Simplified Gas Chromatography of Trimethylsilyl Esters of C₁ through C₅ Fatty Acids in Serum and Urine

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All the metabolically important C₁ through C₅ fatty acids and lactic, pyruvic and β-hydroxybutyric acids can be gas chromatographed surprisingly well on the common 2-meter 3% methylphenylsilicone (e.g., "OV-17") column when a late-eluting silylating reagent such as trimethylsilylimidazole is used. In these circumstances, the trimethylsilyl esters elute in an interval that is free of the reagent and solvent interferences usually found when the more volatile preparations are used for silylations. Use of the method is demonstrated by its application to a serum from a case of isovaleric acidemia and a urine from a case of methylmalonic aciduria.

Additional Keyphrases: diagnostic aid • inborn errors of metabolism • trimethylsilylation

We have sought a simple and dependable gas chromatographic method for separating and identifying all the metabolically significant saturated and olefinic carboxylic acids in the range C₁ to C₅ together with lactic, pyruvic, and β-hydroxybutyric acids. We find that their trimethylsilyl (TMS) derivatives chromatograph unexpectedly well on the usual 2-meter, 3% dimethyl- or phenylmethyl silicone liquid phases (such as "OV-1" or "OV-17," respectively). The use of undiluted trimethylsilylimidazole as the silylation reagent for these acids is advantageous, because it elutes after lactic acid on OV-1 and even after β-hydroxybutyric acid on OV-17.

Conventional approaches to gas chromatography of the free acids usually involve the use of heavily polyester-loaded supports modified with phosphoric acid (1-4). These methods suffer from sample adsorption, difficult problems of tailing, and the rapid thermal degradation of column performance. These difficulties, along with high bleed rates and frequent column changing necessitated by their narrow range of applicability, restrict their use in coupled gas chromatography-mass-spectrometry.

The TMS derivatives of these acids are superior to the methyl esters because they are easily formed by use of trimethylsilylimidazole and the fact that they can be analyzed on the same highly temperature-stable, low-bleed, relatively low-polarity columns on which the longer chain organic acids are analyzed. Tailing is minimal, and formic and acetic acids elute free of solvent interferences. Column changing is not required to analyze for the less volatile acids (those eluting after lactic acid) because another aliquot of the same extract silylated by one of the more conventional early-eluting reagents may be analyzed on the same column for these longer-chain acids (5).

As examples illustrative of the utility of this approach we present results of gas chromatographic analyses of urine from a patient with hereditary vitamin B₁₂-responsive methylmalonic aciduria and of serum obtained postmortem from an infant diagnosed as having isovaleric acidemia.

Materials and Methods

Chemicals

Solvents and authentic acids were purchased in their highest available purities from a variety of chemical suppliers.

α-Methylbutyric acid was prepared by the reaction of solid carbon dioxide (crushed to a snow) with a dilute ethereal solution of 2-butylmagnesium bromide, and was purified by the appropriate procedures.

The silylating reagents were manufactured by Pierce Chemical Co., Rockford, Ill. 61105.

Standards

For reference purposes, a mixture of silylated standards was prepared by heating at 60 °C for 15 min a solution of 500 µl of trimethylsilylimidazole and 50 µl of a "stock" acid mixture composed of equal weights of the following acids: formic, acetic, propionic, acrylic, butyric, isobutyric, crotonic, methacrylic, valeric, isovaleric, α-methylbutyric, β-methylcroton-
ic, tiglic, lactic, pyruvic, and β-hydroxybutyric.

Aliquots of this silylated mixture were analyzed in a Hewlett-Packard Model 5750 gas chromatograph with flame ionization detection and equipped with two columns, each 2 meters long, one packed with Chromosorb W (80–100 mesh) coated with 3% OV-1, the other coated with 3% OV-17 (these packing materials were from Applied Science Labs., Inc., State College, Pa. 16801). The operating conditions were as follows: injector, 265 °C; detector, 270 °C; helium carrier gas; post injection delay, 2 min; column temperature, programmed from 35 °C at 6 °C/min until the elution temperature of the excess trimethylsilylimidazole was reached (approximately 110 °C on OV-1 and 130 °C on OV-17). Identifications were confirmed by incremental addition and re-chromatography.

Aliquots were also analyzed with an LKB 9000 coupled gas-chromatograph–mass-spectrometer operated with similar columns under conditions corresponding to those described above. The mass spectra of the eluting peaks were in accord with their assignments.

