Journal of Chromatography, 527 (1990) 11–19
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5162

Determination of urinary vanillylmandelic acid by direct injection and coupled-column chromatography with electrochemical detection

BRITT-MARIE ERIKSSON*, BENGT-ARNE PERSSON and MARGARETA WIKSTRÖM

Bioanalytical Chemistry, AB Hässle, S-431 83 Mölndal (Sweden)

(First received September 26th, 1989; revised manuscript received November 22nd, 1989)

SUMMARY

An automated column-switching system for determination of vanillylmandelic acid in urine is described. The liquid chromatographic system was composed of two separation columns with different selectivity properties, an octadecyl column coated with tributyl phosphate as stationary liquid phase and a silica-based anion exchanger. Urine samples were injected directly onto the first column, where vanillylmandelic acid was separated from the main part of the sample matrix. The internal standard isovanillylmandelic acid was co-eluting with vanillylmandelic acid, and a fraction of the eluate containing both substances was switched to the second column, where separation was performed. To assess peak purity, detection was performed with dual working electrodes in parallel mode. A relative standard deviation of 3.5% was obtained for determination of human urine samples containing 3 μM vanillylmandelic acid, and less than 0.1 μM could be detected.

INTRODUCTION

Vanillylmandelic acid (VMA) is the main urinary metabolite of epinephrine and norepinephrine, and is used as a diagnostic tool in various cardiovascular and neurological disorders. Measurement of VMA in a complex urine matrix often requires extensive purification of the samples. Most techniques are based on organic solvent extraction [1–4], anion-exchange resin [5–8] or different types of solid-phase extraction [9–11] before liquid chromatographic separation combined with fluorimetric or electrochemical detection. Direct injection of urine has also been attempted in conjunction with post-column reaction

0378-4347/90/$03.50

[12], gradient elution [13,14] or column-switching technique [15,16]. No internal standard has been incorporated in the methods comprising direct injection [12–16] although, in amperometric assays, it has the advantage of compensating for minor fluctuations in electrode response during the chromatographic process.

The assay presented here is a simple procedure for quantifying VMA by injecting urine directly into a coupled-column system and monitoring the effluent electrochemically.

EXPERIMENTAL

Apparatus

The chromatographic system was composed of two Model 2150 pumps (LKB, Bromma, Sweden), a Model 460 autosampler (Kontron, Zürich, Switzerland) with a refrigerated sample tray, two Model 4270 integrators (Spectra-Physics, San Jose, CA, U.S.A.), a UV detector, SpectroMonitor III (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and electrochemical detectors: an M460 (Waters Assoc., Milford, MA, U.S.A.) with a glassy carbon working electrode or two combined BAS LC-4B with dual glassy carbon working electrodes in parallel mode (Bioanalytical Systems, West Lafayette, IN, U.S.A.) or an ESA 5100 A Coulochem (Environmental Sciences Assoc., Bedford, MA, U.S.A.), with a Model 5011 analytical cell. The switching events were controlled by the integrator and were performed by pneumatically operated six-port valves, Model 7010 (Rheodyne, Berkeley, CA, U.S.A.) and an interface constructed by the Instrument Department, Hässle (Mölndal, Sweden).

Chemicals

VMA (4-hydroxy-3-methoxymandelic acid) was obtained from Sigma (St Louis, MO, U.S.A.) and iso-VMA (3-hydroxy-4-methoxymandelic acid) from Aldrich (Milwaukee, WI, U.S.A.). Tri-*n*-butyl phosphate (TBP), disodium ethylenediaminetetraacetate (EDTA), all buffer substances and inorganic acids were of analytical-reagent grade from E. Merck (Darmstadt, F.R.G.) and methanol of p.a. quality from Rathburn (Walkerburn, U.K.).

