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FLUORESCENCE LABELLING IN TRACE ANALYSIS OF BIOLOGICAL SAMPLES

SIMULTANEOUS DETERMINATION OF FREE FATTY ACIDS AND RELATED CARBOXYLIC COMPOUNDS

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SUMMARY

Free fatty acids in serum, together with representatives of a new class of oxirane carboxylic acids having blood-glucose-lowering activity, were determined simultaneously by fluorescence labelling with 4-bromomethyl-7-methoxycoumarin (Br-Mmc) followed by separation by high-performance liquid chromatography. Esterification kinetics of selected compounds were studied, and separation conditions for analysis of oxirane carboxylic acids, related compounds, and a series of saturated and unsaturated fatty acids as their Mmc esters were established. Three compounds (3, 6 and 7) were determined from serum quantitatively by extraction, derivatization, and analysis by high-performance liquid chromatography. Detection limits from serum (1 ml) were 50 ng/ml. Two hours after intravenous bolus injection of 10 mg/kg of compound 6 in dog, a serum level of 1.1 mg/l of administered compound was determined.

INTRODUCTION

Certain oxirane carboxylic acids[1] exhibit a hypoglycaemic[2] effect after oral or intravenous application in laboratory animals, such as rats and guinea-pigs. The structures of these compounds are shown in Table I, together with the structures of some chemically related compounds (1–9).

Oxirane carboxylic acids are, therefore, expected to be useful as drugs for the treatment of diabetic diseases. Apart from lowering the glucose concentration in blood, they suppress fatty acid oxidation which is supposed to be due to a mechanism of inhibition of carnitine acyl transferase I, thereby hindering the transport of long-chain fatty acids through the mitochondrial membrane to the site where oxidation occurs[3].

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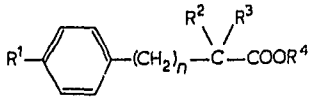
TABLE I

OXIRANE CARBOXYLIC ACIDS AND RELATED COMPOUNDS



Compound	R^1	R^2	R^3	R^4	*n*	*Name*
1	H	OH	CH_2OH	Na	3	Sodium 2-hydroxy-2-hydroxymethyl-5-phenylpentanoate
2	Cl	OH	CH_2OH	Na	3	Sodium 5-(4-chlorophenyl)-2-hydroxy-2-hydroxymethylpentanoate
3	H	OH	CH_2OH	Na	5	Sodium 2-hydroxy-2-hydroxymethyl-7-phenylheptanoate
4	Cl	OH	CH_2OCH_3	H	3	5-(4-Chlorophenyl)-2-hydroxy-2-methoxymethylpentanoic acid
5	Cl	$-CH_2-O-$		Na	3	Sodium 2-[3-(4-chlorophenyl)propyl]oxirane-2-carboxylate
6	H	$-CH_2-O-$		Na	5	Sodium 2-(5-phenylpentyl)oxirane-2-carboxylate
7	Cl	$-CH_2-O-$		Na	5	Sodium 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate
8	H	$=CH_2$		H	5	2-Methylene-7-phenylheptanoic acid
9	Cl	$=CH_2$		H	5	7-(4-Chlorophenyl)-2-methylenheptanoic acid

This paper describes the fluorescence labelling of some representatives of this class of compounds by esterification with 4-bromomethyl-7-methoxycoumarin (Br-Mmc), and separation and determination of the esters by high-performance liquid chromatography (HPLC). As an example, three selected compounds (3, 6 and 7) were determined from serum, thereby establishing a method for the investigation of their pharmacokinetics. Labelling of serum extracts with Br-Mmc automatically yields the Mmc esters of free fatty acids (FFAs). As the levels of FFAs are of importance in the treatment and assessment of diabetic diseases, Mmc esters of a series of both saturated and unsaturated fatty acids were included in the investigations, thus confirming and extending the results of Dünges[4], Dünges and Seiler[5], Lam and Grushka[6] and Lloyd[7], who studied the reaction conditions for esterification of carboxylic compounds with Br-Mmc and analysis of the esters by thin-layer chromatography and HPLC. Lloyd[7] considered the use of Mmc esters of fatty acids in the determination by gradient HPLC to be rather limited because of low fluorescence yield in non-polar solvents and possible formation of micelles at higher water contents of the eluent. Dünges and Seiler[5], however, found quite reproducible results in the determination of Mmc esters of fatty acids in gradient programming, as long as experimental conditions are held rigorously constant. As FFAs occur at relatively high levels in biological samples such as blood (integral values *ca.* 0.5–1 m*M*), the detection limits of Mmc esters, which (depending on quantum yields[7]) may be less favourable in fluorescence than in UV monitoring, are not crucial. The main advantage of our method lies in the simultaneous determination of the pharmacologically active compounds shown in Table I and their possible metabolites, together with the whole pattern of FFAs in serum. Therefore, no separate sample preparation for the gas chromatographic[8,9] or spectrophotometric[10,11] determination of FFAs is necessary. A second important advantage of fluorescence labelling of our compounds as compared to other "pre-column" derivatisation techniques, *e.g.* formation of *p*-nitrophenacyl esters, is expected to enable specific detection with respect to Mmc-labelled blank serum extracts.

Lloyd[12] recommended the formation of phenanthrimidazoles as fluorescent derivatives in fatty acid analysis. The very rigorous reaction conditions needed, however, would probably destroy the oxirane compounds we were interested in; furthermore, the hydroxy compounds of Table I would be phosphorylated and therefore lost. UV monitoring of the free oxirane carboxylic acids via absorption of the phenyl group at 265 nm ($\varepsilon \approx 190$) is much too insensitive; resorting to the intense absorption near 210 nm ($\varepsilon \approx 7500$) raises problems with baseline drift in necessary gradient programming.

