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SEPARATION OF PICOLINYL ESTER DERIVATIVES OF FATTY ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR IDENTIFICATION BY MASS SPECTROMETRY

WILLIAM W. CHRISTIE* and KAMEN STEFANOV*
The Hannah Research Institute, Ayr KA6 5HL (U.K.)
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SUMMARY

The picolinyl ester derivatives of the fatty acids of cod liver oil and of pig testis lipids were separated by high-performance liquid chromatography (HPLC) in the reversed-phase mode on a column containing an octyl-bonded phase. To obtain satisfactory resolution, it was necessary to use a mobile phase containing pyridine, which precluded the use of UV spectrophotometric detection. It was, however, possible to use the mass detector with a stream-splitter. Each of the fractions obtained was then analysed by gas–liquid chromatography–mass spectrometry, with separation on a column of fused silica coated with a methylsilicone phase. From the mass spectra, it was possible to identify 39 and 32 different fatty acid components from cod liver oil and pig testis lipids respectively. A high proportion of these were not resolved when the samples were analysed without pre-fractionated by HPLC.

INTRODUCTION

The simplest approach to the location of double bonds in a fatty acid molecule consists in preparing a derivative such as a pyrrolidide[1] or picolinyl ester[2–4], since these give distinctive fragmentations, which are characteristic of the double bond positions. They are easily prepared and are not too polar for separation by gas–liquid chromatography (GLC). Such methods, together with others that have been devised, have been reviewed[5,6]. Of these derivatives, it has recently been confirmed that the picolinyl esters are the more useful since they permitted unequivocal identifications even with polyunsaturated components[7]. It was also demonstrated that derivatives of this type, prepared from natural mixtures, gave satisfactory resolutions when subjected to GLC on capillary columns of fused silica coated with a non-polar methylsilicone phase, for identification by mass spectrometry (MS). With the picolinyl esters prepared from the fatty acids of cod liver oil and pig testis lipids, for example, 22 and 19 different components respectively were separated and identified by means of

* Permanent addres: Institute of Organic Chemistry, Sofia 1113, Bulgaria.

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their mass spectra alone. It was evident, however, that the resolution was far from complete, and many more compounds are known to be present in both cod liver oil[8] and pig testis lipids.[9] Similar conclusions were obtained by others in a study of the fatty acids in the phospholipids of certain mouse membranes[10]. Accordingly, a method for the separation of picolinyl ester derivatives by high-performance liquid chromatography (HPLC) in the reversed-phase mode has now been developed, so that simpler fractions or concentrates of particular components could be obtained for subsequent identification by GLC–MS.

EXPERIMENTAL

All reagents and solvents were Analar grade and were supplied by Fisons (Loughborough, U.K.). The cod liver oil and pig testis lipids were those described previously[7]. Pyridine was freshly distilled from potassium hydroxide pellets.

The lipids were hydrolyzed and the mixed anhydrides of trifluoroacetic acid and the free fatty acids were prepared as described previously[7]. These were reacted with 3-(hydroxymethyl)-pyridine (a 10-fold excess) and 4-dimethylaminopyridine (1.2 molar proportion) in dichloromethane for 3 h at room temperature. The products were taken up in hexane–diethyl ether (1:1) by volume, and they were washed with 2 *M* hydrochloric acid then twice with water. Finally after removal of the solvent, the pure picolinyl ester derivatives were obtained by elution from a short column of Florisil with diethyl ether. They were subjected to GC–MS as described earlier[7]. In brief, a fused-silica capillary column (25 m × 0.2 mm I.D.), coated with a cross-linked methylsilicone (Hewlett-Packard, Wokingham, U.K.), with helium as carrier gas, was temperature-programmed from 60°C to 220°C at 50°C/min then to 260°C at 1°C/min. The column outlet was connected directly into the ion source of a Hewlett-Packard 5970 mass selective detector, operated at an ionization energy of 70 eV.

For the optimum HPLC separations, a Spectra-Physics Model 8700 solvent delivery system (Spectra-Physics, St. Albans, U.K.) was connected to an ACS 750/14 mass detector (Applied Chromatography Systems, Macclesfield, U.K.). A stream-splitter was fitted between the end of the column and the detector, when it was necessary to collect fractions for analysis by means of GC–MS. A column (250 × 5 mm I.D.) of Spherisorb™ C_8 (5 μm particles) (HiChrom, Reading, U.K.) was eluted with a solvent gradient with reservoir A containing methanol and reservoir B containing water–pyridine–acetic acid (98.5:1.5:0.025, v/v). The mobile pase was A:B = 80:20 for 1 min, then a linear gradient was generated over a further 39 min to A:B = 92:8, and this composition was held for 10 more min. The flow-rate was 0.75 ml/min. When not in use, the column was filled with acetonitrile.

