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# DIAGNOSIS OF NEURAL CREST TUMORS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF URINARY CATECHOLAMINE METABOLITES

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## SUMMARY

Aberrations in the metabolic pathways of catecholamines in patients with neural crest tumors result in characteristic urinary excretion patterns of their catabolites. Tumors such as pheochromocytoma, neuroblastoma and ganglioneuroma usually defy clinical diagnosis because of their rarity, small size, intraabdominal position and clinical symptoms similar to those of essential hypertension.

Quantitative determination of catecholamine metabolites such as vanillylmandelic acid (VMA) and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) offers possibilities for reliable confirmation of diagnosis. However, previous techniques for the assessment of catabolite levels suffered from inadequate sensitivity, reproducibility or specificity, which seriously diminished their usefulness as biochemical determinants in the prognosis of these life-threatening tumors.

Reported in this paper is the analysis of urinary levels of VMA and MHPG using reversed-phase high-performance liquid chromatography with electrochemical and spectrophotometric detection. We present the excretion patterns showing these metabolites in 15 control subjects, 15 patients with pheochromocytoma and 5 patients with neuroblastoma.

## INTRODUCTION

Since the discovery of the metabolic pathways of catecholamines by Armstrong *et al.*[1] in 1957, the determination of catecholamines, their precursors and major metabolites has become of considerable importance in certain disease states and particularly in clinical oncology.

Abnormal secretion patterns of catecholamines and their catabolites have

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contributed considerably to the understanding of parkinsonism[2], essential hypertension[3], muscular dystrophy[4] and neural crest tumors[5–8] such as pheochromocytoma, neuroblastoma and ganglioneuroma.

Pheochromocytoma, a lethal tumor which is a rare cause of hypertension, manifests itself in symptoms commonly associated with elevated catecholamine excretion. Tumors belonging to the neuroblastoma group are one of the most common solid malignant neoplasms of childhood, while ganglioneuromas are rare benign or malignant tumors occurring in young adults.

The relatively small size of these intraabdominal tumors and the possibility of complete remission of patients with malignant neural crest lesions cause great difficulties in clinical and even histopathologic diagnosis.

The deactivation of the catecholamines norepinephrine (NE), epinephrine (E) and dopamine (D) proceeds via 3-O-methylation by the enzyme catechol-O-methyltransferase and oxidation by monoamine oxidase. While homovanillic acid (HVA) is the major metabolite of D, vanillylmandelic acid (VMA) results from deactivation of NE and E in peripheral tissues and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) is the terminal product of NE and E in the brain. The precise quantitative assessment of urinary catecholamines and their catabolites such as VMA, MHPG, HVA and total metanephrines (TM) enables the clinician to differentiate between patients with neural crest tumors and the vast majority of those with hypertension of different etiology.

Due to the complexity of the sample matrix, analytical procedures for the determination of endogeneous levels of urinary catecholamine metabolites demand high degree of sensitivity and great resolving power from the separation technique. Most separation and identification methods such as spectrophotometry[9,10], electrophoresis[11], isotope dilution[12], paper chromatography[1] and thin-layer chromatography[13] are too time consuming and/or not adequately quantitative to permit routine and reliable determination of these compounds in biological samples. Gas–liquid chromatography (GLC) with electron-capture or flame-ionization detection, alone[14] or in tandem operation with mass spectrometry[15] offers great sensitivity and specificity, but requires alaborate sample pre-treatment which makes the technique unsuitable for routine clinical use.

The known ability of high-performance liquid chromatography (HPLC) to analyze thermally-labile, nonvolatile or polar compounds is well documented in the literature. Recently, Horváth and co-workers[16,17] have demonstrated in several excellent articles the advantages of the reversed-phase mode of HPLC in the analysis of urinary constituents. They have separated over a hundred UV-absorbing compounds, showing that a wide spectrum of compounds of different polarity can be separated simultaneously. Combined with the use of highly sensitive electrochemical detection, HPLC, particularly in its reversed-phase mode, affords great speed of analysis and reproducible quantitation which are mandatory in establishing clinically useful assays.

Reported in this paper is the use of reversed-phase HPLC as a diagnostic tool for rapid detection of neural crest tumors such as pheochromocytoma and neuroblastoma.

## EXPERIMENTAL

### *Instrumentation*

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 a continuously variable UV detector and electrochemical detector. The variable wavelength detector was a Spectroflow monitor, Model SF 770 (Kratos, Schoeffel, Westwood, N.J., U.S.A.) with the cell volume of 8 $\mu$l. The detector was also equipped with a SFA 339 wavelength drive and MM 700 memory module which were used for obtaining stopped-flow UV spectra.

The voltammetric/amperometric detector, Model E 611 (Brinkmann, Westbury, N.Y., U.S.A.) operates on a three-electrode potentiostatic system and uses a glassy carbon electrode as the working electrode and the Ag/AgCl as the reference. The effective cell volume is 1 $\mu$l.

The chromatographic column was a prepacked $\mu$Bondapak $C_{18}$ (10 $\mu$m average pasticle size) from Waters Assoc.

### *Materials and methods*

All reagents were of highest purity (A.C.S. certified grade). Reference compounds were purchased from Sigma (St. Louis, Mo., U.S.A.), glusulase from Endo Laboratories (Garden City, N.Y., U.S.A.) and potassium dihydrogen phosphate from Mallinckrodt (St. Louis, Mo., U.S.A.). Solutions of reference compounds were prepared in distilled-deionized water and kept frozen when not in use. Acetonitrile, distilled-in-glass, was purchased from Burdic & Jackson (Muskegon, Mich., U.S.A.) and ethyl acetate (pesticide grade) from Fisher Scientific (Springfield, N.J., U.S.A.).

### *Chromatographic conditions*

Urinary catecholamine metabolites were determined by means of a gradient elution mode of reversed-phase HPLC. The low-strength eluent was 0.1 *F* $KH_2PO_4$, pH 2.50, and the high-strength eluent an acetonitrile–water (3:2) mixture. The low-strength eluent was always filtered through a Millipore membrane filter (Millipore, Bedford, Mass., U.S.A.), pore size 0.22 $\mu$l and the high-strength eluent was regularly degasses under vacuum.

