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Note

Identification of very long polyenoic acids as picolinyl esters by Ag^+ ion-exchange high-performance liquid chromatography, reversed-phase high-performance liquid chromatography and gas chromatography–mass spectrometry

TOMÁŠ ŘEZANKA
Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague 4 (Czechoslovakia)
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Very long-chain fatty acids, *i.e.*, acids with 22 carbon atoms and longer, have recently been investigated[1,2], but they were mostly saturated fatty acids. In very long-chain polyunsaturated fatty acids (VLCPUFA) the situation is much more complicated and few systematic studies of their isolation and identification have been reported. So far, their occurrence has been demonstrated primarily in animal tissues (*e.g.*, the retina and in various brain diseases such as Zellweger syndrome)[3,4] and in marine species such as the herring (*Clupea harengus*)[5] and sponges[6].

The main problem in the identification of these compounds in natural materials is their low concentrations, which usually do not exceed, with a few exceptions (*e.g.*. certain sponges), tenths of a percent. Their identification is also complicated by their sensitivity to heat and aerial oxygen. In previous methods natural fatty acid mixtures were enriched with VLCPUFA and analyses were based mainly on chromatographic methods[4]. The detection of eluted components is complicated as fatty acids hardly absorb in the UV region. Therefore, it is necessary to use either terminal absorption of the carboxylic bond or of double bond(s) or to derivatize the carboxylic group. Picolinyl esters are the only derivatives that can later be used also for gas chromatographic–mass spectrometric (GC–MS) identification. Unlike the use of pyrrolidides, which show only terminal absorption in the UV spectrum, Ag^+ion-exchange high-performance liquid chromatography (HPLC) and reversed-phase (RP) HPLC, the former method separating compounds according to the number of double bonds and the latter according to the equivalent chain number, appear to be the most promising. Ion-exchange HPLC has only been applied to methyl esters[7], whereas by means of RP-HPLC picolinyl esters could also be separated[8]. In this work the advantages of both methods were combined, followed by identification of the eluted components by GC–MS with the aim of preparing standards that are not available commercially.

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EXPERIMENTAL

Materials

Free fatty acids were obtained by basic hydrolysis of total lipids obtained by extraction of fish meat with chloroform–methanol[9]. Picolinyl esters were prepared from free fatty acids by reaction with thionyl chloride followed by 3-pyridyl carbinol as described by Harvey[10]. The reaction mixture was diluted with water, extracted with hexane and evaporated to dryness. The residue was dissolved in diethyl ether and purified on Supelclean solid-phase extraction tubes containing LC-NH_2 (Supelco, Gland, Switzerland). The eluate was evaporated to dryness, dissolved in acetonitrile–hexane and stored at −25°C. Aerial oxygen was removed from the solvents used for HPLC by bubbling with helium; 70 mg/l of butylated hydroxytoluene (Sigma, St. Louis, MO, U.S.A.) were added to other solvents. All the vessels and the rotary evaporator were flushed with nitrogen before use.

HPLC

Preparative HPLC was performed in a Gradient LC System G-I (Shimadzu, Kyoto, Japan) with two LC-6A pumps (4 ml/min), an SCL-6A system controller, an SPD ultraviolet detector (266 nm), a SIL-1A sample injector and a C-R3A data processor. Semi-preparative columns, 30 cm × 7.9 mm I.D. packed with a strongly acidic cation exchanger (SO_3H groups, SCR-101 H, 10-μm spherical particles; Shimadzu) impregnated according Christie *et al.*[7], and 25 cm × 21.1 mm I.D. packed with ODS(5-μm particles; Shimadzu) were employed.

Silver ion exchange

After injection of 10 mg of picolinyl esters, elution was carried out with a gradient from hexane–dioxane–isopropanol (40:10:50) to dioxane–isopropanol (50:50) in 20 min and then to pure isopropanol in a further 25 min.

In the reversed-phase mode, 5 mg were injected and elution was carried out with a gradient from water–acetic acid–triethylamine–methanol–isopropanol (291:3:6:690:10) to the same mixture in the proportions 9.7:0.1:0.2:700:290 in 28 min and then isocratically for 15 min.

Individual classes of picolinyl esters and individual picolinyl esters after HPLC were collected and evaporated.

GC–MS

The fraction containing both 28:7 isomers was separated and identified using a Finnigan MAT (San Jose, CA, U.S.A.) 1020 B instrument. The injector temperature was 100°C and an HP-1 cross-linked methylsilicone (Hewlett-Packard, Palo Alto, CA, U.S.A.) fused-silica capillary column (25 × 0.2 mm I.D. film thicknes 0.11 μm) was used. The temperature programme was 100°C (1 min), then increased at 20°C/min to 230°C and at 4°C/min to 339°C. The carrier gas was hydrogen at a flow-rate of 75 cm/s. The ionization energy was 70 eV and mass spectra were scanned in the range m/z 150–550.

