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Note

O-(2,3,4,5,6-Pentafluorobenzyl)oxime-trimethylsilyl ester derivatives for quantitative gas chromatographic and gas chromatographic–mass spectrometric studies of aldehydes, ketones and oxoacids

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Oxoacids are an important group of intermediary metabolites and show changes which are characteristic and relevant to therapy in acquired as well as in inherited metabolic disorders, e.g. diabetes mellitus [1], uremia [2], maple syrup urine disease [3], phenylketonuria [4] and unspecified mental retardation [5]. Hydroxylamine and its O-substituted derivatives are the reagents commonly used for the stabilization of an oxo group as an oxime or O-substituted oxime prior to derivatization of other functional groups of a compound by silylation [6]. Recently, O-(2,3,4,5,6-pentafluorobenzyl)oximes (O-PFBOximes) were shown to be suitable derivatives for the quantitative determination of aldehydes [7], ketones [7] and oxoacids [8, 9] as methyl esters. Trimethylsilyl (TMS) esters of carboxyl groups and TMS ethers of hydroxyl groups have proved to be more satisfactory derivatives than methyl esters for general organic acid analysis [6]. We have studied a variety of physiologically and pathophysiologically important aldehydes, ketones and oxoacids as their O-PFBOximes-TMS esters and found them to be advantageous to the commonly used O-ethoximes-TMS esters.

EXPERIMENTAL

Reagents

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine (PFBHA) hydrochloride, silicic acid, 100–300 mesh, tricarballylic acid and all oxoacid standards were obtained

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from Sigma (St. Louis, MO, U.S.A.). Ethoxyamine hydrochloride was purchased from Eastman-Kodak (Rochester, NY, U.S.A.). Silylating reagents, bis(trimethylsilyl)trifluoroacetamide (BSTFA) and TriSil, were purchased from Pierce (Rockford, IL, U.S.A.). 2-Methyl-2-butanol and chloroform (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were high-purity HPLC solvent grade.

Oxoacid standards (1 mmol/l) were made up freshly in water. Tricarballylic acid, hexadecanedioic acid and 2-oxocaproic acid [second stereoisomer at methylene unit (MU) 16.71, see Table I] served as internal standards. Hexadecanedioic acid (3 mmol/l) was made up in ethanol (reagent grade). Tricarballylic acid (5 mmol/l) was prepared in an aqueous bicarbonate solution (20 mmol/l). 2-Oxocaproic acid (5 mmol/l) was made up in water. All internal standards were stored for up to three months at −20°C.

Preparation of derivatives

Biological samples, including urine, plasma and amniotic fluid, were processed identical to aqueous standards. There was no need to deproteinize amniotic fluid or plasma samples prior to derivatization and extraction of organic acids. All protein as well as many groups of low-molecular-mass substances, e.g. amino acids, inorganic acids and urea, were retained on the silicic acid column during the liquid column chromatography [10]. O-PFBOximes were prepared by adding 40 μmol (10 mg per 200 μl water) of PFBHA hydrochloride to each sample containing 0.5 μmol of tricarballylic acid, 0.5 μmol of 2-oxocaproic acid and 0.3 μmol of hexadecanedioic acid as internal standards. The pH of the samples was adjusted to between 2 and 5 with sulfuric acid (0.5 mol/l) and pH paper, and the samples were allowed to stand at room temperature for 2 h. These conditions are known to be optimal for the quantitative formation of O-PFBOximes of 2-oxoacids [8]. O-Ethoximes were prepared by adding 4 mg (41 μmol) of ethoxyamine hydrochloride to each sample, which was left at room temperature for 2 h. The samples were then alkalinized to pH 7–9 with sodium hydroxide (0.5 mol/l) using pH paper to form salts and prevent losses of acids and lyophilized.

