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Determination of urinary vanillactic acid and plasma dihydroxyphenylalanine as markers of non-secreting neuroblastoma by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

An accurate and precise isocratic high-performance liquid chromatographic technique for the analysis of urinary vanillactic acid (VLA) and plasma dihydroxyphenylalanine (DOPA), especially at low concentrations (pmol/l) for VLA and nmol/l for DOPA), is described. The compounds were purified in a single step, (on an anion exchanger for VLA and on aluminium oxide for DOPA), separated by ion-pair reversed-phase liquid chromatography, and detected electrochemically. A single analysis was complete within 18 min. Mean recoveries of 103 and 81% were obtained for VLA and DOPA, respectively, and the limits of detection were 42 and 76 pmol/l, respectively. The mean values of the intra-assay coefficient of variation were 14 and 7.1% for VLA and DOPA, respectively, and the mean values of the inter-assay coefficient of variation were 15.7 and 11.6%, respectively. Modifications of the retention times (between 2 and 42 min) induced by changes in the eluent were determined. Reference values for normal children and children with neuroblastoma or various tumours are given.

INTRODUCTION

The excretion of acidic metabolites of catecholamines in urine and plasma is of clinical importance in the diagnosis and follow-up treatment of tumours secreting these compounds [1,2]. The measurement of urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA), the major final products of norepinephrine (NE), epinephrine (E) and dopamine (DA), has been advocated for the detection and monitoring of neuroblastomas [3], but not that of vanillactic acid (VLA) which is the final DOPA metabolite (Fig. 1). Nevertheless, 5% of neuroblastomas considered as metabolically inactive do not secrete VMA, HVA, NE, E, DA and methoxyamines. This percentage decreases if VLA and its precursor DOPA are measured [4,5]. The possible significance of these substances in neuroblastoma has received very little attention. One possible reason for this is the difficulty of determining the levels of VLA in urine.

During the past four years, many high-performance liquid chromatographic

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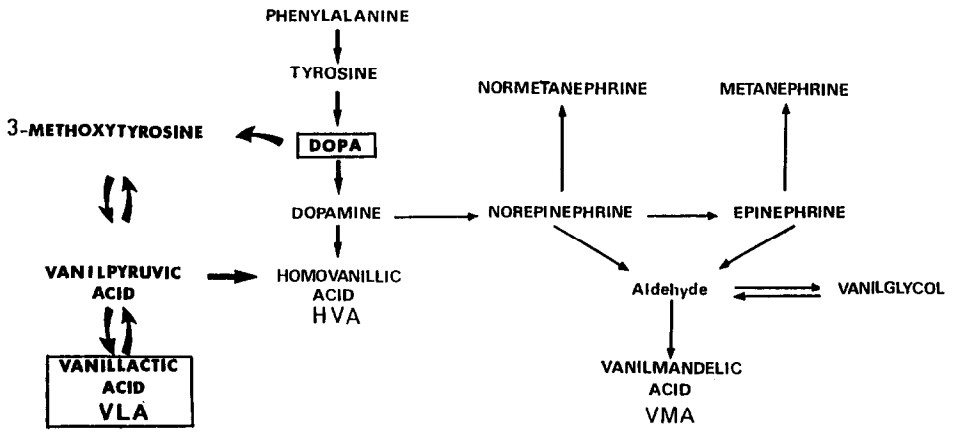

Fig. 1. Simplified scheme of catecholamine metabolism. The metabolic profile of VLA is printed in bold.

(HPLC) methods with electrochemical detection (ED) [6,7] or fluorimetric detection [8,9] have been described for the analysis of catecholamines. Generally, the compounds of interest are extracted from biological samples with organic solvents [10,11], gels [12], gel permeation and solvent extraction [13], anion-exchange resins [14,15], aluminium oxide [16,17] or without pretreatment [18]. HPLC, especially reversed-phase ion-pair chromatography, offers significant advantages over thin-layer chromatography (TLC) and even gas chromatography (GC) [19]. ED provides a high degree of sensitivity for the assay of monoamines.

This paper describes a simple, precise and sensitive HPLC–ED procedure to measure VLA and DOPA after purification.

