Journal of Chromatography, 567 (1991) 11–19
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5856

New analyser for the determination of urinary vanillylmandelic acid, homovanillic acid and creatinine

SEIJI KAWAGUCHI*, NORIO HIRACHI and MITSURU FUKAMACHI

Scientific Instrument Division, Tokyo Research Center, Tosoh Corporation, 2743-1 Hayakawa, Ayase-shi, Kanagawa 252 (Japan)

(First received November 27th, 1990; revised manuscript received January 16th, 1991)

ABSTRACT

We have developed a novel analyser for the determination of vanillylmandelic acid, homovanillic acid and creatinine in urine by high-performance liquid chromatography using three different types of column, cation-exchange, anion-exchange and reversed-phase and the column-switching technique. In this procedure, 10 μl of intact urine were directly injected into the cation-exchange column, and the pass-through fraction, containing vanillylmandelic acid and homovanillic acid was transferred to the anion-exchange column by column switching. The fraction partially purified from endogenous urinary impurities on the anion-exchange column was transferred to the reversed-phase column. Vanillylmandelic acid and homovanillic acid, separated by the solvent-switching technique, were detected fluorimetrically (excitation at 280 nm, emission at 320 nm). Then, creatinine eluted from the cation-exchange column is spectrophotometrically detected (254 nm). Therefore the successive simultaneous analysis of the three could be performed in a 15-min cycle; the within-assay coefficients of variation for normal and patients' urines were $<1.9\%$, $<3.3\%$ and $<3.0\%$ for vanillylmandelic acid, homovanillic acid and creatinine, respectively; the recoveries averaged 100, 103 and 100%, respectively, for supplemented urines.

INTRODUCTION

The determination in urine of vanillylmandelic acid (VMA) and homovanillic acid (HVA), the main metabolic end-products of catecholamines, is indispensable for the diagnosis of neuroblastoma (NB), pheochromocytoma, Parkinson's disease, etc. [1]. In particular, NB can be reliably found by analysing only the two metabolites, VMA and HVA, in urine. If it is detected in the early stages, the disease may be perfectly cured. The mass screening of six-month-old infants commenced administratively from 1985 in Japan [2]. Therefore, many screening techniques have been investigated for the detection of NB. However, some involve semiquantitative and troublesome procedures [3,4]. On the other hand, liquid chromatographic determination is suitable for a reliable mass screening, owing to its high sensitivity and selectivity. Many high-performance liquid chromatographic (HPLC) techniques have been presented for the analysis of urinary VMA and HVA by spectrophotometry [5], fluorimetry [6,7] and amperometry [8–11], but they required laborious sample preparation.

0378-4347/91/$03.50

Furthermore, urine samples collected at all times are commonly used for mass screening. Therefore it is necessary to calculate the concentration ratio of VMA or HVA to creatinine (CRN). However, urinary CRN was usually not measured by HPLC but by the Folin-Wu method, utilizing Jaffe's reaction [12]. Consequently, these techniques are not suitable for clinical needs in emergencies.

Recently, column- and solvent-switching methods have been widely used for the automatic pretreatment of the samples, where one column provides a crude separation of the sample, and a fraction containing the analytes is transferred to another column for the analytical separation. This technique can successfully be used for the separation of catecholamines and their metabolites in biological materials [13,14]. This paper describes a fully automated analyser that uses a new separation technique involving on-line pretreatment of urine samples for the simultaneous determination of VMA, HVA and CRN.

EXPERIMENTAL

Instrumentation

A fully automatic liquid chromatographic analyser (HLC-726VMA), newly developed by Tosoh (Kanagawa, Japan) was used (Fig. 1). We used three different types of column, TSKgel VMA-Cation (5 μm, 150 mm × 4.6 mm I.D.),

