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LIQUID CHROMATOGRAPHIC ANALYSIS OF SEBUM LIPIDS AND OTHER LIPIDS OF MEDICAL INTEREST*

KURT AITZETMÜLLER and JÜRGEN KOCH

*Unilever Forschungsgesellschaft mbH and NATEC — Gesellschaft für naturwissenschaftlich-technische Dienste mbH, Behringstrasse 154, D-2000 Hamburg 50 (G.F.R.)***

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SUMMARY

A technique is described for the high-pressure liquid chromatographic (HPLC) analysis of sebum lipid classes. The lipid classes present in sebum are separated by gradient elution HPLC from a microparticulate silica column and detected using a moving-wire detector. The system described can be linked to a computer. Quantitation can be carried out by comparing peak areas obtained with those of an internal standard. Peak trapping for further investigations of the separated components, for example by gas chromatography—mass spectrometry, is very easy.

Sebum lipids are separated into the following lipid classes: hydrocarbons and squalene, cholesterol esters and wax esters, fatty acids as their methyl esters, triglycerides, 1,3-diglycerides, 1,2-diglycerides, free cholesterol, monoglycerides and other polar materials. Besides to sebum, the method has been successfully applied to other lipid mixtures, such as serum lipids. Examples of other applications are shown.

INTRODUCTION

High-performance liquid chromatography (HPLC) has not yet found widespread acceptance in the field of lipid analysis because of detector performance problems and gradient elution requirements. This situation has recently been reviewed [1]. For a number of analytical lipid class investigations, however, the replacement of thin-layer chromatography (TLC) by HPLC may have definite advantages.

Lipid class analyses of medical interest are still most often carried out by TLC and by enzymatic or colorimetric and photometric assay methods, although gas chromatography will remain the method of choice for the investigation of individual fatty acid or triglyceride compositions. In many cases these

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analytical methods include comparatively lengthy procedures, or they permit the determination of only one substance at a time, such as cholesterol or total glycerol in serum lipids by saponification with subsequent enzymatic analysis.

Adsorption TLC is fairly time-consuming if all the spots have to be scraped off the plate, eluted, and determined separately. Fast TLC procedures, involving densitometry of lipid spots after charring, may (in our experience) often lead to erroneous quantitative results if the various lipid classes present on the plate differ greatly in their contents of unsaturated compounds. Moreover, TLC plus charring is destructive and does not allow further investigation of the separated lipid classes.

For these reasons we have developed liquid chromatographic methods for lipid class analysis where detection is based on the so-called moving-wire detector in the methane conversion form described by Scott and Lawrence [2]. The wide applicability of such a gradient elution—transport—FID system for the analysis of lipids and mixtures of oleochemicals has been shown [3]. One of the first applications for this method was sebum analysis. The method described here is restricted to analyses of the so-called non-polar lipid classes, ranging in polarity from hydrocarbons to monoglycerides. HPLC analyses of polar lipid classes have also recently been described in the literature [4—6], and these may some day lead to new methods for the analysis of brain and membrane lipids of interest to the clinical chemist.

EXPERIMENTAL

The experiment set-up was very similar to those described in earlier reports [7,8]. Major differences are the use of chlorinated hydrocarbon solvents, slurry-packed microparticulate columns and the computer link.

The basic equipment which we use for lipid class analysis by HPLC consists of 3 solvent reservoirs, a solvent-gradient programming device with two solenoid valves (Ultrograd, LKB Produkter, Bromma, Sweden), a high-frequency reciprocating pump (Milton Roy 196-100, Dosapro, Neu Isenburg, G.F.R.) and pump-stroke dampening device, pre-column, pressure monitor, sample injection port (Precision Sampling, Baton Rouge, La., U.S.A.), the chromatographic column, a moving-wire detector (Pye Unicam LCM-2), a recorder (Servogor S) and an electronic integrator or connection to a calculating computer (Hewlett-Packard 3352) via an analog-to-digital converter [7,8].

