Determination of Double Bond Positions in Polyunsaturated Fatty Acids by Mass Spectrometry

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A new method for the determination of double bond positions in polyunsaturated fatty acids is described. Double bonds in polyunsaturated fatty acids were reduced with deuteriodiimide and the resulting saturated fatty acids were converted to pyrrolidine derivatives. The location of deuterium atoms of the pyrrolidines were analyzed by gas chromatography-mass spectrometry. The positions of all the original double bonds could be deduced from mass fragment peaks containing deuterium atoms.

Keywords—polyunsaturated fatty acid; double bond position; gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry offers many advantages for the analysis of double bond positions in unsaturated fatty acids. For such analysis, the unsaturated fatty acid must be converted to a derivative which undergoes characteristic cleavage, giving rise to fragments which indicate the position of the double bonds in the original fatty acid. McCloskey and McClelland suggested the use of O-isopropylidene derivatives of the diols obtained by oxidation of unsaturated fatty acid esters. This method is useful for the characterization of both positional and geometrical isomers of unsaturated fatty acids. However, the application of the method to fatty acids containing more than one double bond has not been reported. Trimethyloxysilyl derivatives have a distinct advantage for the determination of unsaturated fatty acid structure. The problems of this method reside in the increase of the molecular weight due to derivatization. The fragment ions in the high mass range of the derivatives of fatty acids with three or more double bonds were not sufficiently intense to permit deduction of the position of the original double bonds. Several investigators reported that this disadvantage could be alleviated by the use of polymethoxy derivatives of polyunsaturated fatty acids. Andersson et al. showed that pyrrolidine derivatives were suitable compounds for mass spectrometry of unsaturated fatty acids. They observed a simple cleavage pattern for the pyrrolidines of monounsaturated fatty acids and proposed a rule to determine the location of the double bond. However, the spectra of the pyrrolidine derivatives of polyunsaturated fatty acids were not interpretable according to the rule developed for monomonoic acids.

In this study, polyunsaturated fatty acids were reduced with deuteriodiimide and the locations of deuterium atoms of the resulting deuterated saturated acids were determined from the mass spectra of their pyrrolidine derivatives. This procedure has been in successful use in our laboratory for past few years for the determination of the position of double bonds in the absence of authentic fatty acids.

Materials and Methods

Materials—Unsaturated fatty acids were purchased from Gasukuro Kogyo Co., Ltd., Tokyo; acetic acid-d₄
and methanol-O-d were from Merck Sharp and Dohme Canada Ltd. Dipotassium azodicarboxylate was prepared according to the method described by Berson et al. All other reagents and solvents were commercial products of analytical grade.

Reduction of Unsaturated Fatty Acid Esters with Deuteriodiimide

Dipotassium azodicarboxylate (1.0 mmol) was added with stirring to a solution of unsaturated fatty acid methyl ester (0.01 mmol) in methanol-O-d (0.25 ml). The slurry was stirred very rapidly with a mechanical stirrer while a solution of acetic acid-d_4 (0.103 ml, 1.8 mmol) in methanol-O-d (0.2 ml) was added dropwise over a 2-h period at room temperature. The solution was stirred for an additional 30 min at room temperature. The reaction mixture was diluted with water and the product was extracted with n-hexane. The hexane extract was dried over magnesium sulfate and evaporated to dryness under reduced pressure. To increase the yield of saturated fatty acid methyl ester, the above procedure was repeated twice. When the above preparation contained unsaturated fatty acid methyl ester, it was subjected to Lemieux–von Rudloff oxidation to remove the unsaturated ester. Lemieux–von Rudloff oxidation was carried out as described previously.

Preparation of Pyrrolidines of Fatty Acids

Fatty acid methyl ester was dissolved in freshly distilled pyrrolidine (1.0 ml) and acetic acid (0.1 ml). The mixture was heated in a sealed tube at 100 °C for 30 min. The pyrrolidine was taken up in methylene chloride and washed with 5% hydrochloric acid and with water. The methylene chloride layer was dried over magnesium sulfate and evaporated to dryness under reduced pressure.

Gas Chromatography-Mass Spectrometry

Fatty acid pyrrolidine was applied to a 2 m x 3 mm glass column containing 5% Silar 10C on Chromosorb W (AW-DMCS, 80–100 mesh). The column temperature was 260 °C and helium was used as a carrier gas at a flow rate of 30 ml/min. Mass spectra were taken every 6.0 s with a combined gas chromatography-mass spectrometry instrument, GCMS-9000S (Shimadzu-LKB, Kyoto), with an ionizing current of 60 μA, an electron-accelerating voltage of 70 eV and an ion source temperature of 270 °C.