An aliquot of the original “stock” acid mixture diluted with carbon disulfide was also analyzed (with the Hewlett Packard 5750) on a 2-meter column packed with 25% neopentyl glycol adipate and 2% phosphoric acid on Chromosorb W HP 100–120 mesh and temperature programmed from 80 °C at 8 °C/min to 175 °C with a postinjection interval of 2 min.

Urinary Acids: Extraction and Derivatization

Immediately after thawing, 2 ml of urine was made alkaline (pH 13) with a few drops of 5 molar sodium hydroxide, saturated with sodium chloride, and extracted three times with 2 ml of micro-analytical quality diethyl ether. This extract was discarded. The aqueous phase was acidified (pH 1–2) with 5 molar hydrochloric acid and extracted again three times with 2 ml of ether. This extract was dried with anhydrous sodium sulfate and half of it was evaporated to near dryness in a stream of dry nitrogen. The evaporated aliquot was then silylated with about 50 μl of trimethylsilylimidazole. For quantitative purposes, anisole may be used as the internal standard, and is added to the silylate at this point. The sample was gas chromatographed on the 3% OV-17 column under the conditions described above for the mixture of silylated standards.

The remaining half of the extract may be evaporated to near dryness and silylated with “TRI-SIL/BSA.” This silylate may be analyzed on the same OV-1 or OV-17 column, programmed from 80 °C at 6 °C/min for the postlactic acids (up to late-eluting acids, such as 5-hydroxyindolelactic acid for example).

Serum Acids: Extraction and Derivatization

Serum (1 or 2 ml) was deproteinized by adding an equal volume of ethanol. The supernatant fluid was then treated and analyzed in the way described above for urine.

Results and Discussion

Elution Characteristics

The TMS derivatives of all the acids used in the study elute before trimethylsilylimidazole on the usual 2-meter 3% OV-17 column (Figure 1) and yield a useful gas chromatogram free of the reagent and solvent interferences encountered when other silylating reagents are used. Propionic and acrylic acids co-elute on OV-17 as do β-methylcrotonic and tiglic acids. Isovaleric and α-methylbutyric acids are reportedly (1) difficult to separate as free acids on polyester and phosphoric acid columns, but are reasonably resolved here.

On OV-1, the elution order of the acid TMS derivatives is modified by the less polar character of this liquid phase (Figure 2) and trimethylsilylimidazole elutes earlier, as expected, and overlaps with the pyruvic and β-hydroxybutyric acid TMS derivatives. Lactic acid yields the longest retaining derivative still usefully resolvable from the TSIM front. Co-elutions on OV-1 are noted for α-methylbutyric and crotonic acids, with isovaleric acid falling as a shoulder on the peak, and also for tiglic and β-methylcrotonic acids. Acrylic acid yields an inconsistently smaller-than-expected peak possibly because of column absorption or even slow polymerization.

A mixed liquid phase like 1.5% OV-1 and 1.5% OV-17 might retain crotonic acid a little longer and diminish its overlap with α-methylbutyric acid.

Anisole elutes midway between the n-butyric acid peak and the α-methylbutyric/crotonic acid peak on OV-1, and also midway between the n-valeric peak and the β-methylcrotonic/tiglic acid peak on OV-17, and as a result is useful as an internal standard on both of these liquid phases.

The hexamethyldisiloxane peak noted in Figures 1

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**Fig. 1.** Gas chromatogram of a mixture of approximately equal weights of authentic acids silylated with TSIM and analyzed on OV-17 (3%, 2-meter, programmed from 35 °C, 2-min delay, plus 6 °C/min)

The peaks have the following identifications: 1, hexamethyldisiloxane; 2, formic; 3, acetic; 4, propionic; 5, acrylic; 6, isobutyric; 7, methacrylic; 8, butyric; 9, α-methylbutyric; 10, isovaleric; 11, crotonic; 12, valeric; 13, β-methylcrotonic; 14, tiglic; 15, lactic; 16, pyruvic; 17, β-hydroxybutyric; and 18, trimethylsilylimidazole
and 2 is probably the result of the reaction of the trimethylsilylimidazole with residual water, which is difficult to remove from low molecular weight organic acids. This peak is not overwhelming if the ether extracts are well dried with sodium sulfate, and in fact it may be used as a convenient marker for retention time purposes.