Liquid column chromatographic, gas chromatographic and gas chromatographic–mass spectrometric procedures

The liquid column chromatographic (LCC) procedure was derived from the procedure of Sweetman [10]. Silicic acid was sieved to obtain the 200–400 mesh fraction which was dried overnight at 120°C. Dried silicic acid (60 g) was acidified by mixing with 17 ml of 0.1 mol/l hydrochloric acid in methanol, washed with 675 ml of 2-methyl-2-butanol–chloroform (50:50, v/v) and dried overnight at 120°C. The lyophilized samples were acidified with 300 μl of sulfuric acid (0.5 mol/l) and adsorbed onto 0.75 g of dried washed silicic acid. The sample was placed on top of 1 g of dried washed silicic acid that had been hydrated with sulfuric acid (50 ml of 0.05 mol/l sulfuric acid per 92 g of dried washed silicic acid) and eluted with 39 ml of 2-methyl-2-butanol–chloroform (33:67, v/v). The eluate was alkalinized with 0.1 *M* sodium hydroxide in methanol to form the less volatile salts of the acids and evaporated under nitrogen. TMS derivatives were

formed by adding 200 μl of TriSil–BSTFA (1:1, v/v) to the dry residue. After heating for 2 h at 60°C, the samples were ready for analysis by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS). In each instance 1 μl was injected into the GC system. Analyses were performed with a Hewlett-Packard Model 5830A gas chromatograph equipped with a hydrogen flame ionization detector or a Finnigan 4021 quadropole mass spectrometer with the INCOS data system. On both instruments, separations were carried out on a DB-1 wide-bore (30 m×0.53 mm I.D.) 1-μm bonded-film fused-silica capillary column (J & W, Rancho Cordova, CA, U.S.A.). The column temperature was programmed from 70 to 290°C at 4°C/min with helium at 4 ml/min as carrier gas. Temperatures at the injector were 280°C, at the interface to the mass spectrometer 280°C, at the ion source 250°C and at the flame ionization detector 350°C. The ionizing voltage was 70 eV.

RESULTS AND DISCUSSION

The LCC step has the advantage of minimal sample preparation, requiring only lyophilization of dilute samples. Plasma and tissue samples can be processed identically to aqueous standards or urine samples without prior deproteinization, as proteins are retained on the silicic acid during the subsequent elution [10]. There were no significant artefacts due to the formation of derivatives. Any excess of PFBHA was retained on the silicic acid during the following elution. PFBHA would react with silylating reagents yielding a peak at MU 14.55 in our GC system (Table I). The lack of artefact formation and the chromatographic separation of the main constituents of interest are illustrated in Fig. 1, which shows the reconstructed ion chromatogram of a plasma sample of a healthy control human.

The molecular masses and MU values for twenty aldehydes, ketones and oxoacids as their O-PFBOximes-TMS esters are given in Table I. An extensive list of molecular masses and methylene units of O-ethoximes-TMS esters has been published [6]. Acetone, dihydroxyacetone, glyoxylate, pyruvate, oxaloacetate, 2-oxoadipate, 2-oxobutyrate, 2-oxoglutarate, 2-oxooctanoate and succinic semialdehyde all gave single peaks as O-PFBOximes-TMS esters. The other metabolites formed a double peak due to *syn* and *anti* forms of the oxo-pentafluorobenzoxime group. This is an improvement compared to O-PFBOximes-methyl esters of 2-oxoacids, which invariably yield a double peak [8]. The ratios of the two stereoisomers are included below the methylene units in Table I. With the exception of glycolaldehyde, where steric hindrance favouring the formation of one of the isomers over the other can be anticipated to be very small, one or the other isomer greatly predominated in all cases. Therefore, quantification was not unduly affected and could be carried out down to low levels (Table II). As with O-PFBOximes-methyl esters of 2-oxoacids [8], the second peak was generally much more prominent than the first one. This difference increased with an increasing complexity of the R group in the oxoacids (R–CO–COOH). The ratio was reversed for glycolaldehyde, 2-oxoisovalerate, and 2-oxo-3-methylvalerate. In the latter two compounds, steric hindrance due to a methyl group in position

