Determination of pipecolic acid in urine and plasma by isotope dilution mass fragmentography

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Summary

A capillary gas chromatographic method with mass spectrometric detection for the determination of pipecolic acid in urine and plasma (or serum) has been developed. Using a quantification based on stable isotope dilution mass fragmentography the concentration of pipecolic acid was determined in urines of 34 healthy children and 8 patients with Zellweger's syndrome. The urinary pipecolic acid excretion of healthy infants decreases with age. Its concentration in urines of patients with Zellweger's syndrome was not consistently elevated. Normal values for pipecolic acid in plasma were established for 19 healthy children. Pipecolic acid concentrations in 47 urine samples (range 0.02–228.3 mmol/mol of creatinine) and 6 serum samples of Zellweger patients after oral loading with dl-pipecolic acid (range 65–1334 \( \mu \)mol/l) were found to correlate satisfactorily with the results obtained by an amino acid analyzer method. The major advantage of the presented method over the amino acid analyzer method concerns its greater sensitivity and its much shorter analysis time.

Introduction

Raised concentrations of pipecolic acid, a metabolite of lysine, have been described in physiological fluids of patients with hyperlysinemia [1], the cerebro-hepato-renal syndrome of Zellweger [2,3], hyperpipecolatemia [4–6], neonatal adrenoleukodystrophy [7], and infantile Refsum's disease [8]. Pipecolic aciduria was reported in hyperthyroidism [9], prematurity [1,10], and Kwashiorkor [11]. In a
recent study, however, no pipecolic aciduria was found in four hyperthyroid patients [10].

The most commonly applied method for the analysis of pipecolic acid in urine and plasma (or serum) is based on ion-exchange chromatography followed by post-column derivatization with ninhydrine and colorimetric detection, using amino acid analyzers [3,12,13]. Recently, methods based on reversed-phase high performance liquid chromatography with fluorometric detection [14] and gas chromatography with mass spectrometric detection [15,16] have become available. In the latter methods packed columns were used for the analysis of pipecolic acid in rat brain.

Here we describe a capillary gas chromatographic method with mass spectrometric detection for the determination of pipecolic acid in urine and plasma (or serum). A comparison with the results obtained by a previously described amino acid analyzer method [3] is given.

Materials and methods

Reagents

Pipecolic acid and picolinic acid were from Aldrich Europe, Beerse, Belgium. Dowex AG 50-WX-8 (100–200 mesh) was from Serva, Heidelberg, FRG, and heptafluorobutyric anhydride from Pierce Chemical Co., Rockford, USA. All other reagents were from Merck, Darmstadt, FRG.

Synthesis of heptadeuterated pipecolic acid

$[^2H_7]$pipecolic acid (pipecolic acid-d7) was prepared from picolinic acid by catalytic deuteration [17]. Picolinic acid was dissolved in 18 ml deuterium oxide, after which 2 ml deuterium chloride (6 mol/l of deuterium oxide) and 100 mg of platinum oxide were added. This mixture was shaken under 2.5–3.0 bar of deuterium gas until gas uptake ceased. The solution was filtered free of platinum, the solvent was removed under vacuum, and the residue was dissolved in 10 ml of 6 mol/l HCl. The mixture was heated at 110°C for 18 h.

Samples

24-h Urines were collected from 34 apparently healthy children and 8 infants with Zellweger's syndrome. During the collection the samples were kept refrigerated. Blood- or EDTA-anticoagulated blood samples were collected by venipuncture. Either plasma or serum were prepared by centrifugation at 1000 × g for 10 min. All samples were stored at −20°C until analysed.

Patients

The Zellweger patients studied had the clinical characteristics described by Govaerts et al. [19], including severe hypotonia, slow or absent feeding, profound psychomotor retardation and characteristic facial appearance with high forehead, epicanthic folds and large fontanels. In these children the following biochemical abnormalities were consistently observed: defect in bile acid synthesis, increased
excretion of \( p \)-OH-phenyllactic acid in urine, increased levels of saturated long chain fatty acids in serum, increased concentration of pipecolic acid in body fluids.