The pharmacologically active oxirane carboxylic acids can be cleaved to the corresponding open-ringed compounds in acidic media. Although the process is very slow at physiological pH values, it has to be considered as a possible metabolic pathway, because hydrolysis might be catalysed enzymatically, *e.g.* by epoxide hydrolase. For this reason the open-ringed compounds 1, 2, and 3 were included in our investigations.

EXPERIMENTAL

Apparatus

A Hewlett-Packard (HP; Böblingen, G.F.R.) Model 1084B liquid chromato-

graph was used, equipped with a HP 798775A UV detector in combination with a Schoeffel fluorescence detector FS 970. Ready-to-use columns (125 × 4 mm I.D., Hibar, E. Merck, Darmstadt, G.F.R.; 250 × 4.6 mm I.D. HP 79917A) filled with RP-8 (5 μm) were employed. Mmc esters were excited at 320 nm, and the emission filter had a cut-off value of 370 nm. UV monitoring of Mmc esters was performed at 320 nm. The values were processed with a HP 3354 laboratory data system.

Analytical procedures

Mmc esters of oxirane carboxylic acids, related compounds and fatty acids were separated by gradient programming (1%/min) with aqueous acetonitrile, starting at 30% acetonitrile in water of pH 2.7 (acidified with H_2SO_4) after an initial isocratic period of 2 min. Fatty acids from C_6 to C_{24} were analysed with a gradient ending at 95% acetonitrile. For determination of the Mmc esters of oxirane carboxylic acids and related compounds, an upper limit of 70% acetonitrile proved sufficient. Mmc-derivatives of serum extracts were analysed up to 95% acetonitrile, when free fatty acid composition was of interest.

Chemicals and compounds investigated

Oxirane carboxylic acids and related compounds were products from the Research Laboratories of Byk Gulden Pharmaceuticals, Konstanz, G.F.R.; the structures of the compounds are given in Table I. Fatty acids were obtained from commercial sources (Sigma, Munich, G.F.R.). 4-Bromomethyl-7-methoxycoumarin (Br-Mmc) was synthesized according to published procedures[13,14]. 18-Crown-6 was purchased from EGA, Steinheim, G.F.R., and was used without further purification. Acetonitrile (LiChrosolv®) was obtained from E. Merck. Dichloromethane and isopropanol were purified by distillation.

Sample preparation and esterification

The esterification of carboxylic acids with Br-Mmc was performed essentially according to the procedure of Lam and Grushka[6], the reaction being catalysed by addition of 18-crown-6; liberated hydrogen ions were bound by anhydrous K_2CO_3. Br-Mmc in acetone was 10^{-2} *M*, and the stock solution was protected from light stored in a refrigerator and renewed weekly.

For determination of reaction rates, 20–100 μg of each compound were incubated in a small, septum-sealed glass vial together with 1 ml of the above Br-Mmc solution. Anthracene was used as internal standard. After the addition of 18-crown-6–K_2CO_3, the mixture was gently shaken in a water-bath at 60°C. Then 50-μl aliquots were withdrawn at suitable times, diluted 4 times with acetonitrile to stop the reaction, and stored at −20°C until HPLC analysis.

Serum samples (1 ml each) were acidified with 0.2 ml of 1 M HCl and extracted twice with 5 ml of dichloromethane–isopropanol (9:1). The combined organic layers were dried with anhydrous Na_2SO_4 and evaporated to dryness under vacuum at 40°C. The residue was dissolved in acetone and the solution transferred to a 1.5-ml glass vial. After esterification with 200 μl of Br-Mmc–18-crown-6–K_2CO_3 for 30 min at 60°C, the reaction mixture was diluted, centrifuged and evaporated to dryness. The residue was taken up with 200 μl of acetone and analysed by HPLC.

Recoveries and calibration curves of 6, 7, and 3 were performed using "Human

mixture containing the Mmc esters of 1–9 in equimolar amounts at different concen-
6 in an *in vivo* experiment, a male fasted beagle dog was injected 10 mg/kg of 6 intravenously, the drug being dissolved in 0.9% saline. Blood samples were collected at suitable times, serum was separated within 10 min and frozen at −25°C until preparation.

RESULTS AND DISCUSSION

In order to ensure complete esterification of the oxirane carboxylic acids and related compounds shown in Table I, the time-dependent increase in UV-peak area of the esters of 6 and 7 with Br-Mmc was monitored by HPLC analysis and compared with the rate of reaction of palmitic acid. Fig. 1 shows that the reaction takes *ca.* 20 min to complete with 6 and 7, whereas palmitic acid reaches constant peak areas after 5 min. The lower reaction rate of 6 and 7 compared with palmitic acid probably originates from steric hindrance by the substituents in position 2, but electronic influences may also play a part. Esterification of fatty acids occurs more rapidly in our experiments as compared with the results of Lam and Grushka[6], because we applied both higher Br-Mmc concentrations and a larger excess of the reagent. Using an optimal reaction time of 20 min, the other compounds in Table I were also esterified with Br-Mmc. A fluorescence chromatogram of a complete mixture of the crude Mmc esters is shown in Fig. 2, which was obtained by gradient programming, starting at 30% acetonitrile in water of pH 2.7. Excessive Br-Mmc does not appear in the chromatogram because it shows no fluorescence. Peaks that do not correspond to the

