Determination of Urinary Vanillylmandelic Acid and Homovanillic Acid by High Performance Liquid Chromatography for Mass Screening of Neuroblastoma

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SATO, Y., HANAI, J., TAKASUGI, N, and TAKEDA, T. Determination of Urinary Vanillylmandelic Acid and Homovanillic Acid by High Performance Liquid Chromatography for Mass Screening of Neuroblastoma. Tohoku J. exp. Med., 1986, 150 (2), 169-174 — In order to develop a mass screening method for the early detection of neuroblastoma, we measured urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA) by a high performance liquid chromatography (HPLC) equipped with an electrochemical detector. Urinary samples were collected on a piece of filter paper and then pre-treated by a simple method. Measurement of VMA and HVA was performed within a short period of time, and by about 80 samples per a day could be analyzed by automatic system. The method described herein proved to be reliable, and we thus recommend it as a useful method for measurement of both VMA and HVA, leading to more accurate detection of neuroblastoma in mass screening test. ——— VMA ; HVA ; HPLC ; neuroblastoma ; mass screening

Neuroblastoma is noted for its poor prognosis and high frequency in infants. Improvement of the prognosis may rely on early discovery and treatment probably before the year of one (Breslow and McCann 1971; Gitlow et al. 1973; Evans 1980). Therefore, to develop mass screening methods for early detection of neuroblastoma are an important need in pediatrics today.

As the tumor cells of neuroblastoma produce catecholamines, of which the metabolites are excreted into the urine in significantly high quantities, measurement of the amount of metabolites in urine is expected to produce a biochemical diagnosis of neuroblastoma (Gitlow et al. 1973; LaBrosse et al. 1976; Laug et al. 1978).

In 1973, Sawada (Sawada et al. 1982, 1984) began conducting a mass screening test (LaBrosse’s VMA spot test) (LaBrosse 1968) for neuroblastoma by detecting one of the metabolites of catecholamines, VMA, in infantile urine spotted on

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a filter paper. However, some infants do not excrete VMA although they have neuroblastoma (LaBrosse et al. 1976; Laug et al. 1978), and so these patients were undetectable by the spot test. Moreover, the spot test is rendered unreliable by interference by some kinds of food (Sawada et al. 1982).

To overcome these problems, we developed a system employing the HPLC, which make possible the simultaneous measurements of both VMA and HVA as well as the analyses of a large quantity of samples. This method has been applied in a mass screening program conducted in Sapporo city (Sato et al. 1985), some of the results will be reported here.

**MATERIALS AND METHODS**

**Apparatus**

The HPLC system was composed of the following parts: a model 638-50 high performance liquid chromatograph (Hitachi, Tokyo); a model LC-4B electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN, USA); a model 7000B integrator (System Instruments Corp., Tokyo); a model KSST-601 autosampler (Kyowa Seimitsu, Tokyo); and a model 635-0335 column temperature regulator (Hitachi, Tokyo).

**Chromatographic conditions**

The mobile phase contained 0.05 M tartrate buffer (pH 3.7) and acetonitrile (500:70, v/v) and the flow rate was 1.0 ml/min. A stainless steel column, 4 mm i.d. × 250 mm, was packed with Hitachi Gel No. 3013-0 (particular size, 5 μm), and kept at 40°C. The detector potential was set at 0.85 V vs. Ag/AgCl reference electrode and sensitivity was 50 nA at full scale.

**Reagents**

VMA (DL-4-hydroxy-3-methoxymandelic acid), HVA (4-hydroxy-3-methoxy-phenylacetic acid) and other standard compounds of metabolites were obtained from Sigma (St. Louis, MO, USA). Acetonitrile and ethylacetate were used for HPLC grade and other reagents were of reagents grade (Wako, Tokyo).

**Urinary specimens**

Urine was collected on a filter paper (Toyo, No. 2, 10 × 7 cm) that was mailed to mothers, who were instructed to the paper into their infant’s diaper. Wet filter paper was used as the urine samples.

