An Application of Liquid Chromatography/Mass Spectrometry to Determination of Fatty Acids with a Wide Range of Carbon Chain Lengths (C_{12}-C_{54}) in *Mycobacteria*

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Accepted for publication on February 16, 1996

ABSTRACT. We recently developed some new methods for the analysis of fatty acids by liquid chromatography/atmospheric-pressure chemical-ionization/mass spectrometry (LC-APCI-MS) using anilide, N-n-propylamide and 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one derivatives. N-n-propylamide derivatization was applied to the analysis of very long chain fatty acids prepared from *Mycobacterium smegmatis* and *Mycobacterium lepraemurium* using LC-APCI-MS. Fatty acids with a wide range of carbon chain lengths (C_{12}-C_{54}) containing saturated, unsaturated and cyclopropane acids were detected and assigned.

Key words: LC-MS — fatty acids (C_{12}-C_{54}) — cyclopropane fatty acids — *mycobacteria*

We recently developed some new methods for the analysis of fatty acids by liquid chromatography/mass spectrometry (LC-MS).[1-3] Anilide and N-n-propylamide derivatization of fatty acids was useful for the analysis of hydroxy and non-hydroxy fatty acids and analysis of rat brain fatty acids by a single run of LC-MS was achieved. For the quantitative analysis of labile fatty acids, such as polyunsaturated hydroxy and hydroperoxy fatty acids, 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one derivatization was useful. By means of the latter method, some hydroperoxy fatty acids split from photo-oxidized lecithin could be identified.

In this study, we applied our N-n-propylamide derivatization method to the analysis of very long chain fatty acids prepared from *Mycobacterium smegmatis* and *Mycobacterium lepraemurium*. Fatty acids with a wide range of carbon chain lengths (C_{12}-C_{54}) containing saturated, unsaturated and cyclopropane acids were detected and assigned.

EXPERIMENTAL

Reagents

Nonadecenoic acid ($C_{19:1}$) was purchased from Nu-Check prep, Co. through Funakoshi Pharmaceutical Co. (Tokyo, Japan). Palladium (Pd) on charcoal was obtained from Wako Chemicals (Osaka, Japan). High-performance liquid chromatography (HPLC)-grade dichloromethane and acetonitrile were purchased from Nakarai Tesque Chemicals (Kyoto, Japan).

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Thin-layer chromatography (TLC) was carried out with precoated plates of silica gel LK5 (thickness 0.25 mm) (Whatman Co., Clifton, N.J., USA).

Culture conditions

Lactobacillus fermenti was purchased from the Institute of Fermentation (Osaka, Japan) and grown for two days at 37°C in a lactobacillic inoculum broth medium (Nissui Co., Tokyo, Japan). *M. smegmatis* was a gift from Dr. H. Shoji of the Research Institute for Microbial Diseases, Osaka University and was grown for two days at 37°C in a Sauton medium containing 0.06% tween 80. *M. lepraemurium* was a gift from Dr. H. Nomaguchi of the Research Institute for Microbial Diseases, Osaka University and was grown on 1% Ogawa's egg-yolk medium for 60 days at 37°C.[4,5]

Extraction of fatty acids

Saponification of lipids in bacterial cells was carried out by refluxing for 4h in 5% (w/v) KOH in 50% (v/v) ethanol.[5] Fatty acids were extracted with a mixture of n-hexane and diethyl ether (1 : 1, v/v) after acidification with HCl. Then they were applied to TLC plates and developed with n-hexane-diethyl ether-acetic acid (80 : 20 : 2, v/v). Non-polar fatty acids (non-mycolic acids) containing spots on the plates were collected and extracted with chloroform.[5,6]

Derivatization of fatty acids

N-n-propylamidation of the fatty acids was achieved as described previously.[2,7,8] Briefly, to a solution of fatty acids and n-propylamine-HCl in dry dimethylformamide (DMF), diphenyl phosphorazidate or diethylphosphoryl cyanide in DMF was added at 0°C, followed by the addition of triethylamine in DMF. Then the mixture was stirred at room temperature. Thereafter the reaction mixture was applied to LC-MS. The yield of derivatization is quantitative.[2]