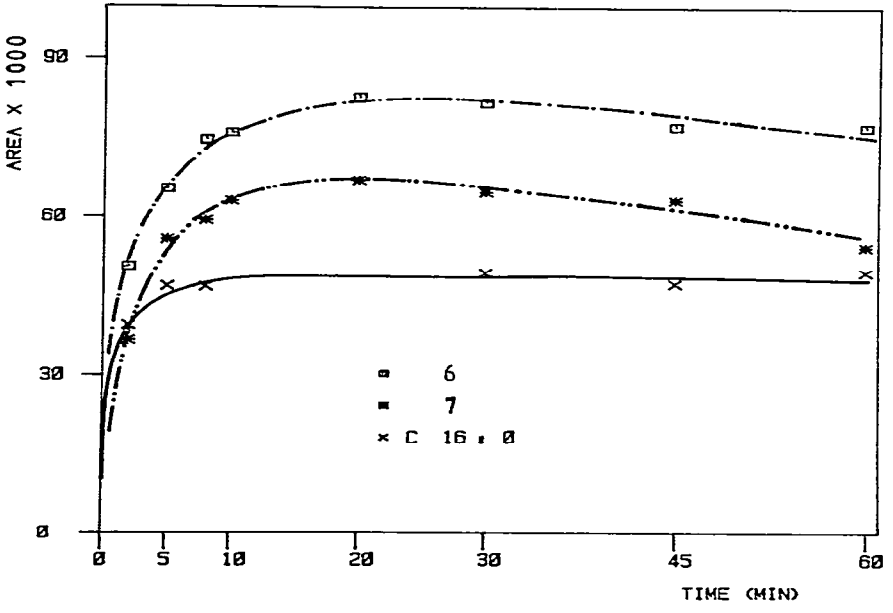


Fig. 1. Reaction kinetics of esterification of 6, 7, and palmitic acid with Br-Mmc. UV areas *vs.* time. For reaction conditions, see Experimental section.

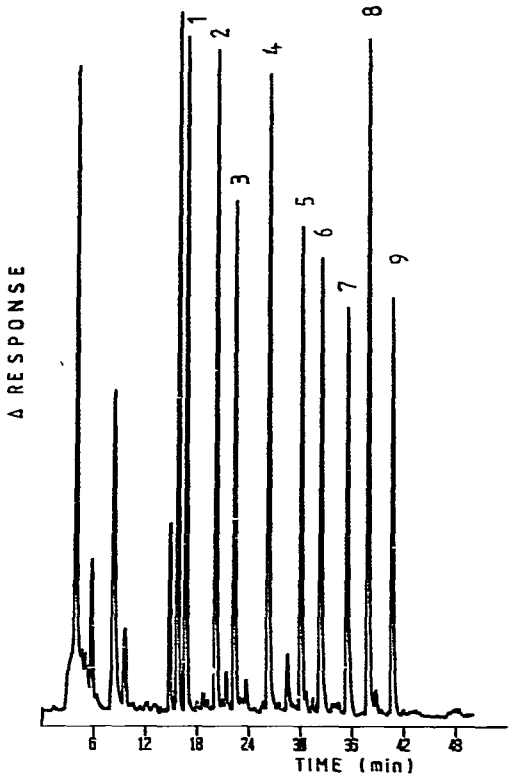


Fig. 2. Separation of Mmc esters of compounds from Table I. Fluorescence excitation at 320 nm, fluorescence cut-off at 370 nm. Column, RP-8, 5 μm, 250 × 4 mm I.D.; flow-rate, 2.0 ml/min; temperature, ambient; gradient, acetonitrile–water pH 2.7, 1%/min; initial isocratic period, 2 min at 30%. Each peak corresponds to *ca.* 500 ng.

esterified compounds are mainly caused by fluorescent degradation products of the reagent used.

Table II lists collected UV and fluorescence areas obtained by analysing a mixture containing the Mmc esters of 1–9 in equimolar amounts at different concentrations which had been adjusted by suitably diluting a concentrated stock solution. UV areas are proportional to amounts over the whole range for each compound, whereas fluorescence areas begin to trail off at *ca.* 40 nmol/ml. Ratios of areas obtained by fluorescence and UV detection were calculated for the linear range of fluorescence, the means of which are given in Table II. The values vary between 2.94 for 1 and 1.22 for 9, *i.e.* fluorescence detection of the Mmc esters is only slightly more sensitive than UV detection. The general trend seems to be that compounds that elute at higher acetonitrile concentrations towards the end of the chromatogram show lower molar fluorescence response than those that appear earlier in the chromatogram at higher water contents. The reason for this probably lies in a decrease of fluorescence quantum yield as the percentage of water in the eluent decreases during gradient programming. This explanation would confirm the results of Lloyd[7], who found a dependence of quantum yields on the water content in methanol–water mixtures with Mmc esters of saturated fatty acids.

The lowering of fluorescence response with changing composition of the eluent during gradient programming is still more marked with Mmc esters of fatty acids. Table III gives UV and fluorescence areas of a series of Mmc esters of saturated fatty acids ranging from C_6 to C_{24}, thus extending the results obtained by Dünges and Seiler[5]. The data were obtained by analysing equimolar amounts of C_6–C_{24} Mmc esters by gradient HPLC at descending concentrations, adjusted in a similar way as in the comparable experiments with 1–9. A representative chromatogram, which in addition contains a number of unsaturated fatty acid Mmc esters expected to partially occur in biological samples, such as palmitoleic, arachidonic, linoleic, oleic, erucic, and nervonic acid, is shown in Fig. 3. Fluorescence was excited at 320 nm, a cut-off filter of 370 nm was used. UV absorption at 320 nm was monitored parallel to fluorescence.

A plot of UV areas *vs.* concentration is shown in Fig. 4 for three representative fatty acids ($C_{10:0}$, $C_{14:0}$, $C_{22:0}$). The straight lines were obtained by regression analysis.

