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DETERMINATION OF FREE FATTY ACIDS AS PENTAFLUOROBENZYL ESTERS BY ELECTRON CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A method for the determination of free fatty acids in minute serum samples has been developed. The acids are esterified by extractive alkylation, using tetrabutylammonium as a counter ion and pentafluorobenzyl bromide as alkylating reagent. The derivatization of palmitic acid required a reaction time of 25 min.

The excess of pentafluorobenzyl bromide is removed by coupling it with a phenolalkylamine and extraction of the product into an acidic aqueous phase. Quantitation is carried out by gas chromatography with electron capture detection.

The precision achieved in the determination of 6.2 μg of palmitic acid in 50 μl of mouse serum was 6.1 % (S.D.).

INTRODUCTION

Free fatty acids in serum are usually determined, after methylation, by gas chromatography (GC) with flame ionization detection. The acids are methylated with diazomethane[1], methanol and sulphuric acid[2], methanol and boron trifluoride[3], by flash methylation[4], with methyl iodide in the presence of potassium carbonate[5,6] and by methanolysis of the imidazolide of the fatty acid[7].

Extractive alkylation is a convenient method for derivatization of acidic organic compounds. The acid is extracted as an anion with a quaternary ammonium ion into an organic phase containing the alkylating reagent. Extractive methylation has been used prior to GC with electron capture detection for the determination of chlorthalidone[8], furosemide[9], hydrochlorthiazide[10], nitrazepam[11], and clonazepam and flunitrazepam[12] in serum samples. The high electron capture response in these instances is due essentially to the contribution from the original molecule. For acidic compounds not exhibiting electron capture properties, an electrophoric group can be introduced by extractive alkylation. After alkylation with pentafluorobenzyl (PFB) bromide, pentazocine[13] and theophylline[14] can be determined in serum by electron capture detection in concentrations down to the ng/ml level. The same derivatization principle has been used in this study for the determination of free fatty acids in minute serum samples.

The excess of alkylating reagent (PFB bromide), however, disturbs the electron capture detector (ECD) and must be removed from the final solution prior to the injection into the gas chromatograph. Several methods for overcoming this difficulty have been proposed. The PFB ether of the aminophenol pentazocine[13] can be separated from the excess of reagent by extraction into an acidic aqueous phase. This principle has been used for the determination of theophylline[14]. The PFB bromide can also be removed by evaporation[15,16] but this may give rise to difficulties caused by its high boiling point (174°). Liquid chromatography has also been used[17].

This paper presents a method for the removal of the excess of the reagent by coupling the PFB bromide with a phenolalkylamine followed by re-extraction of the product into an acidic aqueous phase. A similar procedure has been used by Sumida *et al.*[18] for the removal of excess of 2,4-dinitrofluorobenzene.

EXPERIMENTAL

Gas chromatography

The following apparatus and conditions were used. An Aerograph 600 D and a Varian 1400 gas chromatograph fitted with flame ionization detectors and glass columns (150 × 0.2 cm I.D.), packed with 5% OV-17 on Gas-Chrom Q (80–100 mesh). Column temperature, 220°; nitrogen flow-rate, 30 ml/min.

A Varian 1400 gas chromatograph equipped with a ^{63}Ni detector, operated in the d.c. mode, and a glass column (90 × 0.2 cm I.D.) containing 3% OV-17 on Gas-Chrom Q (80–100 mesh). Column temperature, 195°; detector temperature, 285°; nitrogen flow-rate, 30 ml/min.

A Hewlett-Packard 5710 gas chromatograph equipped with a constant-current ^{63}Ni detector and a glass column (120 × 0.2 cm I.D.), packed with 5% OV-17 on the same support as above. Column oven temperature, 205°; detector temperature, 300°; carrier gas (argon with 5% methane) at a flow-rate of 50 ml/min.

Mass spectrometry

An LKB 9000 instrument with an ionization energy of 70 eV was used. The gas chromatographic separation was performed on a glass column (210 × 0.4 cm I.D.) packed with 6% SE-30 on Chromosorb W (80–100 mesh).

