Journal of Chromatography, 164 (1979) 345—353
Biomedical Applications

CHROMBIO. 397

ROUTINE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MEASUREMENT OF URINARY VANILLYLMANDELIC ACID IN PATIENTS WITH NEURAL CREST TUMORS

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(Received April 23rd, 1979)

SUMMARY

A rapid and simple reversed-phase high-performance liquid chromatographic procedure for the determination of vanillylmandelic acid (VMA) is described. This method was applied in the determination of the VMA content in urine from normal subjects and patients with neural crest lesions. Sample preparation is minimal and the analysis is short (20 min) and reproducible. The sensitivity of the UV detection is in the ng range. By this technique, fourteen adult control subjects were found to excrete a mean of 2.86 μg VMA per mg creatinine, whereas twelve patients with pheochromocytoma excreted a mean of 15.7 μg VMA per mg creatinine.

INTRODUCTION

The urinary analysis of catecholamine metabolites including vanillylmandelic acid (VMA) has given the clinician the ability to separate those patients with neural crest lesions from the vast majority of hypertensive subjects whose

hypertension is of different etiology [1—3]. The discovery of the metabolic pathways of catecholamines by Armstrong et al. [4] in 1957 revolutionized the approach for diagnosis of these lesions. The more stable O-methylated and/or amine-oxidized metabolites are excreted in much larger quantities than their precursors and thus are more easily quantified. In addition, since characteristic excretion patterns are found for different tumor types [5, 6], it is possible to diagnose and differentiate them by determining the levels of urinary VMA content.

Although a variety of 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 pheochromocytoma. Among the analytical methods for measurement of VMA are spectrophotometry [7, 8], electrophoretic techniques [9], isotope dilution [10], paper chromatography [4], thin-layer chromatography (TLC) [11], gas-liquid chromatography (GLC) alone [12] and coupled with mass spectrometry [13], and more recently high-performance liquid chromatography (HPLC) [14—18]. Although GLC methods offer specificity and sensitivity, the procedures are too complex for routine use by the clinical laboratory. Paper chromatographic and TLC procedures, on the other hand, lack precision and require procedures too time consuming for routine use. Unfortunately, the alternative methods, largely colorimetric, are so non-specific as to almost totally lack clinical usefulness [1].

Recent advances in HPLC suggested the usefulness of this technique for the rapid analysis of urinary VMA. The known separating power of HPLC and its ability to analyze directly non-volatile, polar or thermally labile compounds, make it ideally suited for the analysis of urinary acids. Recently developed microparticulate, chemically-bonded reversed-phase packings for HPLC are slowly displacing the ion-exchange mode because of the simplicity of operation of the reversed-phase columns, their longer life times and the ability to analyze simultaneously both nonpolar and polar compounds. In addition this methodology appeared to offer the requisite sensitivity and specifity to detect and quantify VMA. A method utilizing reversed-phase HPLC coupled with UV spectrophotometric detection was developed for analysis of VMA content in urine from control subjects and those with neural crest tumors.

EXPERIMENTAL

Methods and materials

Random urine samples [1] were obtained from 15 control subjects, 11 patients with pheochromocytoma, 1 patient with ganglioneuroma and 1 with neuroblastoma. Fourteen of the control subjects were adults with uncomplicated essential hypertension and one was a normal 7-month-old baby. All samples were acidified with 6 *N* hydrochloric acid (to pH less than 1) and refrigerated until assayed. A 5-ml specimen was extracted with three 5-ml portions of ethyl acetate. The pooled extracts were then evaporated to dryness under a stream of dry nitrogen. The residue was dissolved in 0.5 ml of distilled water in preparation for assay by HPLC or two-dimensional paper chromatography as subsequently discussed.

