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Note

High-performance liquid chromatographic procedure for the analysis of urinary 3-methoxy-4-hydroxymandelic acid

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Measurements of 3-methoxy-4-hydroxymandelic acid (VMA) in urine are a small but important portion of the workload of many clinical chemistry laboratories. VMA is the main metabolite of dopamine and norepinephrine or epinephrine, and determination of VMA in urine has some importance in the diagnosis of neuroblastoma and in treatment of this disease and Parkinson's disease.

Although many techniques have been applied to the assay of VMA, their complexity, inadequate sensitivity, poor reproducibility or non-specificity have seriously compromised their value in the diagnosis of neural crest tumors.

Among methods for separating and identifying VMA in urine are paper chromatography [1, 2], thin-layer chromatography (TLC) [3, 4], spectrophotometry [6—8], electrophoresis [9, 10], gas chromatography [11—13], gas chromatography—mass spectrometry [14—16] and, more recently, high-performance liquid chromatography (HPLC) [17—25].

Paper chromatography and TLC procedures lack precision and require too much time for a routine procedure. Although gas chromatographic methods offer specificity and sensitivity, the procedures are too complex for routine use in a clinical laboratory. The other methods are less specific.

HPLC would appear to be adaptable to VMA assay. Direct HPLC analysis for VMA in urine, after its separation by column chromatography, is complicated because many other urinary compounds absorb at wavelengths of 254 nm or less, where VMA absorbs strongly. Thus, if one uses a highly sensitive UV detector, extensive clean-up of the urine before chromatography is required.

Therefore, VMA determination in urine samples is carried out in two steps: (1) extraction of VMA from the urine matrix by column chromatography with graphitized carbon black (GCB), and (2) quantitative determination of

the eluate by HPLC with reversed-phase column and UV detection.

The present procedure is compared with that developed by Pisano et al. [8].

EXPERIMENTAL

Materials and methods

HPLC apparatus. We used a high-performance liquid chromatograph, series 3, with a Model LC 75 detector (both from Perkin-Elmer, Norwalk, CN, U.S.A.) and a Rheodyne Model 7125 syringe loading sample injector (Berkeley, CA, U.S.A.).

The instrument was operated in the isocratic mode and the absorbance of the eluent was monitored at 280 nm, with the aid of a Perkin-Elmer Model 56 strip-chart recorder. The reversed-phase column of 10 ⅴ

strip-chart recorder. The reversed-phase column of 10 μm average particle size used was a Perkin-Elmer C18/10 (25 × 0.46 cm, No. 258-0184). All the experiments were carried out at room temperature.

Reagents and adsorbent. All the reagents used were of highest purity (A.C.S. certified grade). Methanol (special grade for liquid chromatography), phosphoric acid, potassium dihydrogen phosphate were from Carlo Erba (Milan, Italy). For the VMA standard, a stock solution of VMA (1 g/l, purchased from Sigma, St. Louis, MO, U.S.A.) was prepared in 10 mmol/l hydrochloric acid. This solution is stable for many months at 4°C.

The GCB (Carbopack B, Supelco, Bellefonte, PA, U.S.A.) is characterized by a surface area of 100 m^2 g^{-1} and 80—100 mesh size.

Chromatographic conditions. The low-strength eluent (0.01 M KH$_2$PO$_4$, pH 3.0) was filtered through a 0.22-μm filter (No. GSWPO 4700; Millipore Corp., Bedford, MA, U.S.A.). The high-strength eluent was methanol. The ratio between low- and high-strength eluent was 99:1. The flow-rate was 1.2 ml/min. Chromatographic peaks were quantitated by measuring absorbance at 280 nm.

Evaluation of the chromatograms. Initial peak identification of VMA was performed on the basis of retention times and comparison with the reference compound. Peak identity was also confirmed using stopped-flow UV spectra. The UV spectrum was then scanned in the region between 220 and 320 nm.

Procedure

Urine samples. Urines (24-h) used for the correlation and normal value study were collected in glass bottles containing 10 ml of 6 M hydrochloric acid.

Extraction procedure using GCB column. A glass column (I.D. 0.4 cm) with PTFE cock was filled with 250 mg of GCB (80—100 mesh) to a height of 5 cm. The GCB was retained by two small plugs of silanized glass wool. The column was packed by passing through 5 ml of methanol and 5 ml of distilled water. Then 1 ml of acified urine (pH 2.0 with HCl) was passed through the column by gravitational flow.

After the urine had passed through the column, the column was washed with 0.5 ml of 0.01 M hydrochloric acid. Then VMA was eluted from the

GCB by methanol at a rate of 0.5 ml/min. The first fraction of 0.5 ml was discarded. The methanol fraction between 0.5 and 1.9 ml was collected and dried at 40°C by a gentle stream of nitrogen.

The residue was dissolved in 100 μl of methanol and 10 μl of this solution were analyzed by HPLC.

RESULTS AND DISCUSSION

The majority of clinical laboratories are still utilizing non-specific colorimetric VMA assays. Many of these procedures have too long analysis times, and frequently lack the sensitivity and specificity required. HPLC is potentially a powerful technique for the separation and quantitation of compounds in complex samples.