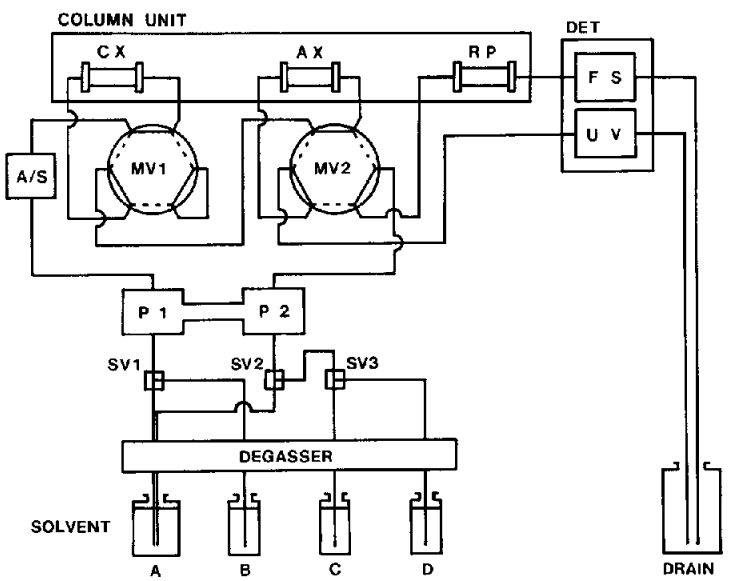

Fig. 1. Schematic diagram of the HLC-726VMA equipment. SV = solvent-switching valve; P = pump; A/S = autosampler; MV = motorized six-way valve; CX = TSKgel VMA-Cation column; AX = TSKgel VMA-Anion column; RP = TSKgel VMA-RP column; DET = detector; FS = fluorescence detector; UV = ultraviolet absorbance detector. Column temperature, 25°C. The positions MV OFF and MV ON are indicated by the solid and the dashed lines, respectively. The flow-rate was 1.0 ml/min for both P-1 and P-2.

TSKgel VMA-Anion (5 μm, 120 mm × 4.6 mm I.D) and TSKgel VMA-RP (5 μm, 150 mm × 4.6 mm I.D.) especially developed for the analyser by Tosoh.

Materials

Pure VMA, HVA and CRN were obtained from Sigma (St. Louis, MO, U.S.A.). Creatinine deiminase (E.C. 3.5.4.21) were from Toyobo (Osaka, Japan). Other reagents of special grade were purchased from Wako (Osaka, Japan).

Mobile phases

Four solvents were used for chromatography and sample clean-up: (A) 50 m*M* citrate buffer containing 0.02% (w/v) sodium azide (pH 4.8); (B) 300 m*M* sodium sulphate in solvent A (pH 4.6); (C) 10% (w/w) acetonitrile in solvent A (pH 5.0); (D) 40% (w/w) acetonitrile in solvent A (pH 5.5). Solvent A serves to elute CRN on the cation-exchange (CX) column and for initially equilibrating the three columns before sample injection. Solvent C completes the rapid and fine separation for HVA on the RP column. Solvent B serves to wash the CX and anion-exchange (AX) columns, and solvent D for washing the reversed-phase (RP) column.

Standards

A stock solution (500 μg/ml each of VMA and HVA) was prepared by diluting 50 mg each of VMA and HVA to 100 ml with eluent A. A working standard containing VMA(5 μg/ml), HVA (5 μg/ml) and CRN (0.5 mg/ml) was prepared by diluting 1.0 ml of stock VMA, HVA standard solution and 50 mg of CRN to 100 ml with eluent A. The working standard was stable for *ca.* three weeks, and the stock standard for at least four weeks at 4°C.

Urine samples

Intact urine samples were directly injected into the liquid chromatograph. The supernatant was also used after centrifugation (2000 *g*, 5 min) in case the urine sample contained insoluble impurities. VMA, HVA and CRN were stable for at least four weeks at −20°C if the urine sample was acidified with 6 *M* hydrochloric acid to a pH of *ca.* 4.

Principle of separation

The following six analytical steps are time-controlled (Table I) by the sequencer (Fig. 1):

(1) Pretreatment step on the CX column. Inject 10 μl of intact urine with an automatic loop injector (A/S) into the pretreatment line, which includes the CX and AX columns connected in series through MV1 (OFF) and MV2 (OFF); most of cationic compounds, including CRN, are adsorbed on the CX column, the pass-through fraction containing anionic VMA and HVA is transferred to the AX column.

TABLE I

SCHEME OF COLUMN AND SOLVENT SWITCHING

Valve	ON (min)	OFF (min)
MV1	3.5	4.0
MV2	4.0	10.0
SV1	9.0	10.0
SV2	6.0	14.0
SV3	13.0	14.0

(2) Pretreatment step on the AX column. Switch the CX column into the drain (MV1 ON) and stop delivering the solvent A to it (CRN remains in the CX column); the fraction containing VMA and HVA is partially purified on the AX column from other anionic impurities.