Hydrogenation and bromination of fatty acid N-n-propylamide derivatives

Unsaturated fatty acid N-n-propylamides were hydrogenated with 200 mg of 5% Pd on charcoal in 20 ml of methanol-chloroform (1 : 2, v/v) at room temperature for 15 min. A 1-ml amount of bromine in ether (1 : 5, v/v) was added to the N-n-propylamides of the fatty acids in 2 ml of ether and was left to stand for several minutes at room temperature.[9,10]

Equipment

A Hitachi M-2000-type double-focusing mass spectrometer-computer system (Tokyo, Japan), equipped with a Hitachi L-6200-type HPLC instrument through a Hitachi APCI interface system, was used. HPLC was performed using a reversed-phase Cosmosil $5C_{18}$-packed column with 5-μm particles (250 mm$\times$4.6 mm I.D., Nakarai Tesque Chemicals). Acetonitrile was used in the starting mobile phase, and dichloromethane was added in a linear gradient if necessary. The drift voltage of the APCI system was 100 V and the temperatures of the vaporizer and desolvator were 250 and 385°C, respectively. The multiplier voltage was 1500 V. Gas chromatography/mass spectrometry (GC-MS) was performed with the electron impact (EI) system of a Hitachi M-80 mass spectrometer at 70 eV as described previously.[11]

RESULTS AND DISCUSSION

Analysis of fatty acids from *Lactobacillus fermenti*

First we attempted to confirm that the hydrogenation procedure under the conditions mentioned in Experimental was selective in that unsaturated acids were converted saturated fatty acids, although cyclopropane acids were not affected. We checked fatty acid composition by the GC-MS. Peaks for n-propylamide derivatives of $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, cyclo$C_{19:0}$, $C_{20:1}$ were observed (data not shown).[12] The n-propylamide derivatives of fatty acids prepared from *L. fermenti* were applied to LC-MS before (Fig 1A) and after (Fig 1B) hydrogenation. Peaks 1, 2, 3, 5 and 6 were identified as $C_{16:1}$, $C_{18:1}$, $C_{16:0}$, $C_{20:1}$ and $C_{18:0}$, respectively, from the retention times on mass chromatograms of authentic samples and the series of each type of fatty acids such as saturated, mono - and di-unsaturated acids, according to a previously described method.[1-3]

