Journal of Chromatography, 431 (1988) 231–238
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4322

IDENTIFICATION OF VERY-LONG-CHAIN FATTY ACIDS IN RAT AND MOUSE HARDERIAN GLAND LIPIDS BY CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

EVA TVRZICKÁ

Lipid Laboratory, Faculty of Medicine, Charles University, 128 08 Prague 2 (Czechoslovakia)

TOMÁŠ ŘEZANKA*

Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague 4 (Czechoslovakia)

and

JAN KRIJT and VÁCLAV JANOUŠEK

Institute of Pathological Physiology, Faculty of Medicine, Charles University, 128 53 Prague 2 (Czechoslovakia)

(First received April 5th, 1988; revised manuscript received May 30th, 1988)

SUMMARY

Lipids of Harderian ophthalmic gland were separated by means of thin-layer chromatography with flame ionization detection in an latroscan apparatus. Wax ester and polar lipids (phosphatidylethanolamine and phosphatidylcholine) were detected as the main lipids in rats and glyceryl ether diester and both polar lipids were the main lipids in mice. Fatty acids were determined in individual lipid classes by means of gas chromatography and gas chromatography–mass spectrometry on capillary columns. The content of fatty acids, the positional isomers of monoenoic acids being predominantly C_{18}, C_{20} and C_{22}, is most interesting. Very-long-chain fatty acids, saturated fatty acids up to C_{30} and even monoenoic acids up to C_{28} were detected. Branched-chain fatty acids, predominantly iso and anteiso, are minority components, although their chain length distribution (C_{15}–C_{27}) is broad.

INTRODUCTION

The mammalian Harderian gland is a large tubuloalveolar gland located within the orbit of many species [1]. It is especially well developed in rodents and lagomorphs. The physiological role of the gland remains unclear, although a number of theories have been published [1,2].

A characteristic feature of the mammalian Harderian gland is its lipid secretion. The major lipid class of the rat gland consists of unsaturated wax ester [3]. Wax esters have not been detected in infantile rats and 1-alkyl-2,3-diacylglycer-

0378-4347/88/$03.50

ols (glyceryl ether diester, GEDE) form the major fraction [4]. The latter also form the major lipid fraction in the Harderian gland of mice [5,6], golden hamsters [7], guinea pigs [8] and rabbits [9]. In the rabbit red lobe, the alkyl chains are hydroxylated and the OH groups are esterified. The white portion of this gland contains primarily a mixture of 2-(O-acyl)hydroxy fatty acid esters [10]. Cholesterol and triacylglycerols have been identified as the minor lipid fractions in most species, although triacylglycerols (TG) were not found in the glands of golden hamsters and guinea pigs. The composition of the phospholipids has been analysed in the glands of rabbits [11], golden hamsters [7] and guinea pigs [8]. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were always present.

The fatty acid composition of the main lipid fractions has been determined in the Harderian glands of rats [3], golden hamsters [7], rabbits [12], guinea pigs [8] and Mongolian gerbils [13] and in mouse Harderian gland tumour [5]. The fatty acid compositions of phospholipid fractions has been determined in the guinea pig [14].

This study was carried out to determine the fatty acid composition of the total lipid and main lipid fractions in the Harderian gland of the mouse and rat.

EXPERIMENTAL

Materials and methods

Wistar rats and ICR mice were decapitated and, after removal of the eyeballs, the Harderian glands were excised and the total lipid was extracted as described by Folch et al. [15]. Preparative thin-layer chromatography (TLC) was carried out on laboratory-prepared plates (silica gel 60 PF; Merck, Darmstadt, F.R.G). Neutral lipids were developed with heptane–diethyl ether–acetic acid (60:30:5). Total lipid and isolated fractions of neutral and polar lipids were saponified using 5 *M* potassium hydroxide in 50% methanol for 1 h. Fatty acids were extracted from the acidified mixture and esterified using 7% BF_3 in methanol. Fatty acid methyl esters (FAME) and hydroxy fatty acids were separated by preparative TLC. Chromarods S-II (Iatron Labs., Tokyo, Japan) for analytical TLC were developed with hexane–diethyl ether–formic acid (52:10:0.1) for 20 min.

