CHROMSYMP. 077

CAPILLARY GAS CHROMATOGRAPHY OF FATTY ACID METHYL ESTERS FROM HUMAN MILK LIPID SUBCLASSES

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SUMMARY

The fatty acid (FA) composition of the human milk lipid subclasses sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and cholesterol esters (CE) were analysed by capillary gas chromatography (GC) on wall-coated open-tubular glass columns. Compared with GC on packed columns, capillary GC was found to be ten times more sensitive (0.1 μg of each individual FA methyl ester could be quantified), and the time needed for the analysis could be reduced by a factor of five. The reproducibility of the analysis was good relative standard deviation (4–7%) and comparable to that obtained by packed column GC.

INTRODUCTION

Lipids account for the bulk of energy in human milk. However, their constituents, such as linoleic (C18:2ω6), linolenic (C18:3ω3) and arachidonic (C20:4ω6) acids, also play other important roles in the metabolism of mammals. These fatty acids (FA) have, therefore, been studied by many investigations in the past[1–5]. Using packed column gas chromatography (GC), we recently showed that human milk triglycerides (TG) and the total phospholipid fraction (PL) each have a characteristic FA composition which, in addition, changes with the length of lactation[5]. More detailed investigations, for example, the analysis of minor lipid fractions, such as PL subclasses and cholesterol esters (CE), which must be separated prior to GC analysis by thin-layer chromatography (TLC), need more sensitive techniques because of the limited amounts of samples available. Capillary GC of FA methyl esters has proved to be especially useful for analysing the FA composition of the milk PL subclasses phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SPH) and for the analysis of CE.

EXPERIMENTAL

Materials

All chemicals and organic solvents were of analytical-reagent grade from Merck (Darmstadt, F.R.G.). TLC plates (Nano-Sil 20) were obtained from Macherey,

Nagel & Co. (Düren, F.R.G.). Standards of FA methyl esters were purchased from Nu Check Prep (Elysian, MN, U.S.A.), Supelco (Bellefonte, PA, U.S.A.) and Analabs (Karlsruhe, F.R.G.).

Isolation of lipid subclasses

Milk lipids were extracted by the method of Bligh and Dyer[6] and FA methyl esters of total lipids were prepared from evaporated extracts by the boron trifluoride method[5].

Human milk

Lyophilized human milk, obtained from a pool containing mature milk from 15 mothers, was used after appropriate reconstitution.

In order to isolate CE and PL in addition to PL subclasses, evaporated lipid extracts were redissolved in an appropriate amount of chloroform–methanol (9:1). A 4-ml volume of this solvent was used for extracts from 4 ml of milk. Volumes of 2 ml were then applied (streaked) on to TLC plates. PL subclasses were separated on the first plate with four different solvent systems, as described by Gentner *et al.*[7]. On the second plate, CE were separated from other lipids with hexane–diethyl ether–chloroform–acetic acid (70:20:10:1). The running distance was 6 cm. After scraping off the bands, transesterification was carried out by the boron trifluoride method[5].

The FA methyl esters to be analysed and the reference standards were dissolved in an appropriate amount of *n*-hexane. An aliquot of this solution, usually 2 μl, were used for capillary GC.

Gas chromatography

Capillary GC was performed on a Sigma 1/10 computerized gas chromatograph (Perkin-Elmer, Überlingen, F.R.G.), equipped with a flame-ionization detector by using wall-coated open-tubular glass columns (50 m × 0.25 mm I.D.) coated with CP-Sil-88 (Chrompack, Middelburg, The Netherlands). The lengths were connecting directly to the injection port and to the detector. The carrier gas was nitrogen (pressure 1.5 bar) and the splitting ratio was 1:60. The oven temperature was increased from 175 to 210°C at 5°C/min and kept at 210°C for 20 min. The injector temperature was 250°C and the detector temperature 290°C. Peak identification was achieved by comparison with authentic standards by capillary GC and silver nitrate TLC[8].

GC on packed columns was performed as described recently[5]. Computerized peak identification and integration were achieved on the Sigma data system with a suitable BASIC program.

RESULTS

The described capillary GC method was five times faster (30 min were required for a chromatographic run) than packed column GC (150 min). The separation of branched-chain, odd-carbon-numbered, *trans-* and long-chain polyunsaturated FA was even better. Elaidic acid (C18:1tω9) could be well separated from oleic acid. The separation of eicosenoic (C20:1ω9) and linoleic acids (C18:3ω3), which is critical on packed columns, was considerably improved.