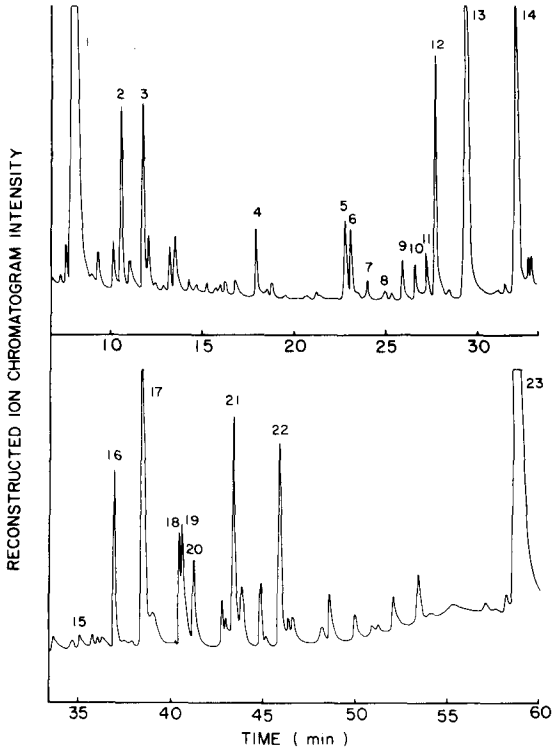

Fig. 1. Reconstructed ion chromatogram from a 2-ml sample of a control plasma. Conditions: analyses were performed with a Finnigan 4021 gas chromatograph-masss spectrometer on a DB-1 wide-bore capillary column. Column temperature was programmed from 70 to 290 °C at 4 °C/min with helium at 4 ml/min as carrier gas. Temperatures at the injector were 280 °C and at the ion source 250 °C. The ionizing voltage was 70 eV. Peaks: 1 = lactate; 2 = 2-hydroxybutyrate; 3 = 3-hydroxybutyrate and 3-hydroxyisobutyrate; 4 = glycerate; 5 = pyruvate; 6 = 5-oxoproline; 7 = 2-oxoisovalerate; 8 = acetoacetate; 9 = 2-oxo-3-methylvalerate; 10 = 2-oxoisocaproate; 11 = 2-oxocaproate (first stereoisomer, internal standard); 12 = oxocaproate (second stereoisomer, internal standard); 13 = tricarballylate (internal standard); 14 = citrate and isocitrate; 15 = 2-oxoglutarate; 16 = palmitate; 17 = uric acid; 18 = linoleate; 19 = oleate; 20 = stearate; 21 = unknown; 22 = hexadecanodioate (internal standard); 23 = cholesterol.

3 can be anticipated. We would therefore postulate that the first peak is generally due to the *syn*-R derivative.

Stereoisomerism is known to make quantitative work with oximes difficult [6]. The formation of double peaks, corresponding to the *syn* and *anti* isomers, was less prominent with O-PFBOximes-TMS esters than with O-ethoximes-TMS esters, e.g. 1:33 versus 1:3 for the two stereoisomers of *p*-hydroxyphenylpyruvate. This phenomena is probably due to a greater steric hindrance of the O-(2,3,4,5,6-pentafluorobenzyl) group as compared to the ethyl group in case of the O-ethoximes to form the *syn*-R derivative. Succinylacetone yielded four derivatized products, in accordance with all possible *syn* and *anti* combinations of two reacting oxo groups.

In accordance with Chalmers and Lawson [6], the earliest O-ethoxime derivative detectable by GC or GC–MS was glyoxylate at MU 10.75 on DB-1. Acetone

TABLE I

METHYLENE UNIT VALUES AND MOLECULAR MASSES OF O-(2,3,4,5,6-PENTAFLUOROBENZYL)OXIMES OF ALDEHYDES, KETONES AND TRIMETHYLSILYL ESTERS OF OXOACIDS ON DB-1 WIDE-BORE CAPILLARY COLUMN

Conditions: analyses were performed with a gas chromatograph equipped with a hydrogen flame-ionization detector or a quadropole mass spectrometer. On both instruments, separations were carried out on a DB-1 wide-bore capillary column. Column temperature was programmed from 70 to 290°C at 4°C/min with helium at 4 ml/min as carrier gas. Values in parentheses show the ratio of the areas of double peaks due to *syn* and *anti* forms of the oxo-benzoxime group.

