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Note

Direct determination of urinary vanillylmandelic acid and homovanillic acid by high-performance liquid chromatography on an anion-exchange column

KYOKO KODAMA* and TOSHIKAZU NAKATA

Nagoya City Health Research Institute, 1-11, Hagiyama-cho, Mizuho-ku, Nagoya 467 (Japan)

and

MITSUKO AOYAMA

Nagoya City University Medical School, 1, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467 (Japan)

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Neuroblastoma is the commonest tumour of childhood [1]. The prognosis of this tumour closely depends on the patient's age at diagnosis [1]. Therefore, early diagnosis is an important factor in the prognosis. It is favourable when asymptomatic infants who are less than one year of age are detected.

Measurements of vanillylmandelic acid (3-methoxy-4-hydroxymandelic acid, VMA) have been advocated in the detection of patients with neuroblastoma [2—4]. But high levels of urinary VMA are excreted in only 75% of patients with this disease [5]. If measurements of urinary homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid, HVA) along with VMA are performed, approximately 95% of patients with this tumour will be detected [5].

Recent methods for the determination of urinary VMA and HVA are based on the use of high-performance liquid chromatography (HPLC). There are methods for the simultaneous determination of both acids [6—12] in addition to the methods for VMA [13—17] and HVA [18—20] separately. The method that uses ultraviolet (UV) detection [6, 7] involves sample concentration and clean-up procedures because sensitivity is low and many UV-absorbing constituents exist in urine. The method with fluorometric detection [8] is more selective than with UV detection. However, sample concentration is

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necessary because of low sensitivity. The method with derivatized VMA and fluorometric detection was successful in the analysis of VMA, but not of HVA [17]. The methods with electrochemical detection [9—12] allow the direct use of urine samples because of high selectivity and sensitivity. Because it is difficult to separate VMA from other urine constituents in these methods which employ an octadecylsilica (ODS) column, paired-ion chromatography [9], gradient elution [10] or organic extraction from acidified urine [11, 12] are used.

This note describes a rapid and simple HPLC method which employs an anion-exchange chromatographic column, electrochemical detection, direct on-column injection of urine and isocratic elution. This method was applied to the determination of the VMA and HVA contents of urine from six-month-old infants.

EXPERIMENTAL

Reagents

Acetonitrile, concentrated hydrochloric acid, formic acid, and ethylenediaminetetraacetic acid disodium salt (EDTA · 2Na) were purchased from Wako (Osaka, Japan). VMA and HVA were purchased from Sigma (St. Louis, MO, U.S.A.). Analytical-reagent grade chemicals were used without further purification. All solutions were prepared from distilled, deionized water. For standard samples, VMA and HVA were dissolved in 0.1 *M* hydrochloric acid.

Instrumentation

A Yanaco (Kyoto, Japan) Model L-4000W high-performance liquid chromatograph with an LA-100 column oven was used. The stainless-steel column (50 × 4.0 mm) packed with Yanaco NB-5801 (anion-exchange, particle size 12 μm) was used. The mobile phase was 3% formic acid, containing 50 μ*M* EDTA for masking iron ion, delivered at a constant flow-rate of 0.9 ml/min. A VMD 101 glassy carbon electrode (Yanaco) with a silver/silver chloride reference electrode was used to oxidize the compounds of interest at 0.80 V potential versus the reference electrode. The resulting signal was recorded at 10 mV using an R3-201 twin-pen recorder (Yanaco). The column was used at 65°C and the column inlet pressure was about 25 kg/cm^2.

Collection of urine samples

The urines were obtained from twenty normal infants (about six months of age) of both sexes and two patients with neuroblastoma. After collection, the pH of the urine was adjusted to less than 2 with 6 *M* hydrochloric acid and refrigerated until assayed.