**Isolation and derivatization**

To 1 ml of urine, plasma or serum was added 5 nmol pipecolic acid-d7 (100 \( \mu l \) of a solution containing 0.05 mol pipecolic acid-d7 per liter of 0.1 mol/l HCl). Plasma and serum were deproteinized by adding 1 ml of a solution containing 0.47 mol/l sulfosalicylic acid, careful mixing on a ‘Vortex’-type mixer, congelation at \( -20^\circ \)C, thawing at room temperature, and centrifugation at 1000 \( \times \) g for 10 min. To the urine and deproteinized plasma (serum) samples was added 5 ml of 0.1 mol/l HCl. Pipecolic acid was isolated from urine and deproteinized plasma (serum) by cation exchange chromatography and converted into its isopropyl-heptafluorobutyryl derivative by a slightly modified method as described by Degres et al. [18]. The pretreated sample was passed over 1 ml of Dowex 50-WX-8, previously washed with 0.1 mol/l HCl and water. The column was washed with 2 ml of 0.1 mol/l HCl and 6 ml of water, and eluted into a 14 ml ‘Sovirel’ tube with 4 ml of 4 mol/l ammonia. The eluate was evaporated to dryness at 110°C under a stream of air. Isopropyl esters were prepared by the addition of 1 ml of an isopropanol/HCl solution (freshly prepared by cautiously adding 10 ml of acetylchloride to 100 ml of mechanically stirred isopropanol), and heating the tightly capped tubes at 80°C for 1 h. After evaporation to dryness at room temperature under a stream of air, 100 \( \mu l \) of acetonitril and 100 \( \mu l \) of heptafluorobutyric anhydride were added, and the tubes were incubated at room temperature overnight (about 18 h). The solution was evaporated to dryness at room temperature under a stream of air, and the residue was dissolved in 1 ml of 0.5 mol/l sodium phosphate buffer (pH 7.0). The derivatives were extracted into 5 ml of dichloromethane. The dichloromethane (lower) layer was dehydrated by treatment with a small amount of solid anhydrous sodium sulfate, and subsequently evaporated to dryness at room temperature under a stream of air. For plasma (serum) the residue was dissolved in 100 \( \mu l \) of ethyl acetate, and a 2 \( \mu l \) portion was analyzed by gas chromatography with mass spectrometric detection. For urine a second post-derivatization clean-up procedure proved necessary: The residue was redissolved in 5 ml of dichloromethane, 1 ml of 0.01 mol/l HCl was added and the solution was mixed carefully. The dichloromethane (lower) layer was dried by solid anhydrous sodium sulfate, and subsequently evaporated to dryness at room temperature under a stream of air. The residue was dissolved in 100 \( \mu l \) of ethyl acetate, and a 2 \( \mu l \) portion was analyzed by gas chromatography–mass spectrometry.

**Equipment**

Gas chromatography–mass spectrometry was performed with a Varian 3700 gas chromatograph directly coupled to a MAT-44-S mass spectrometer and operated under the following conditions: Injector temperature, 250°C; split ratio, 1 : 10; oven temperature program, 90°C, 5°C/min to 220°C; ion source temperature, 200°C; ionization energy, 180 eV. The column was a 25 m cross-linked methyl silicone-coated (film thickness 0.11 \( \mu m \)), siloxane deactivated, fused silica capillary (i.d. 0.2 mm) from Hewlett Packard, Amstelveen, The Netherlands.
Quantification and quality control

In the ammonia chemical ionization mode the ions at m/z 385 and 392 were monitored, corresponding to the \([M + NH_4]^+\) ion of derivatized naturally occurring pipecolic acid and its heptadeuterated analog, respectively. The peak area ratio of the ion at m/z 385 and 392 was calculated using a Finnigan MAT SS-200 data system. Concentrations were computed by means of linear regression analysis using a calibration graph, composed of the corresponding peak-area ratios of various amounts of pipecolic acid (0–25 nmol) added to a fixed amount (5 nmol) of its deuterated analog. The urinary concentration was expressed in relation to that of creatinine.