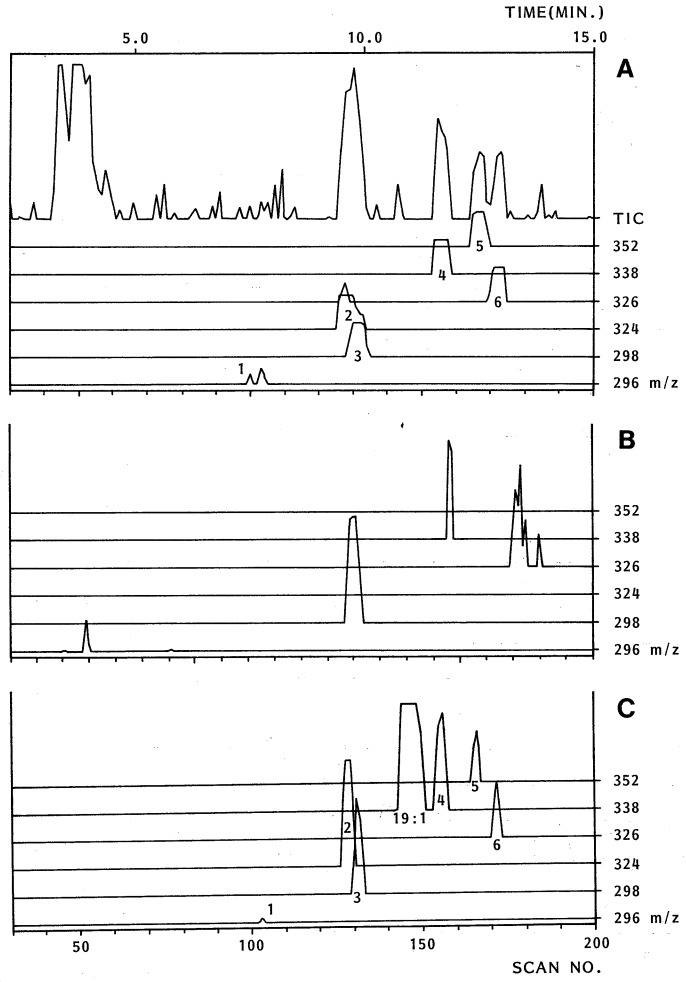


Fig 1. Mass chromatograms of N-n-propylamide derivatives of fatty acids from *L. fermenti* Fatty acids were analysed by LC-MS (A) before, (B) after hydrogenation and (C) simultaneously with authentic $C_{19:1}$.

