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DENSITOMETRIC DETERMINATION OF CATECHOLAMINE METABOLITES AND 5-HYDROXY-INDOLEACETIC ACID AFTER TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY ON CELLULOSE

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SUMMARY

A quantitative two-dimensional chromatographic determination for the catecholamine metabolites vanilglycolic (vanilmandelic) acid, vanilacetic acid, vanillactic acid and vanilglycol is described. The method can also be used for the determination of 5-hydroxy-indoleacetic acid.

The analytical procedure consists of the following steps: 1. extraction (after enzymatic hydrolysis when vanilglycol has to be determined); 2. two-dimensional chromatography on thin-layer cellulose; 3. quantitative evaluation of the spots by means of a chromatogram scanner.

Standard deviations, recoveries, 24-h excretions in 10 normal adults and results in 10 patient suffering from neurogenic tumors are given.

Some of the merits of a multi-component determination of the type described here for the clinical diagnosis of secreting neurogenic and serotonin-producing tumors are discussed.

INTRODUCTION

In patients suffering from a functional tumor of the sympathetic nervous system, the urinary excretion of catecholamine metabolites is significantly increased. For an extensive review of the subject of abnormal and normal catecholamine metabolites see Gjessing[1] and Voûte[2]. Main features can be summarized as follows: In neuroblastoma and ganglioneuroma excessive amounts of vanilglycolic acid (VGA), vanilacetic acid (VAA) and vanilglycol (VG) are excreted. VAA even may exceed VGA. Vanillactic acid (VLA) and 3,4-dihydroxyphenylacetic acid may be present. Also the 3-*O*-methylated norepinephrine and, occasionally, 3-*O*-methylated dopamine and dopa are increased, but the amounts are smaller than of VGA, VAA and VG.

In most patients with benign phaeochromocytoma the main products excreted are VGA and VG. The excretion of VAA, on the contrary, is not abnormal. Metanephrines are increased on a lower excretory level.

For the clinical diagnosis of these tumors two-dimensional paper chromatography and thin-layer chromatography of VGA, VG, VLA and VAA are adequate[2-4]. Two-dimensional chromatograms provide highly specific and quantitative information about multiple metabolic components. Abnormal patterns, characteristic for neurogenic tumors, can easily be recognized and, to a certain degree, even allow a differentiation of these tumors.

For diagnostic purposes visual inspection of chromatograms will suffice in many cases. However, when abnormal excretions are less pronounced, quantitative determinations of all compounds may be necessary. This should also be done for the early evaluation of the response of the tumor to therapy.

At present urinary phenolic substances can be determined by gas–liquid chromatography. Most laboratories, however, are not familiar with the identification and interpretation of the large number of compounds present in such chromatograms. Therefore a simpler method is needed, which can be performed in a routine laboratory, requiring only general laboratory equipment and a minimal background knowledge. Such a method has been developed and is described here. By the same method also 5-hydroxy-indoleacetic acid (5HIAA) can be determined quantitatively, which is an attractive extension, as will be discussed later.

For the separation of the phenolic substances two-dimensional thin-layer chromatography on cellulose plates 10×10 cm is used, having the advantage of being more rapid and less sensitive to temperature fluctuations than two-dimensional paper chromatography. Compact spots are obtained, which are evaluated by densitometric scanning.

MATERIALS

Inorganic reagents and solvents were all of p.a. quality.

Cellulose, microcrystalline (Merck)

β-Glucuronidase/arylsulfatase (Boehringer)

DL-4-hydroxy-3-methoxymandelic acid, bis(4-hydroxy-3-methoxyphenylglycol) piperazine salt, 4-hydroxy-3-methoxyphenylacetic acid and 5-hydroxy-indole-3-acetic acid: all from Sigma.

Thin-layer plates of 10×10 cm. The glass plates are stored in a concentrated solution of Na_2CO_3 for 24 h for removal of fat and dust, rinsed with warm and cold water, next with distilled water and dried.

An 18% cellulose solution is mixed in a mixer at top speed for exactly 1.5 min. Then the thin-layer plates are coated (layer thickness 0.4 mm). The plates are allowed to dry overnight at room temperature.

Preparation of diazotized sulphanilic acid

Fifty g sulphanilic acid are dissolved in 250 ml 10% KOH. The solution is cooled to 0° and then 200 ml 10% $NaNO_2$ is added. While stirring at a temperature below 5°, 120 ml 8.2 *N* HCl is added. The precipitate of diazotised sulphanilic acid is filtered by suction and washed with 20 ml ice-water, 20 ml ethanol and 20 ml diethyl ether respectively. Finally it is dried on filter paper in air and stored in a plastic bottle at 4°.

N.B. Diazotized sulphanilic acid is explosive. Do not scratch in the bottle with a spatula.

