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Conjugated *Versus* "Free" Acidic Metabolites of Catecholamines in Random Urine Samples: Significance for the Diagnosis of Neuroblastoma

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ABSTRACT. Random urine samples were obtained from 31 patients with neuroblastoma (newborn to 8 yr of age) and from 100 children without this tumor (newborn to 10 yr). The urine samples were studied for the presence of sulfate and glucuronide conjugates of homovanillic (HVA), dihydroxyphenylacetic, vanilmandelic, and vanillactic acids. The urinary concentrations of these acids were determined by capillary gas-chromatography before and after enzymatic treatment with glucuronidase and sulfatase. Concentrations of the "free" fraction and "total" urinary content of these acids were determined using the results from untreated and treated urines respectively. Age-related reference values were established for children without neuroblastoma. Fractions of the total content of urinary HVA (18–39%) and dihydroxyphenylacetic acid (36–66%) were excreted as glucuronides and/or sulfates by the control group, with the highest conjugated fractions found in the urine of young infants (0–3 months). Vanilmandelic was excreted mainly as "free" acid (unconjugated), whereas vanillactic acid was undetectable in almost all control samples. Patients with neuroblastoma also excreted a fraction of these acids as glucuronide and/or sulfate conjugates, (25% of urinary HVA, 39% of dihydroxyphenylacetic acid and 45% of vanillactic acid) whereas vanilmandelic acid was excreted only as "free" in controls. Determination of "total" rather than "free" urinary HVA was diagnostic in one neuroblastoma patient with borderline "free" HVA levels, whereas determination of "free" or "total" dihydroxyphenylacetic acid and vanillactic acid did not improve the diagnostic sensitivity in the cases examined. We conclude that it may be clinically useful to determine "total" urinary HVA in patients with borderline "free" HVA levels who are suspected of having neuroblastoma. (*Pediatr Res* 23: 576–579, 1988)

Abbreviations

HVA, homovanillic acid
DOPAC, 3,4-dihydroxyphenylacetic acid
VMA, vanilmandelic acid
VLA, vanillactic acid
UCr, urinary creatinine concentration

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Most children with neuroblastoma excrete higher than normal amounts of catecholamine metabolites in their urine (1–3). The measurement of urinary catecholamine metabolites has been used routinely to establish or confirm the diagnosis of this tumor (4, 5). Three main groups of catecholamine metabolites have been found in elevated concentrations in the urines of neuroblastoma patients *1*) acidic derivatives including HVA, VMA, DOPAC, and VLA; *2*) Alcoholic derivatives including: 4-hydroxy-3-methoxyphenylethylglycol (HMPG) 3,4 dihydroxyphenylethylglycol; and *3*) metanephrines. Of the metabolites listed above, HVA and VMA are used most often as neuroblastoma markers because they are excreted most consistently in elevated amounts by patients with neuroblastoma (5). Of patients with neuroblastoma 92% have elevated concentrations of HVA and/or VMA in random urine samples at diagnosis (6). The use of prolonged urine collections rather than random urine samples is not advantageous in respect of diagnostic sensitivity (7, 8).

Some catecholamine metabolites such as HVA, DOPAC and 4-hydroxy-3-methoxyphenylethylglycol appear in the urine as free acids, and as sulfate and glucuronide conjugates (9–12). Routine methodologies measure only the "free" portion of these metabolites in the urine whereas the conjugated portions remain unaccounted for unless these conjugates are chemically or enzymatically cleaved before analysis (13). This study investigated the clinical implications of measuring the "free" fraction *versus* the total content of HVA, VMA, DOPAC, and VLA in random urine samples for the use in the diagnosis of neuroblastoma. Age-matched normal values were established for "total" and "free" portions of these acids and compared to values obtained from patients with neuroblastoma at diagnosis.

PATIENT POPULATION

Random urine samples obtained at diagnosis from patients with neuroblastoma aged newborn to 8 yr were studied. The diagnosis of neuroblastoma was confirmed histologically in all patients. These patients were at various clinical disease stages at the time of diagnosis. Random urine samples were obtained and studied in a control pediatric population (age newborn to 10 yr) which included healthy children and pediatric patients in whom the diagnosis of neuroblastoma had been excluded. The control population consisted of five different age groups with 20 individuals included in each group. The urine samples were delivered to the laboratory immediately after collection or were frozen at −20° C until delivery or analysis.