(3) Analytical step for CRN. Switch the AX column into line with the RP column; the partially purified fraction is similarly transferred to the RP column. Then, switch the CX column into analytical line (MV1 OFF); deliver the solvent A to it again; the CRN adsorbed on the CX column is eluted and detected at 254 nm (UV).

(4) Washing and initializing step for the CX and AX columns. Switch the AX column again into line with the CX column (MV2 OFF), flush these two columns with solvent B for 1 min (SV1 ON) and return to solvent A (SV1 OFF).

(5) Analytical step for VMA and HVA. VMA and HVA in the fraction partially purified on the AX column are separated by stepwise elution with solvent C (SV2 ON and SV3 OFF) and fluorimetrically detected at an excitation wavelength of 280 nm and an emission wavelength of 320 nm (FS).

(6) Washing and initializing step for the RP column. Prepare the AX and CX columns for the next analysis while the RP column is equilibrated with solvent A (SV2 OFF and SV3 OFF) after flushing with solvent D for 1 min (SV2 ON and SV3 ON).

The CRN, VMA and HVA peaks are eluted in this order, and the ratio of micrograms of VMA or HVA to milligrams of CRN is immediately calculated. The time between analysis of one sample and the next is 15 min, which includes the on-line pretreatment, chromatography and calculation of the ratio of VMA or HVA to CRN.

Calculation of results

The HLC-726VMA analyser used peak-area measurements with a single external standardization to calculate the concentration of VMA, HVA and CRN in urine samples. The ratios of VMA or HVA to CRN were also calculated from their concentration values.

RESULTS

Fig. 2 shows two typical chromatograms obtained by direct-injection analysis of 10 μl of intact urine from a normal infant (left) and an NB patient (right). Good separation and baselines with low background are observed in both cases.

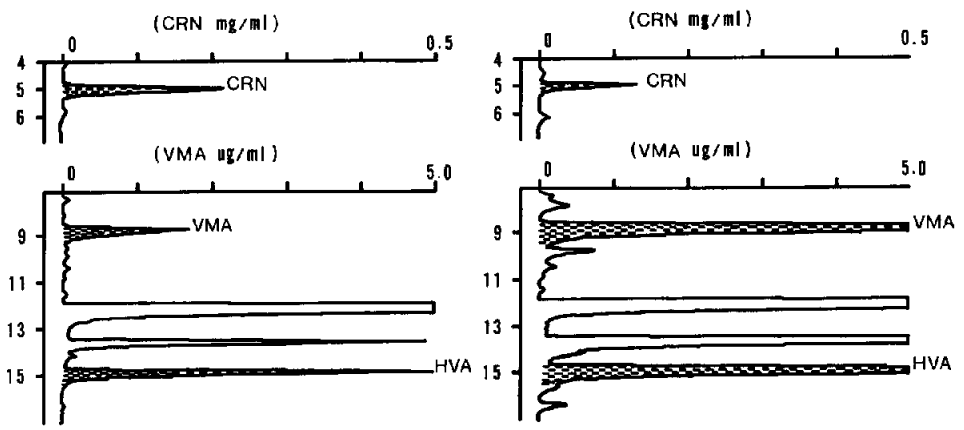

Fig. 2. Chromatograms obtained from urine samples from a normal infant (left) and an NB patient (right). (Left) The concentrations were 0.22 mg/ml, 1.73 μg/ml and 4.29 μg/ml for CRN, VMA and HVA, respectively; μg VMA/mg CRN = 7.9, μg HVA/mg CRN = 19.5. (Right) The concentrations were 0.13 mg/ml, 20.21 μg/ml and 21.92 μg/ml for CRN, VMA and HVA, respectively; μg VMA/mg CRN = 155.5, μg HVA/mg CRN = 168.6.

Peak identity and purity

In this procedure the validity of the assay depends on the complete resolution of the CRN, VMA and HVA from interfering fluorescent or UV-absorbing peaks. The peaks measured were found to contain only pure compounds of interest. Table II summarizes the peak identity and purity test for VMA and HVA.