Plots of UV and fluorescence areas of saturated fatty acid Mmc esters *versus* chain lengths are shown in Figs. 5 and 6 for three selected concentrations (8, 4, and 2 nmol/ml). Surprisingly, the UV areas of the Mmc esters are not constant but decrease in a roughly linear way as the chain length increases from C_6 to C_{24}, the overall change being expressed by a mean factor of 3.2 (Table III). The reason for this behavior is unclear at present. Changes in the polarity of the eluent along the gradient could well cause some shift in the absorption maximum. This, however, should be in the range of *ca.* 5–10 nm. Because of the rather broad absorption band of Mmc esters with a maximum near 320 nm, a small shift of the maximum should not cause the extinction coefficient to fall by a factor of up to *ca.* 3, considering the constant UV-monitoring wavelength. Formation of micelles, which was suspected by Lloyd[7] for fatty acid Mmc esters, might well produce a lowering of fluorescence quantum yield. The influence of micelles on the UV spectra, however, should be very small as the arrangement of the molecules in a micelle is not rigid enough to produce phenomena such as band-splitting.

The loss in fluorescence response from C_6 to C_{24} Mmc esters is even greater than the loss in UV response, resulting in a factor of 14.5 when comparing C_6 and C_{24} (Table III). In contrast to the UV areas, the decrease in fluorescence area is not linear from C_6 to C_{24} (Figs. 5 and 6). Several possible influences will be discussed. In the first place, the dependence of fluorescence quantum yield on the percentage of water need not be linear, as already shown by Lloyd[7]. A second influence, formation of micelles, might occur. This would lead to a reduction of fluorescence quantum yield for the early members of the fatty acid Mmc esters, which elute at short retention times where the concentration of water is high. On the other hand, formation of micelles is more favourable with Mmc esters of long-chain fatty esters which elute at higher acetonitrile concentrations, disfavouring formation of micelles. So the situation is quite complicated, and fluorescence measurements with single compounds of differing chain lengths at a series of acetonitrile–water compositions would have to be made in order to obtain a clearer picture. Anyway, the (not understood) decrease in UV response should also lead to a decrease in fluorescence response, which means that the whole range over which fluorescence response decreases is certainly not due only to losses in quantum yields or formation of micelles.

TABLE II

UV AND FLUORESCENCE (FL) AREAS OBTAINED BY HPLC SEPARATION OF MIXTURES CONTAINING EQUIMOLAR AMOUNTS OF Mmc ESTERS OF COMPOUNDS LISTED IN TABLE I

Column RP-8, 5 μm, 125 × 4 mm I.D., flow-rate 1.0 ml/min. Other chromatographic conditions as indicated in Fig. 2. UV detection at 320 nm, injected volume 20 μl. Areas × 1000.

Concentration (nmol/l)	*Compound*									*UV or FL*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	
200	878.90	1078.37	1048.12	1447.17	1497.00	1427.96	1297.55	1524.40	1420.29	UV
	1009.00	879.90	1061.00	316.70	1103.57	1267.96	1169.33	665.80	815.30	FL
80	307.90	392.31	388.72	516.10	529.30	506.30	478.76	519.50	491.00	UV
	619.20	540.30	539.20	503.00	518.40	645.50	549.00	398.90	453.70	FL
40	144.70	178.47	169.65	228.70	237.80	222.50	207.10	246.60	230.10	UV
	394.50	367.10	361.60	348.50	327.70	400.00	311.20	277.30	268.50	FL
20	75.73	96.41	98.78	128.80	127.30	122.40	112.50	127.60	117.60	UV
	218.10	217.00	202.70	213.70	174.20	211.50	163.30	160.00	138.10	FL

8	26.96	33.66	32.42	44.05	45.15	45.92	38.90	44.06	40.99	UV
	87.16	85.83	73.78	82.46	68.47	81.73	64.61	61.72	53.28	FL
4	13.26	15.64	15.28	23.70	22.47	20.71	19.40	23.89	21.04	UV
	40.22	39.12	35.29	38.25	31.01	37.70	29.26	31.62	25.10	FL
2	6.78	7.48	6.95	11.15	10.58	9.90	9.64	11.40	9.81	UV
	19.18	19.51	16.22	18.85	15.23	18.52	14.36	15.23	12.27	FL
0.8	2.71	2.95	2.66	6.43	4.16	3.47	3.12	4.24	3.92	UV
	7.89	8.22	7.34	7.89	6.36	7.66	6.25	6.02	5.24	FL
0.4	1.70	2.30	–	3.32	2.15	2.24	2.48	2.26	2.32	UV
	5.04	4.67	4.49	4.75	3.77	4.58	3.71	3.22	2.97	FL
0.2	1.05	1.29	1.36	–	–	–	–	–	–	UV
	1.73	2.18	1.96	2.47	1.64	2.96	1.72	1.91	1.26	FL
0.08	–	–	–	–	–	–	–	–	–	UV
	0.67	1.04	0.40	0.72	0.68	0.95	–	0.34	0.36	FL
Ratio: FL/UV										
$\bar{x}$	2.94	2.39	2.55	1.67	1.42	1.80	1.52	1.29	1.22	

TABLE III

UV AND FLUORESCENCE (FL) AREAS OBTAINED BY HPLC SEPARATION OF MIXTURES CONTAINING EQUIMOLAR AMOUNTS OF Mmc ESTERS OF SATURATED FATTY ACIDS

Conditions as indicated in Table II. Areas × 1000.