Instrumentation

Analytical TLC was performed on an Iatroscan TH-10 analyzer (Iatron) equipped with an SP 4100 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.). GC analyses of FAME were carried out on a PU 4900 gas chromatograph (Pye Unicam, Cambridge, U.K.) with a flame ionization detector and capillary column (10 m × 0.32 mm I.D.) with CP WAX 57 CB chemically bonded stationary phase (Chrompack, Delft, The Netherlands); the carrier gas was hydrogen at a flow-rate of 1.7 ml/min and the splitting ratio was 1:30. The injector and detector temperatures were 250 and 260 °C, respectively, and the column temperature was programmed from 120 to 200 °C at 6 °C/min.

The mixture of FAME was analysed on a Shimadzu Model QP 1000 quadrupole gas chromatographic–mass spectrometric (GC–MS) system (Shimadzu, Tokyo,

Japan) using an SPB-1 fused-silica capillary column (Supelco, Bellefonte, PA, U.S.A.) (60×0.25 mm I.D.) with a film thickness of 0.25 μm. The injector temperature (splitting ratio 1:20) was 280°C and the oven temperature was increased from 160 to 250°C at 6°C/min.

The same mixture was analysed on a Finnigan-MAT 90 sector-type GC–MS instrument (Finnigan-MAT, San Jose, CA, U.S.A.), using an HP-1 fused-silica capillary column (Hewlett-Packard, Palo Alto, CA, U.S.A.) (25 mm×0.2 mm I.D.) with a film thickness of 0.22 μm. The injector temperature was 280°C (splitting ratio 1:50) and the oven temperature was increased from 35 to 250°C at 3°C/min. The temperature of the GC–MS interface was 270°C and the electron impact energy was 70 eV.

The positions of the double bonds in the methyl esters were determined on the basis of splitting of pyrrolidides (pyrrolidides were prepared by boiling the methyl esters in pyrrolidine and acetic acid) in the mass spectrum [16]. Methyl esters of fatty acids with more than 21 carbon atoms were separated by high-performance liquid chromatography (HPLC) [17]. Individual methyl esters of monoenoic fatty acids were oxidatively degraded [18] and the monocarboxylic and dicarboxylic acids obtained were converted into methyl esters by reaction with diazomethane and identified by GC–MS [18,19].

Methyl esters of mono- and dicarboxylic acids were analysed according to Řezanka and co-workers [18,19]. Pyrrolidides were separated on an HP-1 fused-silica capillary column (25 m) according to the literature [20].

RESULTS AND DISCUSSION

After gravimetric determination of the total lipids, the individual lipid fractions were separated from mouse and rat samples by the preparative TLC method. Qualitative analysis was carried out by the TLC method with flame ionization detection (FID) (Fig. 1) and the major lipid fractions were quantified gravimetrically. In agreement with the literature, the major nonpolar lipid in mice was found to be GEDE and, in rats, wax. It can be seen from Table I that a higher content of total lipids was found in mouse gland and that the relative contents of

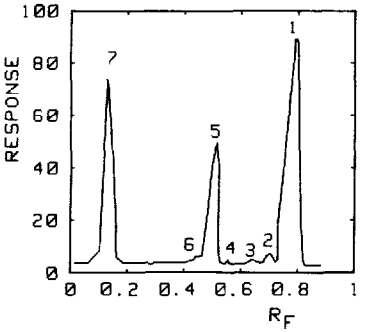

Fig. 1. TLC–FID of rat lipids. For conditions, see Experimental. Peak identification: 1 = wax esters; 2 = GEDE; 3 = TG; 4 = free fatty acids; 5 = internal standard (octadecanol); 6 = cholesterol; 7 = polar lipids.