For quality control, we analyzed in each series 1 ml portions of a pooled urine or pooled plasma and of the same samples enriched with 5 μmol of pipecolic acid per liter. The concentrations of pipecolic acid determined for 47 urine- and six serum samples were compared with those obtained by an amino acid analyzer method [3].

Results and discussion

If low concentrations are expected, which is the case for normal concentrations of pipecolic acid in both urine and plasma (serum), isotope dilution mass fragmentography offers the necessary combination of sensitivity and accuracy for its determination. Ammonia chemical ionization mass spectrometry of derivatized pipecolic acid and its deuterated analog results almost exclusively in the formation of the \([M + NH_4]^+\) ion, which makes the detection very sensitive.

Figure 1 shows mass fragmentograms of pipecolic acid and its deuterated internal standard, monitored at m/z 385 and 392, respectively, for a standard, a urine sample of a healthy infant and for a child with Zellweger's syndrome. For urine a second post-derivatization clean-up procedure was introduced to remove a compound that occasionally gave rise to contamination of endogenous pipecolic acid at the m/z 385 channel (see Fig. 2). Based on its solubility in dilute acid and the moderate contamination experienced in the analysis of urines from young children, the interfering compound was tentatively identified as some heptafluorobutyated derivative of urea.

The within-series precision and recovery for the determination of pipecolic acid was investigated by analyzing 6 portions of pooled urine and plasma from normal adults, together with the same samples enriched with 5 μmol pipecolic acid per liter. Mean concentrations amounted to 0.7 μmol/l (coefficient of variation (CV), 8.3%; range 0.7–0.8 μmol/l) for urine, and 2.5 μmol/l (CV, 2.0%; range 2.4–2.5 μmol/l) for plasma. The mean recovery percentages were 100.3% (CV, 6.7%; range 93.3–110.3%), and 102.8% (CV, 1.4%; range 101.6–105.8%) for urine and plasma, respectively.

The day to day precision and recovery of the method was calculated from the same pooled urine and plasma samples incorporated in 6 series that were analyzed during a period of 4 months. The data for urine were: mean endogenous concentration 0.8 μmol/l (CV, 14.5%; range 0.7–1.0 μmol/l; n = 6), recovery 100.4% (CV, 2.6%; range 97.4–104.7%; n = 6), and for plasma: mean endogenous concentration
Fig. 1. Mass fragmentograms of derivatized pipecolic acid (m/z 385) and its heptadeuterated internal standard (m/z 392) in a standard (S), and urine samples of a healthy child (N) and a child with Zellweger's syndrome (P). Time scale in min and peak intensity in arbitrary units (counts). Note the considerable retention time differences between the endogenous compound and its internal standard, which is due to the heavy labeling (d7) of the latter.

of 2.4 μmol/l (CV, 4.4%; range 2.2–2.5 μmol/l; n = 6), recovery 101.3% (CV, 6.6%; range 93.0–112.8%; n = 6).

The results of the determination of pipecolic acid in urines of 32 healthy children and 15 urines of children with Zellweger's syndrome, were compared with those obtained by an amino acid analyzer method [3]. The data were subdivided into concentration ranges of 0.0–7.0 (Fig. 3A) and 7.0–240 (Fig. 3B) mmol/mol of