Concentration (nmol/ml)	*Fatty acid chain length*										*UV or FL*
	$C_{6:0}$	$C_{8.0}$	$C_{10.0}$	$C_{12.0}$	$C_{14:0}$	$C_{16:0}$	$C_{18.0}$	$C_{20\cdot0}$	$C_{22.0}$	$C_{24.0}$	
80	435.60	472.80	355.80	339.40	305.30	237.30	233.10	188.70	195.60	141.30	UV
	298.00	338.30	343.50	297.30	224.50	151.20	123.40	83.66	71.73	43.53	FL
40	212.40	230.60	174.90	164.70	150.10	117.80	117.10	95.05	96.80	53.99	UV
	218.70	242.00	184.20	149.70	114.60	76.02	63.97	43.89	36.05	17.05	FL
20	99.98	109.50	82.64	78.27	71.25	55.36	55.93	45.30	46.61	36.56	UV
	154.40	144.20	90.39	73.17	55.27	36.84	31.07	21.20	17.98	11.57	FL
8	42.30	45.90	34.61	32.99	30.02	22.99	23.40	18.86	19.58	15.29	UV
	72.20	62.15	38.36	31.08	23.52	15.85	13.48	9.22	7.83	4.84	FL

4	21.12	23.17	17.07	16.22	14.92	11.75	11.62	9.26	9.39	7.68	UV
	36.51	31.56	19.19	15.98	12.07	7.94	6.83	4.67	3.97	2.45	FL
2	10.37	10.85	7.73	7.29	6.71	4.02	5.29	4.38	4.42	3.44	UV
	17.67	15.12	9.37	7.84	5.73	3.77	3.43	2.35	1.85	1.06	FL
0.8	3.72	4.13	2.48	2.55	2.44	1.91	1.72	1.18	1.40	1.01	UV
	7.52	6.85	5.14	2.99	2.26	1.97	1.68	1.75	0.85	0.69	FL
0.4	2.07	2.27	1.51	1.47	1.09	1.15	1.27	0.76	0.56	0.57	UV
	6.82	5.99	3.58	2.78	2.00	1.34	–	–	–	–	FL
0.08	0.80	0.91	0.41	0.52	0.23	0.52	0.19	0.15	–	0.41	UV
	1.47	1.20	0.56	–	–	–	–	–	–	–	FL
Ratio FL/UV											Ratio of areas $C_{6\cdot0}/C_{24\cdot0}$
$\bar{x}$	1.86	1.30	1.19	0.97	0.80	0.72	0.58	0.49	0.40	0.32	UV, $\bar{y} = 3.20$
											FL, $\bar{y}' = 14.53$

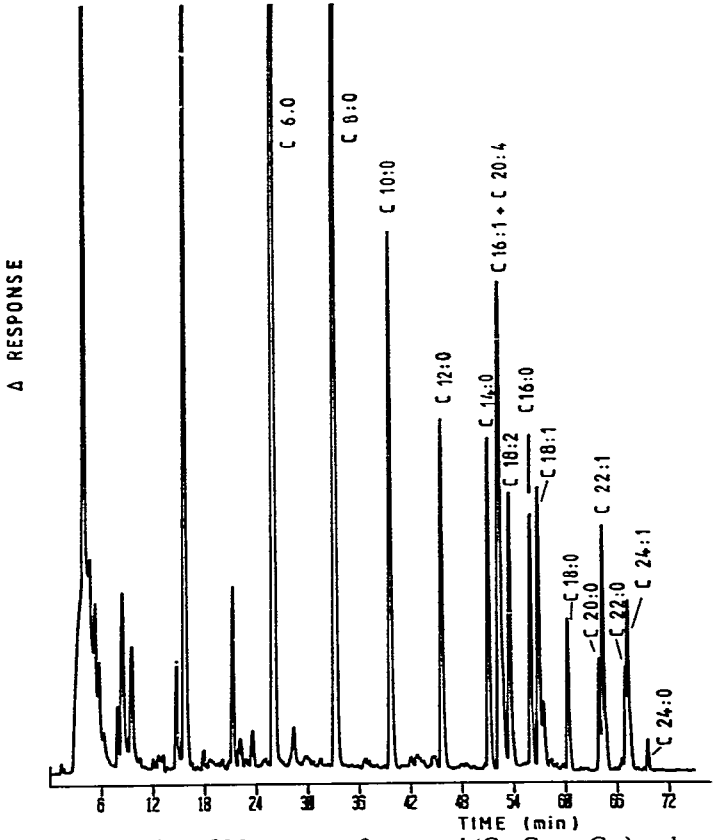


Fig. 3. Separation of Mmc esters of saturated (C_6, C_8 ... C_{24}) and unsaturated ($C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{20:4}$, $C_{22:1}$, $C_{24:1}$) fatty acids; each peak corresponds to *ca.* 500 ng. Conditions as in Fig. 2.

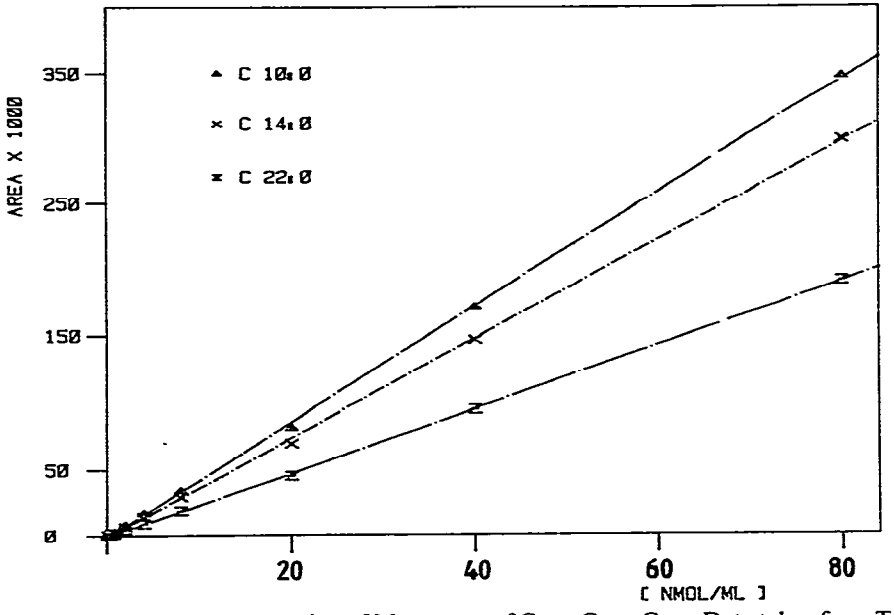


Fig. 4. UV areas *vs.* concentration of Mmc esters of $C_{10:0}$, $C_{14:0}$, $C_{22:0}$. Data taken from Table II and fitted by regression analysis ($C_{10:0}$, $y = 17.33x - 1.38$, $r = 0.9999$; $C_{14:0}$, $y = 14.86x - 1.05$, $r = 0.9999$; $C_{22:0}$, $y = 9.55x - 0.75$, $r = 0.9999$).