TABLE I

CONTENTS OF TOTAL LIPIDS AND MAIN LIPID FRACTIONS IN RAT AND MOUSE HARDERIAN GLAND

Each value represents the mean ± S.D. from five animals.

Species	Sex	Wet tissue (mg)	Total lipids (mg)	Neutral lipid* (% total lipid)	Polar lipids (% total lipid)
Mouse	Male	41.9 ± 3.7	10.2 ± 1.2	56.7 ± 2.0	27.2 ± 4.5
	Female	30.0 ± 2.9	6.7 ± 0.9	54.6 ± 2.2	37.8 ± 5.0
Rat	Male	381.3 ± 11.4	79.3 ± 5.6	53.9 ± 6.1	36.8 ± 8.8
	Female	293.6 ± 9.9	53.7 ± 4.8	46.6 ± 3.8	21.6 ± 2.4

*In rat, wax esters; in mouse, GEDE.

TABLE II

FATTY ACID COMPOSITIONS FROM INDIVIDUAL CLASSES OF LIPIDS FROM MOUSE AND RAT (IN MOLAR %)

tr = Trace.

Fatty acid	GEDE*	TG	Mouse PE	PC	Wax	GEDE	Rat TG	PE	PC
14:0	tr	1.6	0.5	0.5	tr	tr	1.3	0.3	0.2
16:0	0.1	23.9	3.9	37.6	0.2	1.3	12.9	4.8	20.6
16:1**	tr	6.8	1.4	1.0	1.0	2.7	4.3	39.3	25.1
18:1**	5.5	37.1	88.4	57.7	17.7	67.0	74.3	52.6	52.0
9,12-18:2	tr	12.3	1.3	0.8	0.2	3.2	2.0	2.0	1.2
20:0	19.6	1.9	0.2	tr	0.1	0.6	0.1	tr	tr
20:1**	62.6	14.9	3.9	2.4	79.4	20.7	4.6	0.9	0.9
11,14-20:2	1.1	1.4	tr	tr	0.1	tr	0.6	tr	tr
22:0	4.1	tr	tr	tr	tr	tr	tr	tr	tr
22:1**	6.2	tr	tr	tr	1.2	1.9	tr	tr	tr

*For the nomenclature of class of lipids, see text.
**Sum of isomers, see Table III.

neutral and polar lipids are similar in both species. The minor components were found to be free cholesterol and TG; in rats, GEDE was also isolated. The major components of the polar lipids are PC and PE.

Porphyrins or their degradation products make the identification of lipids after TLC impossible. After spraying with agents such as Rhodamine 6G or dichlorofluoroescein, the whole plate exhibits fluorescence, and we therefore used spraying with water for detection. It is not very sensitive and is not a specific reagent and hence does not make the identification of minor compounds possible.

The fatty acid contents were measured using capillary GC of all the isolated lipid fractions. The total lipid fatty acid composition, the main fraction of neutral lipids and the total polar lipid fraction were identified and quantified by GC–MS. Table II gives the fatty acid contents in mice and rats for the individual lipid

TABLE III

FATTY ACID COMPOSITION IN TOTAL LIPIDS AND THEIR FRACTIONS FROM HARDERIAN GLAND OF RAT AND MOUSE (IN MOLAR %)