Three mixed solvents (I, II and III) of increasing polarity are used to produce a sequence of two solvent gradients (from I to II and then from II to III), using the Ultrograd gradient programmer. The sequence of solvents used consisted of the mixtures carbon tetrachloride—isooctane (34:66) in solvent reservoir I, chloroform—dioxane—*n*-hexane (40:11:49) in reservoir II, and chloroform—methanol—diisopropylether (34:36:30) in reservoir III.

The columns used were of stainless steel slurry-packed with microparticulate silica gels such as LiChrosorb SI 60, of particle size 5 or 10 μm (E. Merck, Darmstadt, G.F.R.). In the previous reports [3, 7, 8] we had mostly relied on hydrocarbon—ether—alcohol sequences for generating the polarity gradients. However, the use of carbon tetrachloride and chloroform in the solvents resulted in better resolution between free cholesterol and the 1,2-diglycerides, due to

secondary solvent effects, and the microparticulate columns permitted electronic integration of the peak areas.

The Ultrograd program charts which we have developed for lipid class analysis are cut out of black paper and scanned by a photocell in the Ultrograd master unit, which switches the solenoid valves accordingly [7]. The charts are cut in such a way that column rinsing periods are carried out automatically (usually first with solvent II and finally with solvent I) before a new injection is made.

We have tried to keep analysis times as short as 30 min. This time includes both the sequence of two solvent gradients plus the column rinsing or regeneration periods. For a silica gel adsorption column, this regeneration period is very short [9] and, therefore, injection for analysis should be made only during continuous operation of the column. Whenever the column has been standing idle for some time, it is first started with a blank run.

The eluted lipid class peaks are detected and quantitated by a Pye-Unicam LCM-2 moving-wire detector, linked to an integrator or computer. For the HP 3352 computer we have developed a program for sebum analysis. Response factors are obtained from test chromatograms, and internal standards are used where applicable. In a few cases area percentage calculations were made.

In the LCM-2 detector, a small portion of the sample is transformed to methane, which in turn is detected by a flame ionization detector. It should be noted here, however, that the response of this detector depends not only on the carbon content of the sample, but also on a number of other factors. The most important of those is the solvent flow-rate, which in turn may depend on the column back-pressure if a reciprocating piston pump is used, and which may change during a gradient run. Rather frequent recalibration of the whole set-up is therefore a prerequisite for quantitative work. This is done by injecting a quantitative test mixture, followed by an RC ("recalibrate") order to the computer.

RESULTS AND DISCUSSION

Fig. 1 shows typical lipid class chromatograms as obtained from a mixture of test substances. Squalene appears first, followed by a wax ester, methyl oleate, triolein, two isomers of a monoglyceride diacetate, 1,3-diglyceride, 1,2-diglyceride, cholesterol, and finally monoglyceride.

A question that has often been raised in the recent literature [9] is that of the reproducibility of gradient elution. For a given equipment, this can be checked by looking at the relative standard deviations of peak retention times.

Table I shows that in our case the relative standard deviation (R.S.D.) of single values for retention times was generally around 1—3% and that the reproducibility of peak areas in the investigation was generally around 3—7%. It was found that evaporator oven temperatures play a significant role in this investigation. For example, an increase of that temperature will considerably increase the R.S.D. of both the squalene and fatty acid methyl esters peaks, while leaving the other peaks largely unaffected.