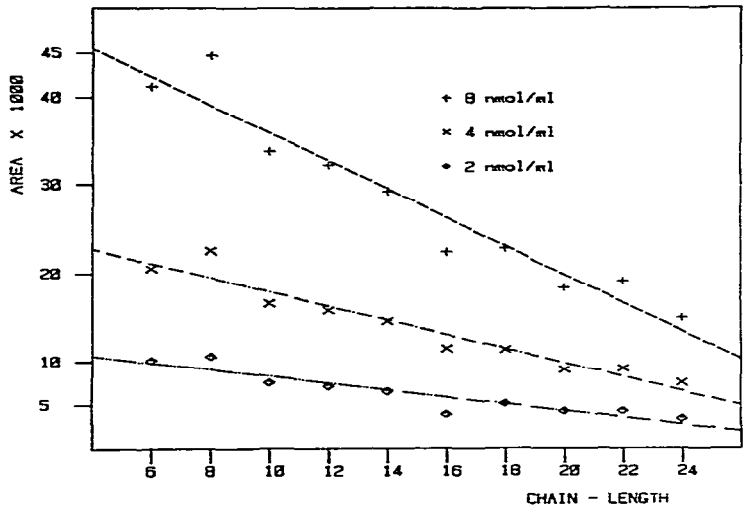


Fig. 5. Plot of UV areas of Mmc esters of saturated fatty acids *vs.* chain lengths; data taken from Table III.

Another interesting result is obtained when comparing UV and fluorescence areas of Mmc esters of unsaturated fatty acids with the data of the respective saturated compounds (Table IV). The areas of the unsaturated compounds were obtained as for the saturated ones. Mmc esters of unsaturated fatty acids ($C_{16:1}$, $C_{18:1}$ and $C_{22:1}$) show both markedly higher UV and fluorescence areas with respect to their saturated counterparts. When comparing the ratios of fluorescence with UV areas of the unsaturated acids, fluorescence areas are already less favourable for $C_{16:1}$ ($\bar{x}$ = 0.76), and the ratio continues to fall as chain length increases ($C_{22:1}$, $\bar{x}$ = 0.49).

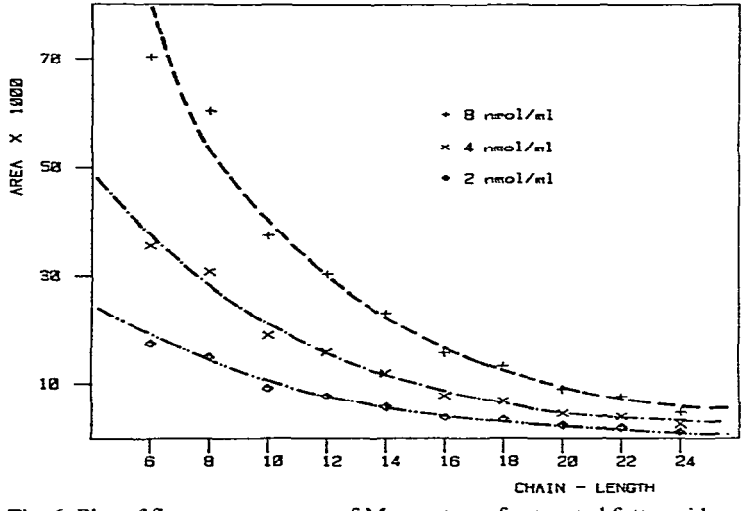


Fig. 6. Plot of fluorescence areas of Mmc esters of saturated fatty acids *vs.* chain lengths; data taken from Table III.

TABLE IV

UV AND FLUORESCENCE (FL) AREAS OBTAINED BY HPLC SEPARATION OF MIXTURES CONTAINING EQUIMOLAR AMOUNTS OF Mmc ESTERS OF UNSATURATED FATTY ACIDS

Conditions as indicated in Table II. Areas × 1000.

Concentration (nmol/ml)	*UV or FL*	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{22:0}$	$C_{22:1}$
80	UV	237.30	485.62	233.10	516.08	195.60	548.18
	FL	151.20	361.20	123.40	323.70	71.73	266.00
20	UV	55.36	99.88	55.93	108.39	44.61	112.37
	FL	36.84	76.42	31.07	70.08	17.98	53.65
8	UV	22.99	40.34	23.40	43.31	19.58	45.12
	FL	15.85	30.99	13.48	27.86	7.83	21.92
Ratio FL/UV							
$\bar{x}$		0.67	0.76	0.56	0.64	0.39	0.49
Ratio $C_{16:0}/C_{16:1}$							
UV, $\bar{y}$			0.54		0.50		0.41
FL, $\bar{y}'$			0.47		0.43		0.31

Again, the reasons for the different behaviour of the Mmc esters of unsaturated fatty acids as compared with the corresponding saturated fatty acids with respect to UV and fluorescence are not clear.