RESULTS

A modified procedure for the preparation of picolinyl esters has now been developed, as the method employed previously[7] gave variable yields with small samples. When dicyclohexylcarbodiimide and 4-dimethylaminopyridine were employed as catalysts, the reliability of the esterification procedure was improved considerably. The former compound was judged to be potentially too hazardous for routine use, so the latter is preferred.

Reversed-phase HPLC has become an extremely useful technique for the isolation of individual fatty acid constituents from natural mixtures. Generally, the preferred approach has been to prepare derivatives with a high extinction coefficient in a region of the UV spectrum that permits specific and sensitive detection. As picolinyl esters have an aromatic moiety, *i.e.* a pyridine ring, it was hoped initially that the separation of such derivatives could be monitored by UV detection. However, it did not prove possible to obtain acceptable resolution of natural mixtures with any combination of solvents, in the mobile phase, resembling those used in comparable work with other fatty acid derivatives with UV chromophores. In particular, components emerged as rather broad bands when acetonitrile on its own or mixed with solvents such as water, isopropanol or tetrahydrofuran, was used. Acetonitrile also appeared to be a poor solvent for picolinyl esters.

Slightly better results were obtained with mobile phases based on methanol, but the separations obtained were again much inferior to those published elsewhere, with other types of fatty acid derivatives. On the other hand, the resolution improved immediately when a small amount of pyridine in water was added to the mobile phase. Presumably, this in some way suppressed unwanted interactions between the picolinyl moiety and the stationary and/or mobile phases. Unfortunately, in these circumstances it was no longer possible to use spectrophotometric detection, so the mass detector, the response of which is not markedly affected by the nature of the solvent, was employed instead. The optimum concentration of pyridine in water was found to be 1.5%. As this was found to have a pH of 8.4, and the recommended maximum pH to which the stationary phase should be subjected was 9.0, a small amount of acetic acid (0.25 ml/l) was added to reduce the pH to a safer 6.6. This had no discernable effect on the nature of the separation. It was necessary to redistill the

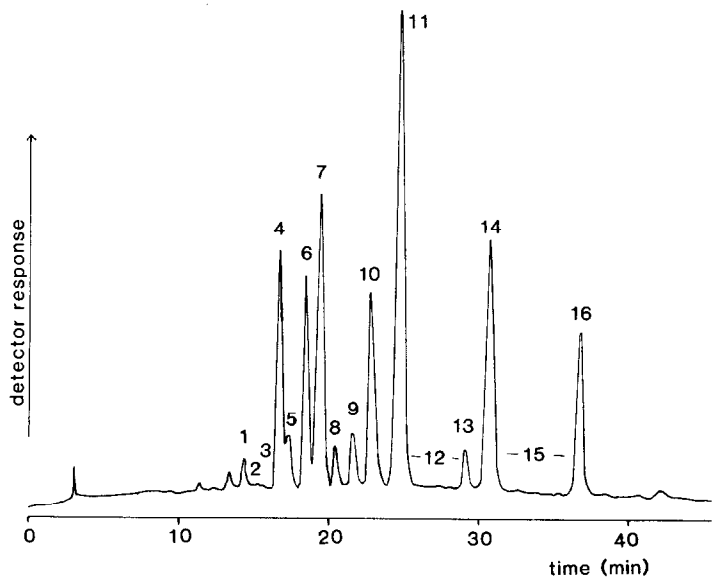

Fig. 1. Separation of picolinyl ester derivatives of the fatty acids of cod liver oil by HPLC in the reversed-phase mode. See the Experimental section for details of the method.

pyridine carefully before use, otherwise high levels of impurities were obtained in the fractions from the HPLC columns. These contaminants could obscure components of interest, especially in the C_{14}–C_{16} region, when the fractions were subsequently subjected to GLC–MS.

In the preliminary work, columns packed with octadecylsilyl phases (Spherisorb™ ODS and ODS-2) were used, but better results were obtained with an octyl-bonded stationary phase. The optimum mobile phase was a gradient of methanol–aqueous pyridine in which the content of the aqueous phase was decreased in a linear manner.

The separation obtained with the picolinyl ester derivatives of the fatty acids of cod liver oil is shown in Fig. 1. As with reversed-phase separations with other fatty acid derivatives, the retention volumes for each component increased with chain length and decreased with the number of double bonds. As the mass detector is destructive to the sample, a stream-splitter was used and 16 different fractions were collected; the solvent was evaporated, and the picolinyl esters were dissolved in hexane for identification by GLC–MS. In each fraction, the nature of the separation was such that components differing in their degree of unsaturation also differed in chain length, so they were well resolved by capillary GLC. Positional isomers of unsaturated fatty acids of a given chain length were in any case well resolved by this technique. Minor components could be concentrated to give acceptable mass spectra without over-loading the capillary column.