Fig. 2. Mass fragmentograms of derivatized pipecolic acid (m/z 385) and its heptadeuterated analog (m/z 392) in a urine sample of a normal person after the first post-derivatization clean-up (A), and the second post-derivatization clean-up procedure (B). The calculated concentration amounted to 1.7 μmol/l and 0.7 μmol/l, respectively. Time scale is in min and peak intensity in arbitrary units (counts).
creatinine. The correlations were satisfactory with correlation coefficients of 0.968 ($n = 39$), and 0.997 ($n = 8$) for the low- and high concentration ranges, respectively. In the low concentration range (Fig. 3A) the results obtained by the amino acid analyzer method were somewhat higher than those determined by the present method, while for 13 urine samples the levels determined by the amino acid analyzer method were at its detection limit (amounting to about 3 pmol/l), and fell below its detection limit for 7 samples. Similar results were obtained by comparing the serum pipecolic acid concentrations (range 65–1334 μmol/l) of 6 Zellweger patients at different times after oral loading with DL-pipecolic acid. The lower limit of quantification by the gas chromatographic-mass spectrometric method is 0.1 μmol/l (peak-background ratio: 7), indicating that, in case low concentrations are expected, the present method is superior over the determination by amino acid analyzer. It should, however, be stressed that there is no known pathophysiological significance of a decreased urinary excretion of pipecolic acid. Until now the use of pipecolic acid determinations in plasma and urine has been confined to the clinical chemical diagnosis of Zellweger's syndrome and related disorders, such as neonatal adrenoleucodystrophy and pseudoreposum syndrome. In all serum samples of patients with Zellweger's syndrome increased concentrations of pipecolic acid were measured soon after birth [19]. The assays of both plasma and cerebrospinal fluid concentrations of pipecolic acid are considered as a reliable clinical chemical diagnostic criteria for the screening of patients suspected of having Zellweger's syndrome. As even in Zellweger's syndrome the cerebrospinal fluid concentrations of pipecolic acid are close to the detection limit of the amino acid analyzer method (0.6–6.6 μmol/l, Ref. 19), the present method may be expected to offer the opportunity to discriminate more accurately between its normal and increased levels. Although the gas chromatographic mass spectrometric equipment is expensive, on the other hand it should be noted that the analysis time on the instrument is completed within 15 min and is much shorter than that on the amino acid analyzer (140 min).
Figure 4 shows age-dependent normal values for the urinary excretion of pipecolic acid, expressed in terms of creatinine, by 34 healthy children, aged 0 to 15 yr. A rather steeply decreasing excretion with age was found, which may only partly be attributed to the rapid increase of creatinine excretion during the first years of life [20]. When compared with older infants the much higher urinary excretion by

TABLE I

Urinary pipecolic acid concentrations in patients with Zellweger's syndrome

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Pipecolic acid mmol/mol of creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>111.2</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>1</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>2.4</td>
<td>9.3</td>
</tr>
<tr>
<td>2</td>
<td>4.1</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>193.9</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>4.8</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>84.6</td>
</tr>
<tr>
<td>4</td>
<td>0.7</td>
<td>2.8</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>29.3</td>
</tr>
<tr>
<td>4</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>3.4</td>
<td>24.8</td>
</tr>
<tr>
<td>4</td>
<td>4.4</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
<td>56.4</td>
</tr>
<tr>
<td>6</td>
<td>0.2</td>
<td>29.4</td>
</tr>
<tr>
<td>6</td>
<td>0.4</td>
<td>228.3</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>26.2</td>
</tr>
<tr>
<td>8</td>
<td>0.3</td>
<td>74.5</td>
</tr>
</tbody>
</table>
neonates is probably for the greater part due to an age-dependent maturation of the reabsorption capacity of pipecolic acid in the kidney-tubular cells [10].

For the establishment of normal values, pipecolic acid was measured in the plasma of 19 healthy children (ages 0–16 yr). No age-dependency was encountered. The mean plasma level was 1.4 μmol/l (CV, 68.8%; range 0.2–4.6 μmol/l) which is in reasonable agreement with data reported by others [3,13,14].

Table I gives the results for urine samples from 8 children with Zellweger's syndrome. A wide range (0.7–228.3 mmol/mol of creatinine) for the urinary pipecolic acid excretion was found. During follow-up the levels were found to vary markedly, occasionally showing normal- and borderline values (patients 1, 3 and 4). The finding of false negative results when only the urinary pipecolic acid concentrations are taken into account is consistent with data reported by others [19].

References