Chromatographic system

A scheme of the chromatographic system is presented in Fig. 1. The first separation column (150 mm×4.6 mm I.D.) contained 5-μm Nucleosil C_{18} (Macherey-Nagel, Düren, F.R.G.), coated with 500 μl of TBP as liquid stationary phase. The application of TBP was performed by injection of 5-μl portions at intervals of a few minutes. The column was kept at 27.0 ± 0.1°C by a thermostatted block constructed by the Instrument Department, Hässle. The mobile phase (pH 3.5; ionic strength, $I = 0.1$), comprising citric acid (108 mM), sodium hydroxide (92 mM) and EDTA (0.3 mM), was saturated to 90% with

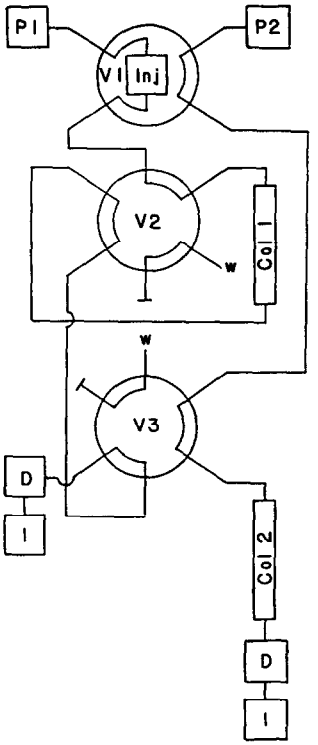

Fig. 1. Coupled-column liquid chromatographic system. P1 and P2 = pumps with mobile phases 1 (citrate buffer pH 3.5, $I = 0.1$, containing 0.3 m*M* EDTA saturated to 90% with TBP) and 2 (citrate buffer pH 3.5, $I = 0.1$, containing 0.3 m*M* EDTA and 5% methanol); Inj = autoinjector; Col 1 = Nucleosil 5 C_{18} + TBP; Col 2 = Nucleosil 5SB; V1, V2 and V3 = six-port valves; D = detector; I = integrator; w = waste. For further details, see text.

TBP by diluting a saturated solution. It was thermostatted at 27.0 ± 0.1 °C in a temperature-controlled water-bath. By recirculating the mobile phase overnight, the chromatographic system was equilibrated and constant retention times were obtained.

The second column, a strong anion exchanger Nucleosil 5SB (150 mm × 4.6 mm I.D.) (Macherey-Nagel) was thermostatted at 26.0 ± 0.1 °C with a temperature-control system similar to that mentioned above. As mobile phase the same buffer solution as for the first column was used but without TBP saturation and with the addition of 0–5% methanol. Deionized and filtered water (Milli Q; Millipore, Bedford, MA, U.S.A.) was used for the mobile phases, which prior to use were degassed and filtered through a 0.45-μm MF Millipore filter.

The flow-rate was 1.0 ml/min, and the eluent from the anion-exchange column was monitored with a single or dual electrochemical detector. Considering

the results from recorded hydrodynamic voltammograms, the dual electrochemical detector (BAS) was operated at +0.75 V and +0.90 V, the single one (Waters) at +0.80 V and the ESA detector at +0.0 V and +0.50 V. Chromatograms for verifying the retention times of the first separation column were obtained by either a UV detector or an electrochemical detector.

Sample preparation

Urine samples were collected in polypropylene bottles and acidified with 5 *M* hydrochloric acid or glacial acetic acid to a pH of ca. 4 before storage at −20°C. After thawing, mixing and centrifugation (1000 *g*, 2 min) of the urine sample, 1 ml was mixed with 1 ml of a solution of iso-VMA (20 μM) in phosphate buffer (pH 6.0) containing 3.0 m*M* EDTA. Standard samples were prepared by mixing a solution of VMA (10 μM) with the same volume of the iso-VMA solution (20 μM). Volumes of 5–20 μl were injected into the liquid chromatograph. Quantitation was performed by comparing the peak-height ratios of VMA and the internal standard for urine samples with those for standard samples.

RESULTS AND DISCUSSION

Chromatography

In a previous study [17], we used the strongly hydrogen-accepting agent TBP as a liquid stationary phase on octadecylsilica in order to increase the retention of hydrogen-donating compounds, such as dihydroxyphenylethylene glycol and dihydroxymandelic acid, relative to amines. This column showed less capability to separate VMA and iso-VMA than an ordinary C_{18} column or an anion exchanger, which is illustrated in Fig. 2. According to the literature [2,10], the content of iso-VMA in human urine is less than 1% of the VMA