Fig. 2 shows chromatograms obtained for samples of human hair surface fat, or sebum. In this case the free fatty acids present in the sebum were meth-

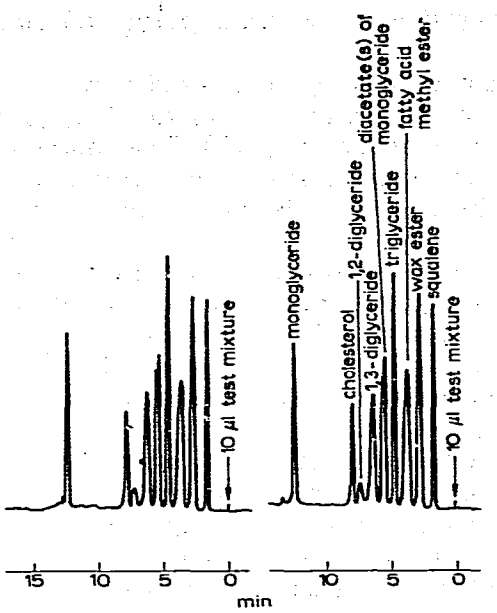

Fig. 1. Lipid class chromatograms of test mixture.

TABLE I

RELATIVE STANDARD DEVIATION OF RETENTION TIMES AND REPRODUCIBILITY OF PEAK AREAS

Ten chromatograms were checked. R.S.D., relative standard deviation. S, squalene (and hydrocarbons); CE, cholesterol esters and/or wax esters; ME, fatty acid methyl esters; TG, triglycerides; IS, internal standard; D, diglycerides; C, free cholesterol; M, monoglycerides (including diols or oxidation products of similar polarity).

	Retention		Peak areas	
	$\bar{x}$ (mm on chart)	R.S.D. (%)	$\bar{x}$ (area %)	R.S.D. (%)
S	13.55	± 1.2	6.23	± 3.4
CE	14.85	± 2.8	17.39	± 2.7
ME	20.00	± 1.7	22.29	± 2.9
TG	29.35	± 2.0	14.28	± 4.6
IS	46.35	± 1.0	12.14	± 3.6
D	52.75	± 1.1	11.60	± 5.3
C	62.80	± 1.1	5.46	± 7.5
M	73.90	± 0.9	8.53	± 6.8

ylated with diazomethane prior to the analysis. A number of individual differences can be detected in the sebum chromatograms. Monoolein diacetate was used as an internal standard for quantitation. The samples were obtained by diethylether extraction from hair of female test persons. The results of this investigation will be reported separately.

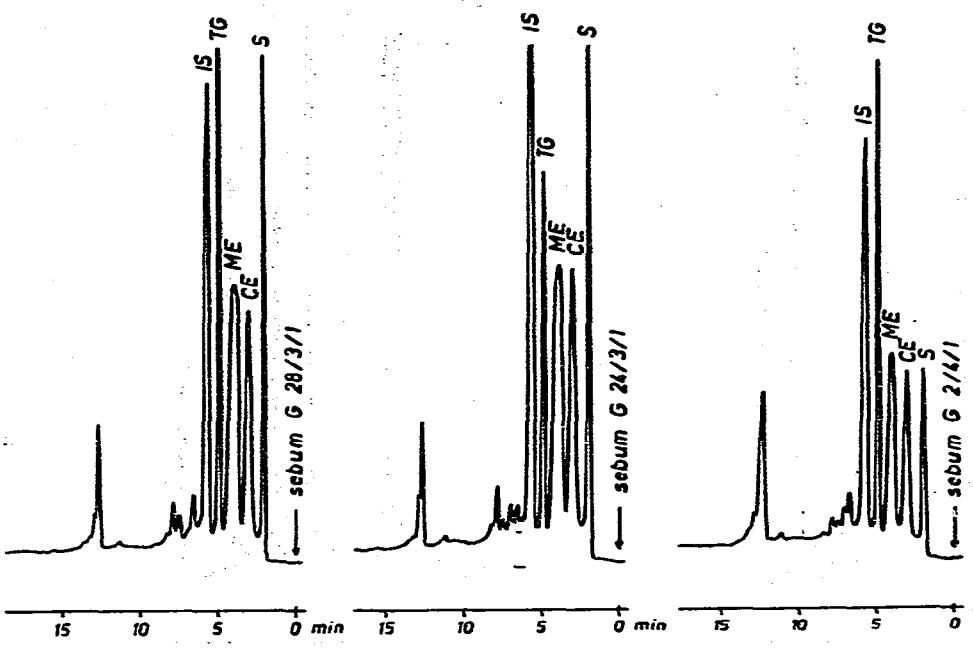

Fig. 2. Chromatograms obtained from various sebum samples. Samples were methylated prior to analysis. See Table I for abbreviations used.

