Journal of Chromatography, 87 (1973) 254–257

CHROM. 6994

Note

Calibration data for the gas chromatographic determination of carboxylic acids as their benzyl esters

D. L. CORINA

Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 5NH (Great Britain)

(Received August 1st, 1973)

Benzyl esters, prepared by esterification with diazotoluene (phenyldiazomethane), have been used in this laboratory for the purification by preparative gas chromatography (GC) of radioactive acetic acid from bacteria[1]. Control experiments on bacterial extracts revealed further esterified components which had retentions different from the benzyl esters of short-chain (C_1–C_{10}) fatty acid standards. These unidentified peaks suggested the possibility that other acids, such as tricarboxylic acid cycle components, were being extracted and chromatographed; GC of their methyl esters has been reported[2]. Previous experience[1] had shown that the lower volatility over methyl esters, good solubility in organic solvents and ease of preparation using diazotoluene of the benzyl esters conferred upon them the advantage of lower losses during extractions from biological material. This therefore prompted a continued investigation into the GC behaviour of the benzyl esters of other standard acids, including many of biological interest. Retention data thus obtained are reported in this paper.

EXPERIMENTAL

Preparation of samples

All acids were standard grade and were used without further purification. Salts were converted to the free acids by dissolving the salt in methanol–water (1:1) and shaking with (H^+) ion-exchange resin followed by filtration and evaporation *in vacuo* to a thin syrup. The free acids as solids, liquids or syrups, were dissolved in a suitable solvent (methanol, diethyl ether) or solvent mixture prior to benzylation. The initial extraction of carboxylic acids from biological material can be performed by established methods[1–6].

Diazotoluene (phenyldiazomethane, $C_6H_5CHN_2$) was prepared from N-benzyl-N-nitroso-toluene-4-sulphonamide[3] by the method described previously[1]. A solution of the free acid (50–100 mg/ml) was added in 10-μl aliquots to a solution of diazotoluene (200 μl, 1:5 v/v in petroleum spirit of b.p. 30–40°) until the red colour was discharged. Diethyl ether (1.0 ml) was added and the solution washed with dilute $NaHCO_3$ solution, water, dried for 1 h over anhydrous Na_2SO_4, concentrated to approximately 300 μl under a stream of nitrogen and finally dried by the addition of

four to five pellets of molecular sieve (2-mm pearl, 4A). The samples were stored at 3° prior to chromatography.

Gas chromatography

The esters were analysed using a Pye 104 chromatograph (flame ionisation detector) on a 1.52 m×4.5 mm (I.D.) glass column packed with 3% E-30 on AW-DMCS Chromosorb G, 80–100 mesh. Carrier nitrogen flow-rate was 60 ml/min. The samples were divided into groups according to molecular-weight ranges and chromatographed at temperatures between 125° and 275°. Column and inlet port temperatures are detailed in Table I.

After attaining suitable chromatographic conditions and the selection of suitable internal reference standards, 1–2 μl of sample and 1–2 μl of reference ester were mixed and chromatographed. Retention times were measured, and after correction the relative retentions of the samples were calculated with respect to the standards, and these are listed in Table I. Controls comprised the chromatography of solutions of the free acids to ensure that the peaks recorded were due to the ester product.

RESULTS AND DISCUSSION

Table I illustrates the four groupings used: (1) 130°, reference benzyl thioglycollate; (2) 175°, reference benzyl salicylate; (3) 190° and 215°, reference benzyl succinate, and (4) 275°, reference benzyl abietate. Some components were chromatographed in more than one group to show approximate relationships in retention between groups.

Most esters were eluted in an order approximating to log retention$\propto$molecular weight[8]. Deviations from the general pattern were observed in certain cases. The presence of nitrogen as ring hetero-atom or as amine (indoleacetic acid and 6-aminocaproic acid esters) produced variation; *p*-nitrobenzoic acid ester had a considerably longer retention than the *o*-nitrobenzoic acid ester, similar to that found[9] for *o*- and *p*-aminobenzoic acid TFA derivatives. Halogen-containing acids also had retention times shorter than expected (*cf.* halogen compounds on a hexadecane phase[10]). Fumarate (*trans*) had a retention slightly less than maleate (*cis*) whereas the position was reversed in the *cis–trans* isomers citraconate and mesaconate. Both pairs are dicarboxylic acids, the latter two acids possessing a methyl group on an allylic carbon atom rather than the hydrogen on the former acids. Unsaturation in these acids decreased the retention with respect to the saturated species.

The homologous series of saturated dicarboxylic acids, oxalic to adipic, is listed separately (group 3B), and the benzyl esters were found to conform to the linear relationship of log retention *vs.* carbon number as do the methyl esters[11]. Insufficient members in other homologous series precluded the observation of other similar relationships, while the benzyl esters of short-chain fatty acids have been shown previously[1] to follow this relationship. In a few cases, little or no resolution was achieved (pairs denoted by an asterisk (*) in Table I).

Several acyl ester derivatives used in GC have been well documented[12–14], but there are apparently few instances[14–16] where aryl esters have been used.

The acids in this study were considered to represent a wide variety of chemical types. However, in common with procedures using diazomethane[7,17] chromatograms

TABLE I

RETENTION DATA FOR BENZYL ESTERS OF CARBOXYLIC ACIDS

Column, 1.52 m × 4.5 mm I.D. glass, 3% E-30 on AW-DMCS Chromosorb G, 80–100 mesh. Temperatures as indicated.