A 45-min linear gradient from 0 to 50% of the high-strength eluent was used. The column was periodically flushed with pure acetonitrile in order to elute compounds which exhibit excessively long retention under the chromatographic conditions used. The flow-rate was 1.4 ml/min, and the temperature was ambient in all cases.

### *Sample preparation*

Random urine samples were obtained from 15 healthy subjects, 15 patients with clinically documented pheochromocytoma and 5 patients with neuroblastoma.

*Determination of VMA and free MHPG.* A 3-ml volume of urine, acidified to a pH of approximately 1 using 6 *N* HCl, were extracted with three portions of ethyl acetate (6, 3 and 3 ml). The extracts were pooled and evaporated to dryness under a

stream of dry nitrogen. The residue was then redissolved in 1.0 ml of distilled-deionized water and the extracts subsequently chromatographed.

*Determination of conjugated MHPG.* Since MHPG is excreted into urine as sulfate and glucuronide conjugate, the aqueous phase, remaining after the initial ethyl acetate extraction had to be enzymatically hydrolyzed. Therefore, the pH of the aqueous phase was adjusted to 5.2 and the sample was incubated for 16 h with 0.1 ml of glusulase (mixture of sulfatase and glucuronidase). The incubated sample was acidified to a pH of approximately 1 and extracted with ethyl acetate (6, 3 and 3 ml). The pooled organic layer phase was then treated as described for VMA and free MHPG.

*Extraction efficiency*

Prior to the analysis of physiological samples, the efficiency of the extraction procedure was determined by means of radioactively-labeled VMA and MHPG. The recovery for both compounds was found to be between 95 and 99%.

*Peak identification*

Initial peak identification was performed on the basis of retention behavior and co-chromatography with the reference solutions.

Separation of acidic compounds from the neutral and basic urinary constituents by means of an ethyl acetate extraction, coupled with the selectivity of the electrochemical detection for species containing an electrophore, gave further information concerning the peak identity. The conditions for the electrochemical detection were optimized in order to maximize the response of the compounds under study without loss of specificity. The optimal oxidation voltage was found to be +1.00 V. In addition, whenever the levels of the compounds under study permitted the use of UV detection, stopped-flow UV spectra of the peaks in urine were obtained and compared with those of the reference compounds. This technique, used in combination with retention behavior and co-chromatography with the reference compounds, has proven to be a valuable aid in identification. The details of the use of UV scanning techniques are given in the literature[18].

*Linearity of response and detection limits*

Linearity of detector response with concentration was determined for the compounds under study over the entire working range. Plots of peak heights *vs.* amount of sample injected were found to be linear at least three orders of magnitude beyond the concentration range of interest. For the electrochemical detection, the lower limit of detection for both compounds was found to be approximately 20 pg.

*Reproducibility of retention times and peak heights*

Ten repetitive injections of urine extracts gave relative standard deviation of 1.0% for the retention times, and 1.2% for the peak heights.

*Interferences*

The described analytical technique was found to be interference-free for the samples analyzed. Whenever possible, the spectroscopic purity of the peaks under

study was checked by means of absorbance ratios at several wavelengths, and periodically by obtaining stopped-flow UV spectra.

## RESULTS AND DISCUSSION

In the absence of reliable biochemical techniques for routine diagnosis of neural crest tumors, the clinicians have often been misled by unreliable colorimetric assays for catecholamine metabolites. This has proven to be particularly dangerous