A Model 6000 A Solvent Delivery System, Model U6K Universal Injector

and Model 660 Solvent Programmer, all from Waters Assoc. (Milford, Mass., U.S.A.) were used in all determinations. The detection system consisted of two Model SF 770 Spectroflow Monitors (Kratos Inc., Schoeffel Instrument Division, Westwood, N.J., U.S.A.) connected in series. Urine constituents were simultaneously monitored at two wavelengths, 254 nm and 280 nm. One UV monitor was also equipped with a Scanning Drive and MM 700 Memory Module used for obtaining stopped-flow UV spectra.

Areas of chromatographic peaks were electronically integrated using a Hewlett-Packard Model 3380 A Electronic Integrator (Hewlett-Packard, Avondale, Pa., U.S.A.).

Stainless-steel columns (30 cm × 4.6 mm I.D.) were prepacked at the factory with 10 μm totally porous silica support, utilizing an octadecyl (C_{18}) chemically-bonded stationary phase (Waters Assoc.).

All reagents used were of highest purity (A.C.S. Certified grade): VMA was purchased from Sigma (St. Louis, Mo., U.S.A.), and potassium dihydrogen phosphate from Mallinkcrodt (St. Louis, Mo., U.S.A.). Solutions of the reference compound were prepared in distilled–deionized water and kept refrigerated when not in use.

Methanol, distilled in glass, was purchased from Burdick & Jackson (Muskegon, Mich., U.S.A.) and ethyl acetate (pesticide grade) from Fisher Scientific (Springfield, N.J., U.S.A.). All solvents and chemicals for two-dimensional paper chromatography were obtained from Fisher Scientific.

Chromatographic conditions

For the determination of VMA in urine samples, a gradient elution mode of the reversed-phase HPLC was employed. The low concentration eluent was 0.1 *M* KH_2PO_4 (pH 2.50) and the high-strength eluent was a 3:2 (v/v) mixture of anhydrous methanol and distilled–deionized water. The low-strength eluent was filtered through a Millipore membrane filter (Millipore Corp., Bedford, Mass., U.S.A.), pore size 0.22 μm, and the secondary eluent was degassed under vacuum. A 20-minute linear gradient from 0 to 100% of the high-strength eluent was used. The flow-rate was 1.2 ml/min, and the temperature was ambient in all cases. Chromatographic peaks were quantified by measuring absorbance at 280 nm.

Peak identification

Initial peak identification of VMA was performed on the basis of retention times and comparison with the reference compound, and an increase in the area of the suspected peak was taken as further proof of peak identity. In addition, since the peaks in HPLC effluents were simultaneously monitored at 254 nm and 280 nm, ratios of absorbances were computed for the VMA reference compound and compared with those of the peaks in the urine extracts.

Finally, peak identity and purity were also confirmed using stopped-flow UV spectra. In order to obtain these spectra, a blank gradient was run and the flow stopped at the point where VMA elutes. The UV spectrum was then scanned in the region between 220 and 320 nm and the scan automatically stored in the memory module. This permits elimination of the spectral background arising from the changing spectral properties of the solvents, flow cells and the monochromator. Next, samples were chromatographed and the spectrum of the peak