Fatty acid	Mouse			Rat		
	Total lipids	GEDE	Polar lipids	Total lipids	Wax	Polar lipids
14:0	0.23	0	0	0.20	0	0.10
i-15:0	0	0	0	0	0	0.02
15:0	0	0	0	0.28	0	0.03
i-16:0	0	0	0	5.45	0.01	0
7-16:1	0.55	0	0.54	11.37	0.16	18.93
9-16:1	0	0	0	0.71	0	0
16:0	3.78	0	18.07	4.03	0.03	17.51
i-17:0	0	0	0	0.47	0	0.58
8-17:1	0	0	0	0	0	0.96
ai-17:0	0	0	0	0.43	0	0.04
10-17:1	0	0	0	0.72	0	0.04
17:0	0	0	0	0.95	0	0.21
9,12-18:2	0	0	0	3.79	0	0.59
i-18:0	0	0	0	0	0	1.58
9-18:1	20.72	0.50	60.82	20.14	11.97	28.03
11-18:1	0	0.13	8.27	2.84	1.21	0
13-18:1	4.65	2.97	5.96	0.97	0.15	25.73
10-19:1	0.01	0	0	0	0	0
c-19-19:0	0.02	0	0	0	0	0
12-19:1	0.21	0	0.03	0	0	0.05
19:0	0.01	0	0.08	0.22	0	0.01
11,14-20:2	0	0	0.28	0	0	0
i-20:0	0	0.33	0	0	0	0
11-20:1	24.41	68.02	4.10	0	0	0
13-20:1	2.96	0	1.17	42.65	86.14	0.80
15-20:1	6.64	0	0	0	0.12	0.01
20:0	12.58	20.04	0.36	0.26	0	0.03
i-21:0	0.03	0	0	0	0	0
12-21:1	0.39	0	0	0	0	0
21:0	0.05	0	0	0.23	0	0
13-22:1	8.14	2.24	0	0.25	0.02	0
15-22:1	6.12	1.86	0	1.66	0.19	0.03
22:0	5.95	3.71	0	0.27	0	0.03
15-24:1	1.24	0.09	0	0	0	0
17-24:1	0.79	0.11	0	0.48	0	0.01
24:0	0.52	0	0	0.70	0	1.00
Unknown	0	0	0	0	0	0.76
Unknown	0	0	0	0	0	0.69

fractions. The fatty acid composition of the GEDE and TG fractions in mice is similar to that given for Harderian gland tumours [5]. Of the polar lipids, the composition of which has not yet been described, oleic acid predominates and palmitic acid is present in large amounts in the PC. In rats, the content of fatty

