/or anion-exchange on disposable columns [12] are generally used. These techniques afford sufficient precision and reproducibility when an internal standard such as iso-VMA (3-hydroxy-4-methoxymandelic acid) is used but are very time consuming.

The Varian AASP (advanced automated sample processor) provides partial automation of the purification procedure. This technique [13, 14], which performs both sample purification and drug (or any metabolite) concentration, is very attractive for many laboratories owing to the quicker sample turn-round times, greater efficiency and better staff motivation, all leading to time and cost savings.

EXPERIMENTAL

Apparatus

HPLC was carried out with a Varian 2010 pump equipped with an extra damper (Touzart et Matignon, Vitry sur Seine, France), a Varian AASP system and a 15×0.46 cm I.D. column packed with LiChrosorb RP-18 (particle size 5 μm) (Merck, Darmstadt, F.R.G.). The column effluent was monitored with a Model AMOR amperometric detector (Chrompack, Les Ulis, France) operating on a three-electrode potentiostatic system with a glassy carbon working electrode and an Ag/AgCl reference electrode. The working potential was set at 0.70 V vs. Ag/AgCl. This detector was connected to a Shimadzu CR-3A recorder–integrator (Touzart et Matignon).

Chemicals

VMA was obtained from Sigma (St. Louis, MO, U.S.A.) and iso-VMA from Janssen (Beerse, Belgium). Other chemicals (Normapur grade) were purchased from Prolabo (Paris, France).

VMA and iso-VMA standard solutions (1 g/l) were prepared in 0.1 *M* hydrochloric acid and stored at 5°C. Dilute working standards (1–10 mg/l) in 0.1 *M* hydrochloric acid were prepared daily. Deionized, quartz-distilled water was used.

Bis-Tris (purchased from United States Biochemical, Cleveland, OH, U.S.A.) was prepared in distilled water at a concentration of 0.05 *M* with a pH of 7.5.

Sample collection and preparation

Human urine samples (24-h) were collected in plastic bottles containing 10 ml of concentrated hydrochloric acid. A portion of the urine was stored frozen until analysis.

Sample preparation was carried out by the AASP system, specifically designed to integrate extraction on bonded-phase columns, with automated on-line chromatographic elution. In the AASP system, each cassette is composed of ten sorbent cartridges, each of which is packed with 40–50 mg of strong anion exchanger

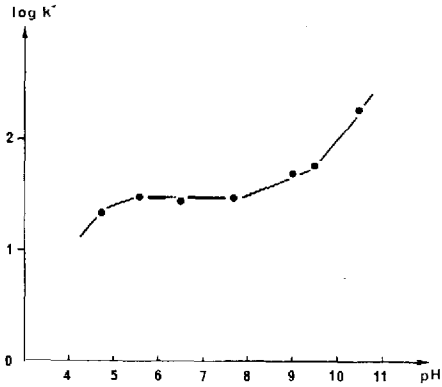

Fig. 1. Variation of log k' of VMA with pH. Column: 10 cm×0.2 cm I.D. Stationary phase: SAX bonded silica gel (20 μm) obtained from AASP cassette. Mobile phase: 0.005 M phosphate buffer at indicated pH values. Flow-rate: 1.2 ml/min. Electrochemical detection: 0.85 V vs. Ag/AgCl.

(SAX) bonded silica gel. Up to ten cassettes (i.e., 100 samples) can be loaded at one time. The sample number and analysis time and also the valve reset time can be programmed.

Procedure

In the first stage, the sorbent cartridge was washed with 2 ml of methanol, 2 ml of water and 1 ml of Bis-Tris buffer (0.05 M, pH 7.5). In the second step, 0.05 ml of urine sample (or standard solution), 0.05 ml of internal standard (iso-VMA, 3 mg/l) and 1 ml of the Bis-Tris buffer for pH adjustment were added to the reservoir placed above the cassette and percolated through the sorbent cartridge using a pneumatic manifold. The cartridge was then washed with 2 ml of water.

