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Note

High-performance liquid chromatographic separation of esters of 4-hydroxymethyl-7-methoxy-coumarin

A method for the determination of acidic compounds in the picomole range

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4-Bromomethyl-7-methoxy-coumarin (Br-Mmc) has been shown to form fluorescent esters with monocarboxylic acids [1,2] and a variety of other acidic compounds of biomedical interest [3]. Although carboxylic acids are readily measured by gas—liquid chromatographic methods [4], a sensitive high-performance liquid chromatographic (HPLC) method may nevertheless find certain applications. In this paper, methods are described for the separation of a series of common fatty acids and of a barbiturate in blood as examples for the application and the limitations of the method.

MATERIALS AND METHODS

Usual laboratory chemicals were of analytical grade. They were purchased from E. Merck (Darmstadt, G.F.R.). Br-Mmc was from Regis (Morton Grove, Ill., U.S.A.). The micro-refluxer [5] and appropriate glassware can be obtained from Regis, or from the Forschungsinstitut Berghof (Tübingen, G.F.R.).

Derivative formation

This was performed by refluxing acetone solutions of the reaction components in the presence of crystalline water-free K_2CO_3 either with [1] or without crown ether as catalyst [2]. Extraction of the barbiturate from small blood samples was performed according to a published procedure [6].

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HPLC Equipment

A Varian 8500 high pressure liquid chromatograph was used in combination with a Perkin-Elmer fluorescence spectrophotometer Model 204 and a Perkin-Elmer spectrophotometer LC55 as detectors. Separations were achieved with methanol—water gradients, using a 25 cm long column with C_{18}-brushes on a 10-μm silica core (Nucleosil 10C-18; Macherey, Nagel & Co., Düren, G.F.R.). (For details of the separation procedure see legends to the figures).

RESULTS

Using a linear water—methanol gradient, 50—100% (Δ = 1% methanol/min) a mixture of the Mmc-esters of the saturated unbranched aliphatic fatty acids between formic and stearic acid can be completely separated within 60 min. (Fig. 1A). Starting with 40% of methanol, a steeper methanol gradient (Δ =

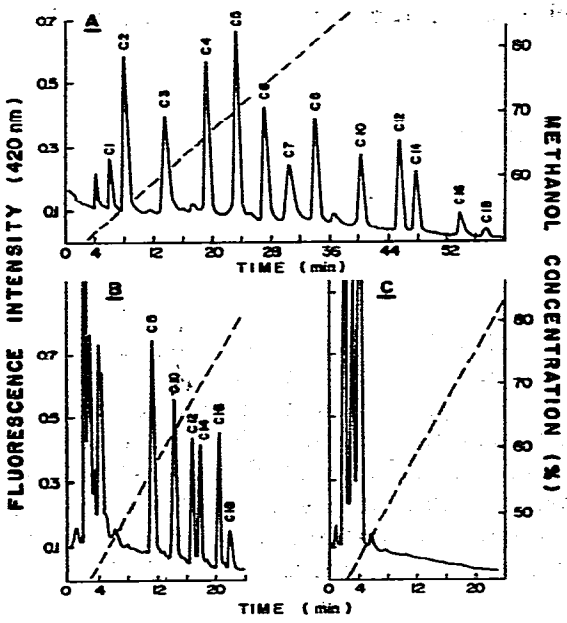

Fig. 1. HPLC separation of Mmc-esters of aliphatic, saturated fatty acids. Equipment: Varian 8500 high-pressure liquid chromatograph; Column: 250 × 2 mm I.D., Nucleosil 10 C_{18}, Macherey Nagel & Co. Detector: Perkin-Elmer fluoroscence spectrophotometer Model 204, with 10-μl flow-cell. Fluorescence activation at 340 nm and fluorescence emission measurement at 420 nm. (A) Mixture of authentic Mmc-esters (about 0.2 nmoles) of formic acid (C_1), acetic acid (C_2) propionic acid (C_3), *n*-butyric acid (C_4), *n*-valeric acid (C_5), caproic acid (C_6), caprylic acid (C_8), capric acid (C_{10}), lauric acid (C_{12}), myristic acid (C_{14}) palmitic acid (C_{16}) and stearic acid (C_{18}). Separation conditions: 3 min elution with 50% methanol, then linear increase of methanol: (Δ = 1%/min). Flow-rate 1 ml/min. (B) Separation of a reaction mixture of fatty acids with Br-Mmc. 10 μl of the solution contained caprylic acid (C_8; 0.16 nmole), capric acid (C_{10}; 0.14 nmole), lauric acid (C_{12}; 0.12 nmole); myristic acid (C_{14}; 0.12 nmole); palmitic acid (C_{16}; 0.17 nmole) and stearic acid (C_{18}; 0.12 nmole). Separation conditions: 3 min elution with 40% methanol, then linear increase of methanol (Δ= 2%/min). Flow-rate 1 ml/min. (C) Blank reaction mixture, containing only reagents, but no fatty acids. Separation conditions as in (B).

