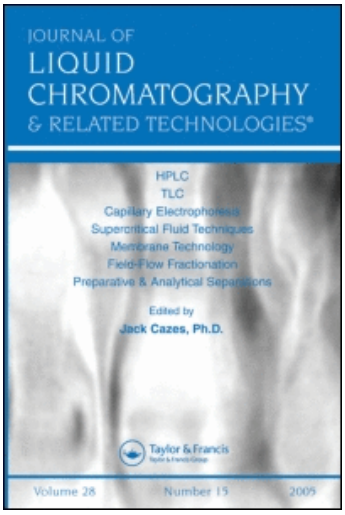

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Fatty Acids as Their p-Bromophenacyl Esters on a C_{30}-Bonded Silica Column by High Performance Liquid Chromatography

K. Takayama[ab]; H. C. Jordi[c]; F. Benson[c]
[a] Tuberculosis Research Laboratory, William S. Middleton Memorial Veterans Hospital, Madison, Wisconsin [b] Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin [c] Waters Associates Inc., Milford, Massachusetts

To cite this Article Takayama, K. , Jordi, H. C. and Benson, F.(1980) 'Separation of Fatty Acids as Their p-Bromophenacyl Esters on a C_{30}-Bonded Silica Column by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 3: 1, 61 — 69
To link to this Article: DOI: 10.1080/01483918008060153
URL: http://dx.doi.org/10.1080/01483918008060153

JOURNAL OF LIQUID CHROMATOGRAPHY, 3(1), 61-69 (1980)

SEPARATION OF FATTY ACIDS AS THEIR p-BROMOPHENACYL ESTERS ON A C_{30}-BONDED SILICA COLUMN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

K. Takayama
Tuberculosis Research Laboratory
William S. Middleton Memorial Veterans Hospital
2500 Overlook Terrace
Madison, Wisconsin 53705

and

Institute for Enzyme Research
1710 University Avenue
University of Wisconsin
Madison, Wisconsin 53706

H. C. Jordi and F. Benson
Waters Associates Inc.
Maple Street
Milford, Massachusetts 01757

ABSTRACT

A new C_{30}-bonded silica column was developed for high performance liquid chromatography. This column was tested for the fractionation of fatty acids as their p-bromophenacyl esters by the reverse-phase mode. Certain pairs of fatty acid esters that are very difficult to separate on a C_{18}-bonded silica column, i.e., arachidonic ($C_{20:4}$)-palmitoleic ($C_{16:1}$); elaidic (trans $C_{18:1}$)-vaccinic (cis $C_{18:1}$); behenic ($C_{22:0}$)-nervonic ($C_{24:1}$); and arachidonic ($C_{20:0}$)-erucic ($C_{22:1}$) esters, were completely resolved on the C_{30}-bonded column using solvent gradients of acetonitrile: water and acetonitrile:p-dioxane. A solvent system of methylene chloride:acetonitrile (2:1, v/v) was developed for this column to achieve good separation of a homologous series of extremely nonpolar C_{76} to C_{82} α-mycolic acid esters from Mycobacterium tuberculosis H37Ra.

INTRODUCTION

Most of the chromatographic separations carried out by high performance liquid chromatography (HPLC) are generally done by the reverse-phase mode. The most commonly used chemically bonded stationary phases are phenyl-, C_8-, and C_{18}- bonded groups. The use of the C_{18}-bonded silica column for the separation of a synthetic mixture of C_2 to C_{24} fatty acids (1) and the C_{24} to C_{82} fatty acids from Mycobacterium tuberculosis H37Ra have been described by Takayama et al. and Qureshi (2-5). Certain problems exist in these separations, i.e., some pairs of fatty acids of a synthetic mixture cannot be separated. The separation of a homologous series of mycolic acids is essentially an analytical, but not preparative method that requires fractionation times of about 2.5 to 3.0 hr (5).

We have now developed a C_{30}-bonded silica column which can resolve some of the problems posed by the use of the C_{18}-bonded silica column in fractionating fatty acids.