Chromatographic conditions

The mobile phase was 0.005 M phosphate buffer (pH 2.6) containing 0.05 M sodium sulphate and 3% (v/v) methanol at a flow-rate of 1.2 ml/min. Electrochemical detection was carried out at 0.70 V vs. Ag/AgCl.

DISCUSSION

Extraction conditions

According to Hanai and Hubert [15], the hydrophobic constant of Rekker of VMA is weak (log P=0.76). This hydrophobic character does not allow the quantitative extraction of VMA through a C_{18} bonded silica column. On the contrary, the acid–base properties (pK_{a1}=3.25; pK_{a2}=9.45) suggest extraction on an SAX column in a basic medium. Fig. 1 shows the VMA capacity factor (k') for silica anion-exchange column (same type as the AASP cassette) chromatography versus pH. The extraction of VMA should be quantitative only at pH above 10.5 where the phenol function is ionized. The ionic interactions between

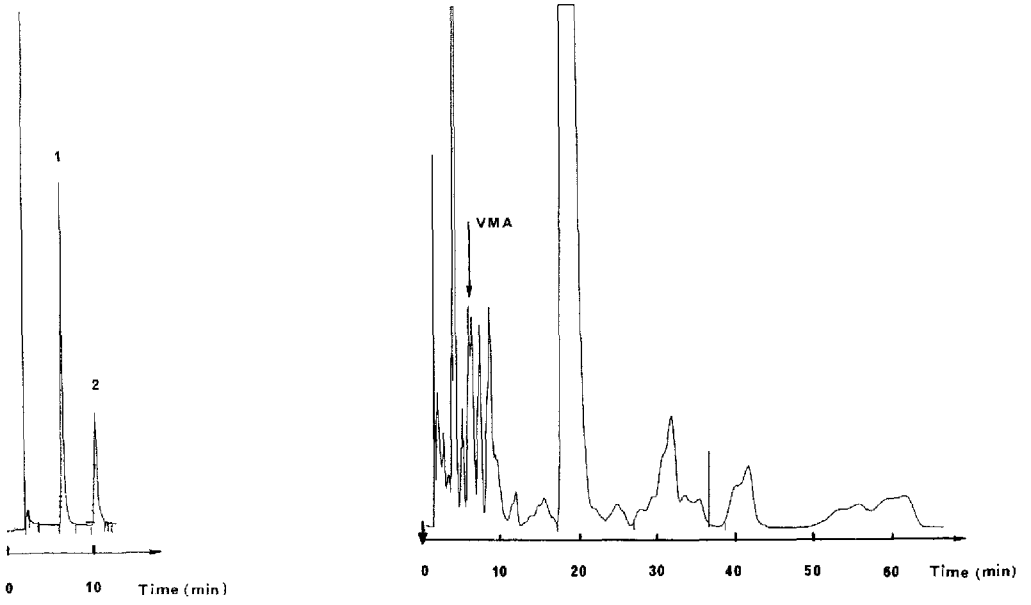

Fig. 2. Separation by reversed-phase chromatography of a synthetic mixture of VMA and iso-VMA. Column: LiChrosorb RP-18, 5 μm (15×0.46 cm I.D.). Mobile phase: 0.005 *M* NaH_2PO_4–0.05 *M* Na_2SO_4 (pH 2.6) containing 3% (v/v) methanol at a flow-rate of 1.2 ml/min. Electrochemical detection: 0.85 V vs. Ag/AgCl. Sensitivity: 1000 nA full-scale. Peaks: 1 = VMA (3 mg/l); 2 = iso-VMA (3 mg/l).

Fig. 3. Chromatogram obtained from a normal human urine sample. Extraction solvent pH: 10.6. Electrochemical detection: 0.85 V vs. Ag/AgCl. Other conditions as in Fig. 2.

the carboxylic group of VMA and the stationary phase were insufficient for quantitative extraction.

In preliminary experiments, we fixed the pH of the extraction solvent at 10.6 using 0.005 *M* phosphate buffer. The extraction recovery of VMA from synthetic mixtures was then 94 ± 3% ($n = 10$) in the range 1–5 mg/l.