Reagents and chemicals

PFB bromide was obtained from Pierce (Rockford, Ill., U.S.A.) and tetrabutylammonium (TBA) hydrogen sulphate was kindly supplied by Hässle (Mölndal, Sweden). Hordenine sulphate [4-(2-dimethylaminoethyl)phenol sulphate dihydrate] was purchased from EGA-Chemie (Steinheim bei Heidenheim, G.F.R.) and margaric acid (pure) from Koch-Light (Colnbrook, Great Britain). Methylene chloride (analytical-grade quality) and *n*-heptane (Uvasol) were supplied by Merck (Darmstadt, G.F.R.). All other reagents were of analytical grade.

Determination of partition properties

The extraction constants of the TBA ion-pairs of lauric acid and hordenine into methylene chloride were determined according to methods outlined by Gustavii and Schill[19].

The partition coefficients of PFB-hordenine between heptane and water was determined at pH 5.9–6.2 (phosphate buffer, ionic strength = 0.1). Quantitation was carried out by GC using tetracosane as internal standard.

Derivatization with PFB bromide

Palmitic acid and the internal standard (docosan) dissolved in 1 ml of methylene chloride were mixed with 1 ml of 0.1 *M* TBA hydrogen sulphate solution (pH 8 or 10) plus 10 μl of PFB bromide in a centrifuge tube. The mixture was shaken in a mechanical shaker operating at 60 strokes per minute.

The reaction was quenched by the addition of 0.5 ml of 1 *M* sulphuric acid and the tube was shaken for 2 min. A 1-μl volume of the organic phase was then injected into the gas chromatograph equipped with a flame ionization detector. The peak height ratio of the PFB derivative to the internal standard was calculated.

The reaction of hordenine with PFB bromide was studied in the same way. The peak height ratio of PFB bromide to internal standard (mesitylene) was measured.

Determination of electron capture detector response

PFB esters of palmitic, margaric, oleic and linoleic acid were prepared in milligram amounts and their solutions in heptane injected into the chromatograph. The minimum detectable amounts, as defined by Walle and Ehrsson[20], were determined at detector temperatures of 220–370° with 4-chlorobenzophenone as reference.

Determination of palmitic acid in serum

(1) A 10–50-μl volume of serum was acidified with 500 μl of 1 *M* sulphuric acid and mixed in a centrifuge tube with 500 μl of heptane containing 4 μg of margaric acid (internal standard). The tube was shaken mechanically for 15 min and centrifuged at 2000 rpm for 2 min.

(2) The heptane phase was transferred to a fresh tube and extracted with 50 μl of 0.1 *M* sodium hydroxide for 15 min. After centrifugation, the heptane phase was discarded.

(3) A 250-μl volume of 0.1 *M* TBA hydrogen sulphate in 0.2 *M* sodium hydroxide and 250 μl of a 0.066 *M* solution of PFB bromide in methylene chloride were added to the remaining aqueous phase. The tube was then shaken for 25 min.

(4) After addition of 50 μl of 0.5 *M* hordenine sulphate in 1 *M* sodium hydroxide, the tube was shaken for 45 min.

(5) The aqueous phase was discarded and the methylene chloride was gently removed with a stream of nitrogen at about 30°.

(6) A 200-μl volume of heptane and 2.5 ml of 0.1 *M* sulphuric acid were added to the residue, and the tube was shaken for 15 min before centrifugation; 2.0 ml of heptane were added to the tube and 1 μl of the heptane phase was injected into the gas chromatograph with ECD.

RESULTS AND DISCUSSION

The pre-chromatographic procedure

The first step in the method for the determination of palmitic acid in serum is the extraction of the acid, from the strongly acidified plasma, into heptane followed

TABLE I

PARTITION COEFFICIENTS, ACID-DISSOCIATION AND EXTRACTION CONSTANTS

$k_d = [HX]_{org}/[HX]_{aq}$ = partition coefficient; $E_{TBAX} = [TBAX]_{org}/[TBA]_{aq} + [X]_{aq}$ = extraction constant of ion pair between TBA and X^-; ionic strength of buffer solutions = 0.1.