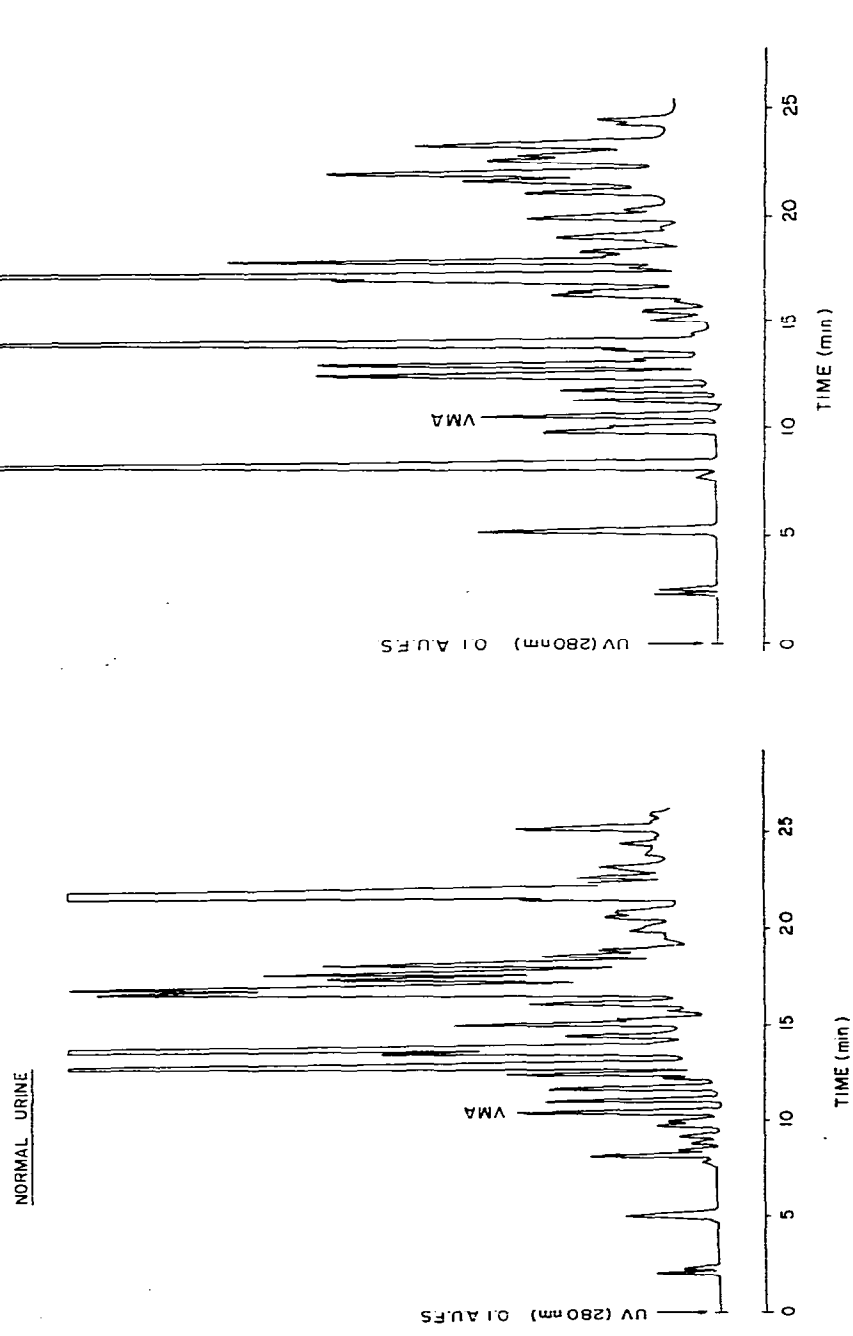

Fig. 1. Chromatogram of the ethyl acetate extract of a urine sample from a control adult subject. Volume injected: 20 µl. Chromatographic conditions: column, µBondapak C_{18} (10 µm particle size); low-strength eluent, 0.1 *M* KH_2PO_4 (pH 2.50); high-strength eluent, methanol—water (3:2, v/v); gradient, linear from 0 to 100% of the high-strength eluent in 20 min; flow-rate, 1.2 ml/min; temperature, ambient.

Fig. 2. Chromatogram of the ethyl acetate extract of urine from a baby. Volume injected: 20 µl. Chromatographic conditions as in Fig. 1