After the chromatographic conditions for the analysis of Mmc esters of pure compounds had been worked out, the quantitative determination of two selected oxirane carboxylic acids (6 and 7), together with the dihydroxy compound 3 from serum samples, was investigated. For this purpose, acidified serum samples containing increasing amounts of each of 3, 6, and 7 were extracted with dichloromethane–isopropanol and esterified as described in the Experimental part. As an internal standard 9 was used. For analysis of the derivatives, a 250 mm column was used in order to achieve better resolution. Fig. 7 shows a typical fluorescence chromatogram obtained by an esterified extract of blank serum from dog. Up to *ca.* 23 min the chromatogram appears to be rather complicated. However, no major peak interferes at the retention times of the Mmc esters of 6 and 7 (*cf.* Fig. 2). Close inspection reveals that even the Mmc ester of the more polar dihydroxyl compound 3, which might occur as possible metabolite of 6 and elutes at about 20 min, can be determined undisturbed by other serum components. Fig. 8 shows the regression obtained by plotting increasing amounts of 6 determined from serum as Mmc ester *versus* calculated amounts. In a similar way, regressions were obtained for 7 and 3; these are also

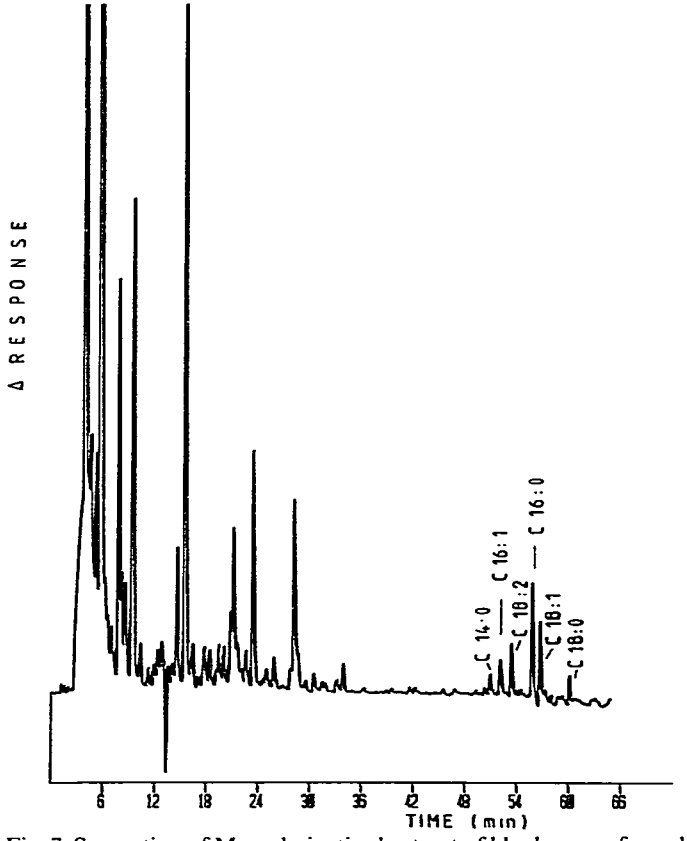


Fig. 7. Separation of Mmc-derivatized extract of blank serum from dog. Reaction mixture obtained from 1 ml of serum was concentrated to *ca.* 200 μl; injected volume, 20 μl. Separation conditions as in Fig. 2.

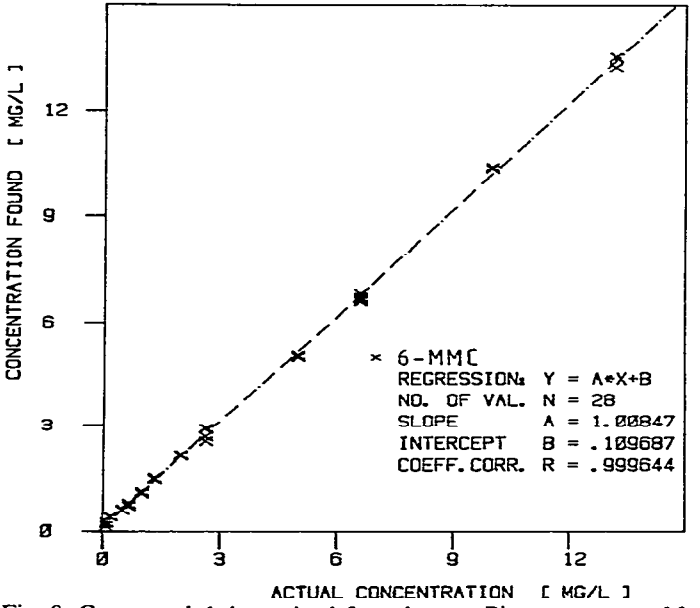


Fig. 8. Compound 6 determined from human Biotest-serum as Mmc ester. Details for extraction and derivatization are given in the Experimental section. Amounts observed *vs.* amounts calculated. Data fitted by regression analysis (3, $y = 0.971x + 0.262$, $r = 0.9983$; 7, $y = 0.976x + 0.2681$, $r = 0.9997$).