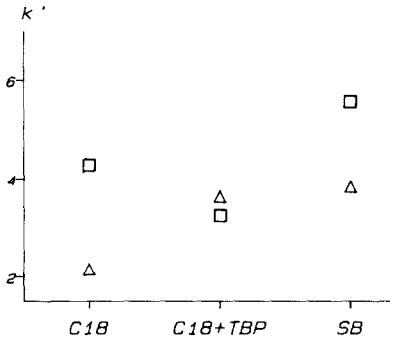

Fig. 2. Effect of stationary phase on the retention (k') of VMA (△) and iso-VMA (□). Stationary phase, Nucleosil 5 C_{18}, Nucleosil 5 C_{18} coated with TBP and Nucleosil 5SB; mobile phase, citrate buffer (pH 3.5, I=0.1).

concentration. Using iso-VMA as internal standard, a fraction (1.5–2.0 ml) of the eluate containing both VMA and iso-VMA could be switched to the anion-exchange column, where separation was performed. No additional band broadening was seen when chromatograms of a standard solution injected into the coupled-column system were compared with those recorded after injection of 20 μl directly onto the anion-exchange column. The mobile phase was saturated with TBP to 90% [18], otherwise the stationary phase was slowly washed off the support, causing decreasing retention times of the analytes. If, on the other hand, the column became supersaturated, the stationary phase was progressively stripped off, which interfered with the detection.

The separation ability of the anion exchanger was unaffected by the TBP-saturated fraction of mobile phase that was transferred to the column during switching. Anion exchangers from various manufacturers (LiChrosorb AN, Merck; RSil AN, Alltech; Spherisorb SAX, Phase Sep; Vydac, The Separation Group; Zorbax SAX, DuPont; Bakerbond, Baker; and Nucleosil 5SB, Macherey-Nagel) showed great differences regarding column efficiency and retention characteristics. Some columns gave broad peaks whereas others were inappropriate in terms of retardation. The material chosen, Nucleosil 5SB, differed to some extent between various batches, and to adjust the retention times the ionic strength or the content of methanol (0–5%) of the mobile phase was varied. A similar buffer solution as for the first separation column was used, otherwise extra peaks may appear in the chromatogram after column switching. A pH of 3.5 was chosen as it gave suitable retention times on both columns, and EDTA was added to the mobile phase as complexing agent, as it seemed to lower the background current. The ion-exchange columns were purchased or packed in our laboratory with methanol, using toluene–cyclohexanol (4:5) or methanol as slurry medium.

The amperometric cell from Waters was occasionally cleaned with acetone and the one from BAS was polished with alumina. During times when the detector cell was not in use the potential was lowered to +0.5 V, which extended the durability of the cell response.

Column switching

To confirm the time schedule for switching of the valves (Table I), a reference solution of the analyte and the internal standard was injected onto the first column before and after each series of samples. With increasing temperature the capacity ratios were decreased (0.05 units/°C), and as constant retention times are a prerequisite, the mobile phase and the column were thermostatted. With these precautions, retention times of 6.21 min ± 0.2% ($n=14$) for VMA and 5.81 min ± 0.2% ($n=14$) for iso-VMA were obtained during a time period of 13 h. Direct injection onto column 2 was performed after the injector and the column had been connected by switching valve V1.

Chromatograms of a standard solution and a human urine sample recorded

TABLE I

SCHEME OF COLUMN-SWITCHING EVENTS

Time after injection (min)	Switch valve No.	Event
0.0		The sample is injected onto column 1
5.5	3	Column 1 and 2 are connected in series and the eluate (VMA + iso-VMA) from column 1 is switched to column 2
7.5	3 reset	The columns are disconnected from each other
7.7	2	Column 1 is cleaned by back-flushing with mobile phase
17.0	2 reset	After the chromatogram is completed and the report recorded the next sample is injected

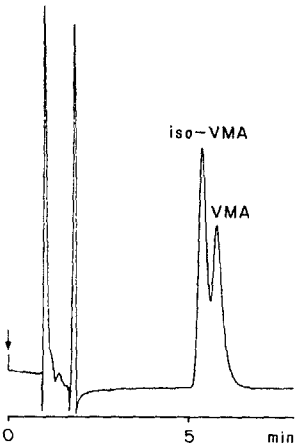

Fig. 3. Reference solution containing 5.1 μM VMA and 6.3 μM iso-VMA injected onto column 1. Stationary phase, Nucleosil 5 C_{18} coated with TBP; mobile phase, citrate buffer (pH 3.5, $I=0.1$) containing 0.3 mM EDTA saturated to 90% with TBP; injection volume, 20 μl; potential, +0.8 V; sensitivity, 12.8 nA full scale.