Fig. 1 shows a typical chromatogram obtained from the PE fraction. A total of

TABLE 1

REPRODUCIBILITY OF CAPILLARY GC OF FA METHYL ESTERS FROM HUMAN MILK LIPID SUBCLASSES

Means of five replicate analyses including lipid extraction, TLC and capillary GC; tr = traces.

| FA	Sphingomyelin		Phosphatidyl-choline		Phosphatidyl-ethanolamine		Cholesterol esters	
	Concentration (%)	S.D.	Concentration (%)	S.D.	Concentration (%)	S.D.	Concentration (%)	S.D.
C12	tr		tr		tr		4.55	0.33
C14	4.03	0.19	1.46	0.14	1.86	0.14	5.77	0.25
C16	26.98	0.95	20.77	0.90	11.76	0.63	21.30	1.19
C16:1ω7	0.73	0.06	1.09	0.13	1.16	0.18	3.59	0.26
C18	19.91	0.69	26.31	0.99	15.57	0.81	8.57	0.32
C18:1ω9	0.64	0.05	1.25	0.15	1.40	0.15	1.94	0.13
C18:1ω9	18.34	0.68	12.27	0.59	14.85	0.35	28.49	1.14
C18:2ω6	12.34	0.25	20.14	0.71	12.96	0.76	6.28	0.10
C18:3ω3	0.77	0.07	1.02	0.12	0.40	0.06	2.23	0.14
C20:3ω6	0.83	0.05	0.68	0.03	1.97	0.36	0.64	0.06
C20:4ω6	1.56	0.11	2.42	0.19	13.44	0.27	0.57	0.06
C22:6ω3	0.35	0.04	0.73	0.13	2.57	0.12	tr	
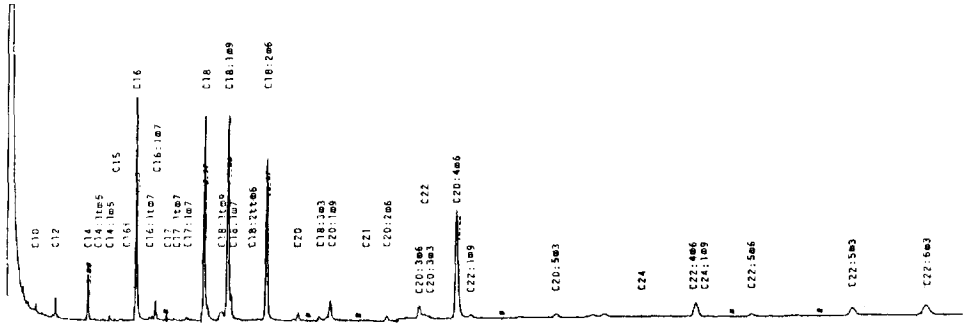
Fig. 1. Capillary gas chromatogram of FA methyl esters from human milk PE.

48 FA methyl esters could be identified. On injecting 2 μl of the FA methyl ester solution (prepared as described under Experimental), 0.1 μg of each individual FA methyl ester could be quantified with a relative standard deviation lower than 2.5 % (ten injections). This is an approximately 10-fold increase in sensitivity over packed columns.

The FA composition of PL subclasses and CE could, therefore, be determined from only 4 ml of milk. Five replicate analyses, including lipid extraction, TLC, methylation and capillary GC, gave relative standard deviations of approximately 4 % for the major and 7 % for the minor FA (Table I). A comparison of packed column GC on Silar C10 and capillary GC, evaluated by five replicate analyses of the FA composition of total lipids from pooled human milk, is given in Table II.

The good reproducibility of the relative retention times obtained by capillary GC allowed computerized peak identification and normalization.

TABLE II

FA DISTRIBUTION OF HUMAN MILK TOTAL LIPIDS DETERMINED BY CAPILLARY GC COMPARED WITH PACKED COLUMN GC

Means of five replicate analyses, including lipid extraction, methylation and GC.

FA	Packed column GC		Capillary GC	
	Concentration (%)	S.D.	Concentration (%)	S.D.
C12	4.49	0.19	4.54	0.15
C14	8.75	0.25	8.23	0.20
C16	27.79	0.93	26.05	1.03
C18	9.12	0.31	9.32	0.25
C18:1ω9	31.19	0.90	30.12	0.31
C18:2ω6	6.96	0.28	6.81	0.29
C18:3ω3	0.98	0.08	1.07	0.07
C20:4ω6	0.50	0.06	0.60	0.05

DISCUSSION

The accurate analysis of human milk lipids, including the evaluation of the FA composition of TG and of the minor lipid subclasses, is very time consuming and often limited by the small amounts of sample available. Therefore, few data exist on the composition of human milk lipids, as obtained from individual samples at different stages of lactation[5,8,9]. In this respect, the capillary GC method described here proved to be a powerful tool, especially for the analysis of the FA composition of CE and PL subclasses, such as phosphatidylethanolamine, phosphatidylcholine and sphingomyelin. The method is very accurate, being about ten times more sensitive than classical packed column GC, and the time needed for the analysis of up to 48 well separated FA methyl esters could be reduced by a factor of five. The good reproducibility of the relative retention times allows computerized peak identification and normalization, so that this procedure offers a great convenience in the analysis of large sample series.

The first results from our laboratory indicate that the method may well be suitable for the analysis of the FA composition of lipid subfractions from sources other than human milk, *e.g.*, lipid subfractions from blood, such as high- and low-density lipoproteins or their subclasses.

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