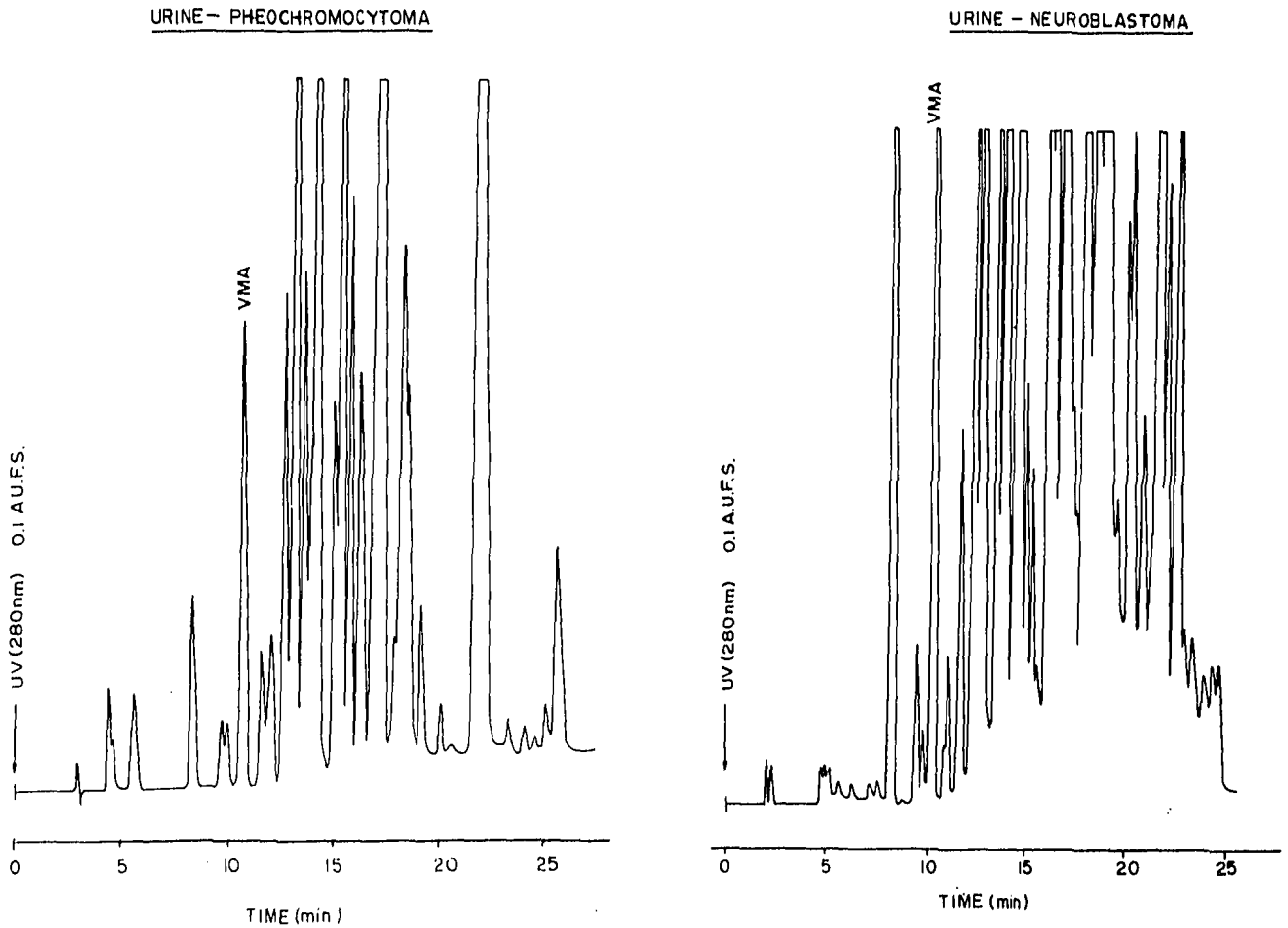

Fig. 3. Chromatogram of the ethyl acetate extract of a urine specimen from a patient with pheochromocytoma. Volume injected: 20 µl. Chromatographic conditions as in Fig. 1.

Fig. 4. Chromatogram of the ethyl acetate extract of a urine specimen from a patient with neuroblastoma. Volume injected: 20 µl. Chromatographic conditions as in Fig. 1.

under study scanned over the same wavelength range. Those spectra were then compared with the spectrum of the VMA reference solution.

Linearity of detector response and detection limits

Linearity of detector response was determined by plotting peak heights or areas versus the amounts of VMA injected. The response was found to be linear over the entire working range. In addition, the lower limit of detection was found to be approximately 10 ng.

