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## Note

# Liquid chromatographic determination of vanillylmandelic acid and homovanillic acid by a column-switching technique involving direct injection of urine

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Measurement of urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA), the major final metabolic products of catecholamine, has been advocated in the detection and monitoring of patients with neuroblastoma. Recently, liquid chromatography (LC) has been adopted to measure the levels of urinary VMA and HVA, because of the greater accuracy of this method. For the determination of urinary VMA and HVA by LC, pretreatment on various types of short column [1–4] or an organic extraction procedure [5,6] are commonly used, because it is difficult to separate these compounds from other urinary constituents without pretreatment. However, these methods not only are time consuming but also often result in low recoveries. Furthermore, a method that identifies as many urinary constituents as possible without pretreatment is suitable for the acquisition of medical information.

Several LC methods using an anion-exchange column [7] and gradient elution [8] have been reported for the direct determination of VMA and HVA in urine. We perform mass screening of neuroblastoma by LC for all infants who are less than one year of age, and it is of great importance to be able to analyse urinary VMA and HVA more rapidly than by these methods. Recently, column-switching techniques have been widely used [9–12], and it seems that these techniques can be of advantage for biological materials, including catecholamines and their

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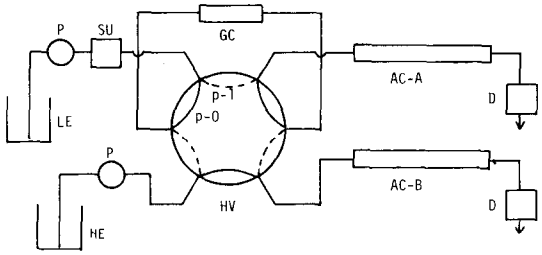


Fig. 1. Flow diagram of the column-switching valve system. HV = high-pressure six-way valve; AC = analytical column; GC = guard column; D = detector; SU = sample injection unit; LE = low-strength eluent; HE = high-strength eluent; P = pump; p-0 = valve in position 0; p-1 = valve in position 1.

metabolites. This paper presents a simple and rapid method for the determination of VMA and HVA in untreated urine.

## EXPERIMENTAL

### *Reagent*

Acetonitrile, potassium phosphate, phosphoric acid and ethylenediaminetetraacetic acid sodium salt (EDTA) were purchased from Nakarai (Kyoto, Japan), VMA and HVA were purchased from Sigma (St. Louis, MO, U.S.A.). The prepared mobile phases were filtered under vacuum through a 0.45-$\mu$m membrane filter. A stock standard solution of VMA and HVA (0.1 mg/ml) was prepared in 0.1 *M* phosphoric acid and stored at 4 °C. A working standard solution was prepared by diluting the solution 100-fold with 0.1 *M* phosphoric acid.

### *LC instrumentation*

We used a Model LC6A system (Shimazu, Kyoto, Japan), with a system controller (Sil 6A). The effluent was monitored electrochemically with a glassy carbon electrode (Kyoto Irika Kogyo, Kyoto, Japan).

### *Sample preparation*

Urine was collected from an infant on a cotton disk pad and placed in a small container, which was mailed to our laboratory for testing. A 0.1-ml aliquot of urine from the container was mixed with 1.4 ml of 0.1 *M* phosphoric acid and centrifuged at 1870 *g* for 10 min. The supernatant (5–30 $\mu$l) was injected directly onto the LC column.

### *LC procedure*

The flow diagram of the column-switching valve system and the time programme of the system controller are shown in Fig. 1 and Table I. The columns, mobile phases and LC operating conditions were as follows: guard column, Cosmosil 5C18 (50 × 4.6 mm I.D., Nakarai); analytical column A, Shimpac CLC ODS (150 × 6.0 mm I.D., Shimazu); analytical column B, YMC-AM 302 ODS (150 × 4.6 mm I.D., Yamamura, Tokyo, Japan); low-strength eluent, 0.02 *M* potassium

TABLE I

SEQUENCE OF HPLC WITH VALVE-SWITCHING OPERATED BY SYSTEM CONTROLLER

| Time (min) | Operation | Effect |
|---|---|---|
| 0.0 | Sample injection; valve position 0 | Sample is loaded onto guard column. Lipophilic constituents are adsorbed; hydrophilic constituents are eluted. |
| 3.2 | Data sampling by integrator start | |
| 3.8 | Valve-switching into position 1 | Lipophilic constituents are eluted from the guard column. Constituents in both groups are separated on the analytical column A and analytical column B. |
| 11.8 | Valve-switching into position 0; time programme stop | Ready for next injection. |

phosphate buffer pH 3.2–acetonitrile (1000:5, v/v); high-strength eluent, 0.02 *M* potassium phosphate buffer pH 3.3–acetonitrile (1000:135, v/v). Both mobile phases contained $2 \cdot 10^{-5}$ *M* EDTA. The flow-rate was 0.95 ml/min, and the temperature of the column oven was 50 °C. The detection was electrochemical at 0.75 V vs. Ag/AgCl.

## RESULTS AND DISCUSSION

### *Chromatography*

The first step of the LC procedure was a rough separation of hydrophilic and relatively lipophilic groups of urinary constituents on a guard column, followed by separation of each group on two different columns with different mobile phases for each constituent (Table I and Fig. 1). The valve was initially in position 0, indicated by the solid line in Fig. 1, and diluted urine was loaded on the guard column with low-strength eluent. The hydrophilic group of urinary constituents was eluted from the guard column within a switching time of 3.2 min and separated on the analytical column A continuously with low-strength eluent. After valve-switching from position 0 to position 1 (indicated by the dashed line), relatively lipophilic constituents in urine retained on the guard column were eluted with high-strength eluent, followed by separation on the analytical column B continuously with the same eluent. Both eluents were monitored by the two electrochemical detectors. The separations of the hydrophilic group, including VMA, and the relatively lipophilic group, including HVA, on each analytical column proceeded almost simultaneously.