MATERIALS AND METHODS

Chemicals. HVA, VMA, DOPAC, VLA, 3,4-dihydroxybenzoic acid, resorcinol, and Helix Pomatia-derived sulfatase + glucuronidase type H-2 were purchased from Sigma Chemical

Company, St. Louis, MO. Ethylacetate and diethylether were purchased from Spectrum Chemical Manufacturing Corp, Redondo Beach, CA. N,O *bis*-(trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane (Regisil RC-3) was purchased from Regis Chemical Company, Morton Grove, IL.

Analysis of "total" and "free" HVA, VMA, DOPAC, and VLA. UCr was determined on each urine sample by the method of Folin and Wu (14). Two urine aliquots of 4 ml of each sample were placed into separate glass tubes (if less urine was available, the total volume was adjusted to 4 ml with deionized water). One ml of 2.5 M sodium-acetate buffer pH 5.3 containing 0.2% resorcinol (an antiseptic, to prevent bacterial growth during incubation) was added to each urine aliquot; and the pH was readjusted to 5.3 with 1 N NaOH or 1 M acetic acid solution. Ten thousand units of glucuronidase + 0.5 Units sulfatase were added to only one of the two tubes. Both tubes were capped and placed in a 37° C water bath for 18 hr. At the end of the incubation, 100 μg of 3,4-dihydroxybenzoic acid (internal standard) were added to each tube. The pH was adjusted to less than 1 with 5 N HCl solution, and 2 g of NaCl were added. The organic acids were extracted from both tubes and prepared for capillary gas chromatographic analysis as described earlier (8). Gas chromatographic conditions for separation and identification of the acids, calculation of the amounts according to internal standard calibration, and normalization of the results to the UCr were identical to the method described for the determination of urinary HVA and VMA (15). In certain samples containing very small concentrations of DOPAC, it was necessary to separate the acids on a non polar 5% methylsilicone capillary column (Ultra I Hewlett-Packard Co., Avondale, PA) to avoid interference with DOPAC separation by hippuric acid. In those instances quantitation was done according to internal standard calibration performed on the Ultra I column. The results of the acidic metabolite levels obtained from the urine aliquot pretreated with glucuronidase/sulfatase enzyme represented the "total" amounts of the respective acid, whereas those obtained from the non-treated samples represented the "free" portion of the acid.

Analysis of results. Normal values (mean and 95% confidence intervals of the mean) of "total" and "free" levels of HVA, VMA, DOPAC, and VLA in random urine samples were established in five age groups. "Total" urinary levels of each acid were compared to the levels of "free" acid in the patients with neuroblastoma and in the control group using a one tail student's paired *t* test (16). The statistical analysis identified the significant differences between the "free" and "total" values in order to assess which of the urinary acids is conjugated to sulfate and/or glucuronate. The degree of conjugation, its variability and age dependence in the control population were analyzed. Results obtained from the control group were compared to those observed in samples of patients with neuroblastoma in order to assess differences in the magnitude of conjugation between controls and patients with neuroblastoma. The clinical implications of measuring "total" *versus* "free" concentrations of the acidic metabolites of catecholamines in random urine samples for the diagnosis of neuroblastoma were evaluated.

RESULTS

The extraction efficiencies of "free" HVA, DOPAC, VMA, and VLA from urine by the method used in this study were 95, 95, 75, and 85%, respectively, and their limits of detection about 0.1 μg/mg UCr. This study was performed with the assumption that conjugated acids are not extractable by the solvent extraction method, and therefore the sulfate and glucuronide conjugates of these acids can be accounted for by our methodology only after cleavage of the conjugate.

Table 1 summarizes the "free" and "total" levels of HVA, DOPAC, VMA, and VLA in random urine samples obtained from children without neuroblastoma. Consistently higher amounts of HVA and DOPAC have been recovered from the urine samples treated with glucuronidase + sulfatase compared to the untreated samples. The results obtained for VMA and VLA did not show a similar pattern. Table 2 shows statistically significant differences between the "free" fraction and "total" content of HVA and DOPAC in the various age groups, whereas no significant differences have been observed for VMA and VLA. A possible exception is the 0- to 3-month age group where a significant difference between "free" and "total" urinary VMA has been observed.