Reproducibility of retention times and peak areas

Reproducibility of retention times and peak areas was determined by repetitive injections of the urine extracts. The percentage standard deviations obtained from ten chromatograms of the same extract gave 1.0% for retention time and 1.2% for peak areas.

Interferences

This reversed-phase HPLC separation of the UV-absorbing constituents in ethyl acetate extracts of urine was found to be interference-free for all the samples analyzed. The purity of the VMA peak was constantly checked by determining the ratios at 280 nm and 254 nm, and periodically by obtaining the stopped-flow UV spectra. This helped to ensure that the correct peaks were quantified.

Two-dimensional paper chromatography

Urine specimens were concomitantly assayed for VMA content by the HLPC technique noted above and the standard two-dimensional chromatography procedure routinely in use at the Catecholamine Research Laboratory [1, 19].

Creatinine determination

Creatinine content of urine specimens was determined by the method of Jaffé [20].

RESULTS

Experiments were conducted in order to obtain the best analytical conditions for the separation of VMA from other UV-absorbing urine constituents. The reversed-phase gradient elution mode of HPLC was tested in the analysis of urine samples from control subjects and patients with neuroblastoma, pheochromocytoma and ganglioneuroma. Typical chromatograms of urine samples from a control adult subject and a 7-month-old child are shown in Figs. 1 and 2, respectively. The chromatograms of the urine extracts from patients with pheochromocytoma and neuroblastoma are presented in Figs. 3 and 4, respectively.

Chromatographic peaks were identified on the basis of evidence accumulated from retention characteristics, co-chromatography with the reference compound, absorbance ratios and stopped-flow UV scanning. The comparison of the UV spectra of the peak in the urine extract from a patient with pheochromocytoma and the VMA reference compound is shown in Fig. 5. A

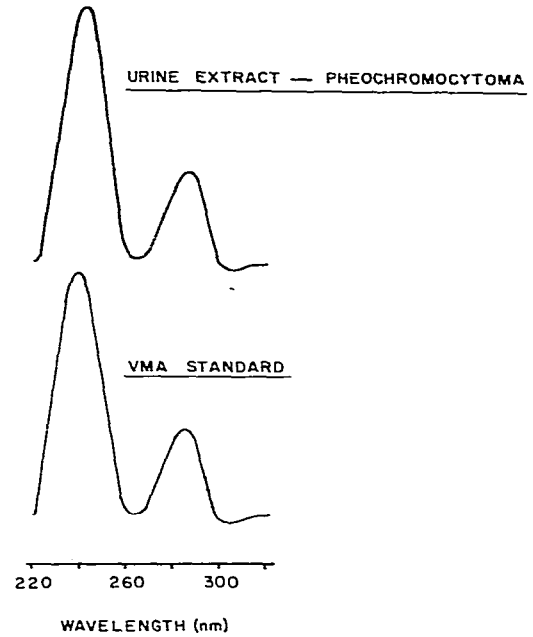

Fig. 5. UV spectra obtained by stopped-flow UV scanning (rate = 100 nm/min).