EXPERIMENTAL

Chemicals

Ethylenediaminetetraacetic acid disodium salt (EDTA), tris(hydroxymethyl)-aminomethane, sodium hydrogenphosphate dihydrate, glacial acetic acid, citric acid, phosphoric acid, sodium disulphite, anhydrous sodium acetate and aluminium oxide (90 active neutral), all of analytical-reagent grade, were obtained from Merck, (Darmstadt, Germany). Absolute ethanol and methanol were purchased from Carlo Erba (Milan, Italy), 1-octanesulphonic acid, sodium salt (Pic B8) from Aldrich (Milwaukee, WI, USA) and β-(4-hydroxy-3-methoxyphenyl)lactic acid, sodium salt (vanillactic acid, VLA), 3-hydroxy-4-methoxybenzoic acid (isovanillic acid, isoVA), L-β-3,4-dihydroxyphenylalanine (DOPA), and 3,4-dihydroxybenzylamine hydrobromide (DHBA) from Sigma (St Louis, MO, U.S.A.).

Aqueous solutions were prepared from twice-deionized water with a Millipore system.

Reagents

Acetate buffer (2 *M*, pH 6.1), adjusted with 0.5 *M* sodium hydroxide or 0.5 *M* acetic acid, 100% sodium disulphite, 5% EDTA, 2 *M* Tris/EDTA (pH 8.7) adjusted with 10 *M* sodium hydroxide, 0.2% Tris/EDTA (pH 8.1) adjusted with 0.5 *M* sodium hydroxide and 50:50 (v/v) 1 *M* acetic acid–ethanol were used.

For the elution of DOPA, a solution was freshly prepared by mixing 100 μl of acetic acid, 50 μl of 10% sodium disulphite, 50 μl of 5% EDTA with twice-deionized water to make a final volume of 10 ml.

Standard solutions

A VLA stock solution (426.9) n*M*) was prepared in 0.01 *M* acetic acid. A dilute working standard (14.23 n*M*) in 2 *M* acetate buffer (pH 6.1) was prepared daily. A working internal standard of isoVA (2.972 μ*M*) was prepared in methanol. A DOPA stock solution (507 n*M*) was prepared in 0.01 *M* acetic acid. A dilute working standard (20 p*M*) in 0.01 *M* acetic acid was prepared daily.

The stock solution of DHBA (454 n*M*) was diluted with 0.01 *M* acetic acid to give a (227 p*M*) working internal standard solution.

Stock and working solutions were stored at −4°C.

Urine and plasma collection

A 48-h diet without bananas, pineapple, tomatoes, chocolate, nuts and products containing vanilla was prescribed before urine and plasma collection. Specimens of 24-h urine were collected in plastic bottles containing 20 ml of 6 *M* hydrochloric acid (pH < 3) and stored at −20°C.

Blood was drawn by direct venipuncture after overnight fasting and collected into heparinized lithium vials (10 I.U./ml). The sample was immediately centrifuged (2000 *g*, 10 min) at 4°C, and the separated plasma was frozen and stored at −70°C prior to analysis.

Extraction procedure

VLA analysis. A 5-ml volume of acetate buffer (2 *M*, pH 6.1) containing 400 μl of internal standard were added to a 3-ml urine sample. The sample was mixed thoroughly, adjusted to pH 6.5 with 1 *M* sodium hydroxide and allowed to percolate through the ion-exchange resin column from Bio-Rad (Ref. 1955005, Bio-Rad Clinical Division, Paris, France). The effluent was discarded. The column was washed with 5 ml of 2 *M* acetate buffer (pH 6.1), followed by 15 ml of reagent 1 *M* acetic acid–ethanol and both washings were discarded. The VLA was eluted five times with 9 ml of reagent acetic acid–ethanol. The collected eluents were pooled, diluted 1:9 (v/v) in mobile phase and filtered through Millipore filter (pore size: 0.45 μm). A 50-μl aliquot was injected into the HPLC instrument. A standard (3 ml of 2 *M* acetate buffer containing 42.6 pmol of VLA) was extracted with the urine using the same amount of internal standard.