The major differences that can be detected in the sebum chromatograms concern the ratios of the first four major peaks, plus a rather variable, but characteristic, region of small peaks in the vicinity of diglycerides and free cholesterol.

Fig. 3 shows a chromatogram of the unsaponifiable portion of human sebum. Major peaks for squalene, wax alcohols and cholesterol are clearly seen. In addition there is a peak in the polar region that may represent a mixture of dihydroxy compounds plus polar oxidation products, from squalene for example.

Fig. 4 shows a chromatogram of methylated atheroma lipids. In this case not all the peaks could be identified conclusively, but it is quite clear that the two major peaks represent free cholesterol and a very polar compound (with a retention time similar to monoglycerides). Some esterified cholesterol, fatty acid and triglyceride may also have been present.

Fig. 5 shows chromatograms of the chloroform-soluble portion of human cerumen, in the methylated and non-methylated form. It contains lipid classes similar to sebum.

Human blood plasma lipids have also been analysed and the result is shown in Fig. 6. Quantitation was achieved with the aid of monoolein diacetate as internal standard. The plasma lipid sample shown contained cholesterol esters, triglycerides and free cholesterol. Apart from the phospholipids, which usually remain on the column, chromatograms obtained from samples of erythrocyte lipids exhibit only one large peak for free cholesterol.

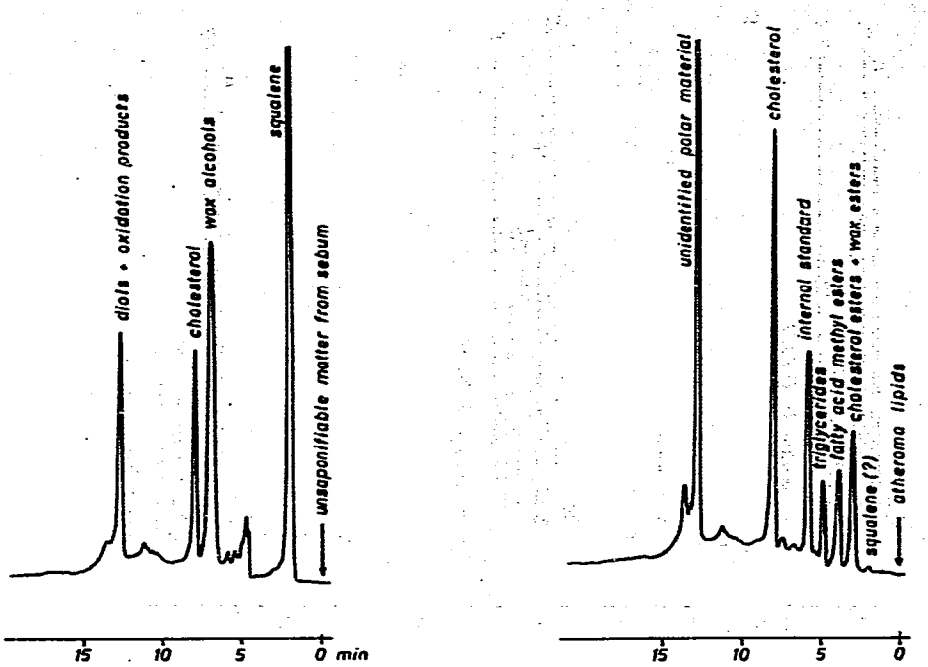

Fig. 3. Chromatogram of unsaponifiable portion of human sebum.

Fig. 4. Chromatogram of atheroma lipids. The extract was methylated by diazomethane prior to analysis.