Results and Discussion

Reduction of Unsaturated Fatty Acid Methyl Esters

When methyl stearate was obtained by catalytic deuteriation of methyl oleate over Adams’ platinum catalyst, Dinh-Nguyen and Ryhage observed the incorporation of more than two deuterium atoms into the acyl chain. We also observed similar extensive incorporation of deuterium atoms during catalytic deuteriation of methyl oleate in the presence of palladium carbon. This extensive replacement is probably related to isomerization of the double bond which is known to occur often during catalytic hydrogenation of olefins. To avoid this extensive incorporation of deuterium atoms, we utilized deuteriodiimide to reduce the double bonds in unsaturated fatty acid methyl esters. Reductions of olefinic bonds with diimide are known to exhibit a very high degree of stereospecificity, cis addition of hydrogen predominating to the extent of 97–98%. Methyl esters of unsaturated fatty acids were reduced to the corresponding saturated esters labeled with deuterium by treatment with deuteriodiimide. The yield of saturated esters was dependent on the numbers of double bonds, ranging from 20 to 80%. The yield of stearate from oleate was 80%, and that of eicosanoate from eicosapentaenoate was 20%. The unsaturated esters were removed by Lemieux–von Rudloff oxidation and the resulting methyl esters of saturated fatty acids were converted to pyrrolidine derivatives. Then the location of deuterium atoms was analyzed by gas chromatography-mass spectrometry.

Mass Spectra of Pyrrolidines of Deuterated Fatty Acids

The mass spectrum of the pyrrolidine of a saturated fatty acid shows simple cleavage patterns, with a base peak at m/z 113 formed by McLafferty rearrangement. Fragment peaks with 14 atomic mass units difference in the high mass region are derived from cleavage at each C–C bond. Andersson and Holman compared the spectra of the pyrrolidine derivatives of monounsaturated fatty acids and formulated the following rule: if an interval of 12 atomic mass units, instead of the regular 14 atomic mass units, is observed between the most intense peaks of clusters of fragments containing n and n−1 carbon atoms of the acyl moiety, a double bond occurs between carbon n and n+1 in the molecule. However, the spectra of polyunsaturated fatty acid pyrrolidines were not all interpretable according to the
above rule. A double bond closer to the carbonyl was indicated by the interval of 12 atomic mass units according to the rule, but a remote double bond did not conform to the rule. Consequently, reference compounds were necessary to identify each positional isomer of polyunsaturated fatty acids.

When one compares the spectra of pyrrolidine derivatives of $[^2]$H$_6$]-octadecanoates, which have been reduced from cis-6, cis-9, cis-12-octadecatrienoate ($\alpha$-linolenate) and cis-9, cis-12, cis-15-octadecatrienoate ($\gamma$-linolenate), there are clear differences, as shown in Fig. 1. In the case of $[^2]$H$_6$]-N-octadecanoylpyrrolidine derived from 6,9,12-octadecatrienoate, there was a series of ions $m/z$ 113, 126, 140, 154, 169, 184, 198, 213, 228, 242, 257, 272, 286, 300, 314, 328, 343. In the case of $[^2]$H$_6$]-N-octadecanoylpyrrolidine derived from 9,12,15-octadecatrienoate, there was a series of ions $m/z$ 113, 126, 140, 154, 168, 182, 196, 211, 226, 240, 255, 270, 284, 299, 314, 328, 343. An interval of 15 atomic mass units, instead of the regular 14, between the most intense peaks of clusters of fragments shows the presence of a deuterium atom. The positions of double bonds in the original fatty acid are easily deduced from mass fragment peaks containing deuterium atoms. Figure 2 shows mass spectra of $[^2]$H$_6$- and $[^2]$H$_{10}$]-eicosanoylpyrrolidines derived from cis-5, cis-8, cis-11, cis-14-eicosatetraenoate (ara-
chidonate) and cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoate, respectively. From the deuterium contents of reagents used in these experiments, the deuterium content of diimide used was considered to be between 90 and 95%. Therefore, the most intense peaks in the molecular ion regions were observed at m/z 372 and 374 instead of 373 and 375, respectively. These spectra also indicate that this method is useful for the determination of double bond locations in polyunsaturated fatty acids.

Ryhage and his coworkers\textsuperscript{15,16} analyzed the mass spectra of methyl esters of various fatty acids labeled with deuterium. They found that the mass spectra were complicated owing to partial hydrogen–deuterium exchange reactions and to rearrangements within the mass spectrometer. On the other hand, pyrrolidines offer several advantages for the analysis of deuterium locations in the fatty acid molecule.\textsuperscript{8} Metastable peaks indicate a direct cleavage from the molecular ion to each principal fragment in a cluster.\textsuperscript{8} All peaks include the pyrrolidide groups and no metastable peaks were detectable for stepwise degradations.\textsuperscript{8} The fragmentation patterns shown in this paper suggested that the pyrrolidines do not measurably undergo hydrogen–deuterium exchange reactions under electron impact. Therefore, the procedure described in this paper can provide information on the locations of double bonds in polyunsaturated fatty acids without the use of authentic fatty acids.
References