**Determination of VMA and HVA**

The wet filter paper with urine was dried and 5 pieces of paper, 10 mm i.d., were prepared and placed in a test tube, to which 1.0 ml of 0.5 N NaOH was added. Urine was eluted by applying ultrasonic wave for 10 min. To the eluent, 1.0 ml of 2.6% HCl was added, gently mixed, and 1.0 ml aliquot was removed for creatinine determination. The residual solution was saturated with NaCl, 2.5 ml ethyl acetate was added and shaken for 10 min. The 1.5 ml of the ethyl acetate layer was removed and put into a sample cup and the extract was evaporated to dryness in a water bath at 40°C. The residue was dissolved in 200 μl of the mobile phase solution and 10 μl aliquot was injected into the column. Urinary creatinine was determined by Jaffé’s method colorimetrically.
RESULTS

Under the HPLC conditions stated above, VMA and HVA were eluted at 3.7 and 12.3 min, respectively, and were successfully separated from other main metabolites of catecholamines, VLA, MHPG, DOPAC, 5-HIAA and VA (Fig. 1). Linear relationship between the current and the amount of VMA (0-2 ng) and HVA (0-4 ng) was detected. As eluates of other compounds were not found after 15 min in the samples examined, the sample could be injected into HPLC every 18 min, and thus about 80 samples could be assayed daily.

Ratio of elution from the filter paper by ultrasonic wave was estimated by the addition of 0.5 μg of standard VMA and 1.0 μg of HVA, and the recovery was 92.4% for VMA and 96.1% for HVA.

Ratio of extraction with ethyl acetate was poorer as the pH increased, and the percentage of extraction of VMA was markedly lower when the pH was higher than 3.5 (Fig. 2). Thus, for extraction, the pH was set at less than 1. As only one extraction was performed, the overall recovery of VMA was 70% on the average and that of HVA 90%. A calibration curve was prepared by the following procedure at each assay. The normal infantile urine of known concentration containing a standard mixture of VMA and HVA was applied to a piece of filter paper. VMA and HVA of each filter paper were determined by the same pretreatment of samples, and a calibration curve was prepared from the values obtained. The lowest limit of the assay by the present method, using 5 pieces of filter paper of 10 mm i.d., were 0.005 μg of VMA and 0.01 μg of HVA.

![Fig. 1. Chromatograms of catecholamine metabolites.](image)

(A) Mixture of standard compounds (1 μg/ml); (B), Urine extract of a normal infant; (C), Urine extract of an infant with neuroblastoma. VMA, vanillylmandelic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; VLA, vanillactic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid; VA, vanillic acid.
Reproducibility using the same specimen was expressed as the coefficient of variation of retention time, 1.17% of VMA and 0.38% of HVA, and as the peak height, 1.3% of VMA and 3.8% of HVA. When the same filter paper was used for 5 consecutive days, the coefficient of variation of VMA and HVA were 7.5% and 7.1%, respectively.

To examine the accuracy of the present method, the assay values obtained by the electrochemical detector method were compared with those by the fluorescence detector method. VMA gave a correlation coefficient of 0.948 and a regression curve of $Y = 0.95X + 1.38$, and HVA gave a correlation coefficient of 0.949 and a regression curve of $Y = 0.93X + 1.10$, which revealed good correlations in VMA and HVA (Fig. 3).
Table 1 shows VMA and HVA levels in the urine of 4,603 infants, who were the subjects of the mass screening. There was no difference of VMA and HVA levels according to the ages examined, 6 months to less than 1 year old, among the present subjects, and the mean value of VMA was 11.7 μg/mg creatinine and HVA 17.1 μg/mg creatinine.

**DISCUSSION**

Measurement of urinary VMA and HVA has been performed on HPLC equipped with ultraviolet spectrophotometric (Dziedzic et al. 1979), fluorescence (Yoshida et al. 1976) and electrochemical (Morrissey and Shihabi 1979a, b) detectors. In these detectors, electrochemical detector has both high sensitivity and specificity for catecholamine metabolites.

However, urine on the filter paper were so far not used as the specimens for the measurement of VMA and HVA. In the proposed method, VMA and HVA in the urine on the filter paper can be assayed within 15 min after a brief pretreatment. Furthermore, no eluates were found after 15 min in the urine extract of 6 to 12 months old infants, thus, the samples can be successively assayed at intervals of 18 min.

Besides, we provided normal levels of urinary VMA and HVA in 6 to 12 months old infants using an automated HPLC. By setting up cutoff values of VMA and HVA based on these results, the mass screening of neuroblastoma can be performed with accuracy and without missing patients. These findings indicate that the present method is useful for the mass screening of neuroblastoma in infancy.

**References**