DOPA analysis. In a 2-ml polypropylene sample tube (Waters Millipore, Mil-

TABLE I

ANALYTICAL RECOVERY, ACCURACY, PRECISON AND DETECTION LIMIT FOR VLA AND DOPA

Sample	Amount present	Amount added	Amount found	Recovery[a] (%)	Intra-assay precision		Inter-assay precision		Detection limit[b] (pmol/l)
					Mean	C.V. (%)	Mean	C.V. (%)	
VLA in urine (n = 5) (pmol/l)									
Urine 1	840	4269	5158	100.9	1693	12	1856	14.5	42
Urine 2	3368	2134	5758	104.6	4221	16	4390	17	
Urine 3	1122	853	2049	103.7	1975	14	2119	15.5	
DOPA in plasma (n = 5) (nmol/l)									
Plasma 1	12.71	20.28	27.03	83.5	14.77	6.5	15.48	11	76
Plasma 2	9.62	10.14	15.80	79.9	11.22	7.8	12.22	9.5	
Plasma 3	23.51	5.07	23.39	81.8	18.17	7.0	19.04	13	

[a] Ratio of (amount found after spiking)/(amount before spiking + amount added in spike).

[b] At a signal-to-noise ratio of 3.

ford, MA, U.S.A.), 500 μl of plasma and 50 μl of DHBA standard solution were vortex-mixed for 30 s and briefly centrifuged at 10 000 *g* at 4°C. Using a sapphire spatula especially provided for this purpose (Waters), 10 mg of aluminium oxide and 400 μl of 2 *M* Tris–EDTA (pH 8.7) were added. The mixture was shaken for 5 min vertically and centrifuged for 1 min at 10 000 *g* at 4°C. The supernatant was removed by aspiration (avoiding the uptake of aluminium oxide particles), 1 ml of 0.2% Tris–EDTA (pH 8.1) was added to the remaining material, and the mixture was shaken for 1 min. After refrigerated centrifugation for 1 min the supernatant was removed. This procedure was repeated twice more. During the final aspiration care was taken to remove all the washing reagent. To the aluminium oxide in the sample tube were added 100 μl of eluent reagent. The mixture was shaken for 5 min vertically and centrifuged for 30 s. The supernatant was removed carefully, and a 5-μl aliquot was injected into the HPLC instrument. A standard (0.5 ml of 0.01 *M* acetic acid containing 10 pmol of DOPA) was extracted along with the plasma using the same amount of internal standard.

Analytical separation, detection and peak identification

A Waters system was used. It consisted of a Model 510 pump, a stainless-steel column (150 mm × 3.9 mm I.D.) packed with Nova-Pak C_{18} (4 μm particle size), and a Model 460 electrochemical detector. This detector was connected to a CR-5A Shimadzu recorder–integrator (Kyoto, Japan).

The mobile phase was a mixture of 15% (v/v) methanol in 0.02 *M* citric acid–0.02 *M* Na_2HPO_4 (2:1, v/v) buffer. To 100 ml of this buffer, $2.5 \cdot 10^{-3}$ *M* sodium octyl sulphate and $5 \cdot 10^{-5}$ *M* disodium EDTA were added [8]. The pH was adjusted to 3.6 with 2 *M* sodium hydroxide or 2 *M* phosphoric acid. Prior to use the mobile phase was filtered through an HVLP 04700 Durapore membrane (Millipore) and helium-degassed. All separations were carried out at room temperature. Once set up, the system was run continuously to ensure stability and sensitivity. During sample injections the flow-rate was usually 0.4 ml/min, and this was reduced to 0.1 ml/min when the system was not in use.

The working electrode potential was 0.8 V *vs.* a KCl reference electrode.

The peaks of VLA, isoVA, DOPA and DHBA were identified from the retention times of the standard peaks and of the peaks from the specimens, after modification of the various components of the mobile phase and after variation of the oxidation potential between 0.5 and 1.1 V.

RESULTS AND DISCUSSION

Recovery, accuracy, precision and detection limits

In order to determine the precision and the accuracy of the entire procedure for analysis of VLA and DOPA, five analyses of three different urine samples, alone or spiked with different amounts of VLA, and three different plasma samples, alone or spiked with different amounts of DOPA (Table I), were analysed on the same day and on different days.

The mean values of the recovery of VLA added to urine was 103%, and the recovery of DOPA added to plasma was 81%.

The mean values of the intra-assay coefficient of variation (C.V.) were 14% for VLA and 7.1% for DOPA, and the mean values of the inter-assay C.V. was 15.7% for VLA and 11.6% for DOPA.

The detection limits, also shown in Table I, were defined as the concentration of the compound necessary to produce a singal three times higher than the background noise under assay conditions.