Similar results were obtained in patients with neuroblastoma for HVA and DOPAC (Table 3). In these children also, there was no statistically significant difference between the "free" and "total" amount of urinary VMA. However, for VLA there was a significant difference between the "free" and "total" values. These results indicate that HVA and DOPAC are excreted both as "free" and conjugated acids in urine of normal children and children with neuroblastoma. VMA was not excreted in a conjugated form by either group except in the first months of life, whereas an appreciable fraction of VLA was excreted in a conjugated form by some patients with neuroblastoma but not by healthy children. The fraction of the total urinary content of HVA, DOPAC and VLA excreted as sulfate and glucuronide conjugates is shown in Table 4.

Young babies appear to excrete a higher fraction of these acids in a conjugated form when compared to older children. The mean conjugated fraction of HVA in urine of patients with neuroblastoma (25%) was similar to the controls (18–39%) whereas the conjugated fraction of DOPAC was somewhat higher

Table 2. *Level of significance of differences (p) by one-tail paired student's t test between means of free fraction and total content of HVA, DOPAC, VMA, and VLA in random urine samples of children without neuroblastoma*

Age	HVA	DOPAC	VMA	VLA
0–3 mo	0.001	0.018	0.017	0.055
3–12 mo	0.000	0.007	0.499	0.388
1–2 yr	0.001	0.042	0.090	0.085
2–5 yr	0.000	0.005	0.458	0.263
5–10 yr	0.001	0.003	0.088	0.083

Table 1. *"Free" and "Total" HVA, DOPAC, VMA, and VLA in random urine samples of children without neuroblastoma**, †*

Age	HVA (f)‡	HVA (t)§	DOPAC (f)	DOPAC (t)	VMA (f)	VMA (t)	VLA (f)	VLA (t)	*n*
0–3 mo	15.5 ± 3.2	26.8 ± 6.8	0.8 ± 1.0	6.5 ± 5.5	9.0 ± 3.9	11.9 ± 4.5	1.3 ± 1.8	2.3 ± 3.1	15–20
3–12 mo	15.9 ± 2.3	21.8 ± 4.0	0.6 ± 0.6	2.9 ± 2.0	13.6 ± 2.7	13.6 ± 2.9	0.2 ± 0.4	0.3 ± 0.6	18–20
1–2 yr	19.4 ± 10.0	25.3 ± 10.3	1.5 ± 1.2	4.3 ± 2.7	13.9 ± 7.1	16.2 ± 8.4	0.0 ± 0.0	0.1 ± 0.0	15–20
2–5 yr	9.4 ± 1.4	11.4 ± 1.6	1.9 ± 0.8	3.2 ± 1.0	8.8 ± 4.9	8.7 ± 4.5	0.5 ± 0.6	0.5 ± 0.8	13–20
5–10 yr	5.8 ± 1.2	7.5 ± 1.6	1.8 ± 1.0	3.1 ± 1.2	4.8 ± 1.0	5.6 ± 1.2	0.1 ± 0.2	0.2 ± 0.2	18–20

* Levels below the limit of detection (<0.1 μg/mg UCr) were calculated as zero values.

† Mean and 95% confidence intervals for the mean in μg/mg UCr.

‡ Free fraction.

§ Total content.

Table 3. *"Free" and "Total" HVA, DOPAC, VMA, and VLA in random urine samples of children with neuroblastoma** †*

Age	HVA (f)‡	HVA (t)§	DOPAC (f)	DOPAC (t)	VMA (f)	VMA (t)	VLA (f)	VLA (t)	*n*
0–3 mo	103.9	149.9	4.6	7.6	162.8	156.8	0.2	2.1	7
3–12 mo	127.5	184.7	6.1	23.0	75.3	91.6	0	0	8
1–2 yr	61.3	91.6	10.4	20.9	50.6	56.5	0.2	0.3	4
2–5 yr	130.1	157.7	16.4	19.0	101.6	99.5	1.6	3.7	10
5–10 yr	200.1	225.0	30.1	34.1	162.9	158.4	4.4	6.3	2
All	119.2	158.7	11.5	19.2	106.0	108.7	1.2	2.1	31
p‖	0.001		0.019		0.286		0.013		

* Levels below the limit of detection (<0.1 μg/mg UCr) were not included in the calculation of the mean values.
† Mean in μg/mg UCr.
‡ Free fraction.
§ Total content.
‖ Significance levels of the differences between the mean "free" and "total" values (all patients excluding those with undetectable values).