TABLE II

PEAK-PURITY TEST FOR VMA AND HVA

Excitation wavelengths (nm)	Calculated fluorescence ratio			
	Standard		Urine	
	VMA	HVA	VMA	HVA
280 and 240	1.82	3.28	1.78	3.24
280 and 260	3.29	3.68	3.29	3.60
260 and 240	0.56	0.89	0.54	0.91

A good indication of peak purity can be obtained by comparison of the fluorescence ratios of the peak areas corresponding to VMA and HVA at two or more specific excitation wavelengths and constant emission wavelength. This suggests that the two peaks detected at the elution positions of VMA and HVA were pure. The identity and purity of the CRN peak were assessed in comparison with the chromatogram from an intact urine and that from the same pretreated with creatinine deiminase. It is well known that CRN is converted into 1-methylhydantoin by catalysis of creatinine deiminase. Therefore urinary CRN was converted into 1-methylhydantoin by addition of creatinine deiminase, and the reaction mixture was injected into the column. The peak corresponding to CRN was completely eliminated. Moreover, no overlapping peaks were found at this position in the chromatogram.

Precision

Table III summarizes the precision data for the direct-injection analysis. The reproducibility was evaluated by the within- and between-assay precision of three and two samples of infant urine, respectively. Good coefficient of variation (C.V.), less than 3.3% for within-assay and less than 2.9% for between-assay, were obtained.

TABLE III

REPRODUCIBILITY OF THE PRESENT ASSAY

Compound	Concentration (mean ± S.D.)	C.V. (%)
Within-assay (n = 10)		
CRN (mg/ml)	0.10 ± 0.000	0.00
	0.23 ± 0.007	3.04
	0.51 ± 0.012	2.35
VMA (μg/ml)	1.14 ± 0.022	1.93
	6.33 ± 0.086	1.36
	13.17 ± 0.200	1.45
HVA (μg/ml)	1.89 ± 0.049	2.59
	5.07 ± 0.170	3.35
	17.50 ± 0.290	1.66
Between-assay (n = 7)		
CRN (mg/ml)	0.24 ± 0.007	2.92
	0.44 ± 0.013	2.95
VMA (μg/ml)	2.36 ± 0.041	1.74
	4.23 ± 0.078	1.84
HVA (μg/ml)	4.65 ± 0.070	1.51
	7.77 ± 0.085	1.09

Linearity and detection limits

The standard curves were linear up to 350 μg/ml, 300 μg/ml and 8 mg/ml for VMA, HVA and CRN, respectively. The detection limits for VMA, HVA and CRN were 300 pg, 300 pg and 30 ng, respectively, at a signal-to-noise ratio of 3.

Recovery

Recovery was assessed from replicate analyses of urine samples supplemented with VMA, HVA and CRN from three normal subjects (endogenous VMA, HVA and CRN levels were 2.04–5.03 μg/ml, 4.06–10.91 μg/ml and 0.20–0.46 mg/ml, respectively). The mean recoveries of VMA (5 μg/ml), HVA (5 μg/ml) and CRN (0.5 mg/ml) were 100, 103 and 100%, respectively.

Correlation with a comparison method

Values (y) obtained with this method were compared with those (x) from solvent extraction with ethyl acetate for 38 samples [9]. For VMA and HVA the correlation coefficient (r) exceeded 0.95, and the equations for the regression lines were $y = 0.75x - 0.05$ ($n = 38$, $r = 0.968$) for VMA and $y = 0.87x - 0.13$ ($n = 38$, $r = 0.955$) for HVA. The concentrations of VMA and HVA in the urine samples ranged from 0.3 to 7.5 μg/ml. The extraction of VMA and HVA was poorer from a standard solution of dilute hydrochloric acid than from acidified urine. Therefore the VMA and HVA values measured after the ethyl acetate extraction were consistently higher (VMA 25%, HVA 13%) than those obtained by the present method.

CRN values (y) obtained by the present method were also compared with those (x) from the Folin-Wu method. The correlation coefficient (r) was 0.991 and the equation for the regression curve was $y = 0.98x - 0.56$ ($n = 100$). The concentration of CRN in these urine samples ranged from 0.04 to 0.6 mg/ml. The correlation was exceedingly good.