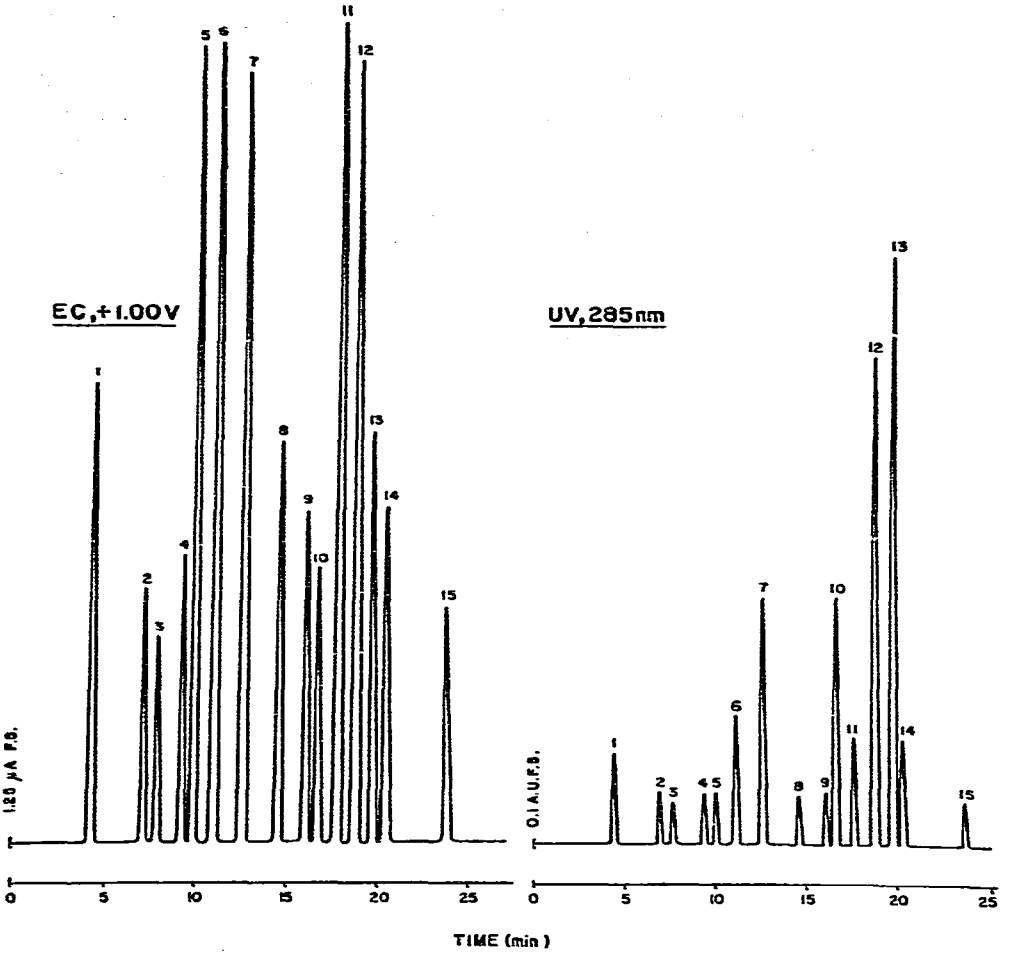


Fig. 1. Separation of a synthetic mixture containing approximately 2 nmoles each of the following: 1 = 3,4-dihydroxymandelic acid; 2 = dopa; 3 = metanephrine; 4 = tyrosine; 5 = vanillylmandelic acid; 6 = 3-methoxy-4-hydroxyphenylethyleneglycol; 7 = 5-hydroxytryptophan; 8 = 3,4-dihydroxyphenylacetic acid; 9 = anthranilic acid; 10 = tryptophan; 11 = 5-hydroxyindole-3-acetic acid; 12 = vanillic acid; 13 = 3,4-dihydroxycinnamic acid; 14 = 3-indoleacetamide and 15 = 3-indole lactic acid. Chromatographic conditions Column: μBondapak $C_{18}$; eluents: (low strength) 0.1 *F* $KH_2PO_4$, pH 2.50; (high strength) acetonitrile–water (3:2); gradient: linear from 0 to 60% of the high-strength eluent in 45 min; flow-rate: 1.4 ml/min; temperature: ambient; detection: electrochemical at +1.00 V and UV absorption at 285 nm.

due to the absence of characteristic clinical symptoms which would enable reliable differentiation of these persistantly secreting tumors from cases of essential hypertension.

In an effort to establish the usefulness of VMA and MHPG as biochemical determinants for reliable diagnosis of neural crest tumors, we have investigated the use of reversed-phase HPLC coupled with electrochemical and spectrophotometric detection.

The separation of some catecholamine metabolites of clinical significance is shown in Fig. 1. As can be seen from this illustration, the UV absorption is considerably less sensitive than the electrochemical detection. This difference is particularly crucial in the analysis of the free levels of MHPG for which the UV absorption is not sufficiently sensitive. In addition, the ethyl acetate extracts of urine samples contain more UV absorbing than electroactive substances which can be oxidized at the selected potential. In order to optimize the conditions for electrochemical detection in terms of sensitivity and selectivity, a reference solution of the compounds under study was chromatographed and detected at different oxidation potentials. Fig. 2 shows the change in response of MHPG with change in oxidation potential. Since optimal response for both compounds was obtained at +1.00 V, this potential was adopted for the protocol procedure.

The developed analytical procedure was then tested in the analysis of urinary VMA and MHPG. In the analysis of free glycol in samples from normal subjects, the use of electrochemical detection is mandatory. A typical separation of the constituents

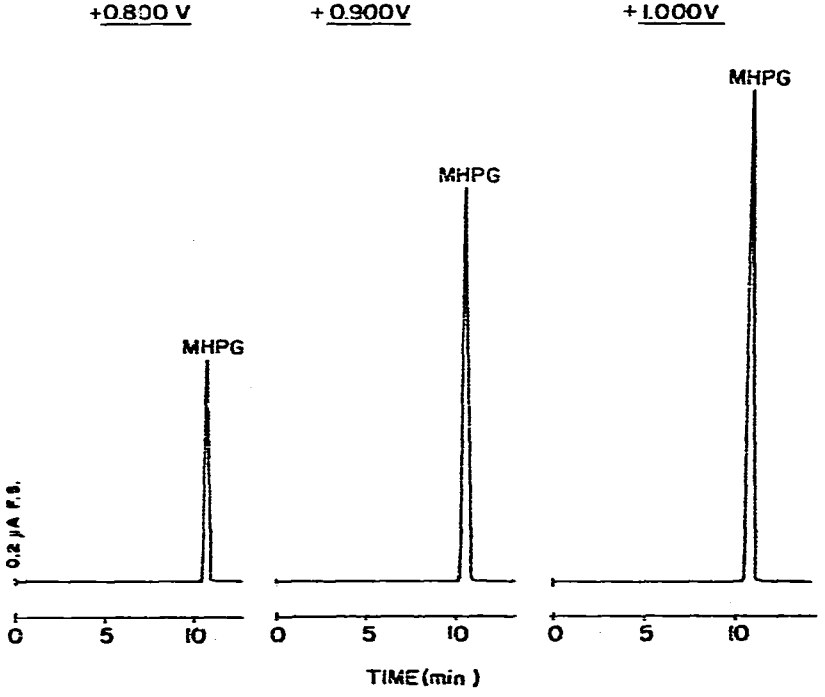


Fig. 2. Change in detector response of MHPG reference solution with change in oxidation potential. Chromatographic conditions as in Fig. 1.

