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SENSITIVE MASS FRAGMENTOGRAPHIC DETERMINATION OF ACIDIC CATECHOLAMINE METABOLITES IN HUMAN BODY FLUIDS

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SUMMARY

A sensitive and simple method for the simultaneous quantitation of homovanillic acid and vanilmandelic acid in small samples (0.1–1.0 ml) of urine, serum, plasma and cerebrospinal fluid is described. The stable dimethylthiophosphinyl methyl ester derivatives are detected specifically by mass fragmentography using the respective deuterated compounds as internal standards. Gas chromatographic separation is performed on a fused-silica DB-1 capillary column combined with a cold injection system for large sample volumes. Linear response curves and a detection limit of 1 ng/ml are obtained. The method has been applied to the localization of pheochromocytoma by selective determination of venous vanilmandelic acid.

INTRODUCTION

The phenolic acids homovanillic acid (HVA) and vanilmandelic acid (VMA) are stable metabolites of the catecholamines dopamine and noradrenaline or adrenaline, respectively. Quantitation of these compounds in urine is of great importance for the diagnosis and therapy of catecholamine-producing tumors, *e.g.*, neuroblastoma and pheochromocytoma. Quantitation of HVA in cerebrospinal fluid (CSF) is of interest in the case of some neurologic disorders and for neuropathobiochemical investigations. Muskiet *et al.*[1] have determined serum levels of HVA and VMA from patients with neurogenic tumors and other diseases. However, the diagnostic value of these determinations in serum or plasma has still to be proven.

A series of gas chromatographic (GC) methods have been described for the analysis of HVA and VMA in human body fluids[2]. Quantitation of urine from patients with increased levels of HVA and VMA can easily be performed with a flame ionization detector, whereas the specificity is often too poor for normal urines due to the large number, approximately 500, of concomitant acids[3]. In this case additional clean-up steps[4,5] or appropriate derivatization procedures[6] have to be carried out.

In serum, plasma and CSF, HVA and VMA are found at the ppb level. Therefore, their quantitation requires a highly sensitive and specific detection mode. Mass fragmentography offers both high sensitivity and specificity. Another advantage of

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this detection technique is the possibility of using deuterated internal standards, which can compensate for losses during the extraction and derivatization step.

In this paper we describe a new mass fragmentographic method for the simultaneous analysis of HVA and VMA in urine, serum, plasma and CSF. As derivatives of these acids we chose the dimethylthiophosphinyl methyl esters, which we have applied successfully to the determination of HVA in minute samples of CSF[7]. The overall sensitivity of our method was increased remarkably by means of a large volume split–splitless capillary column cold injection system[8,9]. As an application of this new method we attempted to localize pheochromocytoma by the determination of VMA levels from samples obtained by selective venous catheterization.

EXPERIMENTAL

Reagents and materials

Ethyl acetate, dried methanol and dried diethyl ether were of analytical grade and purchased from E. Merck (Darmstadt, F.R.G.). HVA and VMA were obtained from Merck, d_3-HVA and d_3-VMA (both deuterated in the methoxy group) from Bruker-Franzen Analytik (Bremen, F.R.G.). Boron trichloride was used as a 10% (w/v) solution in methanol (Serva Feinbiochemica, Heidelberg, F.R.G.). Dimethylthiophosphinic chloride was from Riedel-de Haën (Seelze, F.R.G.) and distilled prior to use. Triethylamine (Merck) was distilled over potassium hydroxide. Sodium sulphate was dried at 250–300°C in a stream of nitrogen.

Apparatus

Gas chromatographic–mass fragmentographic analyses were performed with a Model 1440 gas chromatograph (Varian, Palo Alto, CA, U.S.A.) coupled to a Model MAT 311A double-focusing mass spectrometer (Finnigan-MAT, Bremen, F.R.G.). The gas chromatograph was equipped with a split–splitless injector (Werner Günther Analysentechnik, Griesheim, F.R.G.) as described previously[8,9]. A 30-m DB-1 (0.32 mm I.D.) fused-silica capillary column (J & W Scientific, Rancho Cordova, CA, U.S.A.) was used with helium as carrier gas at a flow-rate of 2.4 ml/min. The column was coupled to the ion source of the mass spectrometer with an all-glass open split-type connection[10] consisting of a 50-cm glass capillary (0.15 mm I.D.), temperature 230°C.

Mass fragmentography was carried out either with the multiple-ion detection unit of a Model MAT SS-200 data system (Finnigan-MAT) or a four-channel hardware selected-ion-monitoring device (integration time 0.1 sec). The mass spectrometer was operated with an ionization energy of 70 eV, resolving power $R = 1000$ and ion source temperature of 200°C.

