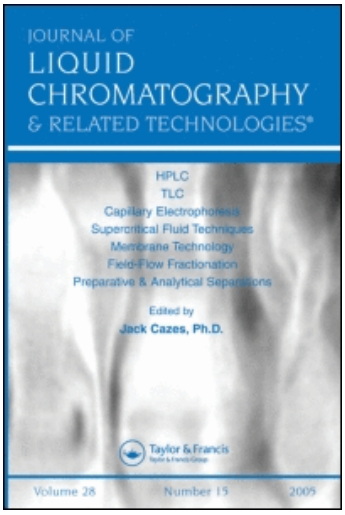

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J. Stein[a]; V. Milovic[a]; S. Zeuzem[a]; W. F. Caspary[a]
[a] Division of Gastroenterology Department of Internal Medicine, Johann- Wolfgang-Goethe-University, Frankfurt, Germany

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FLUOROMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FREE FATTY ACIDS USING PANACYL BROMIDE

J. STEIN*, V. MILOVIC,
S. ZEUZEM, AND W. F. CASPARY
Division of Gastroenterology
Department of Internal Medicine
Johann-Wolfgang-Goethe-University
Theodor-Stern-Kai 7, D-6000 Frankfurt 70, Germany

ABSTRACT

The application of panacyl bromide, a fluorescence labeling reagent for carboxylic acids, was examined. Fatty acids are separated by reversed-phase high-performance liquid chromatography. The derivatives of series of both saturated and unsaturated fatty acids ($C_{10:0}$-$C_{20:4}$) are simultaneously separated by a continuous gradient elution method using a methanol-based solvent containing acetonitrile. The quantitative detection is linear over a range of 10-500 pmol per injection. The method was demonstrated to be able to quantify free fatty acids in blood samples from normal subjects and diabetic patients.

INTRODUCTION

In general, non-aromatic organic acids do not produce strong fluores-cence or absorption in the ultraviolet or visible regions. Therefore, derivatisation with suitable labeling reagents had to be necessary for sensitive derivatisation of

*to whom correspondence should be addressed.

carboxylic compounds. A number of derivatisation methods have recently been described either for UV-absorbing or fluorescence labeling of compounds with carboxylic site groups. The fluorogenic reagents are usually derived from poly-aromatic hydrocarbons, *e.g.* anthryldiazomethan (1), and bromoacetylpyrene (2), or from 7-methoxycoumarin (3-5), as *e.g.*, 4-bromo-7-methoxycoumarin. However, it must be taken into account that these reactions are frequently non-specific since other functional groups, such as phenols, thiols, and imids, are also able to react if the derivatisation procedure is carried out with alkyl halides (6-8). We recently demonstrated panacyl bromide as an useful specific and sensitive precolumn derivatisation reagent for determination of biotin in biological materials (9).

The purpose of the present study was to establish panacyl bromide (-9-anthroyloxy)phenacyl bromide) as a unique precolumn fluorescent labeling reagent of a wide spectrum of free fatty acids prior to HPLC analysis. To demonstrate the applicability of this assay to biological samples, free fatty acid levels in plasma from normal human subjects and diabetic patients were determined.

MATERIALS AND METHODS

Chemicals. Fatty acids were obtained from Sigma (Deisenhofen, FRG). Dibenzo-18-crown-6 (2,3,11,12-dibenzo-1,4-7,10,13,16-hexaoxacyclooctadeca-2,11-dien) and triethylamine (TEA) was obtained from Aldrich (Steinheim, Germany). Panacyl bromide (p-9-anthroyloxy-phenacylbromide) was purchased from Molecular Probes (Oregon). All other chemicals and solvents were purchased from Merck (Darmstadt, F.R.G.). All substances used were of the highest purity (HPLC grade). Water was purified with a Milipore Q system (Waters, Eschborn, F.R.G.).