Compound	M^+	Methylene units
Acetone	253	11.46
Glycolaldehyde	327	14.11 14.18 (1.3:1)
PFBHA TMS★	357	14.55
Glyoxylate	341	14.66
Pyruvate	355	14.98
2-Oxobutyrate	369	15.36
2-Oxoisovalerate	383	15.37 15.56 (4.4:1)
Acetoacetate	369	15.70 15.92 (1:1.5)
2-Oxovalerate	383	15.96
2-Oxo-3-methylvalerate	397	16.04 16.13 (6.4:1)
2-Oxoisocaproate	397	16.05 16.29 (1:3.3)
2-Oxocaproate	397	16.52 16.71 (1:22)
Succinic semialdehyde	369	16.74
Dihydroxyacetone	429	16.84
2-Oxooctanoate	425	18.37
Oxaloacetate	471	18.52
β-Phenylpyruvate	431	19.48 19.76 (1:18)
2-Oxoglutarate	485	19.61
2-Oxoadipate	499	20.50
p-Hydroxyphenylpyruvate	519	22.59 22.75 (1:33)
Succinylacetone	620	24.34 24.46 24.61 24.70 (3.2:2.7:13:1)
Tricarballylate★★	392	17.33

★TMS derivative of O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine.
★★Internal standard.

and glycolaldehyde eluted in the solvent front. The formation of O-PFBOximes caused a significant increment in molecular mass and shifted the oxoacids to between MU 14.66 for glyoxylate and 24.61 for succinylacetone. The molecular mass of the O-PFBOximes-TMS esters exceeds the O-ethoximes-TMS esters by 152 mass units resulting in a shift of 3 to 4 methylene units later than the O-ethoximes for all compounds. Not only was glyoxylic acid moved far out of the

TABLE II

DATA ON STANDARD SAMPLES CONTAINING 0.01–1 μmol OF NINE DIFFERENT METABOLITES

The peak-area ratios of the compound peaks to that of the internal standard (tricarballylate) were used to calculate linear regression curves for the different metabolites. The concentrations of each metabolite were then calculated from the area data with these curves. The values given are the means and the relative standard deviations derived from the nine individual values for a given concentration. Conditions: analyses were performed with a Finnigan 4021 gas chromatograph–mass spectrometer on a DB-1 wide-bore capillary column. Column temperature was programmed from 70 to 290°C at 4°C/min with helium at 4 ml/min as carrier gas. Temperatures at the injector were 280°C, at the interface to the mass spectrometer 280°C and at the ion source 250°C. The ionizing voltage was 70 eV.

Expected value (μmol)	Calculated mean	Relative standard deviation (%)
0.01	0.019	78
0.02	0.026	48
0.05	0.038	26
0.10	0.082	16
0.20	0.213	15
0.50	0.514	7
0.75	0.746	1
1.00	0.992	2

solvent front, but all oxoacids were moved into a region of the gas chromatogram, where we found relatively little interference from other relevant compounds in complex biological fluids (Fig. 1). The oxoacids were well separated from each other and the other constituents of the plasma, some of which were present in much higher amounts, e.g. lactate and free fatty acids. These chromatographic properties allowed quantification of pyruvate and all three branched-chain oxoacids from the reconstructed ion chromatogram in plasma samples of healthy control humans (levels: 10–100 μmol/l). Acetoacetate and 2-oxoglutarate were just detectable in the reconstructed ion chromatogram of normal plasmas (levels: <20 μmol/l). For these metabolites, quantification could only be carried out on single characteristic ions by means of the mass spectrometer. The molecular mass shift on derivatization of the oxo groups permitted the detection and quantification of aldehydes and ketones in addition to oxoacids, e.g. compounds which elute in the solvent front with other O-substituted oxime derivatives. This allows the evaluation of a new group of metabolites, which are normally not detectable simultaneously with organic acid analyses. So far, we mainly detected large quantities of acetone in samples of patients with ketoacidosis.