Infrared spectroscopy

For scanning infrared spectroscopy of the fraction 28:7$n-6$ and 28:7$n-3$ (in

carbon tetrachloride), a Perkin-Elmer Model 1310 infrared spectrophotometer was used.

RESULTS AND DISCUSSION

As described previously[9], herring is a suitable source of VLCPUFA, even if only of $n-3$ isomers. In that work, two 28:7 positional isomers, *i.e.*, $n-3$ and $n-6$, were identified in preliminary tests. In the present work, attention was therefore focused on a conclusive demonstration of the previously proposed structure. Combination of HPLC–GC of picolinyl esters having satisfactory chromatographic properties, either in HPLC or GC, *i.e.*, with an aromatic ring showing intensive UV absorption and not too high an elution temperature, can accelerate both qualitative and quantitative analyses. Separation of picolinyl esters by means of Ag^+ ion-exchange HPLC has not previously been described. It was necessary to solve two problems, *viz.*, detection of the eluted compounds and the question of whether the contribution of the double bond is sufficient for separation as the picolinyl ester already contains an aromatic system. Picolinyl esters fulfil very well the first condition, absorbing at 266 nm ($\varepsilon = 10^4$). When solving the latter problem we utilized literature data[11] on the chromatography on benzoyl derivatives of non-hydroxy fatty acid ceramides. Although the molecule of ceramides after derivatization contained a benzene ring, it was not difficult to separate compounds with 0–3 double bonds.

On the chromatogram obtained by means of Ag^+ ion-exchange HPLC (Fig. 1a), seven distinctly separated peaks can be seen. Unfortunately, the peak 6+7 contains compounds with six and seven double bonds. RP-HPLC as described by Christie and Stefanov[8] was used for an additional semi-preparative separation. However, it was found that the separation of picolinyl esters without a buffer, *i.e.*, in pyridine–acetic acid, is poor and therefore a mass detector had to be used instead of a UV detector. The problem can be solved by replacing pyridine with triethylamine as the elution mixture

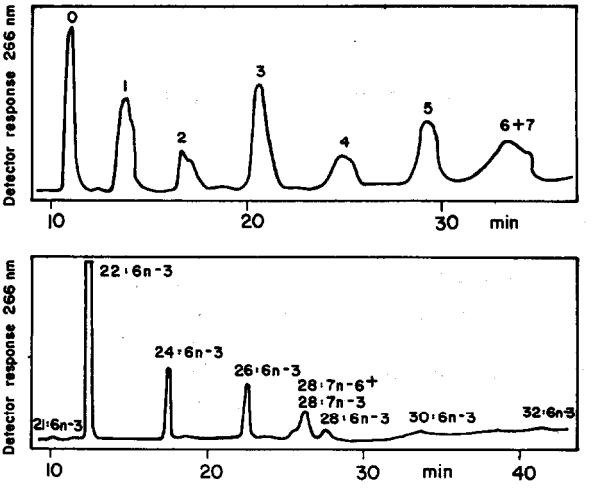

Fig. 1. Preparative chromatography of picolinyl esters from herring. For conditions, see text. Top: preparative Ag^+ ion-exchange HPLC. Bottom: preparative RP-HPLC.

no longer absorbs above 230 nm. By applying RP-HPLC, individual esters could be separated to the baseline, except for 28:7 positional isomers.

Common methods for the determination of configurations of double bond(s) include infrared spectroscopy. Some peaks at 967 cm^{-1}, *i.e.*, the characteristic absorbance for *trans* isomers, were not identified. From the above argument is to be expected that double bonds are *cis*.

Separation of $n-3$ and $n-6$ methyl esters and of free PUFA is in general difficult. Aveldano *et al.*[4] separated 22:5$n-3$ from 22:5$n-6$, whereas Narce *et al.*[12] could not separate 18:3$n-3$ and 18:3$n-6$. With picolinyl esters, none of the positional isomers, *e.g.*, 18:3$n-3$ and $n-6$, 20:3$n-3$ and $n-6$ and 20:4$n-3$ and $n-6$) could be separated[8]. It follows from Fig. 1b that both 28:7 isomers are partially separated, but the separation is not sufficient for further identification. GC of VLCPUFA on capillary columns has been reported only a few times[13,14] and only using non-polar columns. In previous work[9] both 28:7 isomers were separated as methyl esters on a polar capillary column for the first time. However, picolinyl esters of 28:7 isomers were not eluted from the same column. Christie *et al.*[15] also found that it is necessary to increase the elution temperature of picolinyl esters by 50°C above that for methyl esters. Therefore, in the case of peaks 6+7, it was necessary to use a non-polar phase. The elution order $n-9$, $n-6$, $n-3$ (22:3$n-9$, 22:3$n-6$ or 35:6$n-6$, 36:6$n-3$) was found for methyl esters[14] and the same order was also observed for picolinyl esters (22:5$n-6$, 22:5$n-3$)[15].