MATERIALS AND METHODS

Fatty acids (C_3 to C_{24}) were purchased from Sigma Chemical Co., St. Louis, MO; Applied Science Laboratories Inc., State College, PA; and Calbiochem, San Diego, CA. Spectroquality distilled-in-glass acetonitrile, p-dioxane and methylene chloride were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. The p-bromophenacyl ester derivatization kit was purchased from Applied Science Laboratories Inc.

Cells of M. tuberculosis H37Ra were grown at 37°C in glycerol-alanine-salts medium in a New Brunswick 28-liter fermentor (6). Procedures for isolation and purification of the mycolic acids from M. tuberculosis H37Ra were previously described by Takayama et al. (5). The free acids were converted to the p-bromophenacyl ester by a modified method of Durst et al. (7) as described by Takayama et al. (5).

HPLC was performed with an instrument containing the following components: two Waters model M6000A solvent delivery systems, a Waters model 660 solvent programmer, Waters model U6K universal liquid chromatograph injector and model 440 UV detector. The p-bromophenacyl esters were detected at 254 nm.

Preparation of C_{30}-Bonded μPorasil

Twenty-five g of μPorasil (Waters Associates Inc., Milford, MA), 120 μ particle size, were dried overnight at 150°C and suspended in 500 ml of dry toluene. At this point 25 ml of redistilled and dry triethylamine were added. Twenty g of C_{30}-trichlorosilane (Petrarch Systems Inc., Levittown, PA) were suspended in 500 ml of dry toluene by stirring for about 4 hr. The C_{30}-trichlorosilane suspension was added to the μPorasil suspension and refluxed for 1 hr. The resulting C_{30}-bonded μPorasil product was then washed with toluene and diethyl ether. The above silylation was repeated on the treated μPorasil with 10 g of hexamethyldisilazane (Pierce Chemical Co., Rockford, IL). The final product yielded 37.3 g

C_{30}-bonded material was also prepared from 10 μ spherical silica with a 500-Å pore size (LiChrospher Si 500, EM Laboratories Inc., Elmsford, NY).

Packing HPLC Columns with C_{30}-Bonded Silica

The C_{30}-bonded silica was slurry-packed using a Micromeritics model 705 stainless steel slurry packer (Micromeritics Instrument Corp., Norcross, GA). The bonded silica was slurried in isopropyl alcohol and pushed into 3.9 mm x 30 cm stainless steel columns at 6000 psi with a Waters M6000A solvent delivery system (Waters Associates Inc., Milford, MA), using isopropyl alcohol as the pushing solvent. After packing, each column was flushed with acetonitrile-water (3:2, v/v) and tested.

RESULTS AND DISCUSSION

A synthetic mixture of 40 (C_3 to C_{24}) fatty acid p-bromophenacyl esters (Table 1) was fractionated on two C_{30}-bonded silica columns placed in series (Fig. 1). Total fractionation time was 3.5 hr. Separations followed the same principles as described previously for the C_{18}-bonded column (1). It showed baseline resolution of most of the components. The esters which did not separate were lauric-myristoleic, α-linolenic-γ-linolenic-tridecanoic, myristic-linoleic, and oleic-vaccinic esters. Only partial separation was achieved with docosahexaenoic-α-linolenic, elaidic-palmitic and arachidic-nervonic esters.

The separation of this mixture of fatty acid esters with a C_{18}-bonded silica column was reported by Jordi (1). All pairs of fatty acid esters not separated by the C_{18}-bonded column, including arachidic-palmitoleic, elaidic-vaccinic, behenic-nervonic and arachidonic-erucic esters, were completely resolved by our C_{30}-bonded silica column. Thus, the C_{30}-bonded silica column complements the C_{18}-bonded column in the separation of C_3 to C_{24} fatty acid esters.