DISCUSSION

The main advantage of this method is the simultaneous determination of CRN with VMA and HVA following a single injection; thus the exact concentration ratio of VMA or HVA to CRN can be immediately obtained. Another is the high efficiency of the sample clean-up with two different types of column (CX and AX), facilitating the very clean fluorimetric detection of VMA and HVA. In particular, complete separation of VMA from neighbouring peaks could be achieved, in contrast to the manual extraction method.

Conventional LC methods used for analysing biological samples required manipulative extraction steps. Ethyl acetate extraction, commonly used for the assay of urinary catecholamine metabolites, is very selective. However, the VMA value thus measured is not sufficiently precise because of the low extractability of VMA from aqueous solution into ethyl acetate. This problem can be solved with direct on-column injection and on-line purification of urine samples.

The CRN molecule has an absorbance maximum at 234 nm. However, when we detected it at this wavelength with a detector cell of 10-mm path length, we could not obtain a linear calibration curve up to 1 mg/ml. This was due to exceeding the upper detection limit of the UV detector. A linear calibration curve up to 3 mg/ml CRN is absolutely required for urine analysis. Hence, we set the detector wavelength at 254 nm and used a cell with a 1-mm path length for the purpose of lowering the sensitivity, so as to obtain a satisfactory linearity and detection limit.

The lifetime of HPLC columns is influenced by the choice of eluent components, the method of sample pretreatment, the extent of mechanical damage caused by high back-pressure, etc. We tested and evaluated the performance of the columns during 2000 repetitive direct injections of an adult intact urine. The result was that *ca.* 1200 injections could be made without significant loss of resolution. Thereafter, a broadening peak with a small shoulder was observed for VMA after 1300 injections, and a slightly splitting peak was also found for HVA after 1600 injections. The reduction of the VMA and HVA peak heights reflects a decrease in the number of theoretical plates (N) but a change in N does not influence the peak area. However, from the chromatogram after 1600 injections it is difficult to measure the areas of the VMA and HVA peaks accurately because the separation of the two from neighbouring peaks gradually deteriorates.

CONCLUSION

The HLC-726VMA analyser offers a new technique for the analysis of urinary VMA and HVA, including a simultaneous assay for CRN, by direct on-column injection. It is suitable for the mass screening of NB in routine laboratories because of its high selectivity and full automation.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Wada of Osaka Medical Center and Research Institute for Maternal and Child Health, and to Dr. Koremura, Dr. Hoson and Dr. Nakada of St. Marianna University School of Medicine, for evaluating our instrument and for arranging for the supply of urine samples.

REFERENCES

1 E. H. Labrosse, C. Com-Nougue and J. M. Zucker, *Cancer Res.*, 40 (1980) 1995.
2 T. Sawada, M. Hirayama and T. Nakata, *Lancet*, ii (1984) 271.
3 J. J. Pisano, J. R. Crout and D. Abraham, *Clin. Chim. Acta*, 7 (1962) 285.
4 G. Gumboldt, *Clin. Chem.*, 23 (1977) 1949.
5 A. Yoshida, M. Yoshioka, T. Sasaki and Z. Tamura, *J. Chromatogr.*, 227 (1982) 162.
6 T. G. Rosano, H. H. Brown, and J. M. Meola, *Clin. Chem.*, 27 (1981) 228.
7 K. Lindgren and N. Rodopoulos, *Clin. Chem.*, 32 (1986) 693.

8 S. J. Soldin and J. G. Hill, *Clin. Chem.*, 26 (1980) 291.
9 M. H. Joseph, B. V. Kadam and D. Risby, *J. Chromatogr.*, 226 (1981) 361.
10 G. S. Mayer and R. E. Shoup, *J. Chromatogr.*, 255 (1983) 533.
11 T. Tokuda, M. Yoshioka, Z. Tamura and K. Yokomori, *Jpn. Soc. Anal. Chem.*, 33 (1984) 331.
12 R. W. Bonsnes and H. H. Taussky, *J. Biol. Chem.*, 158 (1945) 581.
13 S. Yoshikawa, S. Okuda, T. Ohe, K. Kamohara, O. Maeda and T. Sawada, *J. Chromatogr.*, 421 (1987) 111.
14 B. M. Eriksson, B. A. Persson and M. Wikstrom, *J. Chromatogr.*, 527 (1990) 11.