Diet

For the elimination of exogenous aromatic amines and other dietary compounds which cause urinary excretion of phenolic acids, the patient is given a special diet on the day before the urine is collected and the day of collection.

Omitted from the diet are: vegetables, fruit and fruit juices, coffee, tea, cocoa, spices and aromatic flavourings. The patient may take: meat, fish, eggs, milk, cheese, bread, potatoes, rice, butter, sugar and flour products[5].

Collection of urine

Twenty-four-h urine is collected in a bottle containing 25 ml acetic acid.

METHODS

The procedure consists of the following steps:

A. Extraction; B. Two-dimensional chromatography; C. Scanning. For extraction and chromatography mainly the directions given in refs. 2–4 were followed.

A. Extraction procedures

A1. Method for visual assessment of VGA, VLA, VAA and 5HIAA. This step is also used for the quantitative determination of 5HIAA. Five ml of a 24-h urine sample is acidified with conc. HCl to pH 1.5. Then 5.0 ml saturated NaCl is added and the mixture is extracted twice with 20 ml ethyl acetate. The combined ethyl acetate portions are extracted twice with 5.0 ml 10% $NaHCO_3$. The combined bicarbonate portions are acidified with conc. HCl to pH 1.5 at 0° and then extracted twice with 15 ml ethyl acetate. The ethyl acetate portions are dried with Na_2SO_4 for at least 10 min. The suspension is centrifuged, the ethyl acetate decanted into a 100-ml Rotavapor flask and evaporated to dryness under reduced pressure in a water-bath at 40°.

The residue is extracted three times with 2 ml ethyl acetate; the combined ethyl acetate portions are then evaporated in a Rotavapor tube in the same way. The residue is dissolved in 0.10 ml ethanol 96% and 5.0 μl of this solution are applied to the thin-layer plate with the help of an automatic device.

A2. Method for the quantitative determination of VGA, VAA, VLA and VG. VG occurs in a conjugated form and must be liberated by enzymatic hydrolysis. To 5.0 ml of a 24-h urine sample 2.5 *N* NaOH is added until the pH is 11. Then inorganic sulphate and phosphate are precipitated with 0.2 ml saturated $BaCl_2$. The precipitate is centrifuged, the liquid decanted and the latter acidified with 2 *N* HCl followed by 0.1 *N* HCl to pH 6.5. Then 0.1 ml β-glucuronidase/arylsulphatase is added and incubated at 37° for 24 h, whereafter the solution is centrifuged and decanted.

The decanted portion is saturated with NaCl and extracted twice with ethyl acetate. The ethyl acetate extract (A) contains VG and a part of the phenolic acids. The water layer is acidified with 6 *N* HCl to pH 1.5 and extracted twice with 20 ml ethyl acetate. This extract (B) contains the greater part of the phenolic acids. The combined ethyl acetate fractions (A) and (B) are dried with Na_2SO_4 for 10 min. For further treatment see A1.

B. Chromatography

The chromatograms are developed according to Stahl[6]. Two glass rods (10 mm

i.d., 100 mm length), one of which is wrapped in filter paper, are placed in a Petri dish of 180 mm i.d.

The thin-layer plate is placed onto the glass rod in such a way that the solvent can migrate into the direction of the unwrapped glass rod. The dishes have to be closed tightly, for which vacuum grease can be used.

Solvent I: 100 ml of a mixture of isopropanol, 5% ammonia (4+1, v/v). The time of development is about 2 h.

Solvent II: 100 ml of a mixture of benzene–acetic acid–water (140:73:2, v/v). The time of development is about 45 min.

After development, the chromatograms are dried in a cold air stream.

C. Scanning

The dried plates are sprayed with a solution of 100 mg diazotised sulphanilic acid in 20 ml 10% Na_2CO_3. The isolated spots are scanned quantitatively with a Vita-