Development of the precolumn derivatization procedure. Standards as well as sample extracts were dried under a nitrogen stream and dissolved in 100 μl acetone containing 250 nmol panacylbromide and 50 nmol dibenzo-18-crown-6. In all cases 2.5 nmol hexadecanic acid was added as an internal standard. After adding 20-30 mg K_2CO_3 the reaction tubes were closed and transferred to a water bath at either 37°C or 57°C. At certain intervals 20 μl were withdrawn from the tube and analysed by thin-layer chromatography as mentioned above.

Chromatography. Separation of panacylesters was carried out on a Merck-Hitachi HPLC system consisting of a gradient former L-5000, a solvent metering pump 655A-11, a fluorescence detector F-1000 and an AS-2000 automatic sample injector. All analyses were performed at room temperature. We used a 4.6 x 150 mm column filled by the upward slurry technique (10) using 2-propanol for preparing the slurry. For reversed-phase analyses Hypersil ODS 3 μm (Shandon, Frankfurt, Germany) was used as a stationary phase. Gradient elution was performed with water/methanol/acetonitril gradient as follows: solvent A, acetonitril-water(1:1,v/v); solvent B, metanol. The following gradient program was used: isocratic at 90% B and 10% A for 10 min; a linear gradient from initial conditions to 94% B and 6% A in 3 min, followed by isocratic at 94% B and 6% B for 12 min; a linear gradient from preciding conditions to 100% B in 5 min followed by a further isocratic step at 100% B for 10 min. Columns were re-equilibrated to initial conditions for 5 min between each run. Flow rate was 0.8 ml/min at ambient temperature (25°C). The solvent program was started simultaneously with the injection (50 μL).

Detection. Fluorescence maxima of the free fatty panacylesters were determined using a Hitachi F-2000 fluorescence spectrometer. Peaks were recorded at excitation and emission wavelengths of 380 and 470 nm, respectively. Calibration curves were calculated on the basis of peak area using least square regression analysis.

Determination of free fatty acids in human blood plasma. Extraction of free fatty acids from plasma was performed by a column extraction method as described by Ikeda et al. (11). To 0.5 ml of plasma 0.1 ml of a methanolic solution containing $C_{17:0}$ (1 nmol) was added as an internal standard and then mixed with 1.4 ml of 0.1 N PBS buffer (ph 7.4). The mixture was vortexed for 1 min and 1 ml was transferred on an Extrelut-mini column. After absorbing 20 min fatty acids were eluted with 6 ml chloroform. After evaporation, the residue was redissolved in 0.5 ml aceton and the derivation was performed as described above.

Statistical methods. All values given are the mean $\pm$ SD for group of animals. Number (n) used for each variable is given in the tables and figures. Statistical analysis for all experiments was by the one-tailed Student *t* test (7).