after the first column and after column-switching are shown in Figs. 3–5. Many late-eluting peaks appeared in the chromatogram of a urine sample recorded after the first column (Fig. 4). To get rid of these peaks the flow direction of the mobile phase through column 1 was reversed after ca. 8 min, when the desired eluate fraction had been switched to column 2. The strongly retarded compounds were then forced backwards to waste, while separation of VMA and iso-VMA was performed on the anion exchanger. Without back-flushing of the column the time between sample injections was 30 min or more.

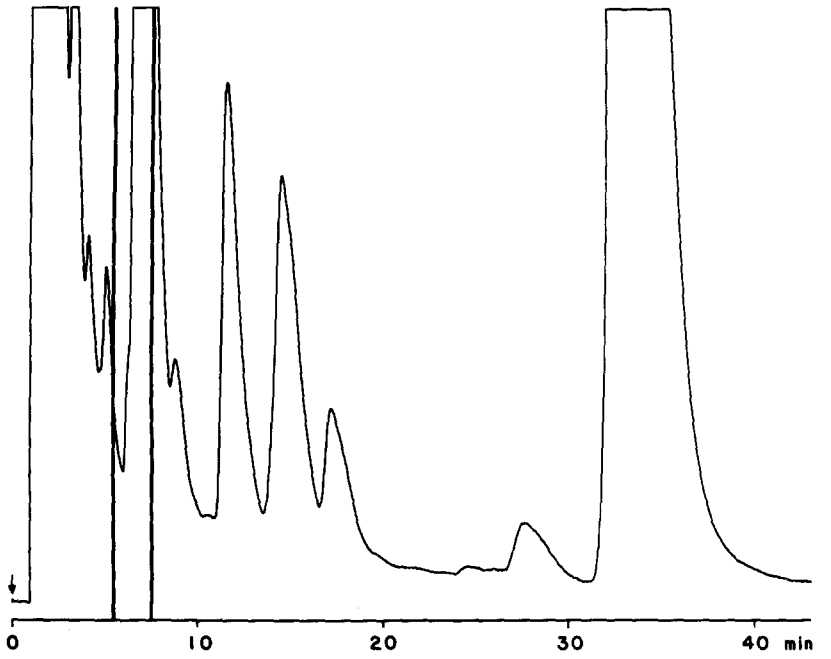

Fig. 4. Human urine sample chromatographed on column 1. The marked fraction is switched to column 2. Injection volume, 10 μl. Other conditions as in Fig. 3.

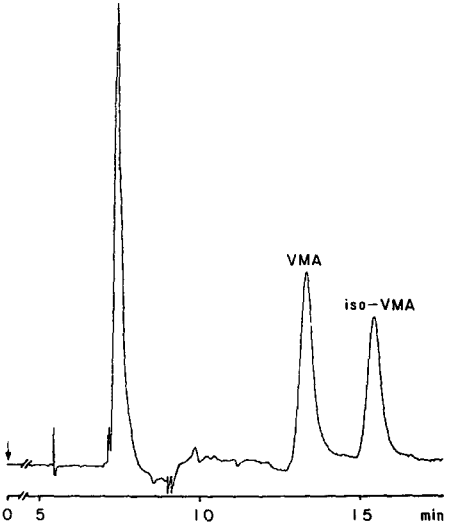

Fig. 5. Chromatogram (after column-switching) of a human urine sample containing 18.3 μM VMA. The content of iso-VMA in the internal standard solution was 12.6 μM. Injected sample, 20 μl; potential, +0.8 V; sensitivity, 12.8 nA full scale. Chromatographic conditions as in Fig. 1.

TABLE II

DUAL-CELL OPERATION IN PARELLEL MODE

Response ratios at 0.90 and 0.75 V for VMA and iso-VMA in standard samples ($n=19$) and urine samples ($n=19$) (2–36 μM), injected over six days.