The use of a purification procedure with the GCB column and subsequent analysis by HPLC shows the following advantages: short analysis time, selectivity, sensitivity and the possibility to be automated.

Experiments were conducted in order to obtain the best analytical conditions for the separation of VMA from other UV-absorbing urine constituents. The possibility to operate in the isocratic mode and the use of a UV detector was obtained by the clean-up step for urine using GCB as adsorbent. This material allows the isolation of VMA in 1 ml of acidified urine and, in the subsequent elution, a marked selectivity with respect to the other UV-absorbing constituents.

Elution and recovery curves

It is possible to determine the minimum amount of solvent necessary for an efficient recovery from the elution and recovery curves.

In Fig. 1 the elution curve of VMA from urine passed through the GCB column is shown. On the ordinate, q_i is the amount recovered within the portion of solvent indicated; on the abscissa, q_t is the total amount of VMA.

The elution curve is only slightly tailing in the right part. This allows the total recovery of VMA simply by using small amounts of methanol (fraction between 0.5 and 1.9 ml).

Choice of mobile phase

Use of highly polar mobile phase is required to elute VMA as a sharp, symmetrical band, when chromatographed on a C18 reversed-phase column. The retention time of VMA depends on the pH of the mobile phase. We chose a pH of 3.0 because it results in a favorable retention time for VMA and is well within the buffer range for orthophosphate.

Chromatographic peaks were identified by retention time with the reference compounds and stopped-flow UV scanning. The use of stopped-flow scanning coupled with the identification retention time is very helpful in a complex biological matrix such as urine.

Recovery

Recovery studies were performed by adding specified quantities of VMA to aqueous solutions of urines. Then 1-ml aliquots were submitted to the
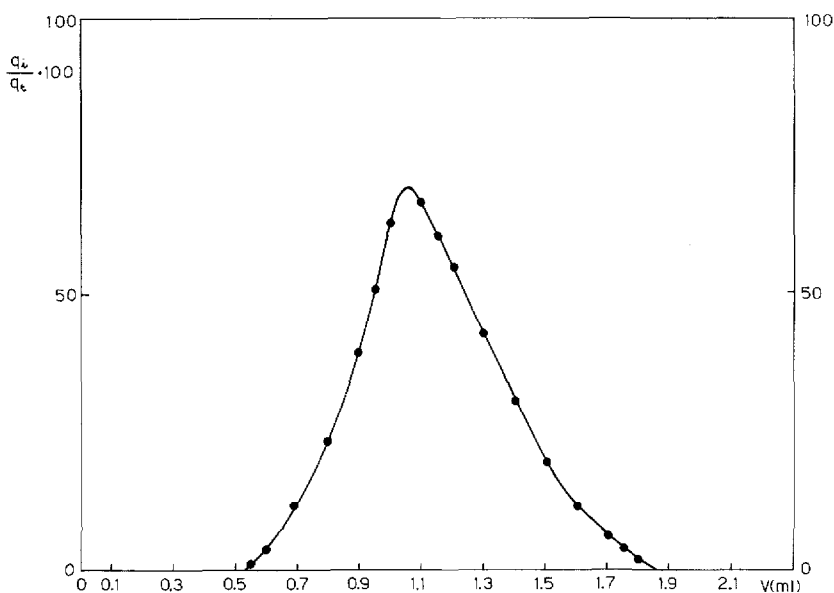
Fig. 1. Elution curve of VMA from urine.

purification procedure. We performed analytical recovery studies using aqueous standard to determine the absolute recovery of VMA. Recoveries of VMA from aqueous solution using the adsorption column method varied between 97% and 102% with a mean of 98 ± 2.4% (n = 6) for VMA concentrations in the range 1—50 μg/ml.

Table I shows the analytical recovery of VMA added to urine. Recoveries are always more than 97%. The recoveries shown in Table I indicate that VMA can be measured in aqueous solution or urine, with equivalent results. The analytical recovery studies indicate the excellent efficiency and reproducibility of this procedure.

Linearity of detector response and detection limit

Linearity of detector response was determined by plotting peak heights or areas versus the amount of injected VMA. The response was found to be linear over the entire working range (1—50 μg/ml). The detection limit per 1 ml of urine is 0.5 μg.