Compound	*Organic phase*	*Aqueous phase*	pK_{HX}	$Log\ k_{d(HX)}$	$Log\ E_{TBAX}$
Palmitic acid	Heptane	(carbonate buffer)	4.8	5.7 (ref. 21)	—
PFB-hordenine	Heptane	phosphate buffer	10	4.4	—
Lauric acid	Methylene chloride	0.1 *M* sodium hydroxide, 2×10^{-4} *M* TBA		—	4.1 (ref. 22)
Palmitic acid	Methylene chloride			—	7.5*
Hordenine	Methylene chloride	0.1 *M* sodium hydroxide, 1×10^{-3} *M* TBA	9.8, 10.0 (ref. 23)	—	0.2 (ref. 22)

* Estimation based on the assumption that log E_{TBAX} changes 0.6 unit per alkyl carbon[24].

by its re-extraction into 0.1 *M* sodium hydroxide. The partition coefficient in Table I indicates that quantitative extractions will be obtained in both instances. It should be noted that Westerlund and Söderqvist[25] by re-extraction from methylene chloride obtained a recovery of about 80% at the actual concentration level (10^{-5} *M*).

The palmitate is extracted into methylene chloride as an ion pair with TBA. The high extraction constant (Table I) indicates that a complete transfer is obtained.

Palmitate is alkylated with PFB bromide in the methylene chloride solution and the remaining PFB bromide is coupled with hordenine, which is transferred to the organic phase as an ion pair with TBA. The extraction constant is rather low (Table I) but the concentration of the hordenine ion pair is sufficient to give a quantitative reaction within 45 min (Fig. 1).

After evaporation of the methylene chloride, the residue is dissolved in heptane and the product formed by the reaction between hordenine and PFB bromide is removed by extraction into sulphuric acid.

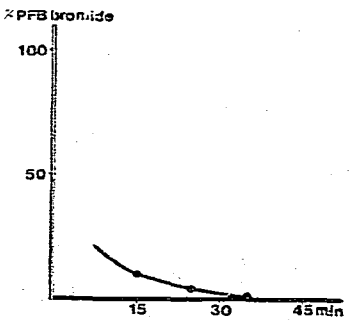

Fig. 1. Disappearance of PFB bromide when caused to react with hordenine. For experimental conditions, see *Derivatization with PFB bromide*. Sample: 0.066 *M* PFB bromide in methylene chloride, TBA (pH 13) (1 ml each) and 200 µl of 0.5 *M* hordenine solution.

Extractive alkylation of palmitic acid with PFB bromide

The extractive alkylation of organic acids has been the subject of investigation[8,26]. The influence of the reaction medium, pH and the nature and concentration of the counter ion and alkylating reagent has been studied to some extent.

The reaction between palmitate and PFB bromide has in this study been followed by GC with flame ionization detection. Before injection of the reaction solution, remaining ion pairs of palmitate were removed by extraction with sulphuric acid in order to prevent further alkylation in the injector of the gas chromatograph.

By extractive alkylation, the pH of the aqueous phase is usually brought to about 13 so as to ensure that the acid is mainly extracted as an ion pair. In this instance a rather wide pH range can be used without significant influence on the time for complete reaction. An illustration is given in Fig. 2, which shows the results obtained with palmitic acid at pH 8 and 13 with 0.1 *M* TBA hydrogen sulphate as aqueous phase.

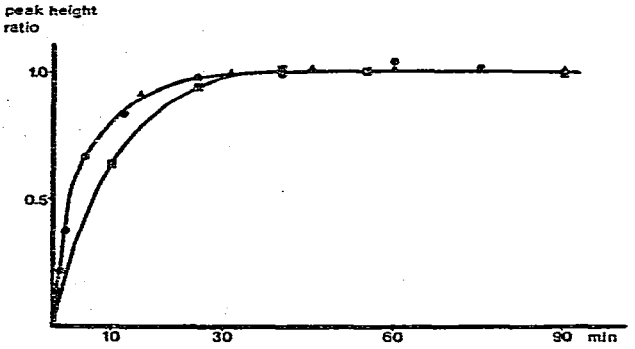

Fig. 2. PFB alkylation of palmitic acid. For experimental conditions, see *Derivatization with PFB bromide*. ▲, Palmitic acid, 4×10^{-3} *M*, and pH of aqueous phase 13; ●, palmitic acid, 1.3×10^{-5} *M*, and pH of aqueous phase 13, ECD; ■, palmitic acid, 4×10^{-3} *M*, and pH of aqueous phase 8

The yield of PFB palmitate was constant at reaction times between 25 min and 3 h, which indicates that no hydrolysis takes place.