Greater separation of the saturated and unsaturated fatty acid esters of the same chain length occurred with the C_{30}-bonded than the C_{18}-bonded column. This was shown by comparing the oleic (C_{18}) and nervonic (C_{24}) esters to their respective saturated esters. The C_{18}-bonded column allowed the monounsaturated C_{24} ester to migrate like a saturated C_{22} ester whereas in the C_{30}-bonded column, it migrated like a saturated C_{20} ester. Similarly, the oleic ester migrated near a saturated C_{16} ester (between C_{16} and C_{18} esters) in the C_{18}-bonded column but in the C_{30}-bonded column, it migrated between the saturated C_{15} and C_{16} esters.

Previously, we suggested that a C_{30}-bonded column could be superior to the C_{18}-bonded column in separating mycolic acid esters (5). The C_{30}-bonded column was tested for the separation of α-mycolic acid esters from *M. tuberculosis* H37Ra using the solvent system of methylene chloride-acetonitrile (2:1, v/v). The result

TABLE 1

Composition of the Fatty Acid Standards Used as the p-Bromophenacyl Esters in the HPLC Fractionation on a C_{30}-Bonded Silica Column.

Standard Number	Fatty acid	Carbon Number
1	Propionic	C_3
2	Butyric	C_4
3	Valeric	C_5
4	Caproic	C_6
5	Heptanoic	C_7
6	Caprylic	C_8
7	Nonanoic	C_9
8	Capric	C_{10}
9	Undecanoic	C_{11}
10	Lauric	C_{12}
11	Myristoleic	cis-Δ^9-$C_{14:1}$
12	5, 8, 11, 14, 17-Eicosapentaenoic	$C_{20:5}$
13	4, 7, 10, 13, 16, 19-Docosahexaenoic	$C_{22:6}$
14	α-Linolenic	$C_{18:3}$
15	γ-Linolenic	$C_{18:3}$
16	Tridecanoic	C_{13}
17	Arachidonic	$C_{20:4}$
18	Palmitoleic	$C_{16:1}$
19	Myristic	C_{14}
20	Linoleic	$C_{18:2}$
21	Palmitelaidic	$C_{16:1}$
22	Linoledaidic	$C_{18:2}$
23	11, 14, 17-Eicosatrienoic	$C_{20:3}$
24	Pentadecanoic	C_{15}
25	Oleic	$C_{18:1}$
26	Vaccinic	$C_{18:1}$
27	Elaidic	$C_{18:1}$
28	Palmitic	C_{16}
29	Heptadecanoic	C_{17}
30	cis-5-Eicosenoic	$C_{20:1}$
31	Stearic	C_{18}
32	Erucic	$C_{22:1}$
33	Nonadecanoic	C_{19}
34	Brassidic	$C_{22:1}$
35	Arachidic	C_{20}
36	Nervonic	$C_{24:1}$
37	Heneicosanoic	C_{21}
38	Behenic	C_{22}
39	Tricosanoic	C_{23}
40	Lignoceric	C_{24}