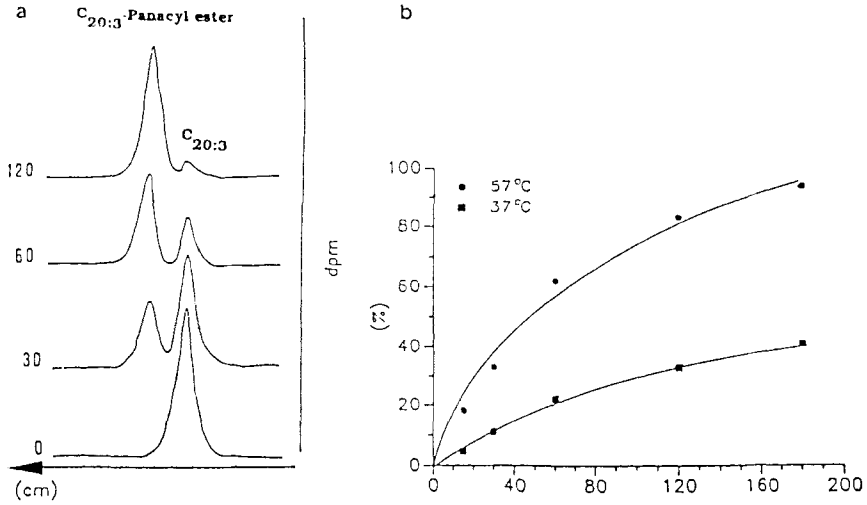

Fig. 1. Conversion of cis-11,14,17-eicosatrienic acid (20:3) to the corresponding panacyl ester as examined by thin layer chromatography. (a) Typical radiochromatograms of cis-11,14,17-eicosatrienic acid $C_{20:3}$ and cis-11,14,17-eicosatrienic acid $C_{20:3}$- panacyl ester obtained at distinct intervals underlining the conversion of cis-11,14,17-eicosatrienic acid (20:3) to its panacyl ester. (b) Time dependent formation of cis-11,14,17-eicosatrienic acid (20:3)-panacyl-ester at 37°C and 57°C.

RESULTS AND DISCUSSION

Derivatization procedure. As can be seen from the radiochromatograms in Fig. 1a, conversion of free fatty acids into the corresponding panacylester is a function of incubation time. The derivatization kinetics (Fig. 1b) emphasize that about 90 % of the free fatty acids could be converted within 3 h at 57°C but efficiency varied from 73 % to 98 %. Nevertheless, when correcting these variations using heptadecanoic acid as an internal standard the technique was highly reproducible. Results of derivatizing the same sample ten times indicated a coefficient of variation of 7.8 %.

Chromatography and detection. As shown on Fig. 2a, reversed-phase HPLC was well suited to achieve complete separation of panacylesters of the

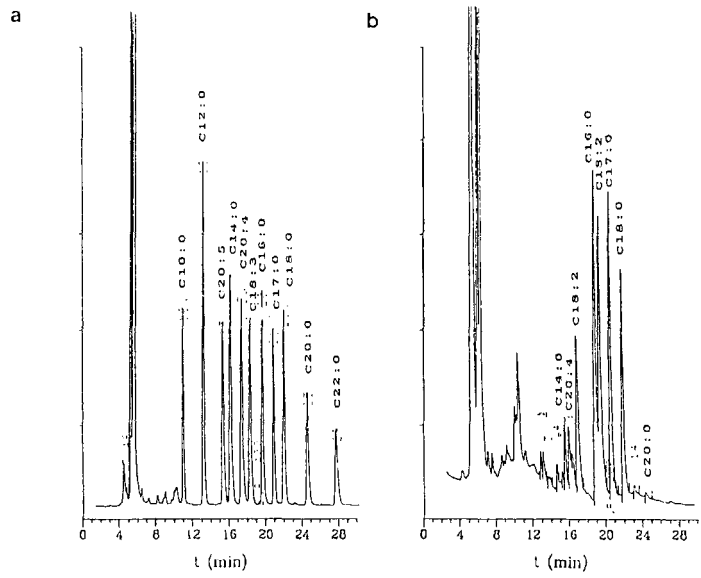

Fig. 2. (a) Chromatographic separation of a select mixture of free fatty acids containg 100 pmol of the panacylesters; (b) A representative chromatogram of plasma free fatty acids obtained from a normal human subject.

series of both saturated and unsaturated fatty acids ($C_{10:0}$-$C_{20:4}$) within 30 min. The fluorescence maxima of both derivatives were found to be 380 nm and 470 nm for excitation and emission wavelength, respectively.

The standard curves of FFAs shows a good linear correlation between peak area ratios and the corresponding standard FFAs over the range from 10 pmol to 500 pmol with r values of 0.993. Reproducibility of duplicate injections was found to be within 3% CV, when the area ratios of the SCFAs:internal standard were calculated. The lower limits of detection were in the range of 10 pmol/ml, when a 50 μl injection was made (signal-to-noise ratio 5:1). This detection limits are in the same range as those observed by other authors for GC (9,11) or HPLC coupled with a conductivity detector (13).