Optimization of chromatography, standard chromatograms and urinary chromatograms

The complete elution of VMA from the cartridge to the analytical column was carried out with the mobile phase used for the analytical separation, 0.005 *M* NaH_2PO_4 (pH 2.6)–methanol (97:3, v/v). At this pH VMA is partially undissociated and, consequently, its retention on the anion-exchange cartridge is very weak. Further, phosphoric acid (0.25 *M*) was used to eliminate trapped air from the cartridge bed and to obtain VMA and iso-VMA in the molecular form. The addition of sodium sulphate as an electrolyte to the mobile phase allowed the complete elution of VMA and iso-VMA from the SAX cartridge by increasing the ionic strength and also provided sufficient conductivity in the effluent when it was monitored electrochemically. Hence quantitative elution is always obtained.

The detection potential was first set at 0.85 V vs. Ag/AgCl based on the VMA hydrodynamic voltammogram obtained in this study.

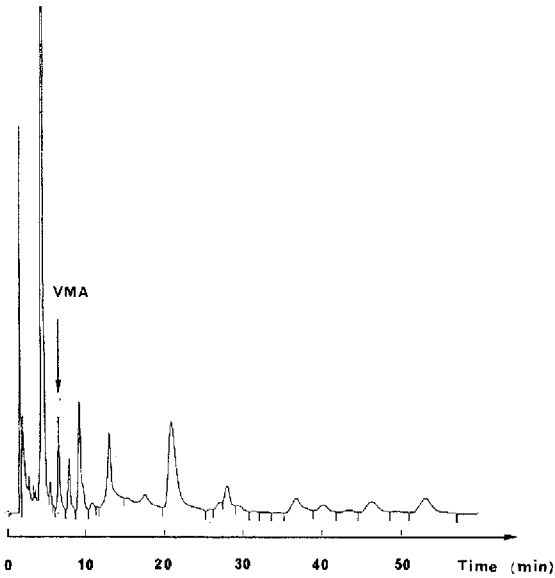

Fig. 4. Chromatogram obtained from a normal human urine sample. Extraction solvent pH: 7.5. Electrochemical detection: 0.70 V vs. Ag/AgCl. Other conditions as in Fig. 2.

A typical chromatogram of a synthetic mixture is shown in Fig. 2 and Fig. 3 shows a chromatogram of a urine sample. Many interferences affect the VMA peak. In order to reduce this drawback, a less basic pH (7.5) was used for VMA extraction with the SAX column (the extraction recovery from a synthetic mixture was then $81 \pm 3\%$, $n = 10$ in the range 1–5 mg/l) and, at the same time, the detection potential was set at 0.70 V vs. Ag/AgCl, which constituted a good compromise between sensitivity and selectivity. A chromatogram of the same urine sample is shown in Fig. 4. The number of peaks decreased, particularly near that of VMA.

Extraction recovery from urine, final method

The extraction recovery was independent of the VMA level in the urine samples but varied from one sample to another, as shown in Fig. 5, where the VMA and iso-VMA peak areas are plotted against concentration (spiked urine samples). The various salt (especially anion) concentrations in different urine samples could be a tentative reason for this variation in extraction recovery.

Iso-VMA was absent from human urine and its extraction recovery from a spiked urine sample on an SAX sorbent cartridge was very similar to that of VMA (its curve is parallel to that of VMA in Fig. 5); for these reasons it can be used as an internal standard. Moreover, we noticed that when the cassette was kept for a long period (5 h) at room temperature, the amounts of VMA and iso-VMA decreased in the same proportion (by 4.8% per hour).

