URINARY CATECHOLAMINE METABOLITES: CAPILLARY GAS CHROMATOGRAPHY METHOD AND EXPERIENCE WITH 12 CASES OF NEUROBLASTOMA

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ABSTRACT

We propose a rapid, simple methodology for routine analysis of human urine to detect vanillylmandelic and homovanillic acid related to neuroblastoma. The assay were specific capillary gas chromatography with flame ionization detection. In this methodology an internal standard is used and the procedure involves ethyl ester formation without isolation of the compounds of interest. The run time is 36 minutes. We also report quantitative results for urinary vanillylmandelic and homovanillic acid in neuroblastoma patients, demonstrating the diagnostic value of this method.

KEY WORDS

Capillary gas chromatography, diagnostic aids, Homovanillic acid, neuroblastoma, vanillylmandelic acid.

INTRODUCTION

About one quarter of cancers in the first year of life are neuroblastomas, making this the most frequent histological type of infant cancer (Gurney *et al.*, 1995 and Gao *et al.*, 1997). The incidence rate of the disease in children under 1 year of age is about 35 per million but declines rapidly with age to about 1 per million between ages 10 and 14 (Stiller and Parkin, 1992). Males appear to be affected slightly more commonly than females, with about 5 cases occurring in boys to every 4 occurring in girls. Screening is performed through biochemical tests for metabolites of norepinephrine and dopamine, i.e., vanillylmandelic acid (VMA), and homovanillic acid (HVA). Seventy-five percent to 90 % of cases of neuroblastoma excrete these substances into the urine, and these can be measured in urine specimens (Williams and Greer, 1963).

We present a relatively simple, rapid method for quantification of HVA and VMA by capillary gas chromatography (GC). This report shows a marked simplification of the two ways: in sample preparation

and derivative formation. Earlier used methodology for quantitative analysis of VMA and HVA in urine is based on ether extraction and silyl ether formation (Seviour *et al.*, 1988). The use of ethyl chloroformate (ECF) as derivatizing agent presents a completely new approach to profiling VMA and HVA in urine (Grkovic and Drazic, 2003).

In this study we also report quantitative results for urinary HVA and VMA in neuroblastoma patients, demonstrating the diagnostic value of this method.

MATERIALS AND METHODS

Organic solvents such as acetonitrile, ethanol, chloroform, pyridine and ethyl chloroformate were of analytical grade and all other chemicals, including the standards of vanillylmandelic and homovanillic acids were obtained from Sigma-Aldrich.

Sample preparation. Samples used in the study was urine. The first morning urine was processed immediately or stored at -20°C. Four hundred microliters of acetonitrile and 200 µL of ethanol were added in 10 µL of malonic acid used as an internal standard (IS) (10 mmolL⁻¹ in water) and 200 µL of urine (approximate creatinine contents 8 µmolL⁻¹). If a precipitate was formed during 10-15 min of standing, it was subsequently centrifuged down at 2000 g for 5 min. To remove amines and amino acids the medium was pushed through a bed of AG 50W-X8 cation exchange resin (100-200 mesh, H⁺ form, 25 mg). Five hundred microliters of the acidified eluent were then treated with 20 µL of ethyl chloroformate and 40 µL of

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pyridine. After the addition of 0.25 ml of chloroform and 0.5 ml of 1 molL⁻¹ aqueous solution of sodium bicarbonate-carbonate (2:1), the two phase system was vortexed and after reaching phase equilibrium the upper phase was aspirated and organic phase dried by adding sodium sulfate (Husek, 1997). The volume was subsequently reduced to 80-100 µL by nitrogen blowing at room temperature, and a 3 µL aliquot was injected onto the column via splitless technique. The same procedure was carried out for the chromatographic standards by addition of 200 µL of water instead of sample (urine). A 3 µL aliquot was injected onto the column in the split-splitless mode (1:10).

Gas chromatography. The analysis succeeded on capillary column HP5 30 m x 0.32 mm I.D. (0.25 mm film thickness; crosslinked 5 % phenylmethyl siloxane, Hewlett Packard, USA), in a temperature range of 60-280°C at 5°C/min. The carrier nitrogen was used at head pressure of 50 kPa, the injector and detector temperatures were set at 250 and 280°C. Instrument was used HP6890 gas chromatograph with a flame ionization detector (Hewlett Packard, USA).