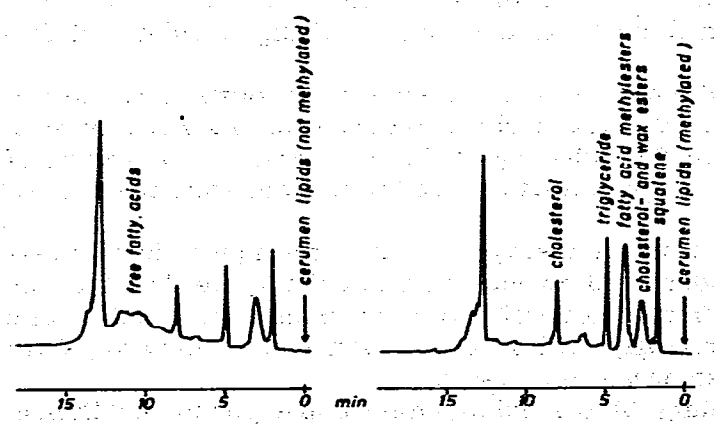

Fig. 5. Chromatograms of cerumen lipids. Methylated and nonmethylated chloroform extracts.

Fig. 7 shows two chromatograms obtained from *vernix caseosa* lipids. The lipid class composition, calculated as area percentage (HPLC, uncorrected), compared well with published results [10]. Diol lipids, which are found only in the surface lipid of the human newborn, appear as a small peak just before the triglycerides.

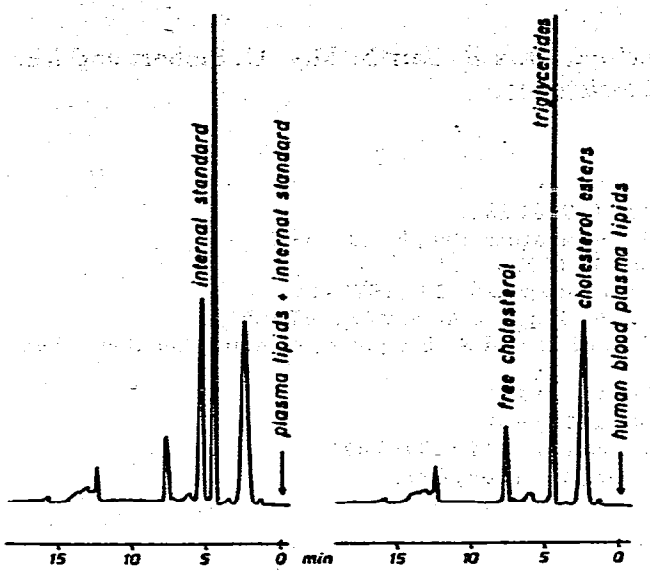

Fig. 6. Chromatograms of a sample of human blood plasma lipids, with and without addition of internal standard.

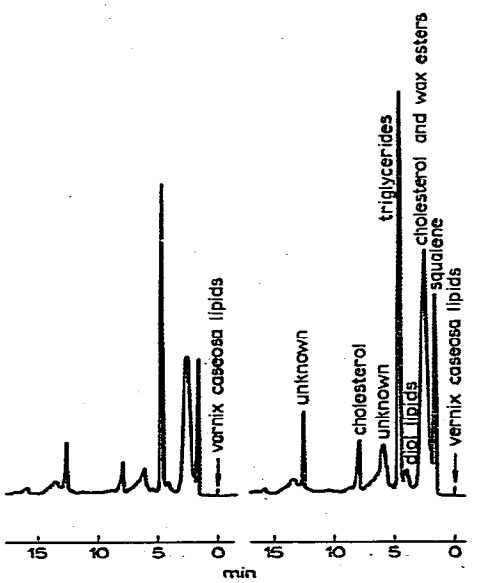

Fig. 7. Chromatograms of a sample of *Vernix caseosa* lipids. Diol lipids appear as a small peak just before the triglycerides.

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