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Short Communication

Analysis of pipecolic acid in biological fluids using capillary gas chromatography with electron-capture detection and [$^2H_{11}$]pipecolic acid as internal standard

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ABSTRACT

A sensitive and accurate stable isotope dilution assay was developed for the measurement of pipecolic acid in body fluids using capillary gas chromatography with electron-capture detection. The method utilizes [$^2H_{11}$]pipecolic acid as the internal standard. Sample preparation consisted of derivatization in aqueous solution (pH 11.5) of the amine moiety with methyl chloroformate to the N-methylcarbamate, followed by acidic ethyl acetate extraction at pH $\leq$ 2 and further derivatization of the carboxyl moiety with pentafluorobenzyl bromide, the excess of which was removed by solid-phase extraction. Control values have been determined in the plasma of at-term infants, age > 1 week (n = 21, mean = 1.36 μM, range = 0.47–3.27 μM). The utility of the method was demonstrated by quantitating pipecolic acid in biological fluids derived from patients with peroxisomal disorders. The method was validated against an established electron-capture negative ion mass fragmentographic technique.

INTRODUCTION

Pipecolic acid (PA) catabolism is disturbed in patients with various inherited peroxisomal disorders (Zellweger syndrome, infantile Refsum's disease, neonatal adrenoleukodystrophy and hyperpipecolic acidemia). These patients accumulate PA in plasma, urine and cerebrospinal fluid (CSF). Determination of PA in plasma and urine can be carried out with an amino acid analyser or high-performance liquid chromatography using the acid-ninhydrin method [1–3]. However, these techniques require relatively large sample volumes, and the sensitivity and selectivity are not sufficient to determine low control PA levels. More sensitivity and selectivity are obtained applying gas chromatography–mass spectrometry (GC–MS) [4–6]. Of the different available GC–MS techniques, the selected-ion monitoring method developed in our laboratory, using the pentafluorobenzyl (PFB) derivative, GC with electron-capture negative ion (ECNI) MS and [$^2H_{11}$]PA as the internal standard (I.S.) [5] appears to be the most sensitive and selective technique.

Owing to the isotope effect caused by the nine deuterium atoms left in the final derivative, partial chromatographic separation between labelled and unlabelled pipecolic acid is observed. This paper describes an optimized capillary GC method for the separation of the two forms of PA using the PFB derivative. This separation meth-

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od, combined with electron-capture detection (ECD), offers a less expensive and more generally available alternative technique for the determination of PA in biological samples, without loss of sensitivity or accuracy compared with the existing GC–ECNI-MS technique.

EXPERIMENTAL

Experimental design

After method development, incoming plasma, urine and CSF samples of suspected peroxisomal diseases to be screened for PA by GC–ECNI-MS were analysed by GC–ECD simultaneously. Urine, plasma or CSF samples collected were stored at −20°C. All pediatric controls were born at term and showed no renal or liver disorder.

Reagents and chemicals

L-Pipecolic acid was purchased from Sigma (St. Louis, MO, USA), and DL-[1,2,2,3,3,4,4,5,5,6,6′-$^2H_{11}$]pipecolic acid ([$^2H_{11}$]PA) from MSD Isotopes (Pointe-Claire, Dorval, Canada). Methyl chloroformate and triethylamine were obtained form Merck (Darmstadt, Germany) and pentafluorobenzyl bromide from Pierce (Rockford, IL, USA). All organic solvents and chemicals were of analytical grade. Disposable silica solid-phase extraction cartridges (100 mg bed volume) were purchased from Analytichem International (Harbor City, CA, USA).

Sample treatment

Aliquots of 100 μl of plasma, urine or CSF were treated as described previously for the GC–ECNI-MS technique [5]. For this purpose the amino group of CA was derivatized in aqueous solution at pH 11.5 with methyl chloroformate, and after extraction with ethyl acetate the carboxy group was derivatized with PFB bromide (PFB-Br).

In addition, PFB-Br and interfering compounds were removed from the sample by solid-phase extraction on silica cartridges. The silica columns were rinsed with 5 ml of hexane before the samples were introduced. The columns were washed with 5 ml of hexane and the derivatives were eluted with 9 ml of 3% ethyl acetate in hexane. The effluents were evaporated to dryness under nitrogen at 40°C and dissolved in hexane (for plasma and urine 200 μl, for CSF 20 μl). A volume of 1 μl of the hexane solution was used for analysis. It should be noted that the deuterium atoms at the 1′ and 6′ positions of [$^2H_{11}$]PA are replaced during derivatization by methyl chloroformate and PFB-Br respectively.