RESULTS AND DISCUSSION

The most commonly assayed catecholamine metabolites are vanillylmandelic acid (VMA) and homovanillic acid (HVA), which are metabolites of norepinephrine and dopamine, respectively (Peaston and Weinkove, 2004). Urinary levels of these metabolites can be accurately measured from a single urine specimen. Gas chromatographic techniques are more accurate to detect these metabolites than colorimetric methods, which require avoidance of tea, coffee, fruit and vanilla from the diet for 72 hours

before urinary sampling (Tuchman *et al.*, 1983 and Seviour *et al.*, 1988). Catecholamine metabolites are most useful in diagnosing and monitoring patients with neuroblastoma (Weinkove, 1991).

Two hundred urine samples of ill children with suspicion diagnosis of neuroblastoma were analyzed. Twelve of them have a diagnosis of neuroblastoma (6%).

Retention times (RT) and response factors (RF) of the individual catecholamine metabolites on the HP5 column were determined with respect to malonic acid. The overall reproducibility of the procedure, expressed as percent variation from the mean, was determined from six independent analyses of the standards (Table 1). The average CV of the within day reproducibility for standard mixture was 4.8%. As shown in chromatogram in Fig. 1, catecholamine metabolites listed in Table 1 were separated on HP5 column on conditions described in Material and Methods. Chromatogram of the standard mixture shows that separation power of the polar phenylmethyl siloxane phase for this kind of derivatives is excellent. A pathological urine profile of catecholamine metabolites is shown in Fig. 2. Identification of the analytes was based on comparison of the retention times with those of standard solutions. The structure of the other peaks remains unknown, GC-MS studies should be performed to elucidate structure of the unidentified metabolites and this will be the next task. In Table 2 are given concentration of HVA and VMA in urine of patients with neuroblastoma. Our recommendation is to use a VMA cut-off of 25 mg/g creatinine and an HVA cut-off of 32 mg/g creatinine. Based on the amounts of VMA and HVA excreted, in all patients was markedly

Fig. 1. GC profile of an equimolar standard mixture of catecholamine metabolites analyzed on the HP5 column. Injected amount 0.73 nmol at 1:10 split ratio

The numbered peaks represent a following acids:

- 1: Malonic (internal standard)
- 2: Homovanillic
- 3: Vanillylmandelic

Fig. 2. GC profile of urinary catecholamine metabolites from patient with a neuroblastoma

The numbered peaks represent a following acids:

- 1: Malonic (internal standard)
- 2: Homovanillic
- 3: Vanillylmandelic

Note the elevated level of HVA (208.3 mg/g creatinine) and VMA (145.6 mg/g creatinine) in urine.

Table 1. Chromatographic parameters of derivatized standards of catecholamine metabolites on HP5 column.

Elution order	Catecholamine metabolites	RRT	RRF	CV (%) n=6
1	Malonic acid (IS)	1.0	1.0	3.850
2	Homovanillic acid	3.514	1.110	5.150
3	Vanillylmandelic acid	4.304	0.340	5.500

increased VMA and HVA (Table 2). Reproducibility of the urine profiling was surprisingly good, slightly lower values were found for VMA than for HVA. Analytical recovery of VMA averages 87.6 % and of HVA 105.1 %. Within run CV's did not differ too much from those resulting from the mere derivatization only, being < 6 % (n=6) of the analytes. The diagnosis of neuroblastoma was confirmed in 12 patients by histological examination of tumor tissue.

Serial monitoring of urinary levels of catecholamine metabolites is extremely important after treatment for neuroblastoma. The failure of urinary excretion of catecholamine to normalize after initial therapy is associated with uniform mortality within two years. Subsequent recurrence of tumor is heralded by elevated urinary levels of catecholamines before clinical or roentgenologic signs in more than 90 % of

instances. Thus, urinary catecholamine metabolites are useful indicators of the extent of disease, prognosis and recurrence in patients with neuroblastoma.

CONCLUSION

Our study indicates that measuring VMA and HVA with a specific procedure is the single most reliable screening test for all patients suspected of having neuroblastoma.

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Table 2. Initial concentrations of VMA and HVA in urine of patients with neuroblastoma

Patient no. and sex	Age at diagnosis, year	Concentration of urinary VMA (mg/g creatinine)	Concentration of urinary HVA (mg/g creatinine)
1, F	3	304.06	488.14
2, M	2	145.60	208.30
3, M	3	73.32	75.20
4, M	4	59.50	177.60
5, M	1	226.22	78.43
6, M	2	26.56	51.71
7, F	5	350.10	370.8
8, F	2	190.50	210.2
9, M	1	70.90	90.70
10, M	7	115.20	136.50
11, M	1	67.80	89.60
12, F	1	210.70	250.60

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