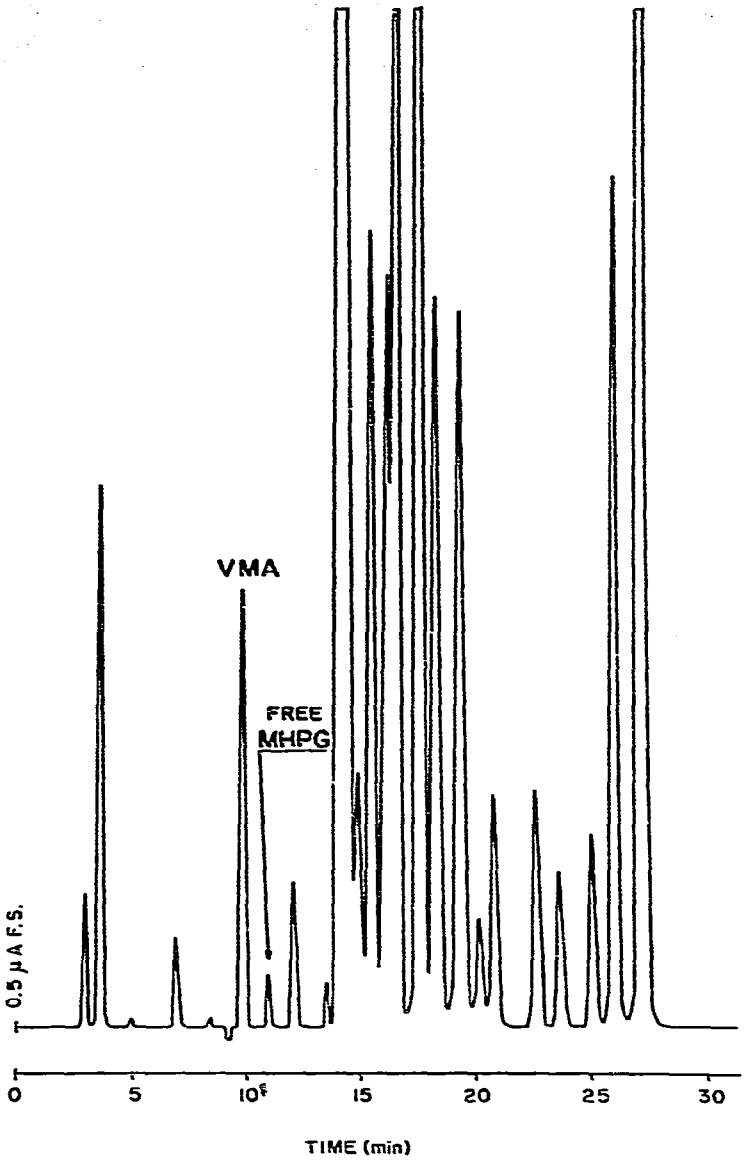


Fig. 3. Chromatogram of the extract of a urine sample from healthy subject. Chromatographic conditions as in Fig. 1; detection: electrochemical at +1.00 V; volume of extract injected: 5 μl.

of the ethyl acetate extract of a urine sample from a normal subject, detected electrochemically at +1.00 V, is shown in Fig. 3. In order to determine the level of the conjugated MHPG in the same sample, the sulfate and glucuronide conjugates of MHPG have to be enzymatically hydrolyzed. A chromatogram of the ethyl acetate extract of the incubated sample is shown in Fig. 4.

The levels of VMA and free MHPG in the ethyl acetate extract of the urine sample from a patient with pheochromocytoma is shown in Fig. 5. Both catabolites

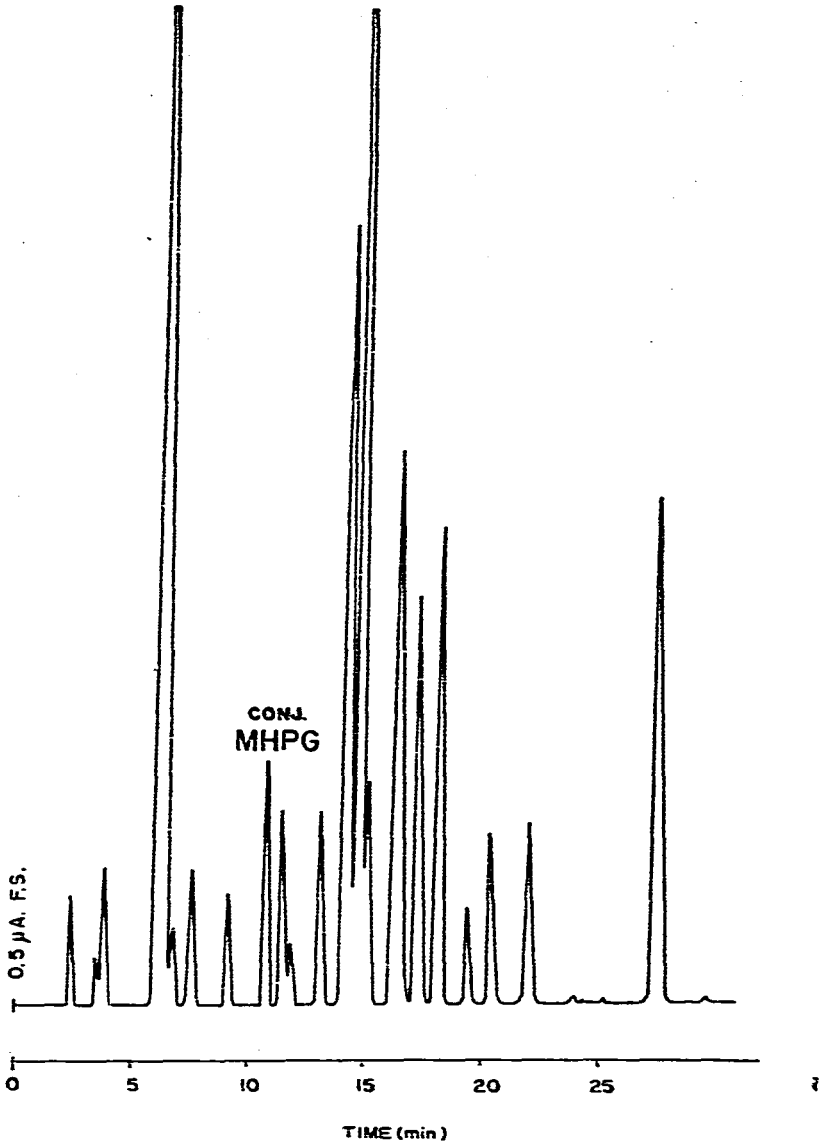


Fig. 4. Chromatogram of the extract of the urine sample from a healthy subject after incubation with glusulase following initial extraction of free acidic compounds. Chromatographic conditions as in Fig. 1; detection: electrochemical at +1.00 V; volume of extract injected: 5 μl.

were found to be elevated in this sample. As can be seen from Fig. 6, deconjugation of MHPG in the urine sample from the same patient also showed an elevated level of MHPG.