The same collection of free acids when chromatographed on a 25% neopentyl glycol adipate-2% phosphoric acid column, a type frequently used for the free acids, yielded the chromatogram illustrated in Figure 3. Formic acid was not detectable in this mixture owing to its very poor sensitivity for flame ionization detection, while acetic and even propionic acid show the effects of reduced response factors (6). Lactic, pyruvic and β-hydroxybutyric acids are not eluted from this column. In our view, the only advantage of using this particular type of liquid phase is that tiglic and β-methylcrotonic acids are well separated, although crotonic and n-valeric and α-methylbutyric and isovaleric acids, respectively, coelute.

Serum and Urinary Organic Acids

_Isovaleric acidemia._ The serum and urine of the isovaleric acid patient contained the organic acids previously reported as being characteristic for this disorder. The serum extract silylated with trimethylsilylimidazole and analyzed on OV-17 (Figure 4a) yielded the expected elevated isovaleric acid (1) (260 µg/ml) along with an apparently normal acetic acid peak. The concentration of lactic acid was also elevated (it was about 1 mg/ml) and is probably the result of postmortem glycolysis (pyruvic acid was notably absent). When silylated with TRI-SIL/BSA, the serum extract revealed again the elevated lactic acid and only a slightly increased amount of β-hydroxyisovaleric acid. Not even a trace of isovalerylglycine could be detected in the serum.

The urine extract silylated with TSIM was unremarkable except for a slightly abnormal amount of isovaleric acid. When silylated with TRI-SIL/BSA, however, the longer-chain acid chromatograms was dominated by gross isovalerylglycine (8) and β-hydroxyisovaleric acid (7) elevations (unquantitated, but in about a 5:1 ratio, respectively). Concentrations of lactic and β-hydroxybutyric acids were near normal.

_MethyImalonic aciduria._ The trimethylsilylimidazole silylated extract of the urine from the patient with hereditary methylmalonic aciduria revealed a relatively normal urinary acetic acid concentration and a greatly elevated propionic acid concentration (15 mg/g creatinine) (Figure 4b). When silylated with TRI-SIL/BSA, the urinary extract was found to have a characteristically very large methylmalonic

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2 This acid has previously been detected in the urine when serum levels of isovaleric acid became excessive and it was proposed that β-hydroxylation becomes an important detoxification pathway in these circumstances (7).
acid concentration (9, 10) (38 mg/g creatinine). A large adipic acid peak was also noted, a phenomenon usually associated with the ketotic state (11) and arising probably from altered fatty acid metabolism (12). Neither propionylglycine nor N-(methylmalonyl)-glycine were found in the urine.3

The serum and urine from two patients with pernicious anemia were also found by this method to contain large concentrations of propionic acid in addition to methylmalonic acid, as has been previously noted (4, 14).

We find this approach best for determination of the short-chain fatty acids owing to a very useful distribution of their retention times on the common 2-meter 3% OV-1 or OV-17 columns. The same column (either OV-1 or OV-17) may also be used for the longer-chain acids silylated with TRI-SIL/BSA. On OV-17, lactric, pyruvic, and β-hydroxybutyric acids elute before trimethylsilylimidazole and after the TRI-SIL/BSA reagents and solvents. This interval of overlap ensures that all the organic acids from formic up to those having limiting volatilities (such as 5-hydroxyindolelactic or arachidic acids) are detected in either the trimethylsilylimidazole or TRI-SIL/BSA silylates. OV-1 has a narrower overlapping interval, which easily accommodates only lactic acid (and closely co-eluting glycolic and α-hydroxyisobutyric acids) and therefore is less advantageous.

The sensitivity with which these acids can be detected depends somewhat on the volume of fluid extracted, as larger volumes yield correspondingly more concentrated extracts. There is very little confusion or interference in this range of very volatile compounds (ketones and other volatile neutrals are excluded in the extractive process), and the number of isomers is restricted to those acids that can be assembled from a small number of carbon atoms. Based upon a 2-ml fluid sample, careful drying of the extract, and economy of silylation volume (15 μl), a conservative estimate of the lower limit would be 50 ng/ml of fluid for each acid.

3 Nyhan et al. (13) have noted the common appearance of acylglycinuria in patients suffering from inborn errors of metabolism in which the coenzyme A derivatives of the corresponding short-chain fatty acids accumulate.

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References