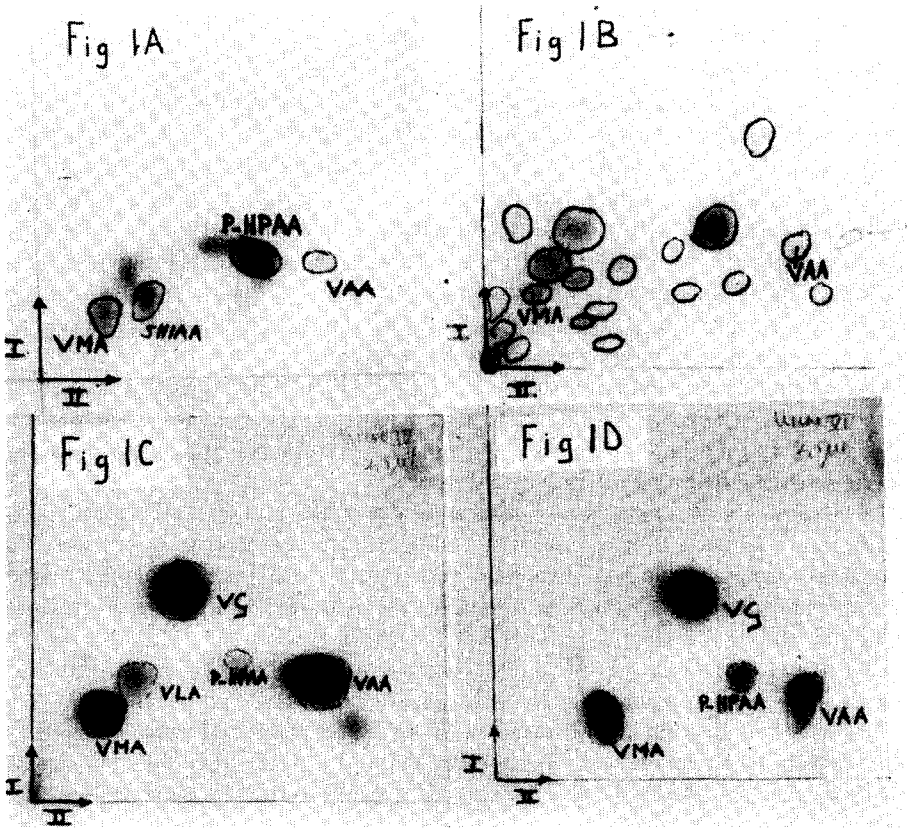

Fig. 1. The I and II = solvents I and II. VMA = VGA (orange red), VAA (reddish purple), VG (orange red), VLA (purple), 5HIAA (carmine), P-HPAA = *p*-hydroxyphenylacetic acid (purple). A. Normal pictures according to extraction method 1. B. Picture according to extraction method 1 on a free diet. C. Chromatogram from patient No. 4 with neuroblastoma, according to extraction method 2. Urine diluted 10 times. VLA is present on the chromatogram. D. Chromatogram from patient No. 6 with neuroblastoma according to extraction method 2. Urine diluted 10 times. VLA is not present on the chromatogram.

tron densitometer TLD 100 (filter 499). The obtained integration units are compared with the corresponding data from standard solutions which have passed through the whole procedure.

RESULTS

Extraction procedure A1 is sufficient for an orientational investigation of VGA, VLA and VAA, whereas 5HIAA can be determined quantitatively by this method. For a quantitative determination of VG together with VGA, VLA and VAA the more complicated method A2 is used, since an enzymatic hydrolysis must be inserted.

Chromatograms obtained from normal persons and patients are shown in Fig. 1.

With standard solutions, which have passed through the whole procedure, VGA, VAA and VG (VLA not checked) give linear responses up to concentrations of 20 nmoles on the chromatograms. The response of 5HIAA (extraction method 1) is not linear. Standard solutions of 5HIAA which were directly applied to thin-layer plates, also did not give a linear response, indicating that decomposition of the diazo-reaction product on the chromatogram or its incomplete formation are responsible for the low colour yield rather than losses during the extraction procedure.

The colour yield is highest for VGA and VG; from VAA and 5HIAA lower yields are obtained (Fig. 2).

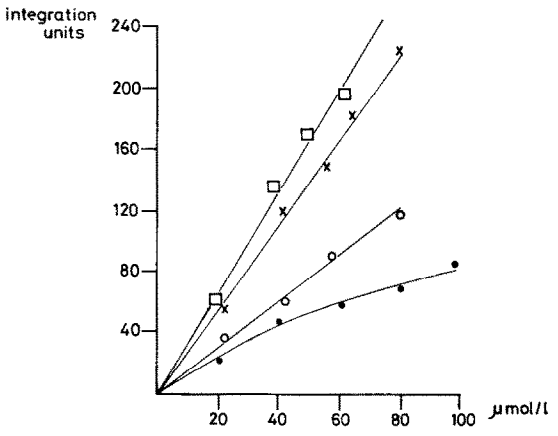

Fig. 2. Integration units *versus* concentration in µmole/litre: –□–□–□–□–□ VGA; –○–○–○–○–○ VAA; –×–×–×–×–× VG; –●–●–●–●–● 5HIAA.

Experiments were done in order to investigate the reproducibility of the method and also recoveries were determined. The standard deviation of the whole procedure, obtained from a tenfold analysis, is given in Table I. For the standard deviation of the scanning step 5.5% was found (also obtained from a tenfold experiment).

Recoveries, obtained from a fivefold determination are given in Table II. It can be seen that the yield of VG is poorest, whereas 5HIAA is slightly better recovered than VGA and VAA. In Table III 24-h excretion values obtained from ten normal adults are given. The results are in good agreement with those given in the literature (refs. 1,2,7).