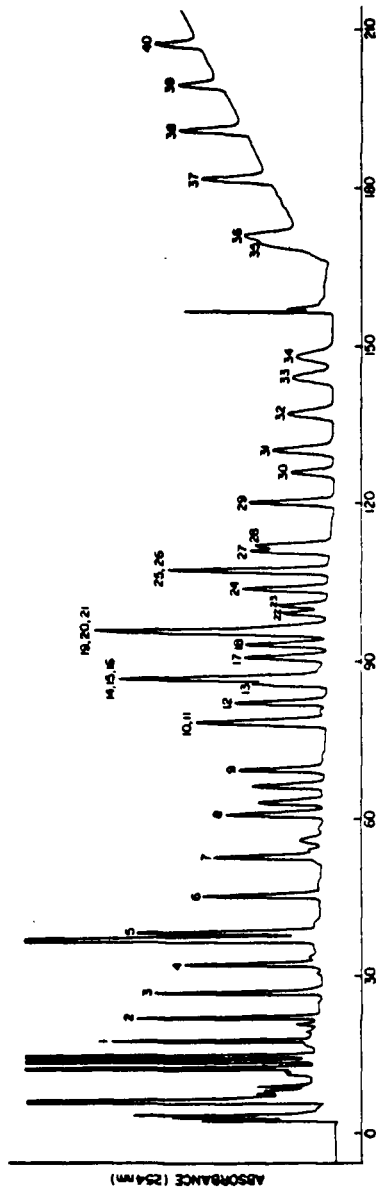

FIGURE 1. HPLC of C_3 to C_{24} fatty acids as their p-bromophenacyl esters. Column: Two 3.9 mm x 30 cm C_{30}-bonded silica. Solvent: convex gradient (gradient curve 5) of acetonitrile-water (2:3, v/v) to 100% acetonitrile for 150 min followed by a linear gradient from acetonitrile to p-dioxane for 150 min. Flow rate, 1 ml/min.

is shown in Fig. 2. Fractionation with the C_{30}-bonded column was almost as good as that with a C_{18}-bonded column reported previously (5). As expected, when a C_{18}-bonded silica column was used with the same solvent system, the entire sample eluted from the column in 16 min with little resolution.

Since a less polar solvent system must be used with a C_{30}-bonded column, the advantage of using this column over a C_{18}-bonded column was that a greater sample load could be applied. Mycolic acid esters are minimally soluble in the p-dioxane-acetonitrile (3:2, v/v) solvent system that is used with the C_{18}-bonded column. This allows the fractionation of mycolic acid esters on the C_{30}-bonded column to become not only analytical, but preparative.

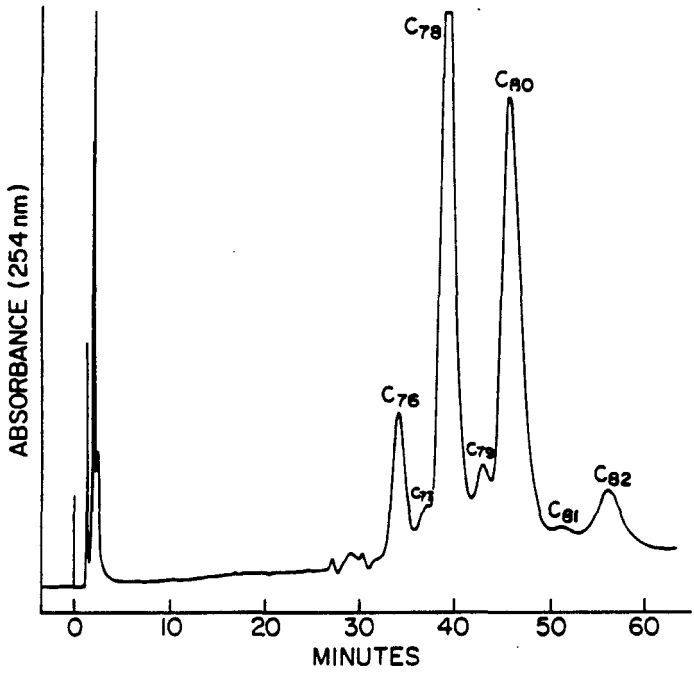

FIGURE 2. HPLC of α-mycolic acids as their p-bromophenacyl esters on a C_{30}-bonded silica column. Column dimension: 3.9 mm x 30 cm. Solvent: methylene chloride-acetonitrile (2:1, v/v). Flow rate, 1 ml/min. The peaks are identified by their carbon number.

We illustrated this feature by comparing semi-preparative 7.9 mm x 30 cm columns of a C_{30}-bonded silica and a C_{18}-bonded silica for their ability to handle large sample sizes. The C_{18}-bonded column could only be loaded with about 800 µg of α-mycolic acid esters before loss of separation occurred. The C_{30}-bonded column could separate at least 5 mg of esters. The even- and odd-numbered series of mycolic acid esters separated differently on both columns. The odd-numbered series appeared as the front shoulder of the even-numbered series on the C_{30}-bonded column and as the back shoulder on the C_{18}-bonded column. It is likely that this difference in elution properties is due to some basic structural differences in the two series.