Parent acid	*Relative retention***	*Mol. wt. of benzyl ester*
Group 1 —130° column, 150° injector		
Glyoxylic	0.34	148
Glycollic	0.60	166
Lactic	0.63	180
Isovaleric	0.72	192
Crotonic	0.93	176
Thioglycollic	1.00[a]	182
4-Hydroxybutyric	1.15	194
Group 2 —175° column, 210° injector		
Trichloroacetic	0.29	243
Iodoacetic	0.33	276
Benzoic	0.64	196
Nicotinic*	0.78	197
Phenylacetic*	0.78	210
o-Toluic	0.89	210
Salicylic	1.00[b]	228
Mandelic	1.15	242
Phenylpropionic	1.24	240
6-Aminocaproic*	1.33	221
Quinaldic*	1.33	263
o-Nitrobenzoic	1.70	257
Acetylsalicylic	1.93	270
p-Nitrobenzoic	2.60	257
Group 3A —190° column, 220° injector		
Thioglycollic	0.72	182
Fumaric	0.82	296
Maleic	0.88	296
Succinic*	1.00[c]	298
Citraconic*	1.00	310
Citramalic	1.12	328
Malic	1.33	314
3,3-Dimethylglutaric	1.47	340
Mesaconic	1.54	310
Indoleacetic	1.59	265
Tartaric	1.81	330
Phthalic	3.22	346
Group 3B —215° column, 250° injector		
Oxalic	0.50	270
Malonic	0.66	284
Succinic	1.00[d]	298
Glutaric	1.36	312
Adipic	1.85	326
Group 4 —275° column, 300° injector		
Phthalic	0.59	346
Stearic	0.66	374
Abietic*	1.00[e]	391
Arachidic*	1.00	402
Behenic	1.65	430
cis-Aconitic	2.08	444
Citric	2.24	462
Isocitric	2.41	462

* Pairs between which poor or no resolution was achieved.

** Retention times (min) of reference esters: [a] 7.2, [b] 7.45, [c] 12.3, [d] 7.7, [e] 7.1.

obtained from diazotoluene-treated keto-acids were generally unsatisfactory, and are not reported; no significant interference or by-products were observed in the case of the unsaturated acids[18].

The lower volatility of the benzyl esters in comparison with methyl esters also necessitated the use of higher chromatographic temperatures, but, possibly in part due to the presence of the benzyl group(s), good separations were achieved on silicone phases. These can be used up to 350°, although such a temperature was not necessary in the separations described here. Because of the higher temperatures required, chromatography was not investigated on phases generally used for esters. It has been reported[11] that certain dicarboxylic acid esters decompose on polyester phases.

This method of analysis could be considered to be complementary to the use of methyl esters. Preliminary experiments have indicated that with the different selectivity and wider range of temperature programming possible on silicone phases the interference by long-chain fatty acids in, for example, tricarboxylic acid cycle component analyses[2] is less with benzyl esters than with the corresponding methyl esters. Hence the method suggests the potential to perform a complete analysis of non-ketonic carboxylic acids extracted from biological material.

REFERENCES

1 D. L. Corina and P. M. Dunstan, *Anal. Biochem.*, 53 (1973) 571.
2 E. M. Da Silva, *Anal. Chem.*, 43 (1971) 1031.
3 H. G. Henkel, *J. Chromatogr.*, 58 (1971) 201.
4 D. B. Drucker, *J. Chromatogr. Sci.*, 8 (1970) 489.
5 M. Rogosa and L. L. Love, *Appl. Microbiol.*, 61 (1968) 285.
6 L. D. Quin and M. E. Hobbs, *Anal. Chem.*, 30 (1958) 1400.
7 C. G. Overberger and J. B. Anselme, *J. Org. Chem.*, 23 (1963) 592.
8 A. B. Littlewood, *J. Gas Chromatogr.*, 1, No. 11 (1963) 16.
9 K. Blau, in L. R. Mattick and H. A. Szymanski (Editors), *Lectures in Gas Chromatography 1966*, Plenum Publ., New York, 1967, p. 193.
10 A. B. Littlewood, *Anal. Chem.*, 36 (1964) 1441.
11 R. G. Ackman, M. A. Bannerman and F. A. Vandenheuvel, *Anal. Chem.*, 32 (1960) 1209.
12 J. Bezard and M. Bugant, *J. Chromatogr. Sci.*, 7 (1969) 639.
13 A. J. Appleby and J. E. O. Mayne, *J. Gas Chromatogr.*, 5 (1967) 266.
14 B. M. Craig, A. P. Tulloch and N. L. Murty, *J. Amer. Oil Chem. Soc.*, 40 (1963) 61.
15 J. R. Watson and P. Cresuolo, *J. Chromatogr.*, 52 (1970) 63.
16 H.-P. Klemm, U. Hintze and G. Gercken, *J. Chromatogr.*, 75 (1973) 19.
17 H. O. House, E. J. Grubbs and W. F. Gannon, *J. Amer. Chem. Soc.*, 82 (1960) 4099.
18 W. Stoffel, F. Chu and E. H. Ahrens, *Anal. Chem.*, 31 (1959) 307.