2% methanol/min) is still sufficient to separate within about 30 min all homologues up to C_{12} and the even-numbered members of the series up to C_{18}. Higher homologues of the saturated aliphatic fatty acids have not been included in these experiments. Side products of the derivative-forming reaction normally interfere under these conditions with the separation of the first four members of the series so that only the fatty acids with $C_{n>5}$ are separable by immediate application of an aliquot of the reaction mixture to the reversed-phase column. It should be pointed out, moreover, that Br-Mmc, the derivative-forming reagent, elutes in this region as well. Although it is non-fluorescent, it causes fluorescence-quenching, due to its relatively high concentration in the reaction mixture. Fig. 1 B shows the separation of caprylic, capric, lauric, myristic, palmitic and stearic acids, which were reacted with Br-Mmc in 0.275 nmole amounts. One fifth of the reaction mixture was applied immediately to the column. It is obvious from this figure, and it was confirmed by numerous repetitions of the reaction with amounts of fatty acids varying between 60—275 pmoles, that the peak areas of the Mmc-derivatives of the homologous fatty acids, as recorded by fluorescence were not identical, however, they were readily reproducible. Even peak height measurements gave satisfactory quantitative results, if the time of column equilibration with 40% methanol was kept constant at 10 min.

TABLE I

RELATIONSHIPS BETWEEN AREAS OF THE RECORDED PEAKS, SUBSTANCE AMOUNT AND LENGTH OF THE CARBON CHAIN OF THE ALIPHATIC FATTY ACIDS

(For details of the separation see legend to Fig. 1). The figures in the table are the mean values ± S.D. (peak height × width at half height) of four measurements of four reactions. The amounts of the fatty acids refer to the amount present in the total reaction mixture.

Amount of fatty acid (pmole)	Carbon chain length					
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}
60	72 ± 1	63 ± 15	45 ± 5	42 ± 9	47 ± 9	18 ± 4
180	201 ± 22	157 ± 16	123 ± 9	112 ± 9	131 ± 6	49 ± 5
200	231 ± 17	173 ± 15	138 ± 10	128 ± 7	144 ± 9	53 ± 5
275	324 ± 13	252 ± 14	199 ± 11	187 ± 10	205 ± 14	76 ± 1

As can be derived from the data of Table I, mean standard deviation in the range of 60—300 pmole was less than ± 10%. The recorded peak areas were directly proportional to the amounts of fatty acids in the reaction mixtures. Fig. 2 shows this for caprylic and palmitic acid. Fluorescence quantum yields of the Mmc-esters have not yet been established. The reasons for the differences in peak areas, starting derivatization with equimolar amounts of the acids are, therefore, not known.

Another example of application of the method is the determination of a

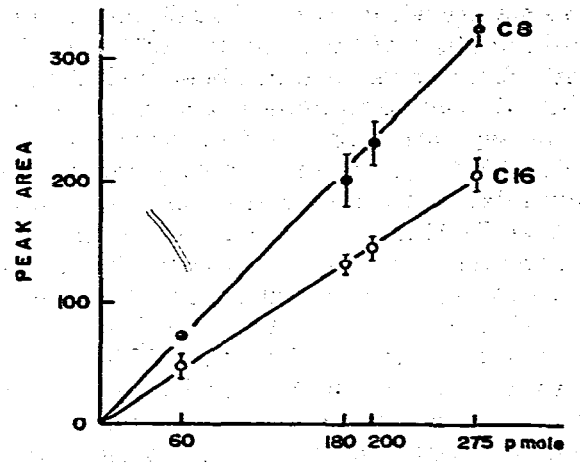

Fig. 2. Standard graph for Mmc-caprylate (C_8) and Mmc-palmitate in the range of 60—275 pmoles; (Table I).

barbiturate in blood. Since details of the extraction and derivatization with Br-Mmc, and thin-layer chromatographic separation have been described [6] only the HPLC-separation of the extract of a 40-μl blood sample containing 2 nmoles of barbital is shown (Fig. 3). The separation of a blood extract free of barbital (blank sample) shows that blood constituents do not interfere with barbital separation. Absorbance recorded for the same sample shows the higher sensitivity of the fluorescence measurement. Preliminary results with HPLC separations of the Mmc-derivatives of some prostaglandins are promising. Due to its high affinity for the reversed-phase used in the present work, Mmc-arachidonic acid was eluted only with methanol, well behind palmitic acid. Its separation from other fatty acids by this method should render a simple and sensitive procedure for its estimation.

