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Short communication

Simple isotope dilution assay for propionic acid and isovaleric acid

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Abstract

A gas chromatographic–mass spectrometric method is described for the assay of propionic acid and of isovaleric acid in physiological fluids by isotope dilution. The acids are derivatized to the pentafluorobenzyl esters to decrease volatility to render them suitable for GC–MS analysis. The following reference values were found. Propionic acid: plasma 0.54 ± 0.38 μmol/l ($n = 13$, range 0.03–1.38 μmol/l), urine 1.7 ± 1.6 μmol/mmol creatinine ($n = 9$, range 0.1–4.9 μmol/mmol creatinine). Isovaleric acid: plasma 0.89 ± 0.93 μmol/l ($n = 10$, range 0.01–3.03 μmol/l), urine 0.38 ± 0.51 μmol/mmol creatinine ($n = 10$, range 0.01–1.70 μmol/mmol creatinine).

1. Introduction

Propionyl-CoA is an intermediate in the catabolism of the amino acids valine, isoleucine, methionine and threonine, of odd-chain fatty acids and of cholesterol. It is metabolized further to methylmalonyl-CoA by the enzyme propionyl-CoA carboxylase. Propionic acid accumulates in physiological fluids as a result of an inherited deficiency of propionyl-CoA carboxylase activity [1] (propionic acidemia), an inherited deficiency in the supply of the cofactor biotin (biotinidase deficiency or holocarboxylase synthase deficiency) or, secondary, an inherited deficiency in the further metabolism of the product of the propionyl-CoA carboxylase catalyzed reaction (methylmalonic acidemia). Isovaleryl-CoA is an intermediate in the catabolism of the amino acid leucine. An inherited deficiency of the enzyme isovaleryl-CoA dehydrogenase leads to the accumulation of isovaleric acid [2] (isovaleric acidemia). Each of these conditions is characterized by the excretion of specific metabolites, such as 3-hydroxypropionic acid, propionylglycine, 3-hydroxyisovaleric acid, tiglylglycine, and the methylcitrates for propionic acidemia and isolvalerylglycine, 3-hydroxyisovaleric acid and 4-hydroxyisovaleric acid for isovaleric acidemia. The diagnosis for these conditions is, therefore, made through analysis of these metabolites in urine. Monitoring of propionic acid and isovaleric acid in plasma and urine is useful in the treatment and management of propionic acidemia and isovaleric acidemia patients. Methods for the assay of propionic and isovaleric acid have been described [2–10]. They are based on the gas chromatographic separation on phosphoric-acid-coated column packings, which give

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easily rise to ghost peaks and need frequent washings with water and/or formic acid to eliminate these fractions. Furthermore, extensive solvent extraction or steam distillation is necessary prior to gas chromatographic analysis.

An HPLC method has been described [11], but it involves similarly considerable sample preparation. The same applies to an HPLC–GC–MS isotope dilution assay, measuring the propionic acid as the bromophenacyl derivative [12].

As highly volatile compounds, propionic acid and isovaleric acid pose specific problems in extraction, concentration and derivatization for gas chromatography–mass spectrometry when an isotope dilution assay is designed for the accurate and sensitive assay of these metabolites. The challenge is not to increase volatility, but to derivatize it to compounds of lesser volatility and to avoid extraction and its associated evaporation of extraction solvent. Pentafluorobenzylation [13] employing an extractive alkylation method, proved to satisfy the requirements mentioned above.

2. Experimental

2.1. Chemicals

[3,3,3-^{2}H]Propionic acid (98%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA), [$^{2}H_9$]isovaleric acid from CDN Isotopes (Veaudreuil, Quebec, Canada), pentafluorobenzylbromide from Pierce (Rockford, IL, USA) and tetrabutylammonium hydrogen sulfate from Sigma (St. Louis, MO, USA). All other chemicals used were of the highest purity grade available.

2.2. Equipment

A Varian-Saturn 3 system (Varian Instruments, Palo Alto, CA, USA) was used. It consisted of a Model 3400 gas chromatograph, 8200 autosampler and a Saturn 3 ion trap.