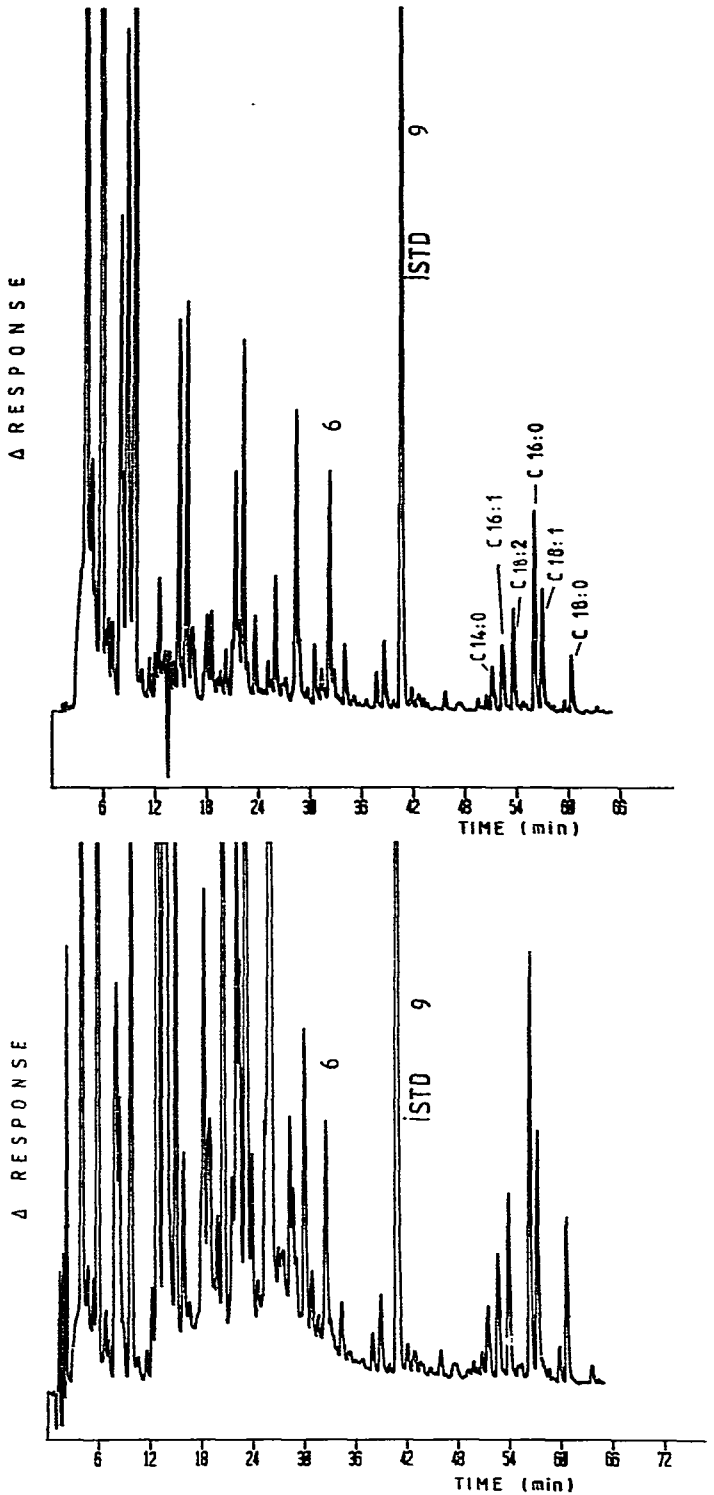


Fig. 9. (a) Separation of Mmc-derivatized extract of serum from dog. Serum collected 2 h after i.v. administration of 10 mg/kg of 6. Volumes as indicated in Fig. 7, HPLC conditions as in Fig. 2; fluorescence detection. Compound 6 corresponds to 1.1 mg/l, no 3 was detected (<50 μg/l). Individual fatty acid concentrations (nM) were determined as follows: $C_{14:0}$, 4.48; $C_{16:0}$, 24.77; $C_{18:0}$, 8.42; $C_{16:1}$, 4.96; $C_{18:1}$, 8.58; $C_{18:2}$, 6.53; sum, 0.057 mM. iSTD = Internal standard. (b) As (a), except UV detection at 320 nm.

presented in Fig. 8. Detection limits were 20 ng/ml at a signal-to-noise ratio of *ca.* 3 for pure compounds. Detection limits for determination from serum are *ca.* 50 ng/ml, as the derivatized extract of blank serum shows traces of fluorescent compounds at the critical retention times. Above 15 μg/ml, fluorescence becomes non-linear.

Fig. 9a shows a fluorescence chromatogram of a Mmc-derivatized sample of dog serum obtained 2 h after intravenous injection of 10 mg/kg of 6. The Mmc ester of the administered drug appears at 32.36 min, quantitative evaluation yields a serum concentration for 6 of 1.10 μg/ml. The internal standard (Mmc ester of 9) appears at 40.67 min. Fig. 9b shows a UV chromatogram of the same sample as in Fig. 9a, clearly demonstrating the advantage of fluorescence monitoring which results in a much simpler chromatogram.

The peaks at the high retention times in Fig. 7, 9a and 9b correspond to the Mmc esters of FFAs in serum. Main components are myristic, palmitoleic, linoleic, palmitic and stearic acid, which have been determined quantitatively by means of a calibration curve (see Fig. 9b). The sum of FFAs (0.057 m*M*) is within the range of usual serum levels. Medium- and short-chain FFAs do not appear to occur at higher concentrations in serum, but should be examined more closely.

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REFERENCES

1 *German Patent Application* P 30 32 669.3, Byk Gulden Lomberg Chemische Fabrik GmbH; K. Eistetter and H. P. O. Wolf, in preparation.
2 H. P. O. Wolf, K. Eistetter and G. Ludwig, *Diabetologia*, in preparation.
3 H. P. O. Wolf, K. Eistetter and G. Ludwig, *16th Annual Meeting of the German Diabetes Society, Bremen, 1981.*
4 W. Dünges, *Anal. Chem.*, 49 (1977) 442.
5 W. Dünges and N. Seiler, *J. Chromatogr.*, 145 (1978) 483.
6 S. Lam and E. Grushka, *J. Chromatogr.*, 158 (1978) 207.
7 J. B. F. Lloyd, *J. Chromatogr.*, 178 (1979) 249.
8 E. Jellum, *J. Chromatogr.*, 143 (1977) 427.
9 H. Heckers, K. Dittmar, F. W. Melcher and H. O. Kalinowski, *J. Chromatogr.*, 135 (1977) 93.
10 W. G. Duncombe, *Biochem. J.*, 88 (1963) 7.
11 W. G. Duncombe, *Clin. Chim. Acta*, 9 (1964) 122.
12 J. B. F. Lloyd, *J. Chromatogr.*, 189 (1980) 359.
13 J. A. Secrist, J. R. Barrio and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, 45 (1971) 1262.
14 W. Baker, C. N. Haksar and J. F. N. McOnise, *J. Chem. Soc.*, (1950) 170.