In spite of the lower sensitivity and specificity of the UV detection, its use is still possible in the analysis of urinary levels of catecholamine metabolites in patients with catecholamine-secreting tumors. Fig. 7 shows a typical UV profile (detected at

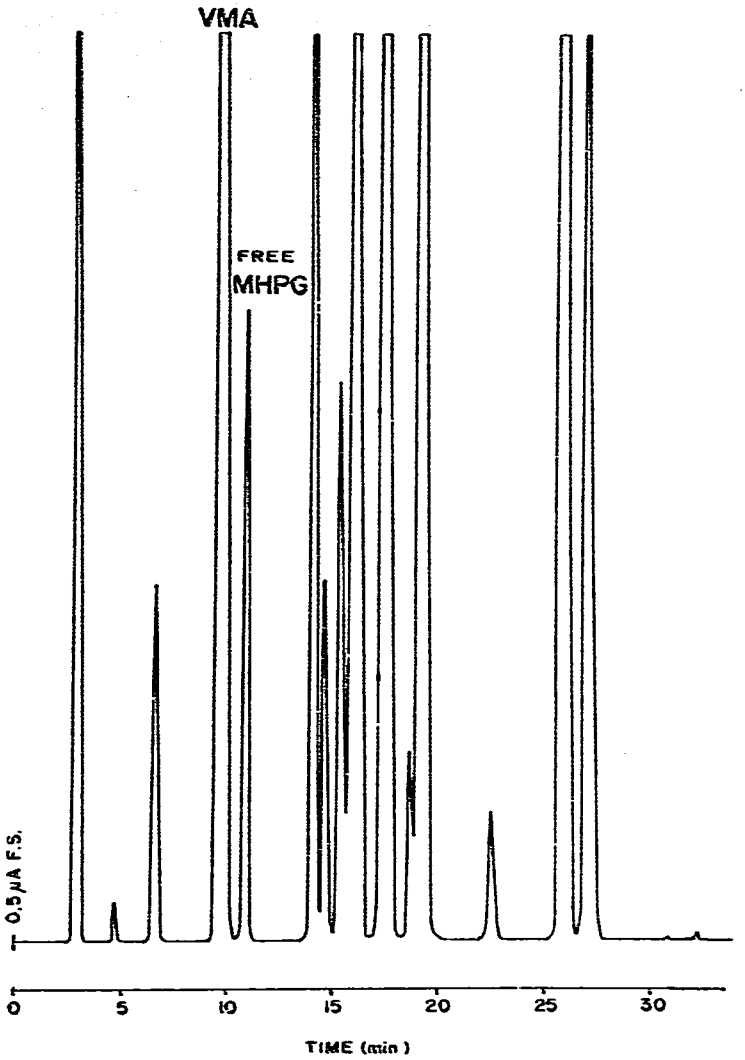


Fig. 5. Chromatogram of the urine extract of a sample from a patient with clinically diagnosed pheochromocytoma. Chromatographic conditions as in Fig. 1; detection: electrochemical at +1.00 V; volume of extract injected: 5 μl.

285 nm) of the ethyl acetate extract of a urine sample from a patient with pheochromocytoma. Due to the high levels of VMA and MHPG it was possible to obtain stopped-flow UV spectra which, although lacking in fine structure, are nevertheless an important fingerprint of the absorber. The comparison of the stopped-flow UV spectra of the reference compounds and the corresponding peaks in the urine extract (Fig. 5) are shown in Fig. 8. It should be noted that this method is more reliable than the use of absorbance ratios since it permits examination of the entire UV spectrum, rather than comparison of several points along the absorption curve.

Analogous results were also found in the case of patients harboring tumors of

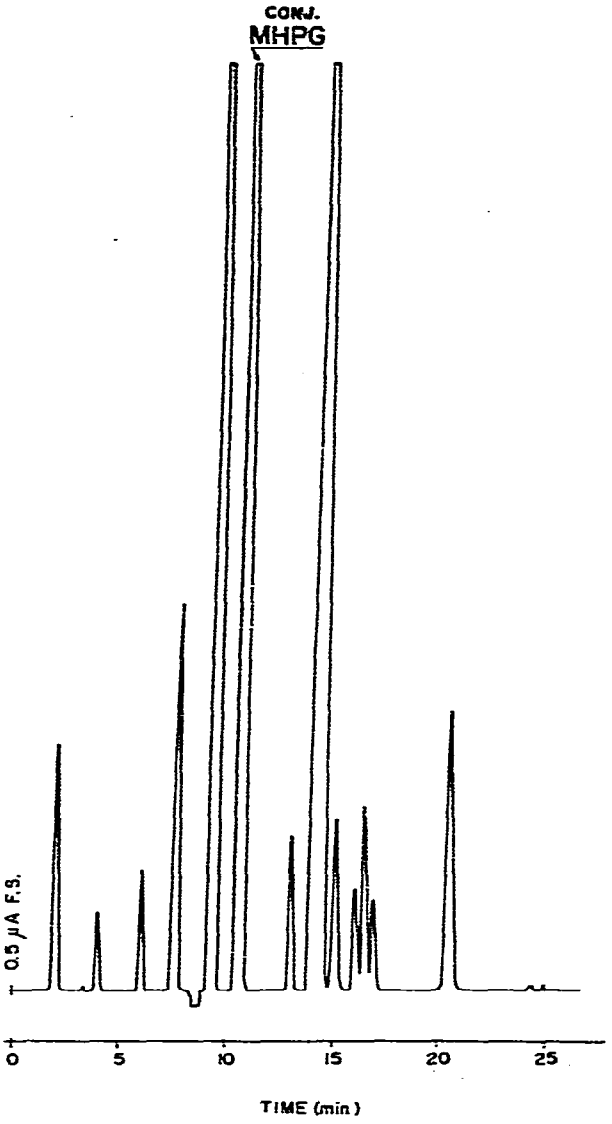


Fig. 6. Chromatogram of the extract of a urine sample from a patient with pheochromocytoma, incubated with glusulase, following initial extraction of free acidic compounds. Chromatographic conditions as in Fig. 1; detection: electrochemical at +1.00 V; volume of extract injected: 5 μl.

the neuroblastoma type. A typical chromatogram of the urine extract from a patient with this tumor are shown in Fig. 9.