Aqueous sample solutions containing 0.01–1 μmol of nine metabolites (acetoacetate, glycolaldehyde, glyoxylate, *p*-hydroxyphenylpyruvate, *β*-phenylpyruvate, pyruvate, 2-oxoadipate, 2-oxoglutarate and 2-oxoisocaproate) were prepared and processed by GC–MS following the described procedure. The combined reconstructed ion chromatogram areas of the *syn* and *anti* isomers were used for quantification of glycolaldehyde, acetoacetate and 2-oxoisocaproate against tri-

carballylate as an internal standard. Linear regression curves were calculated for each metabolite and used to calculate the concentrations of each metabolite from the raw data. Table II shows the mean and the percentage standard deviation derived from the nine individual values for a given concentration. All curves were linear in the range 0.2–1.0 μmol, but in the lower range 0.01–0.2 μmol the curves showed losses. Therefore, the *y*-intercepts were negative, and sample values were overestimated, when calculated from linear regressions (Table II). We suspect that this was mainly due to variations and losses on oxime formation at very low levels. The percentage standard deviation for the mean of all metabolites increased substantially in the lower concentration range. These values also reflect differences among the nine metabolites. As losses did not seem to be identical for each individual compound, we would expect the percentage standard variation for a single metabolite to be smaller. The same phenomena were observed for the compounds as O-ethoximes-TMS esters (data not shown). In general, we felt confident to quantify O-PFBOximes-TMS esters down to 50 nmol per sample by GC alone and down to 20 nmol by GC–MS. However, below 0.2 μmol the results were obtained manually from the standard curves and not calculated from the linear regression curves. The higher sensitivity on the GC–MS system was primarily achieved by visually examining a narrow region of the chromatogram on the data system display. Baseline points before and after the peaks were selected manually and the areas calculated by the computer. Preliminary results indicate that quantification on single characteristic ions will allow a further increase in sensitivity.

CONCLUSIONS

2-Oxoacids can be very sensitively quantified as their O-TMS-quinoxalinol or O-*tert.*-butyldimethylsilyl-quinoxalinol derivatives by GC alone or GC–MS [11–13]. Because of the absence of stereoisomerism, they are eluted as a single peak. However, 3-oxoacids (e.g. acetoacetate and oxaloacetate), other carboxylic acids, aldehydes or ketones cannot be determined simultaneously. The simultaneous analysis of as wide a range as possible of intermediary metabolites in multicomponent mixtures of biological origin is the basis for the determination of metabolic profiles. These allow a confident diagnosis and the pathobiochemical evaluation of either acquired or inherited metabolic disorders and facilitate rationale therapy. We found O-PFBOximes very useful derivatives for the stabilization of an oxo group prior to derivatization of other functional groups by silylation. O-PFBOximes-TMS esters are more suitable than O-ethoximes-TMS esters, because the O-PFBOximes are shifted to higher retention times. Interferences from other relevant compounds in complex biological fluids were less problematic in this region of the gas chromatograms. The formation of a double peak, corresponding to the *syn* and *anti* isomers, was less pronounced with O-PFBOximes-TMS esters than with the corresponding O-ethoximes-TMS esters. Finally, the higher-molecular-mass shift on derivatization permits the detection and quantification of aldehydes and ketones in addition to oxoacids, e.g. compounds which will elute in the solvent front with other O-substituted oxime derivatives.

O-PFBOximes are known to be about 1000 times more sensitive with electron-

capture detectors as compared to flame ionization detection [8], and we would predict the adaptation of O-PFBOximes-TMS esters for sensitive quantification of small-molecular-mass metabolites with an oxo group by GC–electron capture detection. This analysis could follow a GC analysis with flame ionization detection or GC–MS analysis without any modifications of the samples prepared according to the described procedure.

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