Sample preparation

Test urine and acetonitrile were pipetted into glass tubes in a ratio of 1:1. Standards were prepared similarly using the mixture of VMA and HVA in 0.1 *M* hydrochloric acid. Routinely, standards in the range 25—110 μmol/l were used. Each tube was stoppered, shaken for 2 min by hand, and centrifuged at

550 *g* for 5 min. The supernate was injected directly into the column. The sample volume (in μl) of injection is approximately given by 44/(urinary creatinine, mmol/l). Prepared samples can be stored for as long as one week at 4°C.

RESULTS AND DISCUSSION

Chromatography

Chromatograms resulting from the analysis of urine from a normal infant (six months of age) and from a patient with neuroblastoma are shown in Fig. 1. VMA and HVA were eluted from the column at about 10 and 15 min, respectively, as asymmetric peaks.

The retention time was inversely dependent on the concentration of formic acid in the mobile phase; increasing the concentration from 1.0 to 10.0% resulted in shorter retention times. The flow-rate of the mobile phase affected the separation of the peaks.

All samples in which VMA or HVA was above the normal range were re-run with the same mobile phase at a flow-rate of 0.5—0.7 ml/min.

The following compounds were checked and found not to interfere in the method: 3-methoxy-4-hydroxyphenylglycol, dihydroxyphenylacetic acid, dihydroxymandelic acid and 3-methoxy-4-hydroxybenzoic acid (vanillic acid).

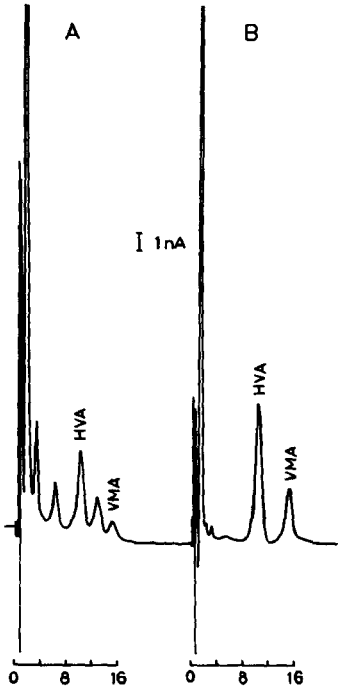

Fig. 1. Chromatograms of urine from a normal infant (A) and a patient (B). Concentrations of HVA and VMA in samples (μmol/l): (A) HVA 18.1, VMA 7.6; and (B) HVA 65.9, VMA 64.1.

Linearity

Calibration graphs for VMA and HVA were rectilinear up to 1 nmol; the detection limits were 50 and 14 pmol, respectively.

Choice of mobile phase

At an early stage of this study, acetate buffer (3 *M*, pH 3.7) was used as the mobile phase. Although the results were satisfactory, this mobile phase has not been used routinely because the concentration of the buffer was too high.

Formic acid was chosen as the mobile phase because it results in a favourable retention time for VMA and HVA, and its concentration is suitable as the mobile phase.

Recovery

Recovery studies were performed by adding specified quantities of VMA and HVA to aqueous solutions of urines obtained from eight normal infants (Table I). The recoveries of VMA and HVA were 96.6% and 94.8%, respectively.

Reproducibility

Reproducibility of the method was determined using two urine samples (Table II). The maximum coefficient of variation (C.V.) was 7.0%.

TABLE I

RECOVERY OF VMA AND HVA FROM URINE

Results obtained by analysis of eight urines from normal six-month-old infants.

	n	Concentration range (μmol/l)	Added (μmol/l)	Recovery (mean ± S.D., %)
VMA	7	5.0—18.9	40	96.6 ± 3.8
HVA	8	6.1—33.3	44	94.8 ± 2.4

TABLE II

REPRODUCIBILITY OF THE METHOD

Results obtained by analysis of ten urine replicates at two concentrations on one day.

	n	Concentration (mean ± S.D., μmol/l)	C.V. (%)
VMA	10	63.5 ± 1.90	3.0
	10	11.2 ± 0.79	7.0
HVA	10	65.8 ± 0.92	1.4
	10	19.8 ± 0.82	4.1