Recovery and Reproducibility. Analytical recovery was estimated by adding a standard mixture of fatty acids to 1 ml of plasma. The resulting

TABLE I

FFA CONCENTRATION IN HUMAN PLASMA DETERMINED BY HPLC

Free fatty acid	normal		diabetic	
	Concentration (mean±S.D.,n=6) (nmol/ml)	%	Concentration (mean±S.D.,n=6) (nmol/ml)	%
$C_{14:0}$	13.2±4.5	7.42	29.4±12.1	2.62
$C_{16:0}$	43.5±9.1	24.41	258.9±63.1	23.1
$C_{16:1}$	11.8±4.5	6.42	20.9±12.1	1.86
$C_{18:0}$	18.2±4.2	10.16	62.8±29.3	5.60
$C_{18:1}$	59.2±16.2	33.22	506.9±111.4	45.2
$C_{18:2}$	30.7±6.8	17.23	239.4±56.4	21.35
$C_{20:4}$	1.7±0.4	0.95	3.1±1.1	0.28
Total	178.2	100.00	1121.4	100.00

recoveries and coefficients of variation were 90 to 102 %, when the area ratios of the SCFAs versus the internal standard were calculated.

The precision of the method was determined for each fatty acid when 20 1000 pmol were injected. The coefficients of variation (C.V.) ranged from 5.9 to 8.6% (intra-assay variability) and from 8.9 to 13.1% (inter-assay variability). For < 10 pmol injected, the C.V. rose to > 20%.

The method was applied to the determination of free fatty acids (2 nmol of each) in human plasma samples. Solid phase extraction was chosen for the pre-column purification. The chromatograms obtained from human sample are shown on Fig. 2b. It is evident that there were few interfering peaks with the free fatty acid derivatives, which were identified by comparison with those of standard derivatives.

The results of derterminations of free fatty acids in plasma samples are summarized in Table I. They are in good agreement with other workers (12,13). The concentrations of certain kind of free fatty acids such as $C_{16:0}$, $C_{18:2}$, $C_{18:1}$, $C_{18:0}$ are significantly increased in diabetic patients.

Problems in free fatty acids analysis mainly arise from the lack of a suitable detection system which permits the quantification of the molecule in the picomolar

range. Therefore, formation of UV-absorbing or fluorescing derivatives is essential when chromatographic procedures are to be used for free fatty acids analysis. Reaction of free fatty acids with panacylbromide leads to an increase in the formation of highly fluorescing esters of these substances and can be used for HPLC determination of free fatty acids in biological materials.

A derivatization reagent for carboxylic functions should have two properties: the derivatives formed should permit sensitive detection and the reaction should be relatively specific for carboxylic funtions. These requirements are fulfilled by several fluorescent labels including panacylbromide. When standard solutions of free fatty acids were analyzed after carrying out the whole clean-up procedure the detection limit was about 10 pmol using reversed-phase HPLC.

A third important factor responsible for adequate derivatization is the reactivity of the labeling reagent in the picomolar range which is necessary for the analysis of biological samples. This was reported to be sufficient with panacylbromide only (16) and could be confirmed during the method development by derivatizing very small amounts of free fatty acids (ca. 25 pmol, Fig. 1).

Formation of free fatty acids esters such as panacyl derivatives may be disturbed by the poor reactivity of the carboxylic function. Reactivity can be enhanced by adding crown ether as a catalyst as first described by Durst et al. (15). Crown ethers complex metal salts i.e. they are also able to form the anionic form of free fatty acids. Furthermore, they contribute to the dissolution of the anionic molecule into an aprotic eluent. Thus the nucleophilic properties of the free fatty acid anions are enhanced and nucleophilic attack of free fatty acids on panacylbromide is facilitated. Furthermore, in contrast to other chemicals used for initiating the reaction such as triethylamine (16), crown ether catalyzed derivatization is not disturbed by traces of water remaining in the sample.

In conclusion, a sensitive HPLC method for free fatty acids was developed by employing panacyl bromide as a precolumn fluorometric labeling reagent. It was demonstrated that high reactivity and high sensitivity is useful for samples containing small amounts of free fatty acids.

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