Determination of urine samples

A 5-μg amount of d_3-HVA in 100 μl of 0.06 mol/l HCl and 5 μg of d_3-VMA in 100 μl of 0.06 mol/l HCl were added to 0.1–1.0 ml of urine in 6-ml conical glass tubes. The mixtures were then adjusted to pH 1 with 6 mol/l HCl. After addition of 1 ml of saturated sodium chloride solution, each sample was extracted by slow rotation for 15 min with three 2-ml portions of ethyl acetate. Phase separation was performed by centrifugation for 5 min at 1000 *g*. The combined extracts were con-

centrated at 35–40°C with a stream of nitrogen to a final volume of 2 ml. The residues were dried over anhydrous sodium sulphate for at least 12 h. The supernatants were transferred in portions to a 1-ml Reacti-Vial (Pierce, Rockford, IL, U.S.A.) and evaporated to dryness at 35–40°C in a stream of dry nitrogen. The residues were methylated with 50 μl of 10% methanolic boron trichloride solution and 50 μl methanol for 30 min at 80°C, then evaporated with a stream of nitrogen. The residues were treated with 100 μl of a 20% (v/v) triethylamine solution in diethyl ether and 100 μl of a 3% (v/v) solution of dimethylthiophosphinic chloride in the same solvent for 30 min at 50°C. The volatiles were removed with a stream of nitrogen. Methanolysis of the residues was carried out with 100 μl methanol and 1 mg sodium hydrogen carbonate by vortexing for 5 min at 20°C. The supernatants were transferred quantitatively with methanol to 2-ml conical glass tubes, evaporated with nitrogen and the residues dissolved in 100 μl ethyl acetate. A 1-μl volume was injected into the GC–MS system using a splitting ratio of 1:10. Other conditions: injector temperature, 255°C; column temperature, 210–300°C; heating rate, 2°C/min.

Mass fragmentographic detection of the HVA derivative was performed at *m/z* 288.06 and of the d_3-HVA derivative at *m/z* 291.08. The VMA derivative was detected at *m/z* 259.05 and the d_3-VMA derivative at *m/z* 262.07.

Determination of serum, plasma and CSF samples

A 100-ng amount of d_3-HVA in 100 μl of 0.06 mol/l HCl and 100 ng (20 ng for CSF samples) of d_3-VMA in 100 μl of 0.06 mol/l HCl were added to 0.1–0.5 ml serum, plasma (obtained from heparinized blood) or CSF. After addition of 0.5 ml of 0.1 mol/l HCl, the pH was adjusted to 1 with 6 mol/l HCl. Sodium chloride (0.2 g) was added, and the mixtures vortexed for 1.5 min. Precipitates were removed by centrifugation for 30 min at 1500 *g*. The supernatants were extracted with three 1-ml portions of ethyl acetate, the combined extracts concentrated and dried as described above. The residues were methylated with 25 μl of 10% methanolic boron trichloride solution and 25 μl methanol for 45 min at 80°C. Phosphinylation was carried out with 50 μl of a 1.25% (v/v) solution of triethylamine in diethyl ether and 50 μl of a 0.25% (v/v) solution of dimethylthiophosphinic chloride in diethyl ether under the same conditions as described above. After treatment with 50 μl methanol and 1 mg sodium hydrogen carbonate, the residues were dissolved in 50 μl ethyl acetate. A 5-μl volume was injected by the split–splitless technique as described previously[7]. The evaporation temperature of the injector was 45°C with the split fully opened. After 15 sec the splitting valve was closed and the injector heated immediately to 255°C. After 1 min the temperature program was being from 150 to 300°C at a heating rate of 6°C/min. Heating of the injector was stopped 3.5 min after injection of the sample. Mass fragmentographic detection was performed as described above.

RESULTS AND DISCUSSION

Extraction and internal standardization

Extraction of HVA and VMA from human body fluids with ethyl acetate at pH 1 after saturation with sodium chloride is a well established procedure[1]. Phase separation problems can be avoided by a slow rotational extraction technique.

Internal standardization with isotopically labelled compounds compensates

widely for losses during the extraction step. No D/H exchange is observed in the case of a deuterium-labelled methoxy group. Storage of VMA standards in organic solvents very rapidly resulted in considerable decomposition. However, no losses occurred over several weeks when the standards were stored in diluted aqueous hydrochloric acid according to Bauersfeld *et al.*[11].