As an example, the total ion current trace from fraction 7 of the cod liver oil derivatives is illustrated in Fig. 2. The main component was the picolinyl ester of the 22:6 ($n-3$) fatty acid, with some residual 16:1 from the previous fraction. In addition, 20:4 ($n-6$) and 20:4 ($n-3$) were clearly resolved and gave mass spectra with diagnostic ions, which permitted the structures to be deduced. When the unfractionated cod liver oil picolinyl esters were subjected to GLC–MS previously[7], these components were obscured by overlapping peaks of C_{20} trienes and pentaenes. (The noisy

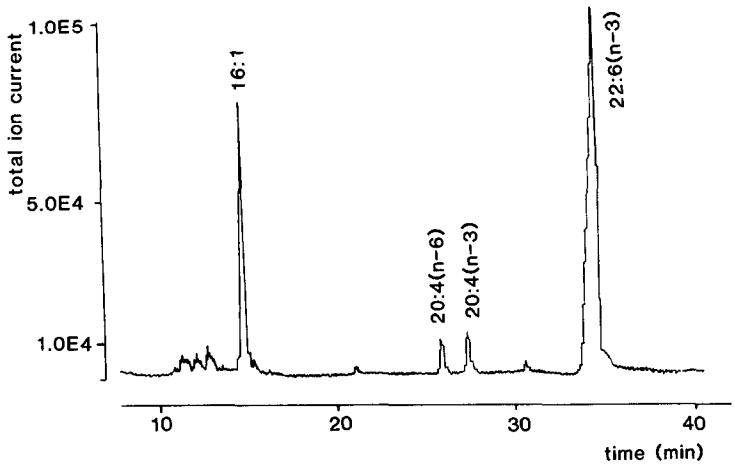

Fig. 2. Total ion current chromatogram obtained from fraction 7 in Fig. 1, when analysed by GLC–MS. See the Experimental section for details of the method.

TABLE I

FATTY ACID COMPONENTS (PICOLINYL ESTER DERIVATIVES) IN HPLC FRACTIONS FROM COD LIVER OIL (FIG. 1) AND IDENTIFIED BY GLC–MS

Fraction	*Major components*	*Minor components*
1	14:1 (*n*−5)	14:1 (*n*−7)*,**
2		18:4 (*n*−3), 13:0
3		16:2 (*n*−4)*
4, 5	14:0, 20:5 (*n*−3)	18:3 (*n*−3)*, 18:3 (*n*−6)*, 20:5 (*n*−6)*,**
6	16:1 (*n*−7)	16:1 (*n*−5), 16:1 (*n*−9)*
7	22:6 (*n*−3)	20:4 (*n*−6)*, 20:4 (*n*−3)*
8	18:2 (*n*−6)	18:2 (*n*−4)*,**
9	22:5 (*n*−3)	17:1 (*n*−9), 7-methyl-16:1 (*n*−7)*,**,***
10	16:0	20:3 (*n*−6)*, 20:3 (*n*−3)*, 22:5 (*n*−6)*
11	18:1 (*n*−9), 18:1 (*n*−7)	18:1 (*n*−5)
12		15-methyl-16:0, 5-methyl-16:0*,**
		19:1, 20:2 (*n*−6)*
13	18:0	
14	20:1 (*n*−9), 20:1 (*n*−11)	20:1 (*n*−7)
15		phytanic acid*,**
16	22:1 (*n*−9), 22:1 (*n*−11)	

* Not found by GLC–MS alone[7].
** Not reported by Ackman *et al.*[8].
*** Identified incorrectly earlier[7].

base-line in the 11–13 min region of the trace was due to residual impurities in the pyridine of the mobile phase).

The fatty acid components identified in each of the fractions are listed in Table I. In each instance, the mass spectra gave a substantial molecular ion, and characteristic ions were generally present that permitted the location of the double bonds

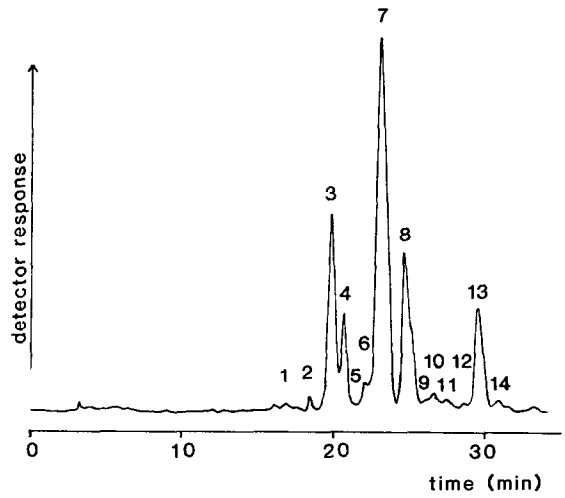

Fig. 3. Separation of picolinyl ester derivatives of the fatty acids of pig testis lipids by HPLC in the reversed-phase mode. See the Experimental section for details of the method.