2.3. Column

The column was a DB-5, 30 m × 0.25 mm I.D., 0.25 μm film, fused-silica capillary column (J&W Scientific, Folsom, CA, USA), and for the assay of propionic acid operated at 85°C with a temperature gradient to 95°C at a rate of 0.8°C/min, followed by a gradient to 260°C at 50°C/min. The column was maintained at that temperature for 20 min. The helium (ultra high purity) flow was 1.0 ml/min. The conditions for the assay of isovaleric acid were essentially the same, except that the column was operated at 100°C, with a temperature gradient to 110°C at a rate of 0.8°C/min.

2.4. Detector

The ion trap was programmed to collect data for the mass ranges 50–70 for the propionic acid derivative and 50–100 for the isovaleric acid derivative, from 10.00 to 13.10 min and from 8.60 to 13.50 min, respectively.

2.5. Sample preparation

To 250 μl of sample or standard solution was added 250 μl 1.0 mmol/l [3,3,3-^{2}H]propionic acid or 250 μl 1.0 mmol/l [$^{2}H_9$]isovaleric acid, followed by 1 ml 0.1 mol/l tetrabutylammonium hydrogen sulfate in 0.2 mol/l sodium hydroxide, 1 ml 1,2-dichlorobenzene and 20 μl pentafluorobenzylbromide. The container was tightly capped and vigorously stirred for 1 h at 57°C. After cooling to room temperature the mixture was centrifuged to separate the phases. The clear bottom layer was removed and 0.5 μl was injected into the gas chromatograph–mass spectrometer (splitless mode).

3. Results and discussion

Conditions were established to maximize the yield of the acid specific signals. Using the method as originally described [13] a poor re-

sponse for the acid specific signals was obtained. The temperature of alkylation was, therefore, increased, with better yields, but the methylene chloride (b.p. 40°C) used in the original method had to be replaced by a solvent with a higher boiling point. 1,2-Dichlorobenzene (b.p. 180°) proved to be satisfactory. The optimum temperature proved to be 57°C.

Fig. 1 shows the chromatograms of the pentafluorobenzyl esters and the intensity of the $m/z = 57$ and 60 signals for propionic acid and the $m/z = 57$ and 66 signals for isovaleric acid, respectively. Electron ionization was used rather than chemical ionization (with ammonia) because the latter yielded a poor response in this case. There is a slight, but significant, difference in retention time between the derivatives of propionic acid and [$^2H_3$]propionic acid, the deuterated derivative emerging slightly earlier from the column. This was also the case for the derivatives of isovaleric acid. Similar differences have been observed with this column in retention times of trimethylsilyl derivatives of some other deuterated compounds as compared to the non-deuterated parent compounds, presumably because the deuterated compounds are somewhat more bulky and are, therefore, less strongly bound.

Calibration curves for propionic acid and for isovaleric acid showed a linear response over the concentration range 4 μmol/l to 1 mmol/l for the $m/z = 57$ and 60 for propionic acid and for $m/z = 57$ and 66 for isovaleric acid. The correlation coefficients (method of least squares) was 0.996 for both acids. For isovaleric acid the $m/z = 57$ and 66 were chosen for quantitation rather than the $m/z = 85$ and 94, representing the 2-methylpropyl group and the 3-methylbutanoyl group, respectively. The latter