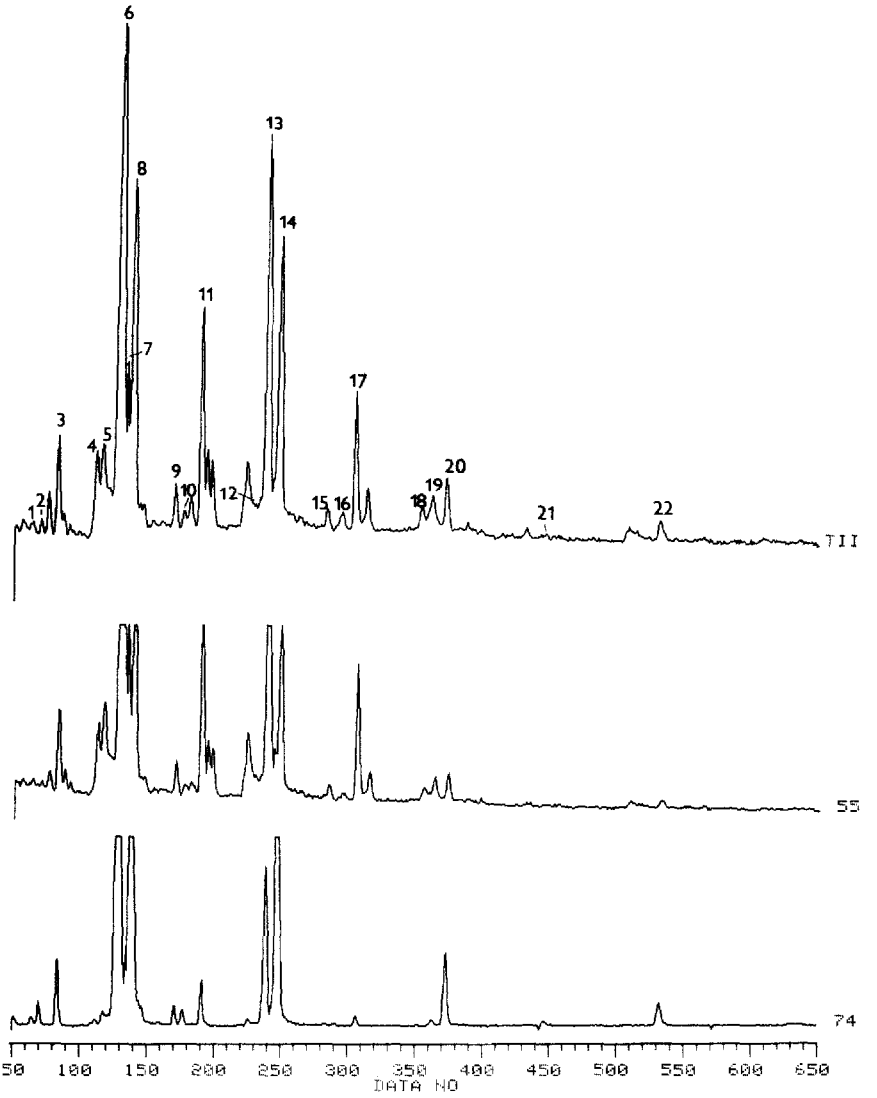

Fig. 2. GC–MS of FAME of mouse total lipids. TII = total ion current; 55 = SIM for ion of *m/z* 55 (base peak for monoenoic FAME); 74 = SIM for ion of *m/z* 74 (base peak for saturated FAME); Data No. = scan number expressed in seconds (one scan per second). For conditions, see Experimental. Peak identification: 1 = i-23:0; 2 = 14-23:1; 3 = 23:0; 4 = i-24:0; 5 = 15-24:1; 6 = 17-24:1; 7 = 19-24:1; 8 = 24:0; 9 = i-25:0; 10 = 16-25:1; 11 = 25:0; 12 = i-26:0; 13 = 17-26:1; 14 = 26:0; 15 = i-27:0; 16 = 18-27:12; 17 = 27:0; 18 = 19-28:1; 19 = 21-28:1; 20 = 28:0; 21 = 29:0; 22 = 30:0.

acids in wax and GEDE is also comparable to the published data [3,4]; eicosenoic acid or oleic acid predominates. The 16:0, 16:1, 18:0 and 18:1 acids predominate in the polar lipids. Table II gives the most important and highest content fatty acids.

The use of GC–MS with a capillary column permitted both the identification and the quantitation of three times as many fatty acids (Table III). In contrast

to guinea pigs, the content of methyl-branched fatty acids is much lower. Iso and anteiso acids are present, including acids with a cyclopropane ring, but no fatty acids branched in the centre of the chain, which are common in guinea pig lipids. On the other hand, at least two or three positional isomers of monoenoic fatty acids were always found.

The pyrrolidide method for the determination of double bond positions is limited primarily by the chromatographic behaviour of pyrrolidides. For the elution of these derivatives a temperature up to 50°C higher than that for the elution of methyl esters must be used. In addition, pyrrolidides of fatty acids with more than 24 carbon atoms can only be chromatographed by means of on-column injection, which is not suitable when using GC–MS.

On the basis of the above findings and useful experience [18] in the determination of double bonds after oxidation as mono- and dicarboxylic acids, we decided to use this method in studies of fatty acids with more than 21 carbon atoms. The greater capabilities of GC–MS are obvious, especially when a capillary column is used, compared with GC on a capillary column. The main advantage is peak identification without the use of standards that are not commercially available for these samples.

Two unknown peaks were also found in the total polar lipid fraction of rats; mass spectrometry identified them as the methyl esters of hexadecadienoic and octadecadienoic acid. However, these structures are in disagreement with the retention times of these substances, as they are eluted between the methyl ester of monoenoic acid and the saturated acid that is one carbon atom longer (e.g., apparently 18:2 between 19:1 and 19:0). The only satisfactory explanation could depend on the unusual positional isomer of an acid with a triple bond, as the mass spectra of 18:2 and a-18:1 are almost identical; this would also correspond to the retention times.