GC–ECD

GC–ECD analysis was performed on a Carlo Erba (Milan, Italy) HRGC 5300 instrument equipped with an ECD-40 electron-capture detector. The detector was used in the constant-current mode (pulse width 1 μs, pulse voltage 50 V, reference current 1 nA). Nitrogen was used as make-up gas at 250 kPa (75 ml/min). The GC column was a 25 m × 0.16 mm I.D. CP Sil-5 chemically bonded fused-silica capillary column (Chrompack, Middelburg, Netherlands). Helium was used as carrier gas with a linear flow of 36 cm/s. The detector temperature was maintained at 300°C, and the injector temperature was 290°C. The GC oven temperature programme was: 180°C to 194°C at 2°C/min, then to 290°C at 40°C/min, and was kept there for 5 min in order to clean the column. Samples were introduced onto the GC column using a split injector (split vent 17.4 ml/min, split ratio 1:40).

GC–ECNI-MS was used as the reference technique and performed on a Kratos MS-80 magnetic sector instrument as described previously [5].

Quantitation and quality control

Two calibration curves have been used for the calculation of the concentrations of PA in plasma and urine samples (curve 1) and CSF samples (curve 2). Calibration curve 1 covered the range 0.1–5.0 nmol PA using 1.0 nmol I.S. and curve 2 the range 0.05–0.5 nmol PA using 0.1 nmol I.S. The calibration samples were prepared in water and were carried through the whole procedure.

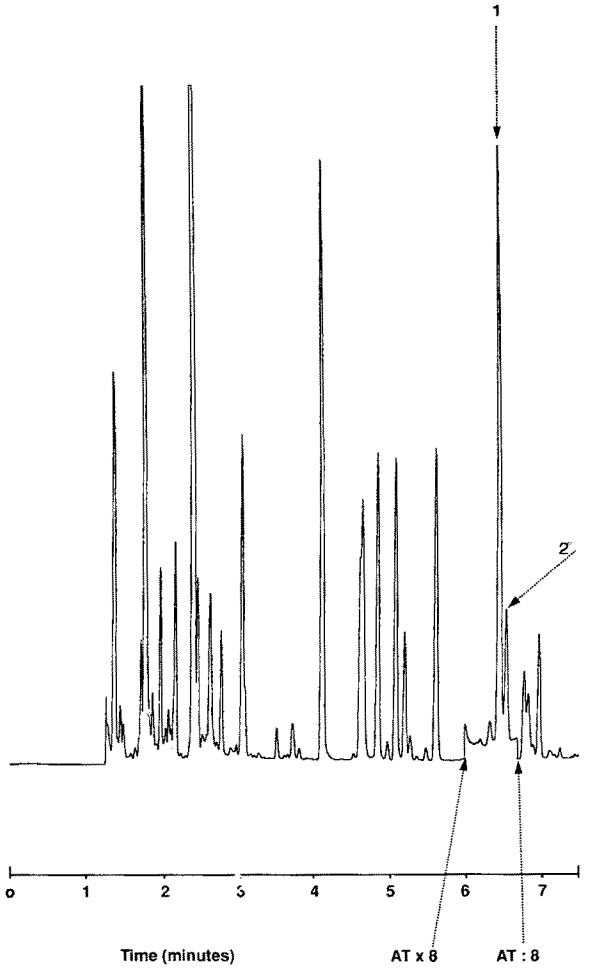

Fig. 1. GC–ECD chromatogram of pipecolic acid in a control plasma sample. Between 6 and 6.7 min the attenuation (AT) was increased by a factor 8. Peaks: 1 = [$^2H_{11}$]pipecolic acid (I.S.); 2 = pipecolic acid. The actual concentration of pipecolic acid in this plasma sample was 2.1 μM.

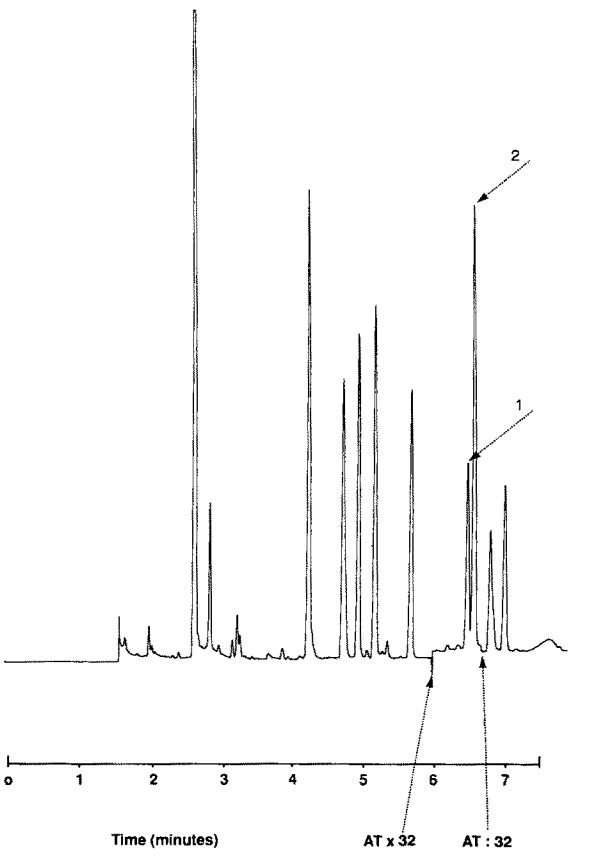

Fig. 2. GC–ECD chromatogram of pipecolic acid in plasma of a patient with peroxisomal disease. Between 6 and 6.7 min the attenuation was increased by a factor 32. Peaks: 1 = [$^2H_{11}$]pipecolic acid (I.S.); 2 = pipecolic acid. The actual concentration of pipecolic acid in this plasma sample was 28.7 μM.