Derivative formation

The dimethylthiophosphinyl methyl ester derivative of HVA is easily formed by the method described previously[7]. However, the derivatization of VMA with this method is complicated by the presence of the benzylic hydroxy group which can be either methylated or phosphinylated. Esterification with methanolic boron trichloride at 80°C enables the simultaneous methylation of this benzylic hydroxy group, leading subsequently to a monophosphinylated compound with dimethylthiophosphinic chloride and triethylamine. This VMA derivative shows similar GC properties to those of the HVA derivative. The bisphosphinylated VMA derivative exhibits an indesirably long retention time.

Prior to the methylation step, the reaction solution has to be dried carefully in order to prevent the formation of free HCl, which can completely destroy the underivatized VMA at elevated temperature. The dimethylthiophosphinyl methyl ester derivatives of HVA and VMA are very stable to moisture and air, and can be stored for several weeks without decomposition.

Gas chromatographic–mass fragmentographic detection

Gas chromatographic separation is performed on a 30-m DB-1 fused-silica capillary column. Due to the relatively large amounts of material available, urine samples are injected by the normal split mode. Injection of serum, plasma and CSF samples is carried out by the split–splitless technique resulting in a nearly solvent-free chromatographic separation[8]. The overall sensitivity of the method is increased remarkably by this cold sampling technique, which is a prerequisite for the analysis of such low concentrations of HVA and VMA. The high specificity of mass fragmentography allows the quantitation of crude reaction solutions without additional purification steps. Detection of HVA and its d_3-HVA derivative occurs via the molecular ions at m/z 288 and m/z 291. VMA and its d_3-VMA derivative are monitored with the intense fragment ions at m/z 259 and m/z 262.

Methodological criteria

The method for the quantitation of urine samples results in linear response curves in the range of interest, from 1 to 15 μg/ml, using either peak height ratios (r = 0.9996 for HVA, r = 0.9997 for VMA) or peak area ratios (r = 0.9995 for HVA, r = 0.9984 for VMA). Coefficients of variation between 4 and 5% are obtained for the peak height ratios, whereas the use of peak area ratios leads to smaller coefficients of variation between 2 and 3% as expected. For nanogram amounts (0–10 ng), a linear response curve is obtained for HVA with the peak height ratios (r = 0.9985), and for VMA in the case of peak area ratios (r = 0.9996). The overall detection limit of the method is 1 ng/ml.

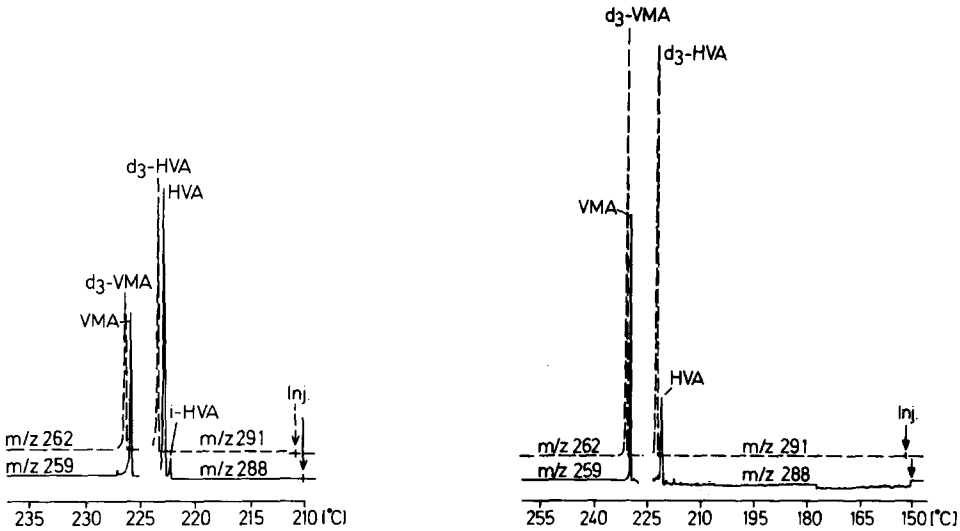

Fig. 1. Mass fragmentograms obtained from a normal urine sample. Temperature program: 210 to 300°C, heating rate 2°C/min. Detected ions: *m/z* 288 (HVA and iso-HVA derivative); *m/z* 291 (d_3-HVA derivative); *m/z* 259 (VMA derivative) and *m/z* 262 (d_3-VMA derivative).

Fig. 2. Mass fragmentograms obtained from a plasma sample. Temperature program: 150 to 300°C, heating rate 6°C/min. Detected ions as in Fig. 1.

Application to human materials

Fig. 1 shows a typical mass fragmentogram of a normal urine sample. The ions of the HVA derivatives are monitored in the first part of the chromatogram, and those of the VMA derivatives in the second part. The small peak of the iso-HVA derivative is well separated under the chromatographic conditions applied. No interfering peaks are observed in the regions of interest, demonstrating the high specificity of detection for dimethylthiophosphinyl methyl ester derivatives.