Linearity

The linearity of the response was investigated for urine and plasma samples after the addition of increasing amounts of VLA (3–30 pmol) to 3 ml of urine, or of DOPA (5–40 pmol) to 500 μl of plasma. At each point, five determinations were made. Linear relationships between the VLA concentration and the peak height, and between the DOPA concentration and the peak height, were observed over the concentration ranges studied. The equations for the calibration curves obtained were: for VLA, $y = 10.3x + 3$; for DOPA, $y = 4.7x + 2.5$. The coefficient of correlation (r) was 1 for both VLA and DOPA.

Quantification of VLA and DOPA concentrations

The concentration of VLA or DOPA in each sample was calculated by determining the peak-area ratio relative to isoVA or DHBA, and comparing them with those obtained with synthetic standards.

Optimization of the chromatographic conditions

The chromatographic conditions described were checked after a further study of the different parameters that might have changed the retention time, the pH, the concentration in 1-octanesulphonic acid (Pic B8), the concentration in methanol or the concentration in Na_2HPO_4 (PO4).

It was important to evaluate the pH in the assay, because the pH (3.6) of the mobile phase determines the concentration of the ionic forms of DOPA or VLA and thus the degree of ion-pairing with citrate and 1-octanesulphonic acid [20,21]. When the pH was changed from 4.5 to 3.2, the retention time of DOPA increased from 4.5 to 13.2 min and that of VLA from 8.4 to 13.9 min. A low pH should be used to obtain a good separation of all substances. At the pH values studied here, the retention times of all compounds were decreased upon addition of methanol.

The addition of Pic B8 increased the retention times for amines (DHBA) [22], but slightly decreased the retention times for acids (VLA, isoVA); an increase followed by a decrease was observed for DOPA, which has two functional groups, one acidic and one basic.

As may be seen in Fig. 2, the Na_2HPO_4 concentration also had an influence on the retention times of these substances.

Figs. 3 and 4 show how these substances were separated using an eluent of pH

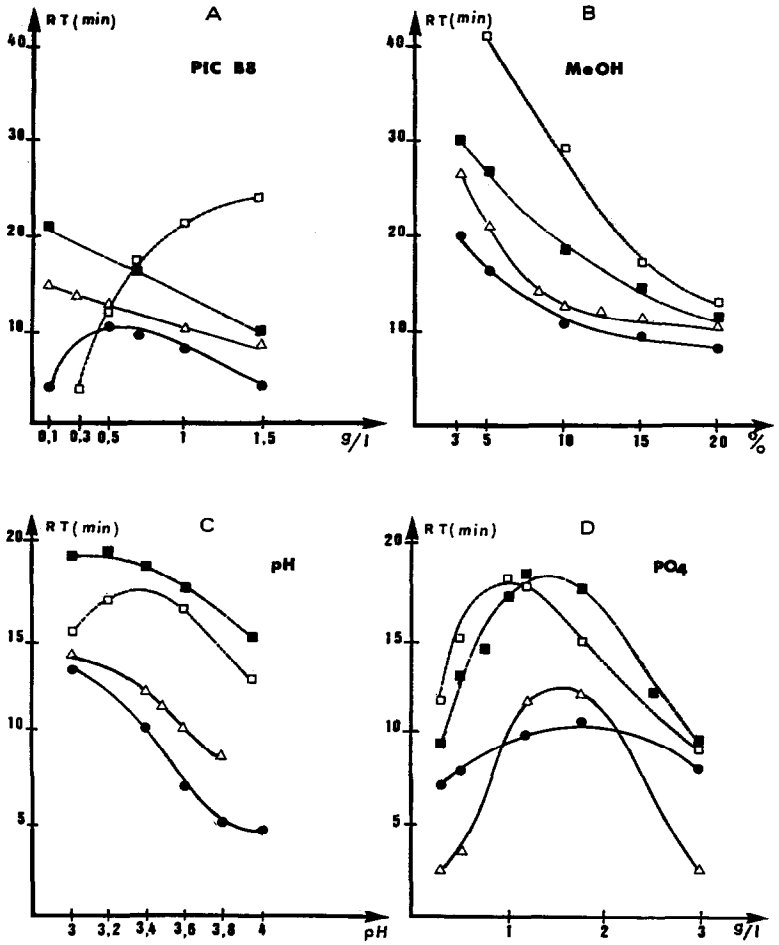

Fig. 2. Modification of the retention times induced by changes in (A) the concentration of octanesulphonic acid (g/l); (B) the percentage of methanol; (C) the pH; (D) the concentration of Na_2HPO_4 (g/l). Symbols: (●) DOPA = dihydroxyphenylalanine; (□) DHBA = dihydroxybenzylamine; (△) VLA = vanillactic acid; (■) isoVA = isovanillic acid.