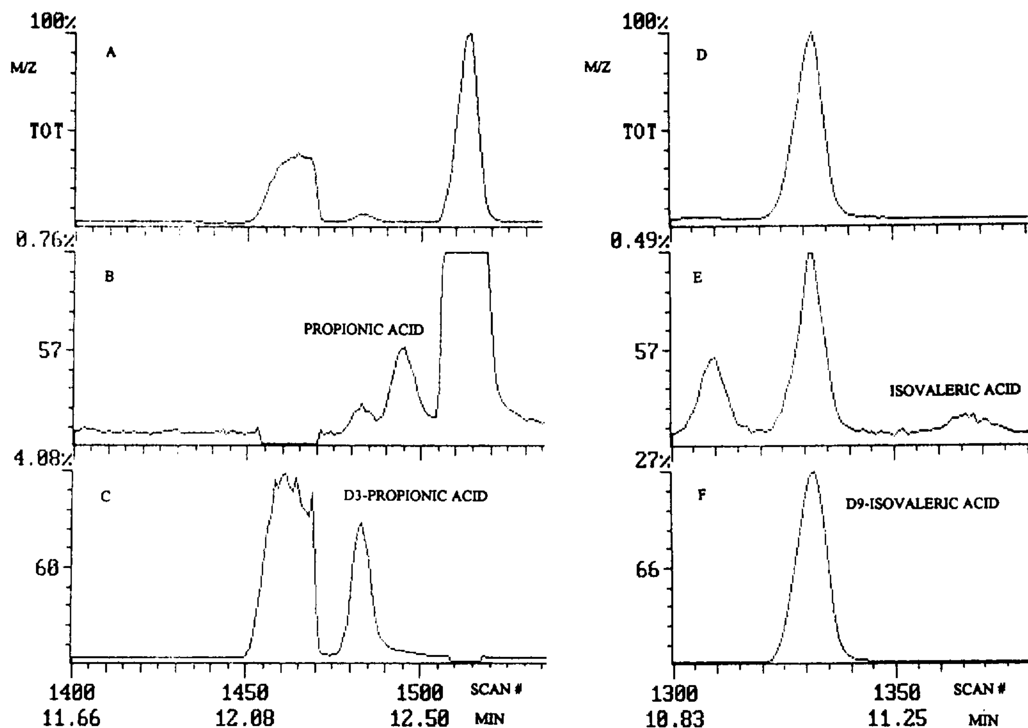

Fig. 1. Selective ion monitoring for propionic acid and for isovaleric acid of plasma. The preparation of the pentafluorobenzyl esters and the GC–MS conditions were as described in the text. (A, D) Total ion count for the propionic acid and isovaleric acid chromatograms, respectively; (B, C) $m/z = 57$ and 60 for the propionyl group and the [$^2H_3$]propionyl group, respectively; (E, F) $m/z = 57$ and 66 for the 2-methylpropyl group and the [$^2H_9$]-2-methylpropyl group, respectively, derived from isovaleric acid.

Table 1
Reference values for propionic acid and isovaleric acid in plasma and in urine

Compound	Plasma (μmol/l)	Urine (μmol/mmol creatinine)
Propionic acid	0.54 ± 0.38 (n = 13)	1.7 ± 1.6 (n = 9)
Range	0.03–1.38	0.1–4.9
Isovaleric acid	0.89 ± 0.93 (n = 10)	0.38 ± 0.51 (n = 10)
Range	0.01–3.03	0.01–1.70

showed equally a linear response, but the yield was considerably lower as compared to the former. The precision of the procedure was evaluated by derivatizing a plasma sample 18 times. The value for propionic acid was found to be 2.1 ± 0.5 μmol/l (range 1.2–2.7). When spiked with two levels of propionic acid (120 μmol/l and 1000 μmol/l), values of 123 ± 2 μmol/l (n = 7) and 1006 ± 4 μmol/l (n = 9) were found, respectively. Similar results were obtained for isovaleric acid. Table 1 summarizes the reference values for plasma and urine.

The method described here allows the accurate determination of plasma or urine volatile organic acids, as exemplified for propionic acid and isovaleric acid. It avoids extensive sample preparation involving steps with potential loss of analyte, such as solvent extraction and evaporation of the solvent, while a more robust GC method is used, rather than columns with phosphoric acid coated supports with give easily rise to ghost peaks.

The $m/z = 57$ signal is not an uncommon signal and could, therefore, interfere with the quantitation of propionic acid and isovaleric acid. In that case, plasma and urine would contain compounds, which after derivatization with pentafluorobenzylbromide, coelute with the propionic acid and isovaleric acid derivatives. It would then also interfere with the determination of the levels of these acids in normal plasmas and urines, resulting in too high values. The normal values established by this method are, however, comparable to those established by other methods [4,12,13]. It is, therefore, highly unlikely that such interference does occur.

Propionic acidemia patients, at various levels of metabolic control, demonstrated plasma propionic acid levels ranging from the upper limits of normal controls to well above those limits (17–918 μmol/l). Urine propionic acid levels of these patients were found to range from 2 to 3947 μmol/mmol creatinine. Similarly, for isovaleric acidemia patients, a range of 13–70 μmol/l for plasma, and 0.4–46 μmol/mmol creatinine for urine was found.

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