Table 4. *Conjugated fractions of HVA, DOPAC, and VLA in random urine samples of children with neuroblastoma and controls**

	HVA	(*n*)	DOPAC	(*n*)†	VLA	(*n*)
Neuroblastoma	24.5 ± 5.0	(31)	39.2 ± 10.6	(21)	45.3 ± 19.6	(5)
Controls						
0–3 mo	38.7 ± 6.5	(16)	66.3 ± 18.6	(4)	31.5 ± 12.7	(3)
3–12 mo	27.1 ± 7.6	(15)	56.0 ± 29.6	(5)	ND‡	
1–2 yr	25.7 ± 7.3	(19)	45.1 ± 28.0	(6)	ND	
2–5 yr	17.7 ± 2.9	(20)	36.2 ± 11.6	(10)	ND	
5–10 yr	23.1 ± 9.2	(17)	41.6 ± 7.1	(8)	ND	

* Percent, mean, and 95% confidence intervals for the mean.
† Undetectable levels of free fraction or total content were not included in the calculations.
‡ Not detectable in any sample.

in controls (36–66%) compared to the patients (39%). One patient with neuroblastoma had normal "free" levels of HVA; however, "total" HVA levels were also normal. Two patients had borderline (2 SD above the mean) levels of "free" HVA. In one of these patients the "total" HVA level was clearly above normal. Thirteen patients with neuroblastoma had normal levels of "free" DOPAC. In three of these patients "total" DOPAC levels were elevated. The "total" DOPAC level showed excellent correlation with the HVA levels and in none of the three patients with normal or borderline "free" HVA and VMA levels was the "total" DOPAC level elevated.

Urinary "free" VLA was detected only in eight patients with neuroblastoma whereas "total" VLA was detected in 12 patients. All but two "total" VLA values were above normal. Moreover, VLA was not elevated in the three patients with normal or borderline HVA and VMA levels.

DISCUSSION

The histological diagnosis of neuroblastoma may be difficult without the demonstration of excessive concentrations of catecholamines and their metabolites in blood or urine of the patients. Measurement of acidic metabolites of catecholamines in the urine is used routinely for the diagnosis of neuroblastoma. However, the extraction methodologies used do not usually recover the hydrophilic sulfate and glucuronide conjugates of these acids. Extraction of the conjugates would have resulted in overestimation of the "free" fraction because derivatization for gas chromatography would lead to deconjugation (17). The presence of DOPAC conjugates in human urine was reported by O'Gorman *et al.* (9) in patients receiving L-DOPA. Other investigators showed that a significant portion of HVA is excreted also in a conjugated form (18–20). VLA is found in elevated concentrations in the urine of some patients with neuroblastoma (21, 22); however, the presence of VLA conjugates has not been studied. Our study reports pediatric age-matched reference values for "free" and "total" DOPAC in random urine sample, which are currently unavailable. Our results confirm the observations of other investigators that a significant portion of urinary HVA and DOPAC is conjugated to glucuronate and sulfate. The data obtained herein suggest that VLA is excreted largely in a bound form especially in patients with neuroblastoma in whom increased VLA production occurs. The clinical implications of the above finding are addressed herein. DOPAC, which is a metabolite of DOPA and dopamine, appeared in elevated concentrations in the urine of most patients with neuroblastoma. However, all these patients also had elevated concentrations of HVA. Thus, determination of urinary DOPAC will probably not add to the diagnostic sensitivity of HVA and VMA analysis. If DOPAC is to be used clinically, "total" rather than "free" DOPAC should be determined because a major portion of urinary DOPAC is conjugated. VLA, a metabolite of DOPA, is elevated in the urine of patients with neuroblastoma and has been measured to diagnose this tumor (22). As DOPAC, it correlated with HVA levels, and its determination does not seem to offer an advantage over the determination of HVA. In cases where "'free" urinary HVA level is borderline, determination of total content of urinary HVA may be of diagnostic value. In such cases it may be useful also to measure VLA, however, we could not demonstrate this in our study.

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