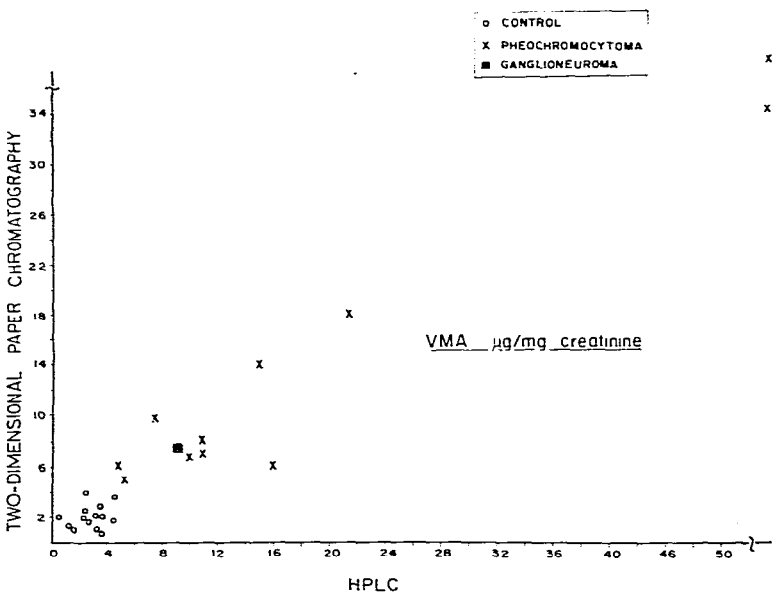

Fig. 6. Correlation of quantitative data obtained by two-dimensional paper chromatography and HPLC (r = 0.96).

scattergram, Fig. 6, depicts the correlation between the techniques. Table I lists the quantitative VMA assay results obtained with each procedure for control subjects and those with pheochromocytoma.

TABLE I

COMPARISON OF HPLC AND TWO-DIMENSIONAL PAPER CHROMATOGRAPHIC QUANTIFICATION OF THE VMA CONTENT IN URINE SAMPLES FROM NORMAL SUBJECTS AND PATIENTS WITH PHEOCHROMOCYTOMA

All values are expressed in μg VMA per mg creatinine.

	n	HPLC		Paper chromatography	
		Mean	Range	Mean	Range
Normal subjects	14	2.86	0.5—4.8	2.25	1—4
Patients with pheochromocytoma	12	15.7	4.5—50	12.5	5—50

DISCUSSION

There is more than ample evidence [1, 21] that the clinical detection and diagnosis of pheochromocytoma can be accomplished but rarely, and that the majority of these life-threatening tumors would remain undetected in the absence of reliable biochemical techniques for determination of their catecholamine by-products. Although assays for VMA have been available to the clinician for over 20 years, many physicians may still be misled by unreliable results [22]. The majority of clinical laboratories in the United States are still utilizing non-specific colorimetric VMA assays. Since by-products of foodstuffs result in falsely elevated values, the normal range of VMA excretion has been set artificially high for such techniques. Such a loss in specificity results in a serious loss in credibility for the practicing physician. In an area where the diagnosis of a highly lethal tumor depends upon factors other than clinical appearance, the absolute reliability of laboratory procedures is essential. In this era of automated clinical laboratories, GLC [12], isotope dilution [10] or two-dimensional paper chromatography [19] have not been welcomed by routine laboratory facilities. It was the above circumstances that led to the development of the present procedure since HPLC is a technique which could ultimately be adapted to automation.

The use of reversed-phase HPLC possesses special advantages: a short analysis time (20 minutes elution time for VMA) and the stability of the microparticulate bonded phases. An average of 400—500 assays can be performed with a single column without deterioration of the plate count or peak distortion. This technique can be used with several on-line methods of peak identification such as absorbance ratios and stopped-flow UV scanning. This is crucial in achieving reliable quantification since in highly populated chromatograms of complex biological matrices such as urine, identification by retention time alone is not sufficient. The electronic integration of peak areas and the high resolving power of HPLC gives highly reproducible results.

Although differences may exist between HPLC and the two-dimensional paper chromatography both techniques can be used for the differentiation of control subjects from those with neural crest lesions. Two-dimensional paper chromatography, while yielding values in close agreement with such reliable

techniques as GLC, must be recognized as being semi-quantitative in nature. If the HPLC method had been compared with the GLC procedure, a more precise correlation might have been anticipated. However, the complexity and difficulty of the assay discouraged its use as a control technique in the present study.

No effort was made to perform a definitive study of the variables which might conceivably influence the present assay procedure. However, as with other specific techniques such as two-dimensional paper chromatography, isotope dilution and gas chromatography, dietary variations do not influence this procedure.

ACKNOWLEDGEMENT

Supported in part by Grant HL-12648 from the National Institutes of Health, and the Scherman Foundation.

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