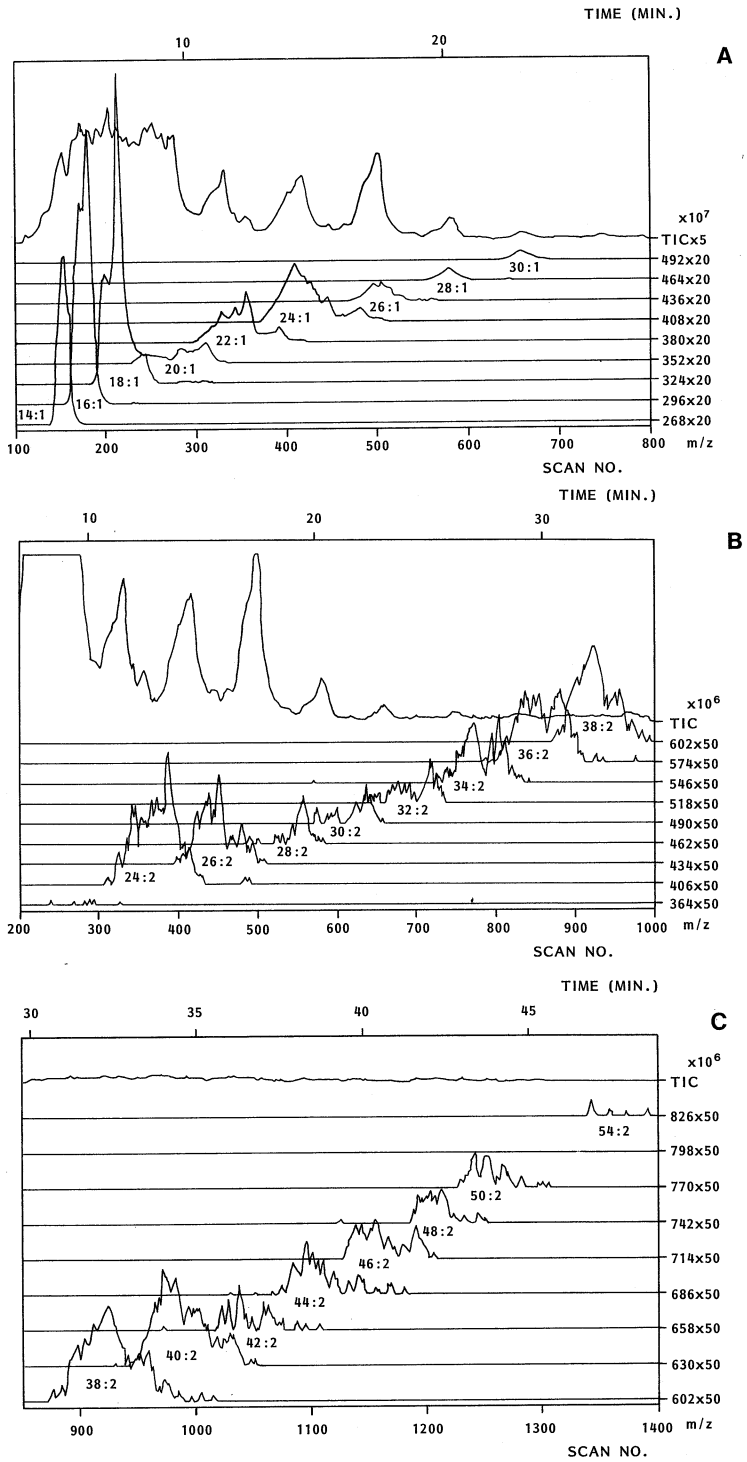


Fig 2. Mass chromatograms of mono- and di-unsaturated fatty acids in N-n-propylamide derivatives of fatty acids from *M. smegmatis*
(A) Mono-unsaturated acids and (B) (C) Di-unsaturated acids

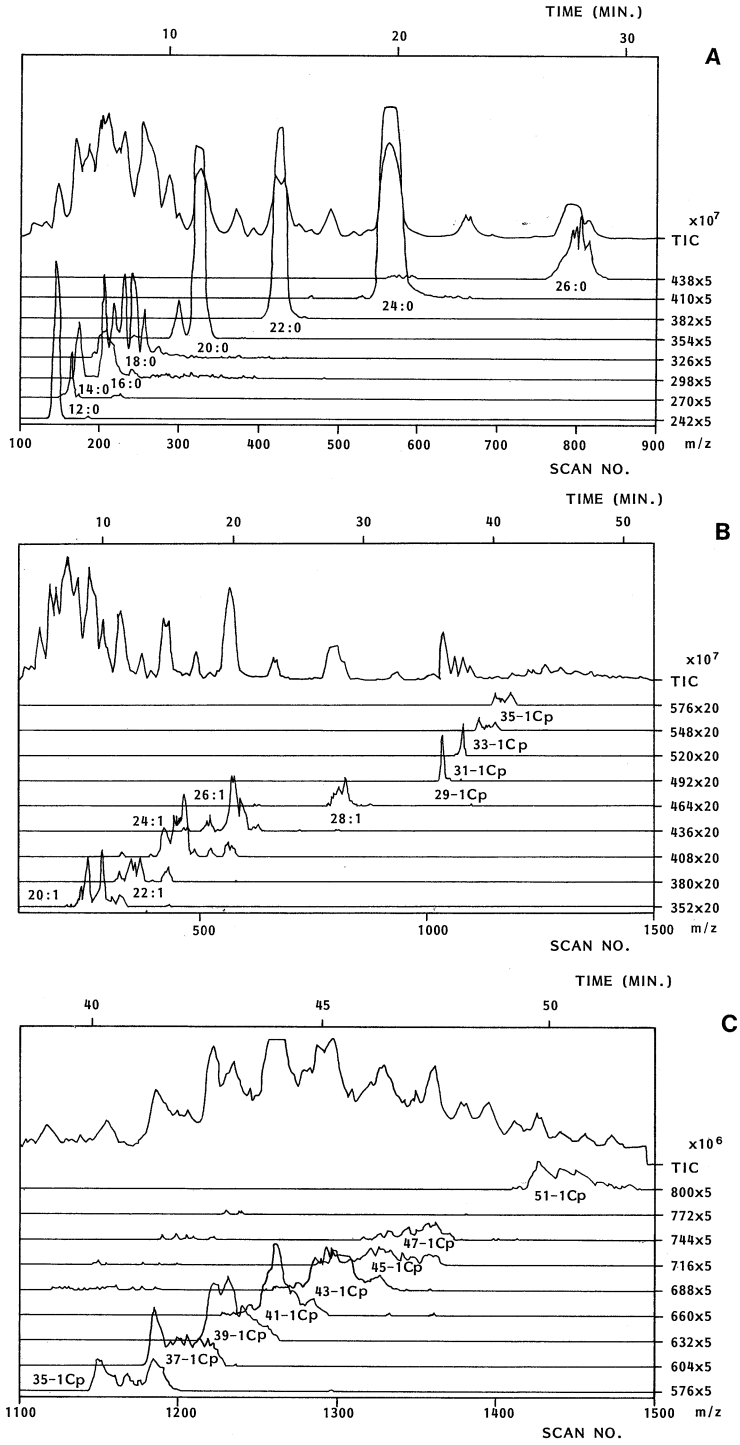
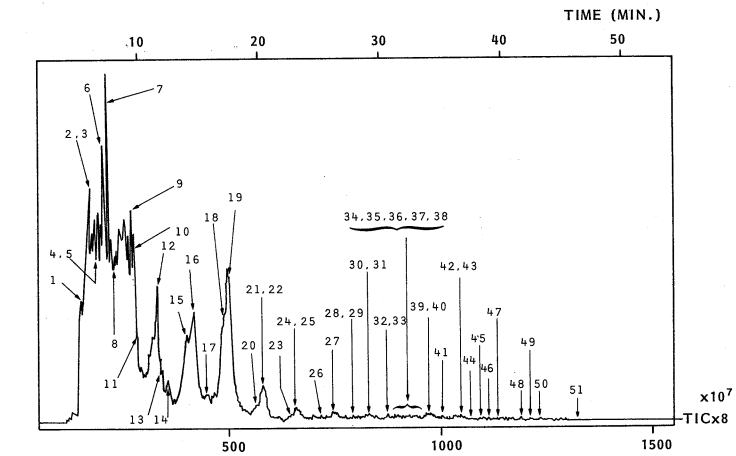