Correlation with a comparison method

Twenty urine samples from normal six-month-old infants and from two patients with neuroblastoma were assayed by the present method and by the method of Miyagawa [8], which employs an ODS column, fluorescence detection and sample extracted with ethyl acetate. The two methods were run in parallel; the results are plotted in Figs. 2 and 3. The data for VMA fit the

regression equation $Y = 0.86X + 0.17$, where X is the method of Miyagawa, with a correlation coefficient of 0.992. The data for HVA fit the regression equation $Y = 0.98X + 0.49$, with a correlation coefficient of 0.998.

The extractability of VMA from aqueous solution into ethyl acetate is low, being lower from dilute hydrochloric acid than from acidified urine [12]. Low extractability causes a large variation in the results. Therefore, VMA in aqueous solution must be extracted three times with ethyl acetate [6, 7] or the method of standard addition (spiking) must be used for each urine [12]. Direct on-column injection of urine samples was studied to solve this problem in our laboratory.

An ODS column has generally been used, but the affinity of VMA for ODS is weak and thus it is difficult to separate VMA from other urine constituents. The difference in affinity between VMA and HVA is large for ODS. Therefore,

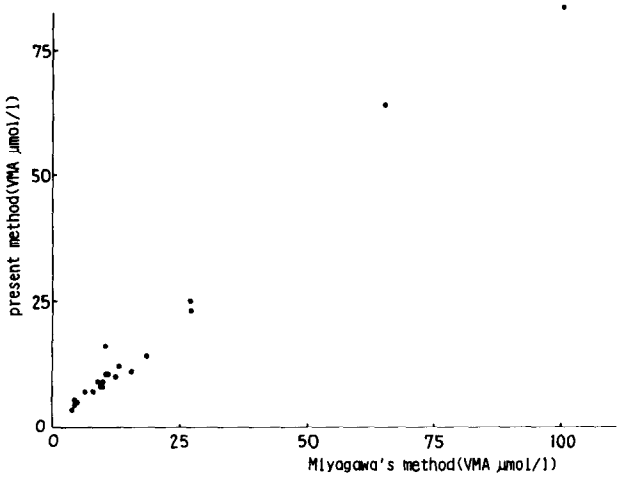

Fig. 2. Correlation between the present method and the method of Miyagawa for VMA assay.

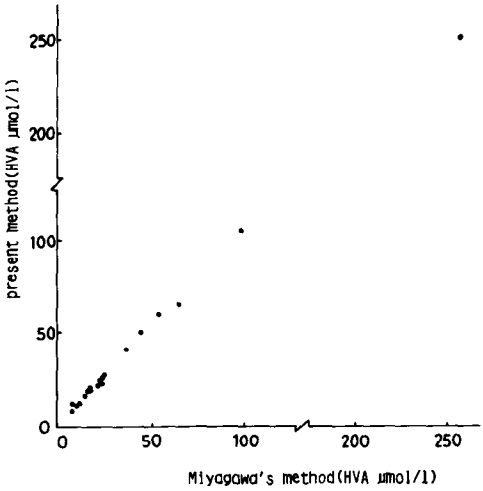

Fig. 3. Correlation between the present method and the method of Miyagawa for HVA assay.

the method reported previously required pre-treatment which extracts VMA (and HVA) with ethyl acetate [11, 12], paired-ion chromatography [9] or gradient elution [10]. An ion-exchange column was studied to solve this problem in our laboratory.

To enable use of a dilute urine sample, electrochemical detection, which has high sensitivity, was studied.

Thus isocratic elution and direct urine sample can be used, and the results obtained by this method were satisfactory.

CONCLUSION

The method described was applied to the determination of the VMA and HVA content of urine from normal infants and patients with neuroblastoma. The method shows the following advantages: short analysis time, selectivity and the possibility to be automated.

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