TABLE I

STANDARD DEVIATION OF THE ANALYSES

	Mean value *μmole/l*	*Standard deviation* % *of mean*
VGA	60	7
VAA	65	6
VG	65	7
5HIAA	55	6

Standard deviation of VGA, VAA and VG (method A2 + B + C) and 5HIAA (method A1 + B + C) $N = 10$.

TABLE II

RECOVERY OF THE METHOD

Compound	*Added* *μmole/l*	*Mean recovery* %	*Range* %
VMA	40	88	± 5
VAA	40	86	± 5
VG	20	76	± 6
5HIAA	45	91	± 4

Recovery of VGA, VAA, VG and 5HIAA, added to urine ($N = 5$).

TABLE III

NORMAL EXCRETION VALUES

	This method *μmole/24 h*	*Ref.*[1] *μmole/g creat.*	*Ref.*[2] *μmole/24 h*	*Ref.*[7] *μmole/24 h*
VGA	35	60	35	
VAA	40	65	52	
VG	17	–	16	
5HIAA	50	–	–	45

Maximal 24-h values obtained from 10 normal adults in comparison with the literature.

TABLE IV

AMOUNTS EXCRETED BY PATIENTS

Patient	*VGA* *μmole/l*	*VAA* *μmole/l*	*VG* *μmole/l*	*VLA*	*Disease*
1. Adult	405	41	245	–	phaeochromocytoma
2. Adult	125	31	270	–	phaeochromocytoma
3. Adult	150	27	40	–	phaeochromocytoma
4. Child	2400	4100	2600	+	fatal neuroblastoma
5. Child	40	220	9	–	fatal neuroblastoma
6. Child	1300	1240	1620	–	non-fatal neuroblastoma
7. Child	300	1380	135	–	ganglioneuroma
8. Child	275	300	120	–	fatal neuroblastoma
9. Child	430	495	190	–	neuroblastoma maturing towards ganglioneuroma
10. Child	300	210	310	–	neuroblastoma

Abnormal values in patients suffering from a tumor. The concentrations are given in μmoles/l as no 24-h samples could be collected.

Abnormal values obtained from eight children suffering from neuroblastoma and three adults suffering from phaeochromocytoma are given in Table IV. In the patients suffering from phaeochromocytoma the concentration of VGA and VG was increased

whereas VAA was normal. In 5 out of 6 children with neuroblastoma and ganglioneuroma VGA and VG were strikingly abnormal, but in one patient approximately normal VGA and VG concentrations were found. All 6 patients showed increased VAA concentrations. VLA was found to be present only in one patient (No. 4).

DISCUSSION

The method described is relatively simple and requires only general laboratory equipment. Therefore it is suited for application in routine laboratories. On the other hand it yields sufficient and specific information necessary for the adequate diagnosis of secreting neurogenic tumors.

For the diagnosis of neuroblastoma other metabolites than VGA alone have to be determined; VAA is an equally important parameter. This is proved by our case No. 5, who showed nearly normal VGA and VG concentrations, whereas VAA was markedly increased.

The same rather unusual excretion pattern has been described by Bohuan[8] and Schweisguth[9], who also found neuroblastoma patients with abnormal VGA together with normal VAA or with both parameters normal. Also phaeochromocytoma patients with normal VGA excretion have been described[10]. Obviously neuroblastoma cannot be excluded by determining only VGA by means of the popular vanillin procedures (refs. 11–13). Methods such as chromatography and electrophoresis from which several parameters can result, are preferable. It must be stressed that, when thin-layer or paper chromatography are used, this should be a two-dimensional technique. One-dimensional separations do not allow a clear-cut recognition of the characteristic metabolites among the numerous phenolic substances often present in urine.

From a clinical point of view it is of importance that 5HIAA can be determined by the same technique as is used for catecholamine metabolites. The advantage of such a combination may become obvious when we deal with the differential diagnosis of unexplained diarrhoea.

In neuroblastoma and ganglioneuroma diarrhoea is a main symptom. Its cause is unknown, but there must be a humoral relationship with the tumor, as after removal of the tumor a prompt cessation of the diarrhoea is observed. In general, excessive production of serotonin is not a causative factor, although Birkenhäger[15] reported on a case of an adult patient with a tumor having the anatomical characteristics of a neuroblastoma, who excreted increased amounts of 5HIAA, serotonin and 5-hydroxytryptophan, whereas VGA was normal.

In patients with carcinoid syndrome, diarrhoea is a main symptom too, in adults as well as in children. Such patients exhibit an increased urinary excretion of 5HIAA at a normal VGA level. Increased urinary 5HIAA has also been described in a patient with oat-cell carcinoma, one of the complaints being diarrhoea[16]. Finally an elevated urinary 5HIAA in (non-malignant) untreated coeliac disease can be mentioned.

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