Good linearity was obtained between peak area and concentration of VMA in both standard solutions and spiked urines in the range 1–10 mg/l (5–50 μmol/l). The coefficient of variation was 2.4% (within-day) for VMA ($n = 8$) at a con-

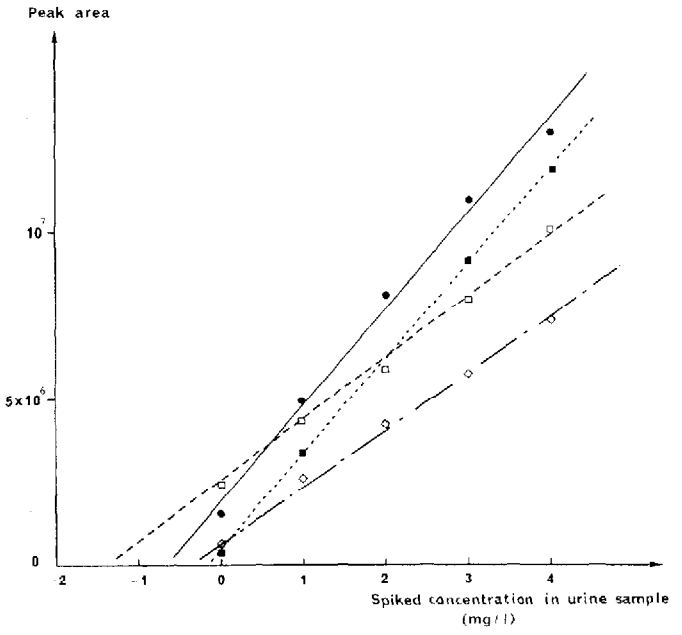

Fig. 5. Plot of VMA and iso-VMA peak areas against their concentrations added to urine for two different urine samples. —, VMA curve for the first urine sample (slope, $2.91 \cdot 10^6$); - - -, iso-VMA curve for the first urine sample (slope, $2.87 \cdot 10^6$); – – –, VMA curve for the second urine sample (slope, $1.85 \cdot 10^6$); —·—·—, iso-VMA curve for the second urine sample (slope, $1.73 \cdot 10^6$).

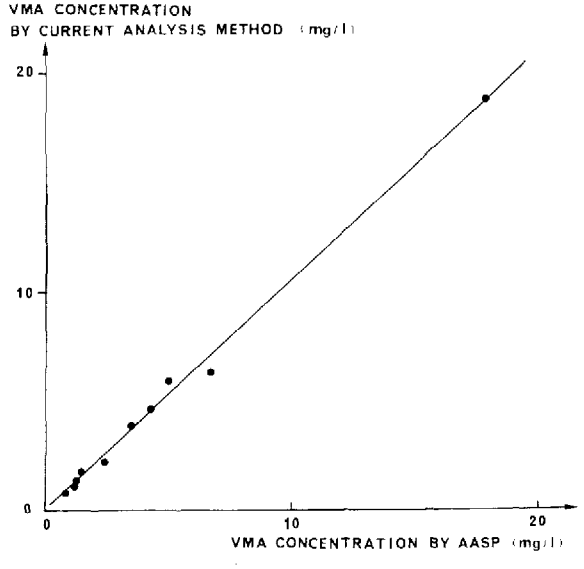

Fig. 6. Plot of VMA concentration in human urine samples by current analysis method [16] versus that measured by the described method ($n = 10$, $r = 0.996$, slope $= 1.06$).

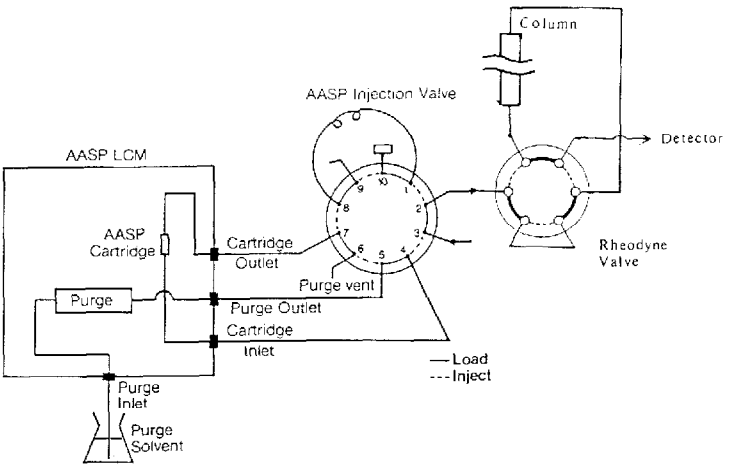

Fig. 7. Back-flushing system using an additional Rheodyne 7010 valve operated by AASP.