GC–MS of the mixed peak 28:7 obtained after RP-HPLC confirmed the assumptions concerning the elution order, i.e., 28:7$n-6$ was followed by 28:7$n-3$, which was demonstrated by the mass spectra (see Fig. 2). Interpretation of the mass spectra of picolinyl esters has been described[10,15,16]. In polyenoic acids containing methylene-interrupted double bonds, gaps of 26 u for any double bond were found. However, Christie[16] showed that it is easier to detect gaps of 40 u between the terminal

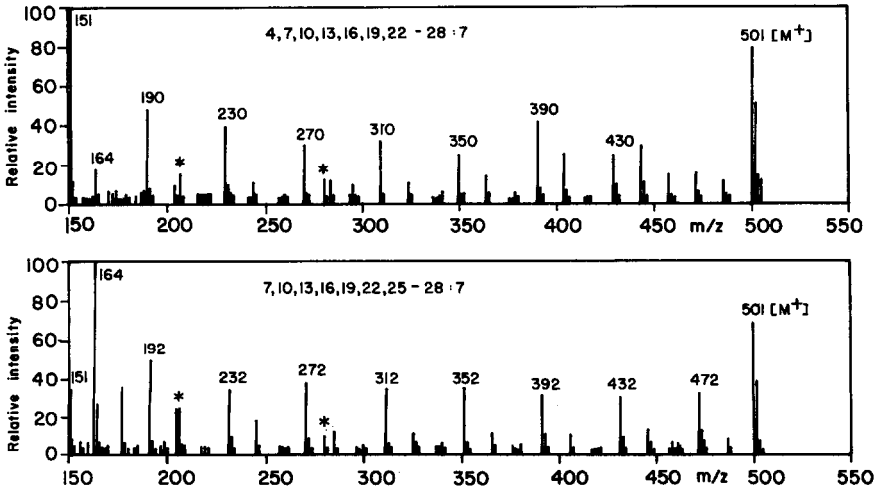

Fig. 2. Mass spectra of picolinyl esters of octacosaheptaenoic acids. Top: mass spectrum of 28:7$n-6$ picolinyl esters. Bottom: mass spectrum of 28:7$n-3$ picolinyl esters. Asterisks indicate peaks of polysiloxanes (stationary phase).

end of any of the double bonds and the methylene group on the carboxyl end. This fact was confirmed also with the two 28:7 isomers. It follows from Fig. 2 (top) that the molecular ion (*m/z* 501) was present and that degradation products (M−15, M−15−14, etc.) could be found. The first double bond from the CH_3 end is in position *n*−6, as also reflected by an ion of *m/z* 430. Gaps of 26 u are not so pronounced as 40 u and, hence, are much more suitable for identification. A certain problem arose with the identification of the Δ^4 double bond, where a gap of only 26 u could be demonstrated. With the second isomer (Fig. 2, bottom) the situation is almost identical, values of diagnostically significant ions differing by only 2 u, being shifted towards higher values. The ratio of ions of *m/z* 151 and 164 is another auxiliary criterion for the position of the double bond. When this bond is not in the Δ^4 position the abundance of the ion of *m/z* 164 is higher than that of the ion of *m/z* 151 (see Fig. 2 and the literature[10,16]). In the presence of the Δ^4 double bond the opposite occurs (Fig. 2). As the abundance of ions above *m/z* 150 is low with these polyenes, as also described by Harvey[10], we scanned the mass spectra beginning with *m/z* 150. Ions having lower *m/z* values are diagnostically not very significant. The chromatographic behaviour of both 28:7 isomers is in agreement with the data obtained so far, *i.e.*, the order is *n*−6–*n*−3. The mass spectra of both isomers of picolinyl esters are clearly different and ions with gaps of 40 u are much more intense and hence more significant for identification than ions with gaps of 26 u. Both isomers therefore yield sufficiently characteristic spectra that can be used for their identification and determination of their structure.

It can be concluded that the above combination of three separation methods, *i.e.*, ion-exchange HPLC, RP-HPLC and GC–MS of picolinyl esters, yields conclusive data even in the identification of unusual VLCPUFA. The use of characteristic gaps of 40 u during the degradation of polyene picolinyl esters can also contribute to their easier identification. Naturally, the combination of the above methods is not limited only to VLCPUFA from fish but can also be used with any biological material containing these rare acids.

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