Fig 3. Mass chromatograms of saturated and cyclopropane fatty acids in N-n-propylamide derivatives of fatty acids from *M. lepraemurium*
(A) Saturated acids (B) Mono-unsaturated and mono-cyclopropane (1Cp) acids (C) Mono-cyclopropane acids



1 12:0, 2 14:1, 3 14:0, 4 15:0, 5 16:1, 6 16:0, 7 18:1, 8 17:0, 9 18:0, 10 19:0
11 20:1, 12 20:0, 13 22:1, 14 24:2, 15 24:1, 16 22:0, 17 26:2, 18 26:1, 19 24:0, 20 28:2
21 28:1, 22 26:0, 23 30:2, 24 30:1, 25 28:0, 26 32:2, 27 32:1, 28 36:3, 29 34:2, 30 34:1
31 35:2, 32 38:3, 33 36:2, 34 37:2, 35 36:1, 36 38:2, 37 40:3, 38 39:2, 39 38:1, 40 40:2
41 41:2, 42 40:1, 43 42:2, 44 43:2, 45 44:2, 46 45:2, 47 46:2, 48 48:2, 49 49:2, 50 50:2
51 54:2

Fig 4. Total ion chromatogram of N-n-propylamides of fatty acids from *M. smegmatis*

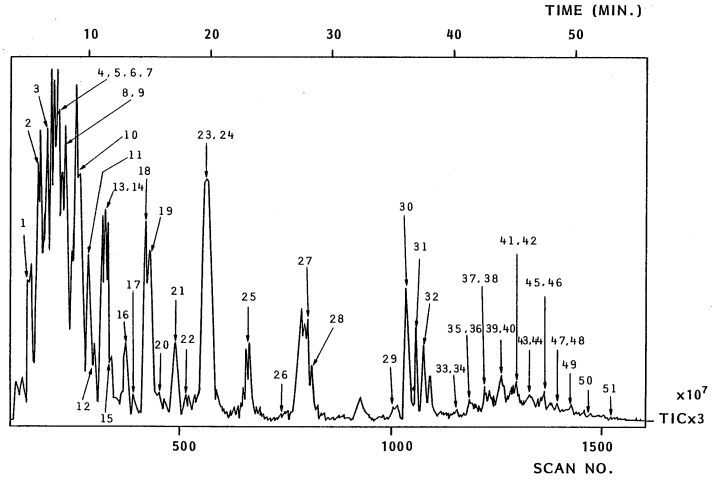
After hydrogenation, peaks 1, 2 and 5 disappeared, but, peaks 3, 4 and 6 did not. After bromination, peaks 1, 2, 4 and 5 disappeared and peaks considered to be brominated acids was observed (data not shown). In addition, peak 4 was clearly discernible from that of authentic $C_{19:1}$, although these molecular ions $[M+H]^+$ are the same. This is because their peaks were observed separately when n-propylamide derivatives of fatty acids from *L. fermenti* and authentic $C_{19:1}$ were applied simultaneously to LC-MS (Fig 1C).