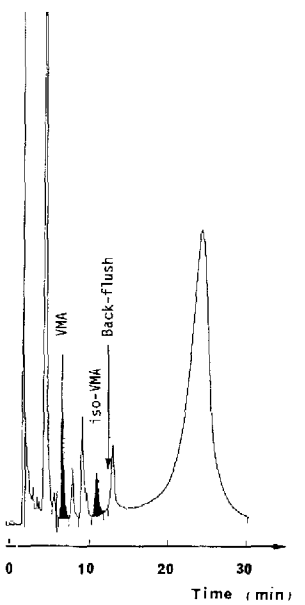

Fig. 8. Chromatogram obtained from a spiked urine sample using the back-flushing technique. Chromatographic conditions as in Fig. 4.

centration of 4 mg/l (20 μmol/l) in spiked urine samples and 3.5% (between-day) at a concentration of 2.6 mg/l (13 μmol/l) in urine samples.

The detection limit of VMA in standard solution was 2 μg/l (10 nmol/l). The signal-to-noise ratio was at least 3:1 for VMA at this concentration.

The described AASP procedure was compared with a current procedure involving anion exchange on disposable column and HPLC [16]. There was good agreement between the values obtained by the two methods (Fig. 6). A correla-

tion coefficient of 0.996 and a slope of 1.06 were obtained for urine samples analysed by these two methods.

Owing to the long analysis time, it is advantageous to back-flush the analytical column after elution of iso-VMA. This is achieved by using an additional Rheodyne 7010 valve (operated by AASP) according to Fig. 7. A chromatogram obtained in this way for a spiked urine sample is shown in Fig. 8. The analysis time is now 30 min, which is half that obtained without back-flushing. In addition, this procedure allows clean-up of the chromatographic column before each injection.

ACKNOWLEDGEMENT

We thank Chrompack France for the loan of the Model AMOR amperometric detector used in this study.

REFERENCES

1 W.M. Manger and R.W. Gifford, Jr., Pheochromocytoma, Springer, New York, 1977.
2 J.J. Pisano, J.R. Crout and D. Abraham, Clin. Chim. Acta, 7 (1962) 285.
3 S.E. Gitlow, M. Mendlowitz and L.M.Bertani, Am. J. Cardiol., 26 (1970) 270.
4 J.S. Annino, M. Lipson and L.A. Williams, Clin. Chem., 11 (1965) 905.
5 W. Von Studnitz, Scand. J. Clin. Lab. Invest., 12 (1960) 30.
6 C. Leiendecker-Foster and E.F. Freier, Clin. Chem., 27 (1981) 2029.
7 J. Chauhan and A. Darbre, J. Chromatogr., 183 (1980) 391.
8 S. Takahashi, M. Yoshioka, S. Yoshiue and Z. Tamura, J. Chromatogr., 145 (1978) 1.
9 A.M. Krstulović, J. Chromatogr., 229 (1982) 1.
10 C. Boujet, C. Drouet, G. Decoux and A. Favier, J. Chromatogr., 228 (1982) 67.
11 M.G. Horning, K.L. Konx, C.E. Dalgliesh and E.C. Horning, Anal. Biochem., 17 (1966) 244.
12 A. Relman, S.C. Gates and J.W. Webb, J. Chromatogr,. 228 (1982) 103.
13 L. Yago, Int. Lab., 18 (No. 9) (1985) 40.
14 R.D. McDowall, J.C. Pearce and G.S. Murkitt, J. Pharm. Biomed. Anal., 4 (1986) 3.
15 T. Hanai and J. Hubert, J. Chromatogr., 239 (1982) 527.
16 C. Fenech, F. Guyon and F. Dauchy, Clin. Chim. Acta, 153 (1985) 49.