When the capillary column was overloaded with a sample of mouse total lipids and for sufficiently long analysis times, two minor peaks appeared with retention characteristics of 26:1 and of 26:0 for the methyl esters. The possible presence of higher homologues was demonstrated using single ion monitoring (SIM) for ions with m/z 55 and 74 (base peaks for the monoenoic and saturated fatty acid methyl esters, respectively). The presence of methyl esters of acids up to C_{30} was demonstrated in chromatography on a quadrupole mass spectrometer by repeated column overloading and spectral recording up to the C_{22} methyl esters.

It can be seen in Fig. 2 (only the FAME are shown) that, in the chromatogram of total mouse lipids, methyl esters of saturated acids with straight chains up to C_{30}, iso compounds to C_{27} and positional isomers of monoenoic acids up to C_{28} are present. It is not surprising that several positional isomers were identified, as it is obvious from Table III that C_{20} and C_{22} also contain three and two positional isomers, respectively. The very-long-chain monoenoic fatty acids were formed by chain elongation of the lower homologues. As compared with the literature [3], we detected not only positional isomers of n – 7 but also n – 9 and n – 5 monoenoic acids. On the basis of the analyses performed it may be stated that the elongation and desaturation system is very complex and requires further investigation.

The function of the Harderian gland is not yet clear, although a number of

hypotheses regarding its importance have been formulated. A study of metabolic changes in the Harderian gland in various physiological and pathological states will contribute to the elucidation of its function and increasing knowledge on the regulation of lipid metabolism.

REFERENCES

1 T. Sakai, Arch. Histol., 44 (1980) 299.
2 J. Hugo, J. Krijt, M. Vokurka and V. Janoušek, Gen. Physiol. Biophys., 6 (1987) 401.
3 U. Murawski and U. Jost, Chem. Phys. Lipids, 13 (1974) 155.
4 J. Zackor, H. Egge and U. Murawski, Hoppe-Seyler's Z. Physiol. Chem., 357 (1976) 288.
5 K. Kasama, N. Uezumi and K. Itoh, Biochim. Biophys. Acta, 202 (1970) 56.
6 M. Watanabe, J. Morphol., 163 (1980) 349.
7 W.L. Lin and M.J. Nadakavukaren, Comp. Biochem. Physiol., 70B (1981) 627.
8 Y. Seyama, in E. Haber (Editor), The Cell Membrane, Plenum Press, New York, 1984, p. 217.
9 K. Kasama, W.T. Rainey and F. Snyder, Arch. Biochem. Biophys., 154 (1973) 648.
10 C.O. Rock, V. Fitzgerald, W.T. Rainey and F. Snyder, Chem. Phys. Lipids, 17 (1976) 207.
11 A. Radominska-Pyrek, Z. Dabrowiecki and L.A. Horrocks, Biochim. Biophys. Acta, 574 (1979) 248.
12 C.O. Rock and F. Snyder, Arch. Biochem. Biophys., 171 (1975) 631.
13 O. Otsuru, H. Otsuka, T. Kasama, Y. Seyama, T. Sakai and T. Yohro, J. Biochem., 94 (1983) 2049.
14 Y. Seyama, K. Ohashi, T. Imamura, T. Kasama and H. Otsuka, J. Biochem., 94 (1983) 1231.
15 J. Folch, M. Lees and G.H. Sloane Stanley, J. Biol. Chem., 226 (1957) 497.
16 B.A. Andersson and R.T. Holman, Lipids, 9 (1974) 185.
17 T. Řezanka and M. Podojil, J. Chromatogr., 346 (1985) 453.
18 T. Řezanka and M. Podojil, J. Chromatogr., 362 (1986) 399.
19 I. Viden and T. Řezanka, J. Chromatogr., 408 (1987) 145.
20 W.W. Christie, E.Y. Brechany, S.B. Johnson and R.T. Holman, Lipids, 21 (1986) 657.