An ultimate proof of the sensitivity of the proposed analytical method is exemplified by the determination of the physiological levels of catecholamine metabolites in samples of amniotic fluid. The knowledge of the exact composition of amniotic fluid as a function of gestational age is useful as an index of maturity of the baby, in elucidation of the nature of solute transfer between the fluid, fetus and the

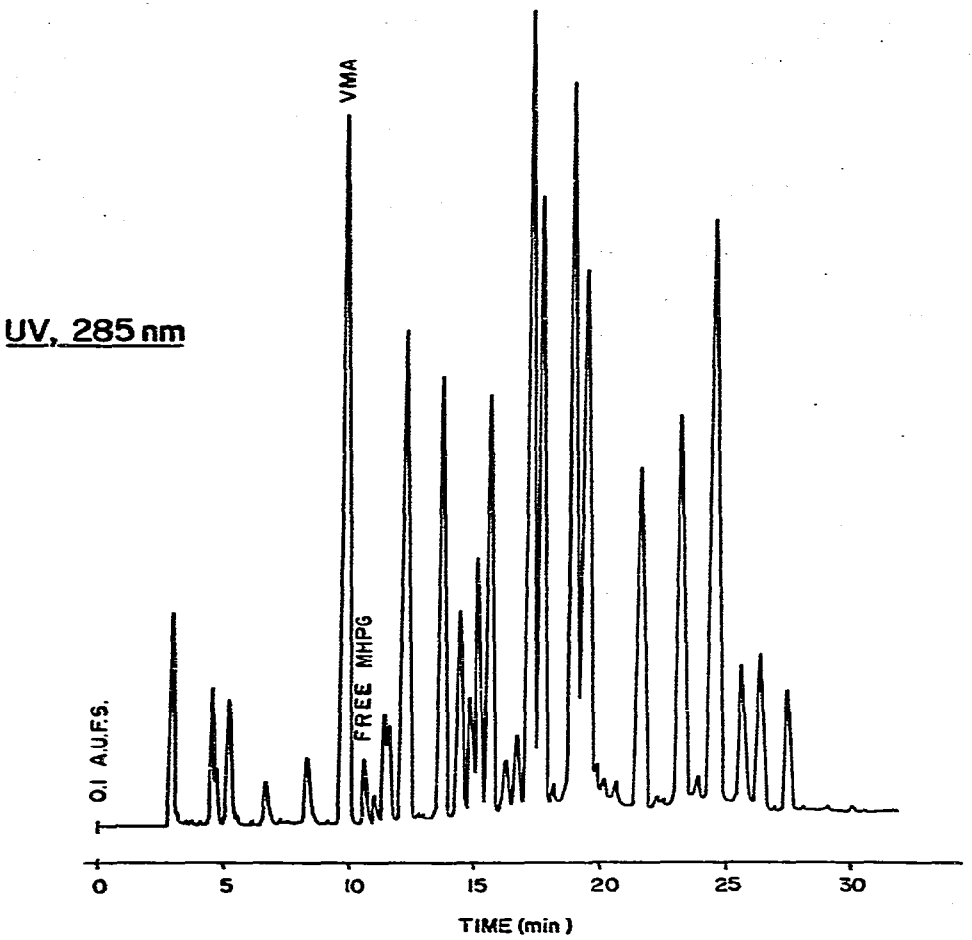


Fig. 7. UV profile of the ethyl acetate extract of a urine sample from a patient with pheochromocytoma. Chromatographic conditions as in Fig. 1; volume of extract injected: 5 $\mu$l.

mother, and also in diagnosis of congenital neuroblastoma and maternal pheochromocytoma. Fig. 10 shows a chromatogram of the amniotic fluid extract detected electrochemically at an oxidation potential of +1.00 V. The concentration of both catabolites is in the ng/ml range.

The establishment of a screening test for neural crest tumors such as pheochromocytoma and neuroblastoma necessitates quantitative assay of VMA and/or MHPG. In the past, the latter catabolite was not often used as an indicator of neural crest lesions due to the significantly higher degree of complexity of the assay procedures.

Previous studies have indicated that the excretion of catecholamine metabolites varied from hour to hour within a narrow range. In order to reduce this variability, it was suggested that rather than using a 24-h urine collection as a reference for excretion values, better reproducibility and reliability could be achieved by using