The concentration and the chain length of the quaternary ammonium ion will influence the degree of extraction[19,24]. In this event 0.1 *M* TBA hydrogen sulphate will give a quantitative extraction as mentioned above, and no improvement in the results was observed by use of the tetrapentylammonium ion. TBA was preferred as it will give rise to less co-extraction of impurities from the sample.

An increase in the concentration of PFB bromide will decrease the time for quantitative reaction, as illustrated in Fig. 3. A PFB concentration of 0.066 *M* will give complete reaction after 25 min, while more than 70 min are required at a concentration of 0.013 *M*.

It is likely that the alkylation rather than the extraction is the rate-determining step. The yield of PFB palmitate was 93% at a concentration of 10^{-5} *M*, determined with a known amount of pure PFB palmitate as reference.

Identification of PFB derivatives

The PFB esters of the fatty acids and the PFB ether of hordenine were identified by mass spectrometry. All esters gave *m/e* 181 (PFB) as the base peak. The PFB ether of hordenine gave a base peak at *m/e* 58 emanating from *α*-cleavage of the dimethylaminoethyl side chain. The PFB ion was the second in intensity but consti-

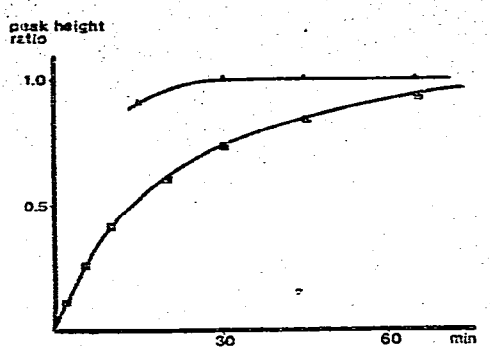

Fig. 3. PFB alkylation of palmitic acid. For experimental conditions, see *Derivatization with PFB bromide*. 0.013 *M* (■) and 0.066 *M* (▲) PFB bromide and pH of aqueous phase 13.

tuted only 8% of the base peak. These data as well as the further fragmentation confirm the identity of the derivatives.

Gas chromatographic separation of PFB esters of fatty acids

The gas chromatographic separation of fatty acids as methyl esters is usually performed on stationary phases of the ester type[1–7,27], and the PFB derivatives of palmitic, stearic, oleic and linoleic acid are separated on the ester phase EGSS-X. This phase could not, however, be used with the electron capture detector owing to a disturbing column bleed.

In the method of determination, OV-17 was used as stationary phase. It is not likely that this phase will separate unsaturated and saturated C_{16} acids.

Electron capture detector response of the PFB esters

The PFB derivatives of acids[15,16,28], phenols[13,17,29,30], primary amines[31] and tertiary amines[32] show high response in the electron capture detector, the minimum detectable amounts being in the order 10^{-16} mole/sec or equivalent to an injected amount of a few picograms.

The PFB esters of the fatty acids give an electron capture detector response of similar magnitude, as illustrated in Table II, and the temperature dependence is slight.

TABLE II

ELECTRON CAPTURE DETECTOR RESPONSE OF PFB ESTERS OF FATTY ACIDS
Apparatus: Hewlett-Packard 5710 A.