Analysis of fatty acids from *Mycobacterium smegmatis* and *Mycobacterium lepraemurium*

N-n-Propylamide derivatives of fatty acids from *M. smegmatis* and *M. lepraemurium* were determined by LC-MS analysis before and after hydrogenation according to the method descibed above. Before hydrogenation, we observed each series of peaks of saturated and mono-, di- and tri-unsaturated fatty acids on mass chromatograms and the peaks of fatty acids on total ion chromatograms. After hydrogenation, all peaks with the molecular ion $[M+H]^+$ of unsaturated acids from *M. smegmatis* disappeared (data not shown). However, two series of peaks remained even after hydrogenation in fatty acids from *M. lepraemurium*. The other peaks with the molecular ion $[M+H]^+$ of unsaturated acids disappeared after hydrogenation. These peaks were assigned as unsaturated acids. The series of peaks remaining after the hydrogenation were the same as those of the corresponding mono- and di-unsaturated acids in the mass numbers, but were different in their retention times. These peaks disappeared after bromination (data not shown). Therefore, they were suggested to be cyclopropane acids. The mass chromatograms of a series of mono- and di-unsaturated acids from *M.*

smegmatis (Fig 2) and a series of saturated, mono-unsaturated and mono-cyclopropane acids from *M. lepraemurium* (Fig 3) are shown. Other series of peaks were similar to these and to previously reported ones[1,2] (data not shown). Notable differences between the acids from *M. smegmatis* and *M. lepraemurium* were observed in the mass chromatograms of mono-unsaturated and mono-cyclopropane acids (Figs 2A, 3B). Thus, the peaks on total ion chromatograms were assigned as cyclopropane, saturated, mono-, di-, and tri-unsaturated acids as shown in Figs 4 and 5. We could detect at least 51 kinds of fatty acids with a wide range of carbon chain lengths (C_{12}-C_{54}) with each single run of LC-MS before and after hydrogenation in fatty acids from *M. smegmatis* and *M. lepraemurium*.

The above results suggest that our methods should be useful for the analysis of the precursor fatty acids of mycolic acids and study of the biosynthetic pathways of mycolic acids in *mycobacteria*.



1 12:0, 2 14:0, 3 16:1, 4 17:1, 5 15:0, 6 16:0, 7 18:1, 8 19:1, 9 17:0, 10 18:0, 11 19:0, 12 20:1, 13 20:0, 14 21:1, 15 22:1, 16 21:0, 17 24:2, 18 24:1, 19 22:0, 20 25:2, 21 23:0, 22 26:2, 23 24:0, 24 26:1, 25 25:0, 26 30:2, 27 26:0, 28 28:1, 29 28-1Cp, 30 29-1Cp, 31 30-2Cp, 32 31-1Cp, 33 32-2Cp, 34 33-1Cp, 35 34-2Cp, 36 35-1Cp, 37 36-2Cp, 38 37-1Cp, 39 38-2Cp, 40 39-1Cp, 41 40-2Cp, 42 41-1Cp, 43 42-2Cp, 44 43-1Cp, 45 44-2Cp, 46 45-1Cp, 47 47-1Cp, 48 46-2Cp, 49 48-2Cp, 50 51-1Cp, 51 52-2Cp

Fig 5. Total ion chromatogram of N-n-propylamide derivatives of fatty acids from *M. lepraemurium* 2Cp : di-cyclopropane acid

ACKNOWLEDGMENTS

This work was supported in part by Research Project Grants 3-411, 4-710 and 5-804 from Kawasaki Medical School.

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