3.6, composed of 10–15% methanol, 0.68 g/l Pic B8 and 1.17 g/l PO4. VLA and DOPA were well resolved from the internal standard and the endogenous urinary compounds.

The electrode potential of 0.8 V *vs.* KCl provided sufficient sensitivity for the determination of picomole amounts of VLA and DOPA with minimal interference from solvent effects and electrical noise.

The main advantages of this technique are: (1) it uses the same eluent with the identical concentration of methanol and the same pH; (2) determination is performed with a single specimen and with a single chromatographic injection; (3) the use of ED results in high sensitivity and allows the detection of very low

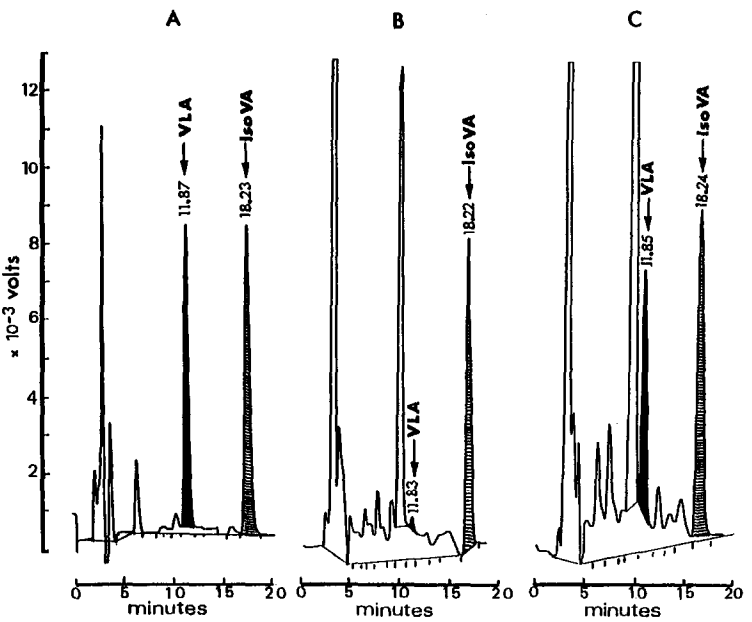

Fig. 3. Chromatograms of (A) a standard mixture of 15 pmol of VLA and 132 pmol of isoVA as internal standard, (B) a urine sample from a five-year-old normal child and (C) a urine sample from a five-year-old child with neuroblastoma.

concentrations; (4) it is a simple procedure and the separation can be performed in less than 20 min. The chromatographic column can be used for a considerable number of injections (more than 500) without showing significant deterioration in retention times or other characteristics of the chromatographic peaks; (5) it is an economical procedure because the products used for the extraction technique and to make the eluent are cheap.

Reference values

Urine and plasma were obtained from forty healthy children, in age groups of 0–5, 5–10 and 10–15 years, who followed the dietary conditions mentioned above. Medication was discontinued. Typical chromatograms of a urine sample and plasma sample from a normal child are illustrated in Figs. 3 and 4.

The reference values presented in Table II are given in pmol/mmol of creatinine for VLA and in mmol/l for DOPA.

The mean excretion was found to decrease between the ages of 0 and 15 years.

Pathology

The urinary and plasma levels of VLA and DOPA, respectively, were determined in children with neuroblastoma or other tumours, prior to treatment, to evaluate the usefulness of these parameters for the diagnosis of neuroblastoma.