Hennion et al. (8) proposed that the variation in capacity factors and selectivity of a bonded reverse-phase column was a function of alkyl chain length of the bonded phase. They found that,in going up from C_4, C_6, C_8, C_{12} and C_{18} in the bonded phase, both the capacity factors and selectivity increased as the alkyl chain was lengthened at constant surface coverage. Thus, the longer the alkyl chain, the better the retention and separation became. We observed the same phenomenon with the C_{30}-bonded phase. We can now use a mobile phase consisting of methylene chloride-acetonitrile (2:1, v/v) for C_{76} to C_{82} mycolic acid esters which cannot be appreciably retained on a C_{18}-bonded phase with the same eluent system. We also found that the increase in retention on the C_{30}- versus C_{18}-bonded phases was not as great for polar as nonpolar samples. This is supported by the finding of Hennion et al. (8) that for nonpolar samples, a plot of the log of k' versus chain length of the bonded phase was linear while for the polar samples, a plot of k' versus chain length was required to obtain a straight line.

ACKNOWLEDGMENTS

This investigation was supported in part by the Medical Research Service of the Veterans Administration and Public Health

Service research grant AI-11297 from the National Institute of Allergy and Infectious Disease.

We thank Cynthia Birch for her valuable editorial assistance with this manuscript.

REFERENCES

1. Jordi, H.C. Separation of Long and Short Chain Fatty Acids as Naphthacyl and Substituted Phenacyl Esters by High Performance Liquid Chromatography, J. Liquid Chromatogr. 1, 215, 1978.

2. Takayama, K., Qureshi, N., Jordi, H.C., and Schnoes, H.K. Recycling HPLC of p-Bromophenacyl Esters of Saturated C_{35-56} Fatty Acids from Mycobacterium tuberculosis H37Ra on a Silica Column, J. Liquid Chromatogr. 1979 (in press).

3. Takayama, K., Qureshi, N., Jordi, H.C., and Schnoes, H.K. Purification of Mono- and Diunsaturated C_{22-47} Fatty Acids from Mycobacterium tuberculosis H37Ra as their p-Bromophenacyl Esters by High Performance Liquid Chromatography, Biological/Biomedical Applications of Liquid Chromatography, Vol. 11, Hawk, G.L., ed., Marcel Dekker, Inc., New York, 1979 p. 375.

4. Qureshi, N. Purification of Very Long Chain Fatty Acids (C_{30}-C_{56}) from Mycobacterium tuberculosis H37Ra by High Performance Liquid Chromatography (HPLC), Fed. Proc. 37, 1707, 1978.

5. Takayama, K., Qureshi, N., Jordi, H.C., and Schnoes, H.K. Separation of Homologous Series of Mycolic Acids from Mycobacterium tuberculosis H37Ra by High Performance Liquid Chromatography, Biological/Biomedical Applications of Liquid Chromatography, Vol. 10, Hawk, G.L., ed., Marcel Dekker, Inc., New York, 1978, p. 91.

6. Takayama, K., Schnoes, H.K., Armstrong, E.L., and Boyle, R.W. Site of Inhibitory Action of Isoniazid in the Synthesis of Mycolic Acids in Mycobacterium tuberculosis, J. Lipid Res. 16, 308, 1975.

7. Durst, H.D., Milano, M., Kitka, E.J., Connelly, S.A., and Grushka, E. Phenacyl Esters of Fatty Acids via Crown Ether Catalysts for Enhanced Ultraviolet Detection in Liquid Chromatography, Anal. Chem. 47, 1797, 1975.

8. Hennion, M.C., Picard, C., and Caude, M. Influence of the Number and Length of Alkyl Chains on the Chromatographic Properties of Hydrocarbonaceous Bonded Phases, J. Chromatogr. 166, 21, 1978.