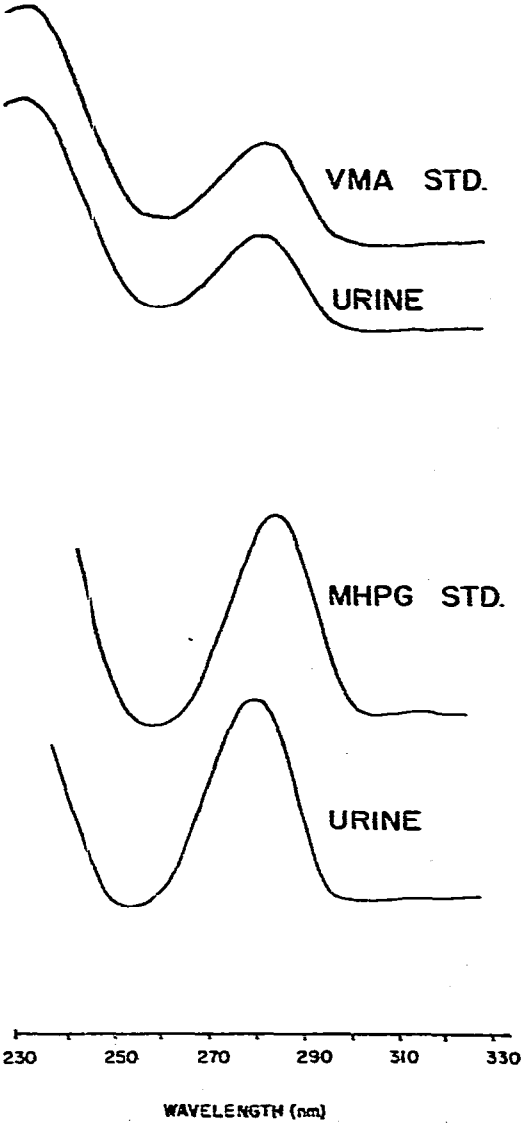


Fig. 8. Corrected stopped-flow UV spectra of the VMA and MHPG reference compounds and the peaks with the same retention times in the sample shown in Fig. 7. Scanning conditions: scanning rate: 100 nm/min; absorbance: 0.1 a.u.f.s.

random urine samples and expressing the catabolite concentration in terms of creatinine content[6,15]. Creatinine, an excretory product of the kidney, is of great clinical importance because of its very constant renal clearance. Quantitative determination of creatinine is most commonly performed by the Jaffé method[20]. Although this reaction has long been an important part of the clinical chemist's armamentarium, it is becoming increasingly apparent that it lacks specificity.

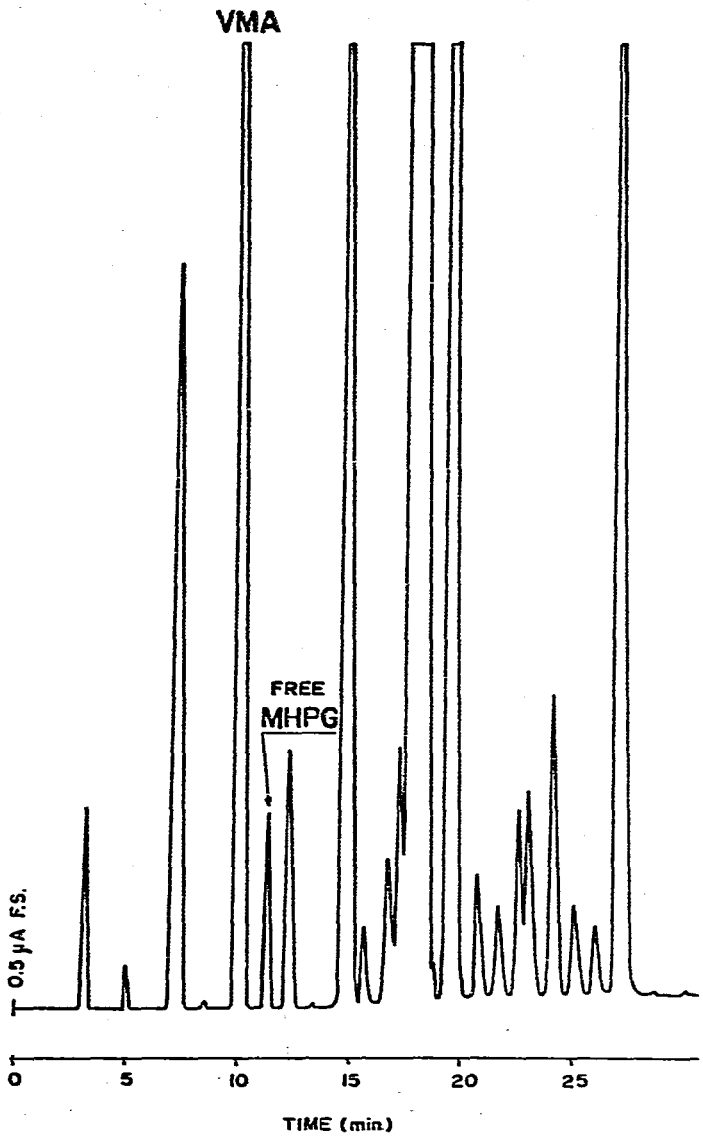


Fig. 9. Chromatogram of the extract of a urine sample from a patient with neuroblastoma. Chromatographic conditions as in Fig. 1; detection: electrochemical at +1.00 V; volume of extract injected: 5 μl.

The use of reversed-phase HPLC with low-wavelength detection as a direct method for measurement of creatinine circumvents problems associated with the reaction of pseudo-creatinine chromogens and affords excellent accuracy and specificity. Fig. 11 illustrates a chromatogram of a diluted urine sample (1:25), detected simultaneously at 235 and 254 nm. Absorbance ratios and stopped-flow UV spectrum of the prominent peak in the chromatogram were used in order to establish peak