For quality control, pooled plasma was prepared for repeated analysis. Samples containing high levels of PA were diluted 10–50 times for accurate quantitation.

RESULTS

Preliminary results applying the sample preparation procedure used for the GC–ECNI-MS analysis of PA showed interference of the PFB-Br reagent peak and coeluting compounds. The clean-up of the derivatization products using silica extraction cartridges removed the interferences but did not affect the quantitative results as tested by the GC–ECNI-MS measurements. The N-methylcarbamate-PFB ester of PA exhibited favourable GC properties and a high sensitivity for ECD. Under the chromatographic conditions used, adequate separation was achieved between the N-methylcarbamate-PFB ester of PA and [$^2H_{11}$]PA. No biological compounds were found to interfere with the I.S. Fig. 1 shows a typical chromatogram of a control plasma sample, and Fig. 2 was obtained from the plasma of a patient with peroxisomal disease. The detection limit of the method corresponds to *ca.* 0.2 μM in plasma. Both calibration curves yielded straight lines that passed through the origin, with correlation coefficients of >0.999. Linear regression analysis was

TABLE I

PIPECOLIC ACID CONCENTRATIONS IN PLASMA, URINE AND CEREBROSPINAL FLUID OF A CONTROL POPULATION AND PATIENTS

		Pipecolic acid concentration in plasma, CSF (μmol/l) and urine (mmol/mol creatinine)			
		GC–ECNI-MS		GC–ECD	
Plasma controls (age > 1 week)	$n = 21$	Mean: 1.52,	range: 0.57–2.73	Mean: 1.36,	range: 0.47–3.27
Plasma patients (age > 1 week)	$n = 5$		range: 22.0–244		range: 20.2–249
Urinary controls (age > 6 months)	$n = 6$	Mean: 0.27,	range: 0.11–0.55	Mean: 0.23,	range 0.04–0.53
Urinary controls (age < 6 months)	$n = 6$	Mean: 3.03,	range: 1.05–5.35	Mean: 2.51,	range: 0.07–5.35
Urine patient (age > 6 months)	$n = 1$	240		229	
CSF controls	$n = 5$	Mean: 0.034,	range: 0.009–0.120	Mean: 0.059,	range: 0.027–0.131
CSF patient	$n = 1$	1.40		1.75	

used to calculate the concentrations of PA in the biological samples.

The coefficient of variation (C.V.) of the PA measurement in pooled control plasma ($n = 6$, mean = 1.54 μM) was 10% within a period of 6 months. The concentrations of PA in plasma, urine and CSF samples of control individuals and patients obtained with both the GC–ECD and the GC–ECNI-MS techniques are shown in Table I. Plasma PA values from patients suffering from peroxisomal diseases involving PA catabolism were found to be much higher using both techniques, and PA concentrations in the urine and CSF of patients were outside the control range of both methods.

DISCUSSION

Quantitation of compounds by GC analysis requires the use of an I.S. The compound to be determined and the I.S. should have similar recoveries during the sample preparation procedure and possess similar detector responses. In this respect labelled homologues of the compound of interest are ideal internal standards and commonly used in quantitative GC–MS analysis. Application of labelled internal standards in GC analysis requires adequate chromatographic resolution from the unlabelled compound as well as from other compounds present in the biological sample. [$^2H_{11}$]PA looses two deuterium atoms in the sample preparation procedure. The final $^2H_9$-labelled derivative and the unlabelled derivative can be adequately separated using a 25 m × 0.16 mm I.D. capillary CP Sil-5 CB column. Sample preparation, including PFB derivatization as described for the GC–ECNI-MS analysis, was insufficient for GC–ECD analysis and had to be extended with an additional clean-up step involving silica cartridges. This extraction procedure removed the excess PFB-Br reagent and interfering biological compounds. Small volumes of plasma, urine and CSF could be analysed by the GC–ECD technique. The control ranges of PA in plasma, urine and CSF obtained with GC–ECD were comparable with the control ranges obtained recently with GC–ECNI-MS [5]. A good comparison between the two methods was also observed for PA concentrations found in biological fluids of patients with peroxisomal disorders.

Using the GC–ECD technique, patient values could be well distinguished from the control ranges ($> \bar{x} + 2\sigma$).

CONCLUSION

It may be concluded that the GC technique described here, using PFB derivatization and

ECD, appears to be a useful way to detect patients accumulating PA in plasma, urine or CSF. It is a less expensive and more commonly available alternative to the expensive GC–MS technique without compromising either the sensitivity or the accuracy.

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