Compound	*Retention relative to 4-chlorobenzophenone*	*Minimum detectable amount ($\times 10^{16}$) at detector temperature*		
		220°	*300°*	*370°*
4-Chlorobenzophenone	1.0	11	25	36
PFB ester of				
palmitic acid	4.4	—	1.7	—
margaric acid	6.3	2.0	2.0	3.3
oleic acid	8.8	—	2.7	—
linoleic acid	9.2	2.4	2.6	4.7

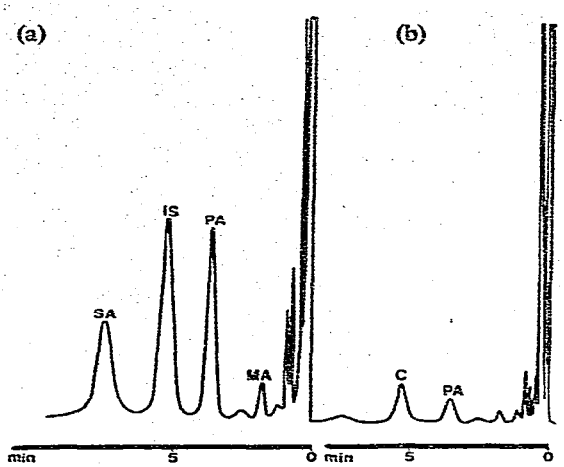

Fig. 4. Determination of fatty acids in serum. For experimental conditions, see *Determination of palmitic acid in serum.* Sample: a, 10 μl of serum from mouse; b, 10 μl of water. Instrument: Hewlett-Packard 5710 A; attenuation setting: 128. MA = myristic acid; PA = palmitic acid; IS = internal standard (margaric acid); SA = stearic acid; C = contaminant from hordenine.

Application of method for determination of fatty acids to biological samples

An analysis of 10 μl of mouse serum according to the method of determination is given in Fig. 4a. Margaric acid (heptadecanoic acid), which is normally present in insignificant amounts in serum, was used as internal standard. A standard curve for palmitic acid is shown in Fig. 5, and was prepared by use of solutions of palmitic acid in heptane (step 1 of the method is omitted).

A blank determination carried out with distilled water as sample gave chromatograms with peaks that had the same retention times as the PFB esters of fatty acids, an illustration of which is given in Fig. 4b. Careful washing of all glass equipment, distillation of the solvents and purification of other reagents did not reduce this

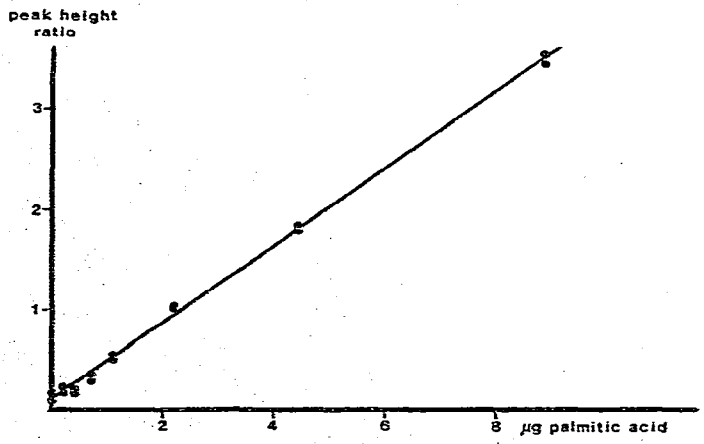

Fig. 5. Standard curve for the determination of palmitic acid. For experimental conditions, see *Determination of palmitic acid in serum.* Instrument: Hewlett-Packard 5710 A. Palmitic acid dissolved in heptane (no serum present). Internal standard: margaric acid, 4 μg.

background level. The disturbing peaks appeared only after alkylation with PFB bromide, which indicates that they represent free fatty acids. The background level in different samples varied in relation to the internal standard, but the relative concentrations of the acids was fairly constant. The background level of palmitic acid corresponded to about 200 ng per sample. Free fatty acid blanks have been observed previously[3,7].

After treatment with hordenine, a disturbing peak appeared in the chromatogram at a position where it interfered with that of the internal standard. Purification of hordenine by batch extraction did not, however, remove the disturbing component. It is therefore necessary to use an accurately known volume of the hordenine solution if high precision is required.

Determination of palmitic acid in six samples of 50 μl of mouse serum gave a standard deviation of 6.1 % and a level of 6.2 μg.

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