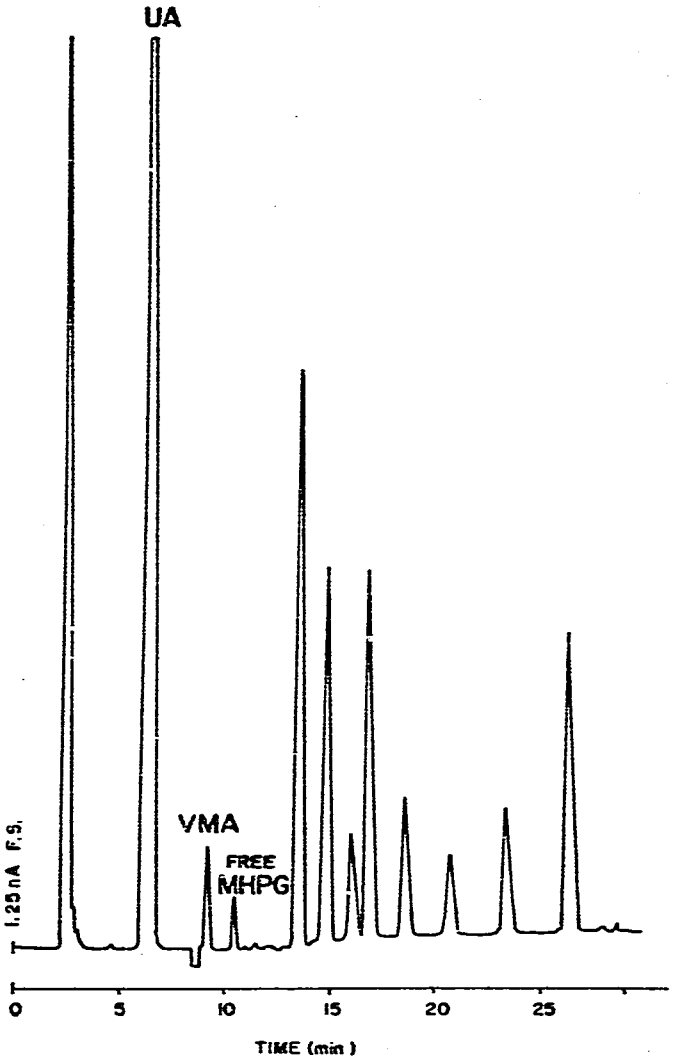


Fig. 10. Chromatogram of the ethyl acetate extract of a sample of amniotic fluid (gestational age: 17 weeks). Chromatographic conditions as in Fig. 1; detection: electrochemical at +1.00 V; volume of sample injected: 100 $\mu$l.

identity. This simple method for assessment of creatinine values facilitates the expression of catabolite excretion in terms of creatinine content.

Quantitative data for VMA, free and conjugated MHPG in urine samples from control subjects and patients with pheochromocytoma and neuroblastoma, expressed in terms of creatinine content, are given in Table I. By inspection, it is immediately evident that both compounds parallel the onset of these tumors. This is to be expected since they both have a common precursor, 3-methoxy-4-hydroxymandelic aldehyde. In the course of this study, no false positives were encountered.

While diet and certain medications[6] are known to influence most of the com-

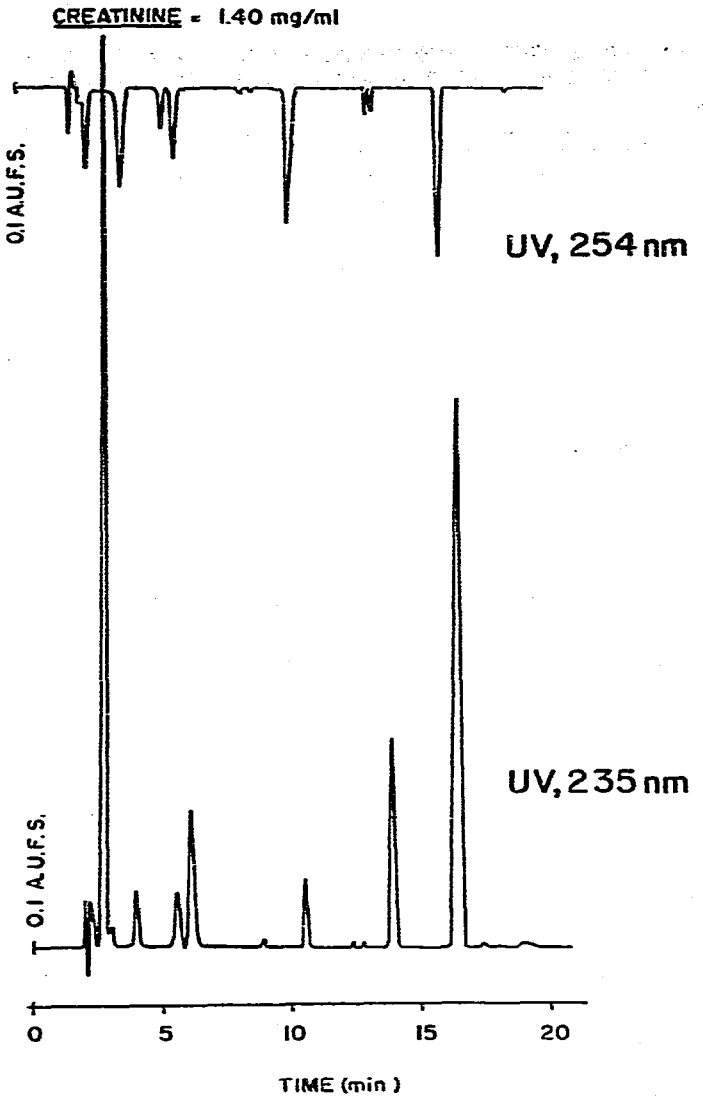


Fig. 11. Chromatogram of a urine sample (1:25 dilution) from a normal subject. Chromatographic conditions as in Fig. 1; detection: UV absorption at 235 and 254 nm; volume of sample injected: 25 $\mu$l.

monly used screening tests, due to its great resolving power the HPLC procedure is not affected by these variables.

The great advantage of the described method is in its ability to monitor simultaneously several compounds of interest. Thus VMA, in addition to MHPG can be measured in the ethyl acetate extract of urinary phenolic compounds.

In addition to its diagnostic merit, the described procedure can also be used for monitoring remission of patients following treatment of surgery.

TABLE I

QUANTITATIVE DATA FOR VMA, FREE AND CONJUGATED MHPG FOR NORMAL SUBJECTS AND PATIENTS WITH PHEOCHROMOCYTOMA AND NEUROBLASTOMA

Results expressed per mg of creatinine.

| | *Normal* | *Pheochromocytoma* | *Neuroblastoma* |
|---|---|---|---|
| *Number of samples* | 15 | 15 | 5 |
| *VMA* | | | |
| Mean | 2.80 | 16.1 | 10.0 |
| Range | 0.5–3.5 | 4.5–50.0 | 5.0–21.0 |
| *Free MHPG* | | | |
| Mean | 0.05 | 0.44 | 0.55 |
| Range | 0.02–0.1 | 0.07–1.15 | 0.09–1.50 |
| *Conjugated MHPG* | | | |
| Mean | 0.6 | 7.7 | 7.4 |
| Range | 0.4–0.6 | 2.4–15.9 | 2.4–16.0 |

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