	Substance	Ratio 0.90/0.75 V (%)
Standard samples	VMA	2.68 ± 8.6
	Iso-VMA	2.26 ± 8.7
Urine samples	VMA	2.66 ± 9.9
	Iso-VMA	2.25 ± 8.7

Accuracy

Dihydroxymandelic acid is a potentially interfering compound as it would elute just after the VMA peak on the first column, and if transferred to the second column it elutes just ahead of VMA. With unchanged retention the separation can be improved by lowering the content of methanol in the mobile phase while increasing the ionic strength. The single amperometric detector (Waters) was used in most of the studies, but to ascertain peak purity we used dual working electrodes (BAS) in parallel mode and recorded simultaneously the peak currents at +0.75 V and +0.90 V. The ratios of the signals at the two potentials were calculated and compared with reference injections. Good agreement was shown, indicating that the samples were free from interfering compounds. In Table II the results from standard samples ($n=19$) and different urine samples ($n=19$) injected over six days are shown. In later studies the ESA detector was also employed, showing satisfactory long-term stability.

Recovery and precision

Peak areas of standard solutions recorded after injection into the coupled-column system were compared with those recorded after injection directly onto the anion-exchange column. The recoveries obtained were 97.7 ± 7.4% for VMA and 94.9 ± 6.7% for iso-VMA ($n=5$). The recovery from urine samples determined by standard addition was 102.2 ± 3.8% ($n=5$), and the relative standard deviation was 3.3% for 10-μl injections ($n=8$) of human urine samples containing 3.2 μM VMA. For aqueous standard samples the corresponding value was 3.7% at a concentration of 10.0 μM. The method as described is linear for both VMA and iso-VMA up to about 100 μM and what limits the amount that can be injected is the linearity range of the integrator.

The method has been used to examine the excretion of VMA from migraine patients. The results showed inter-individual variations of 2.7–6.3 μmol/5-h collecting fractions or 2.0–20.3 μM and are within the range of values reported for normal human urine [4,6,7,13,16].

CONCLUSION

With a coupled-column technique combined with electrochemical detection, endogenous levels of VMA in urine can be determined in a simple way by direct injection of the urine sample.

REFERENCES

1 P. Moleman and J.J.M. Borstrok, Clin. Chem., 29 (1983) 878.
2 P. Frattini, G. Santagostino, S. Schinelli, M.L. Cucchi and G.L. Corona, J. Pharmacol. Methods, 10 (1983) 193.
3 R.T. Brown, K.L. Kirk and J. Oliver, J. Liq. Chromatogr., 9 (1986) 831.
4 K. Lindgren and N. Rodopoulos, Clin. Chem., 32 (1986) 693.
5 S.J. Soldin and J.G. Hill, Clin. Chem., 26 (1980) 291.
6 S.R. Binder and G. Sivorinovsky, J. Chromatogr., 336 (1984) 173.
7 C.N. Ong, B.L. Lee, H.Y. Ong and E. Jaecob, J. Chromatogr., 423 (1987) 278.
8 P. Ni, F. Guyon, M. Caude and R. Rosset, J. Chromatogr., 424 (1988) 255.
9 G.M. Anderson and F.C. Feibel, Clin. Chem., 32 (1986) 709.
10 J.M.P. Holly and N. Patel, Ann. Clin. Biochem., 23 (1986) 447.
11 J. Odink, H. Korthals and J.H. Knijff, J. Chromatogr., 424 (1988) 273.
12 J.G. Flood, M. Granger and R.B. McComb, Clin. Chem., 25 (1979) 1234.
13 K. Fujita, K. Maruta, S. Ito and T. Nagatsu, Clin. Chem., 29 (1983) 876.
14 K. Kodama, K. Yamanaka, T. Nakata and M. Aoyama, Clin. Chem., 32 (1986) 1944.
15 S. Yoshikawa, S. Okuda, T. Ohe, K. Kamohara, O. Maeda and T. Sawada, J. Chromatogr., 421 (1987) 111.
16 P. Nebinger and M. Koel, J. Chromatogr., 434 (1988) 43.
17 B.-M. Eriksson and B.-A. Persson, J. Chromatogr., 386 (1987) 1.
18 K.-G. Wahlund and B. Edlén, J. Liq. Chromatogr., 4 (1981) 309.