The mass fragmentographic detection of a plasma sample with a normal HVA concentration of 14 ng/ml and an elevated VMA concentration of 24 ng/ml is shown in Fig. 2. No peak broadening occurs with the split–splitless sampling technique using a trapping temperature of 150°C. This plasma sample does not contain interfering compounds. In some serum samples from patients with impaired renal function, however, interfering compounds were observed which can prevent an accurate quantitation.

In addition, we have compared the venous HVA and VMA levels for serum and plasma samples, and found no significant differences. Both materials can be analyzed with the method described, whereas the procedure of Muskiet *et al.*[1] can be applied only to serum samples.

The mass fragmentogram of a lumbar CSF sample is shown in Fig. 3. Split–spitless injection and chromatographic separation are performed in the same manner as with the plasma sample. Quantitation of HVA in CSF can be achieved without serious problems since a concentration of 10–70 ng/ml is normally present in this material. Essentially lower concentrations of VMA, ranging from only 0.5 to 5 ng/ml,

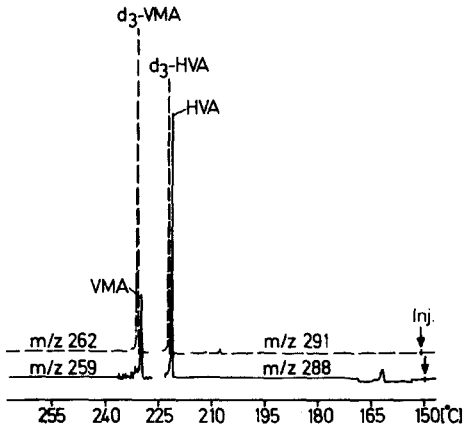

Fig. 3. Mass fragmentograms obtained from a cerebrospinal fluid sample. Temperature program as in Fig. 2, detected ions as in Fig. 1.

are found in CSF[12]. Therefore the quantitation can sometimes be disturbed by interfering compounds.

Localization of catecholamine-producing tumors

Localization of catecholamine-producing tumors can be achieved by several techniques including computerized axial tomography, arteriography and biochemical investigations. Catheterization of vena cava inferior with selective venous sampling and estimation of the plasma catecholamine levels can be very helpful in diagnosis and above all for localization of a pheochromocytoma, especially when other techniques prove uninformative.

Quantitation of plasma catecholamine levels, however, places high demands on methodology. It is rather difficult to perform in comparison with the method for the determination of their acidic metabolites presented here. We therefore hoped to find out whether the quantitation of plasma catecholamine levels can be replaced by determination of the respective VMA or HVA levels as proposed by Muskiet *et al.*[1].

Up to now we have studied two patients with pheochromocytoma. The resulting catecholamine and VMA levels did not correspond completely at all sampling sites. Due to the small number of patients investigated, we cannot yet recommend replacement of plasma catecholamine measurements by determination of their acidic metabolites.

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REFERENCES

1 F. J. A. Muskiet, G. T. Nagel and B. G. Wolthers, *Anal. Biochem.*, 109 (1980) 130.
2 M. Tuchman, P. J. Crippin and W. Krivit, *Clin. Chem.*, 29 (1983) 828.

3 M. Spiteller and G. Spiteller, *J. Chromatogr.*, 164 (1979) 253.
4 H.-U. Melchert and H. Hoffmeister, *J. Clin. Chem. Clin. Biochem.*, 15 (1977) 81.
5 H.-U. Melchert, *Z. Anal. Chem.*, 301 (1980) 224.
6 F. A. J. Muskiet, M. C. Stratingh, G. J. Stob and B. G. Wolthers, *Clin. Chem.*, 27 (1981) 223.
7 W. Vogt, K. Jacob, A.-B. Ohnesorge and G. Schwertfeger, *J. Chromatogr.*, 199 (1980) 191.
8 W. Vogt, K. Jacob and H. W. Obwexer, *J. Chromatogr.*,174 (1979) 437.
9 W. Vogt, K. Jacob, A.-B. Ohnesorge and H. W. Obwexer, *J. Chromatogr.*, 186 (1979) 197.
10 D. Henneberg, U. Henrichs and G. Schomburg, *J. Chromatogr.*, 112 (1975) 343.
11 W. Bauersfeld, U. Diener, E. Knoll, D. Ratge and H. Wisser, *J. Clin. Chem. Clin. Biochem.*, 20 (1982) 217.
12 F. A. J. Muskiet, H. J. Jeuring, J.-P. Ader and B. G. Wolthers, *J. Neurochem.*, 30 (1978) 1495.