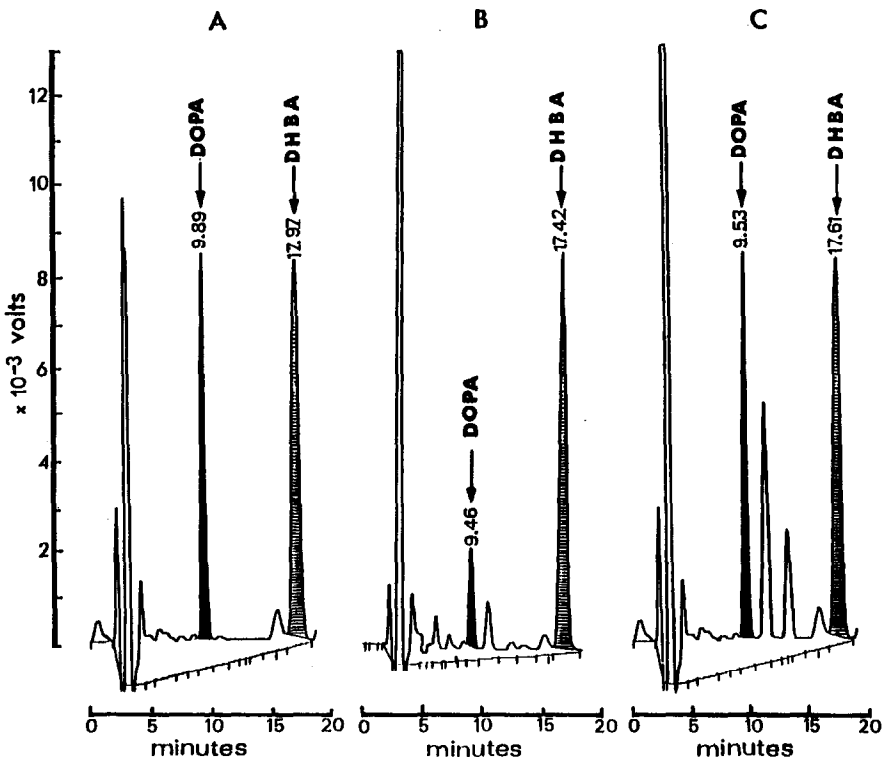

Fig. 4. Chromatograms of (A) a standard mixture of 5 pmol of DOPA and 5.6 pmol of DHBA as internal standard, (B) a plasma sample from a five-year-old normal child and (C) a plasma sample from a five-year-old child with neuroblastoma.

Elevated levels of both metabolites were observed in children with neuroblastoma (Table III) and normal levels were found in children with other tumours. In non-secreting neuroblastoma, the non-differentiated tumour cells do not secrete the usual catecholamines, but only DOPA and its metabolite, VLA. There may

TABLE II

URINARY VLA AND PLASMA DOPA CONCENTRATIONS IN 40 NORMAL CHILDREN

Values are given in pmol/mmol of creatinine for VLA and nmol/l for DOPA.

Compound	Concentration (mean ± S.D.)		
	0–5 years (n = 15)	5–10 years (n = 15)	10–15 years (n = 10)
VLA	363 ± 218	227 ± 96	97 ± 28
DOPA	18.7 ± 3.2	12.4 ± 3.9	11.2 ± 1.8

TABLE III

EXCRETION OF VLA AND DOPA IN PATIENTS WITH NEUROBLASTOMA AND OTHER TUMOURS

Values are given in pmol/mmol creatinine for VLA and nmol/l for DOPA.

Disease	Age	Excretion	
		VLA	DOPA
Neuroblastoma stage I	15 days	888	35.3
Neuroblastoma stage IV	13 years	15850	920
Neuroblastoma stage IV	10 years	9713	641
Neuroblastoma stage IV, relapse after surgery and chemotherapy	3 years	1686	97
Neuroblastoma stage IV, relapse after surgery and chemotherapy	9 years	1050	53
Thoraco abdominal neuroblastoma	1 year	1020	28.5
Askin tumour	10 years	306	9.6
Chondrosarcoma	10 years	330	19.9
Nephroblastoma	5 years	222	18.7

also be a deficiency of the cleavage enzyme DOPA decarboxylase, which has pyridoxal phosphate as a cofactor.

The same coenzyme is involved in γ-cystathionase, a deficiency of which leads to cystathioninuria; thus, we may see the presence of the latter in the urine of a child who has a non-secreting neuroblastoma. Secondary cystathioninuria may also occur with various diseases such as thyroxicosis, liver disease, neuroblastoma and hepatoblastoma [23], because of a lack of vitamin B_6. Therefore we shall be interested to investigate the behaviour of VLA and DOPA in cases of cystathioninuria.

This last assay may obviate many problems of uncertain diagnosis. VLA and DOPA may be markers as good as VMA, HVA, methoxyamines, norepinephrine, epinephrine and dopamine for the management of neuroblastoma.

The clinical course of the disease can easily be followed by the determination of these metabolites, succesful therapy or surgery being accompanied by a corresponding decrease in the plasma levels and relapse by a corresponding increase. The easily conducted VLA and DOPA assays may supply complementary information in uncertain diagnosis of neuroblastoma.

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