TABLE II

FATTY ACID COMPONENTS (PICOLINYL ESTER DERIVATIVES) IN HPLC FRACTIONS FROM PIG TESTIS LIPIDS (FIG. 3), AND IDENTIFIED BY GLC–MS.

Fraction	*Major components*	*Minor components*
1		14:0, 18:3 ($n-6$)*, 20:5
2	16:1 ($n-9$), 16:1 ($n-7$)**	16:1 ($n-5$)*,**
3	22:6 ($n-3$), 20:4 ($n-6$)	15:0*,**
4	18:2 ($n-6$)	
5		17:1 ($n-8$)*,**
6	22:5 ($n-6$), 20:3 ($n-6$)	
7	16:0	20:3 ($n-9$), 20:3 ($n-3$)*
8	18:1 ($n-9$), 18:1 ($n-7$)	22:4 ($n-6$)
9		17:0**
10		20:2 ($n-9$), 20:2 ($n-6$)
11		19:1 ($n-8$)*,**, 24:5 ($n-6$)*,**
12		22:3 ($n-6$)*, 22:3 ($n-9$)
13	18:0	
14	20:1 ($n-9$)	19:0*,**, 20:1 ($n-7$)*,**, 24:4 ($n-6$)*,**

* Not found by GLC–MS alone[7].
** Not reported by Holman and Hoffstetter[9].

or methyl branches, when the rules developed by Harvey[2–4] and confirmed by others[7] were applied. These comprised C_{14}–C_{22} fatty acids with zero to six double bonds, and one or four (phytanic acid) methyl branches. In total, 39 different components were identified unequivocally, compared to the 22 recognized when the intact sample was analysed[7]. Also, one component had been incorrectly identified earlier[7]; thus the fatty acid designated 17:1 ($n-8$) was here found to be almost certainly 7-methylhexadec-7-enoic acid, a known marine oil constituent, from a more intense spectrum. The main straight-chain heptadecenoic acid isomer was in fact 17:1 ($n-9$) as reported elsewhere[8]. Five of the components were not listed in the extensive compilation of Ackman *et al.*,[8] although they may have been recognized by later workers.

The reversed-phase HPLC separation obtained with the picolinyl esters prepared from the pig testis lipids is illustrated in Fig. 3. Each of the 14 fractions was again subjected to GLC–MS and those components, which were identified, are listed in Table II. The fatty acids had 14–24 carbon atoms and zero to six double bonds. In this instance, 31 different components were identified unequivocally from their mass spectra, compared to 19 when the intact sample was studied[7]. Nine of these fatty acids were not recognized in earlier work, in which traditional analytical techniques were employed[9].

DISCUSSION

The value of picolinyl esters as simple derivatives for the structural identification of fatty acids by means of GLC–MS should not be doubted. Positional isomers of unsaturated (including polyunsaturated) and methyl-branched components are readily identified from their mass spectra by applying simple rules. Unfortunately the polarity and relatively-high molecular weight of such compounds has meant that it

has been necessary to use non-polar methylsilicone phases in the capillary GLC columns. This has inevitably resulted in some loss of resolution, in comparison to that obtained with polar phases and methyl ester derivatives especially. Thus, although positional isomers of unsaturated fatty acids were well resolved from each other, they often overlapped with similar components with one more (or fewer) double bond. The most important example was probably the derivatives of the essential 18:2 ($n-6$) and 18:3 ($n-3$) fatty acids, which co-chromatographed. When GLC–MS alone was used to analyse the components in natural mixtures in the form of the picolinyl esters, a large number of different fatty acids were indeed identified[7,10]. On the other hand, it has now been shown that when the technique is used in sequence with reversed-phase HPLC, a considerable gain in the amount of information is obtained.

It was regrettable that HPLC conditions, which might make use of the aromatic chromophore in the picolinyl moiety for detection purposes, could not be devised. Hopefully, means of overcoming this deficiency will be found later. Cod liver oil and pig testis lipids were utilized in this and the previous study, because they contained appreciable amounts of fatty acids of the ($n-3$) and ($n-6$) families, and because they had been well-characterized by other techniques. The methods described here should be of equal value with samples in which the fatty acid composition is not known.

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