DISCUSSION

Mmc-esters are the first fluorescent derivatives suited for liquid chromatographic determination of acidic compounds [7, 8]. Although their properties allow thin-layer chromatographic separation, a system which avoids exposure to light on an active surface is preferable, especially since Br-Mmc is rapidly decomposed under these conditions to fluorescent products. HPLC was therefore the method of choice. It is shown in the present work that a simple, rapid, reproducible separation system is suitable for the estimation of Mmc-derivatives. Since the derivatization reaction can be scaled down to a reaction volume of 5 μl it is possible to determine a few picomoles of the derivatized compounds. This range of sensitivity is comparable with advanced gas—liquid chromatographic methods; it is considerably higher than the post column reaction with Ce(IV) in combination with UV absorptimetry or fluorimetry [8]. The strongly absorbing *p*-bromo-phenacyl esters [9, 10] and the *p*-nitrophenacyl esters [11, 12] are detectable in 0.1 nmole amounts. Dansyl derivatives of barbiturates can be estimated at a comparable level of sensitivity as their Mmc-

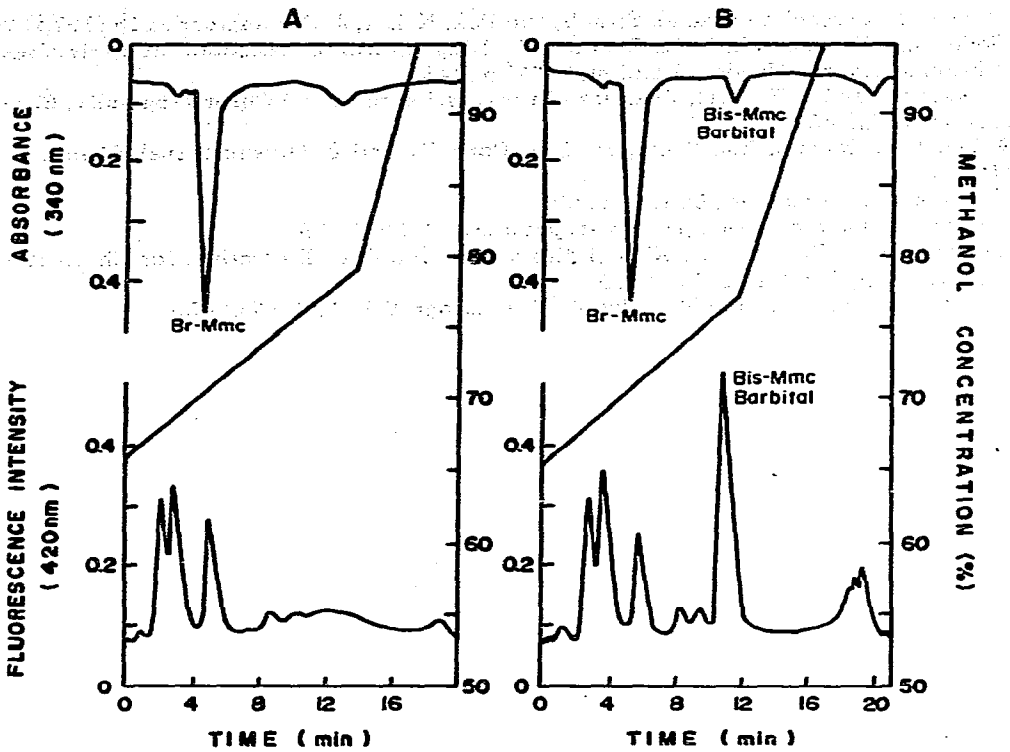

Fig. 3. Determination of barbital in a 40-μl blood sample, by extraction and derivatization with Br-Mmc. (A) Blood sample without barbital (blank); (B) blood sample with 2 nmoles barbital. The reaction volume was 5 μl and 2 μl of the reaction mixture was applied to HPLC separation. Separation conditions: linear water—methanol gradient, starting with 65% methanol (Δ 2%/min); flow-rate 1 ml/min. After the appearance of the bis-Mmc-barbital peak (13 min) the methanol concentration is rapidly increased, in order to wash out impurities from the column. (See also legend to Fig. 1 and Materials and methods).

derivatives [13]. It should be mentioned that UV detectors are well suited to monitor (at 340 nm) Mmc-derivatives. The sensitivity, as compared with the fluorometric method, is lower by about one order of magnitude, if a flow cell of 10 mm path length is used (Fig. 3).

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