The chromatograms resulting from analysis of urine from a normal infant are shown in Fig. 2. Fig. 2A is a chromatogram of hydrophilic urinary constituents and Fig. 2B that of a relatively lipophilic group. The compositions of the mobile phases were chosen after analysis of a large number of urine samples. The reten-

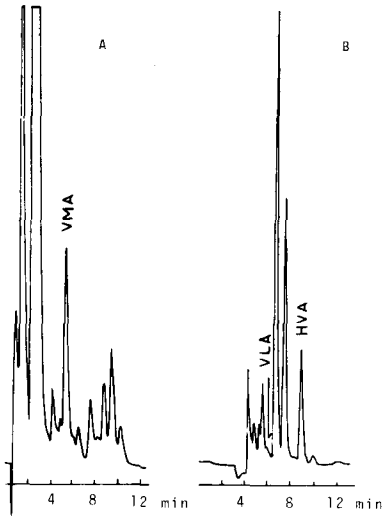


Fig. 2. Chromatogram of urine sample from a normal infant. Recording was started 3.2 min after sample injection. (A) Hydrophilic constituents; (B) lipophilic constituents.

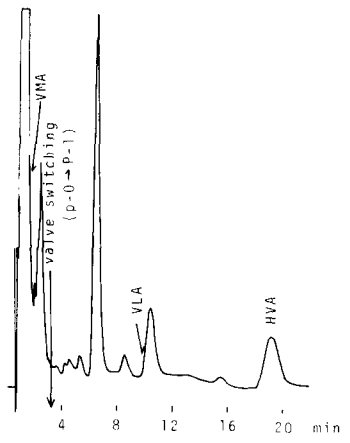


Fig. 3. Chromatogram of urine sample from a normal infant. Analysis was performed with a guard column and a low-strength eluent.

tion time of VMA increases as the pH decreases; however, the retention times of the constituents eluted close to VMA depend on the concentration of acetonitrile rather than the pH of the mobile phase. This relation serves to determine the most suitable composition for the low-strength eluent. Because the retention time of VMA is sufficiently long (ca. 8.5 min) with the low-strength eluent, a clear separation of VMA from other constituents could be achieved without any pretreatment. As shown in Fig. 2A and B, the difference between the retention times of VMA and HVA is very small, ca. 3.5 min, which leads to a reduction of time required for analysis. If VMA and HVA are determined simultaneously on one column with a mobile phase suitable for VMA separation, it takes much too long for the HVA peak to appear. Even if gradient elution is used, with which urinary HVA can be eluted more rapidly after the separation of VMA, more than 22 min are required between injections owing to the time needed for re-equilibration of the column. In addition, gradient elution is not fully compatible with electrochemical detection (unstable baseline).

The time of valve-switching was determined from the results of separation of urinary constituents on the guard column with a low-strength eluent, so that a single constituent would not be divided into different groups (Fig. 3). The separation of HVA from other urinary constituents is easier than for VMA; however, the separation of vanillyllactic acid (VLA), used for the diagnosis of neuroblastoma, from the other constituents was difficult when large peaks appeared close to VLA. The composition of the high-strength eluent and the interval of the sample injection should be determined so that the HVA peak does not overlap with the constituents of the sample injected previously.

According to the questionnaires returned with urine samples answered by the parents of about 4800 infants, the diets of these infants were found to be diverse, including a banana, which would interfere with the VMA spot test, vegetables, cakes, and an egg. However, dietary influence on the separation of VMA or HVA was not observed.

*Sample solution*

Phosphoric acid (0.1 *M*) was used to dilute the urine samples, because the pH values of the urine samples were above 9 in some cases. When the phosphoric acid concentration of the solution used for the dilution of these urine samples was too low, the pH of the sample solution was above 5, which resulted in varying retention time of VMA.

*Electrochemical detector*

Although the detection limits obtained with an electrochemical detector were sufficiently low for VMA (10 ng/ml) or HVA (5 ng/ml), some difficulties must still be overcome. The most important problem is that the stability of the baseline can be influenced by the alteration of pH, ionic strength, and concentration of acetonitrile in the mobile phase. To minimize this difficulty, the time of valve-switching was determined so that the peaks of urinary constituents did not overlap with the peak caused by the change of mobile phase.

The sensitivity of the electrochemical detector was affected by fluctuations of temperature: it dropped 20–30% at ca. 10°C below room temperature. Accordingly, a sensitivity correction was made by measuring a reference sample of VMA and HVA every twenty urine samples.

*Reproducibility and recovery*

Intra-assay precision was determined by analysis ($n=10$) of three urine samples of normal infants. The maximum coefficients of variation were 2.6% for VMA and 2.0% for HVA. Recoveries of VMA and HVA were tested ($n=8$) by adding 5–20 μg/ml of each compound to two urine samples of normal infants. The mean recoveries of VMA and HVA were 102% and 107%, respectively.

## CONCLUSION

A procedure involving direct injection of urinary samples for the determination of VMA and HVA by an automated column-switching technique was developed. Hydrophilic constituents and relatively lipophilic constituents of urine are separated individually with different mobile phases and on different analytical columns, after separation into each group on the guard column. The major advantages include simplification of pretreatment of urine and reduction of the time required.

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