TABLE I

ANALYTICAL RECOVERY OF VMA ADDED TO URINE

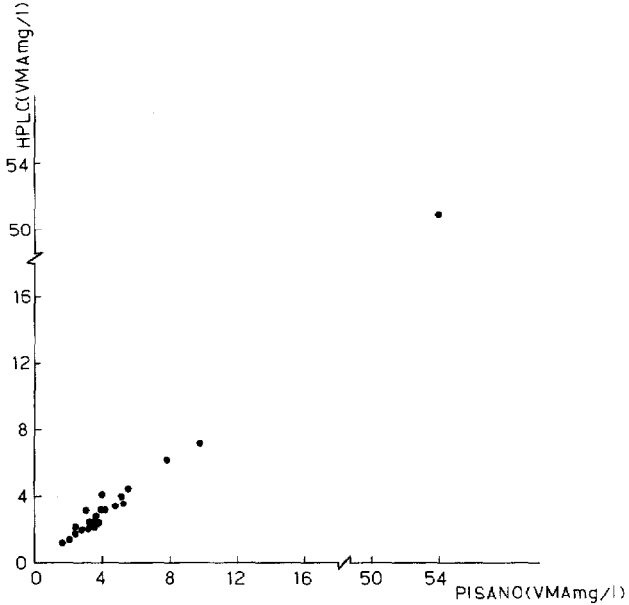| VMA in urine (mean ± S.D., μg/ml)	VMA added (μg/ml)	VMA found (mean ± S.D., μg/ml)	Recovery (mean ± S.D., %)	n
1.15 ± 0.06	2.00	2.05 ± 0.08	97 ± 1.3	6
3.20 ± 0.07	2.00	5.11 ± 0.08	98 ± 2.5	6
2.10 ± 0.06	2.00	4.15 ± 0.09	101 ± 3.3	6
2.71 ± 0.08	5.00	7.60 ± 0.13	98 ± 1.9	6
1.90 ± 0.05	10.00	11.59 ± 0.20	97 ± 2.3	6
2.90 ± 0.09	20.00	22.60 ± 0.38	99 ± 2.1	6

Fig. 2. Correlation between the present method (Y) and the method of Pisano (X).
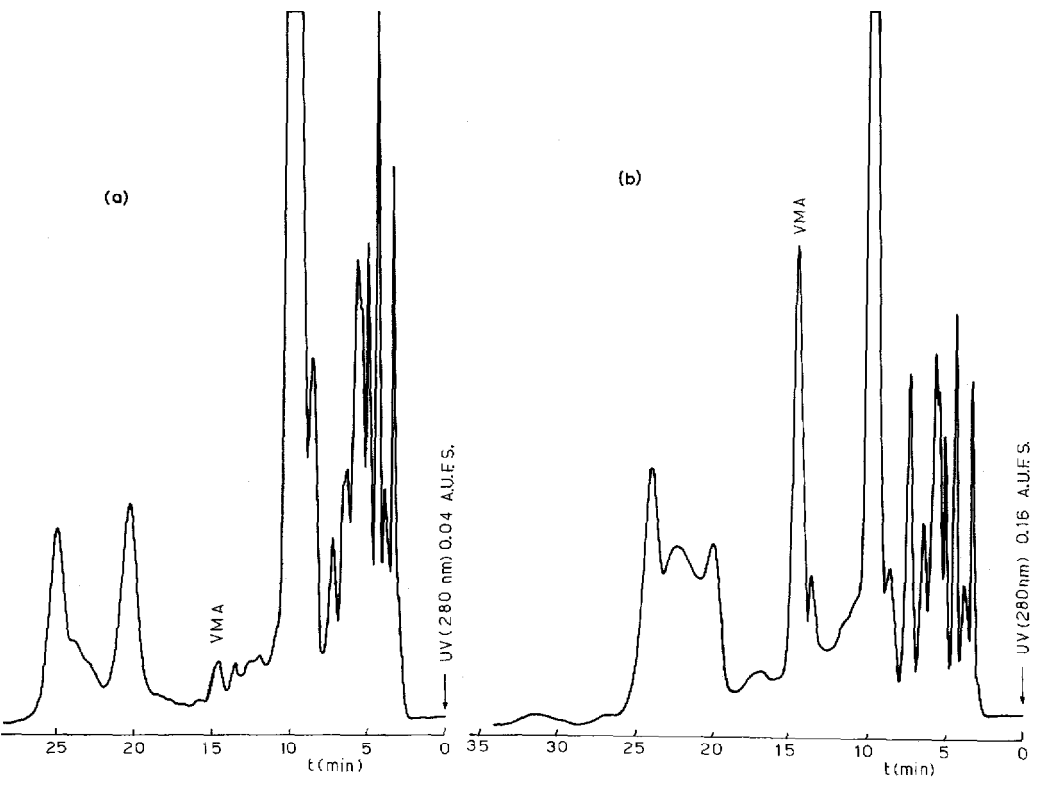

Fig. 3. Chromatograms obtained for 24-h urine samples (a) from a normal person (1.15 μg/ml VMA) and (b) from a patient with neuroblastoma (50.8 μg/ml VMA).

Correlation with comparison method

Twenty-two urine samples were assayed by the method of Pisano et al. [8] and by the present procedure. The two methods were run in parallel; the results are plotted in Fig. 2. The data fit the regression equation $Y = 1.15X - 1.22$, where X is the Pisano method, with a correlation coefficient of 0.987. With few exceptions, this procedure gave lower VMA values than did the classical Pisano method, as shown in Fig. 2. This bias may be attributed to increased specificity in the chromatographic method.

CONCLUSION

The procedure described here shows the advantages of short time, minimal sample, high sensitivity, good accuracy and reproducibility for analyzing VMA concentration in urine. The method is applied to the assay of urine from patients with neuroblastoma, pheochromocytoma and Parkinson's disease. Human urinary VMA values obtained by this method are in agreement with those of other workers [19, 23].

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