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Note

Derivatization of fatty acids with 1-chlormethylisatin for high-performance liquid chromatography

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The determination of fatty acids by high-performance liquid chromatography (HPLC), using a UV detector, is not practicable without prior derivatization because of their low UV absorption. The application of derivatization methods to HPLC analysis has been used widely for detection enhancement and also for improvement of chromatographic properties[1–8].

A new reagent for the derivatization of carboxylic acids with a high reactivity, 1-chlormethylisatin (CMI), has recently been described[9,10] and applied to quantitative thin-layer chromatography[11]. Crown ethers were used as catalysts to shorten the reaction time.

This paper describes the application of this derivatization method to the separation and determination of the isatinylmethyl esters (IM esters) of fatty acids by HPLC.

EXPERIMENTAL

Apparatus

A Perkin-Elmer liquid chromatograph Model Series 2, equipped with a LC 55 UV detector and a Perkin-Elmer recorder 023, was used. Peak areas were calculated with a Perkin-Elmer Minigrator 2.

Separations were carried out on Hibar LiChrosorb Si 60 and Hibar RP-8 (particle size 10 μm) columns (25 cm × 4 mm I.D.) (Merck, Darmstadt, G.F.R.).

Reagents

All solvents and chemicals were of analytical grade (Merck) Dimethylformamide (DMF) was dried over a molecular sieve and distilled. CMI was synthesized as previously described[9]. The reference esters were prepared as described in ref. 10.

Derivatization procedures

Method A. 20 nmol to 2 μmol of a carboxylic acid were dissolved in a 1-ml conical vial together with a 10-fold molar excess of CMI and a 5-fold molar amount of dibenzo-18-crown-6 in dry DMF. A 50-fold amount of finely powdered $KHCO_3$ was then added. The final volume was 50 μl. The reaction mixture was heated at 50° for 10 min. After cooling, 100 μl of water were added to convert excess CMI into

hydroxymethylisatin (HMI). The solution was then extracted with 100 μl of chloroform and an aliquot of 20 μl was injected onto the column. (If necessary, the chloroform extract can be concentrated or diluted to suitable volumes.)

Method B. A 10-fold molar excess of triethylamine was used as catalyst instead of $KHCO_3$–crown ether. After heating at 50° for 45 min, the reaction mixture was treated as described in method A.

RESULTS AND DISCUSSION

The reaction scheme is shown in Fig. 1. The structure of the derivatives has been confirmed by nuclear magnetic resonance and mass spectrometry[11]. Several catalysts were tested. Crown ether–$KHCO_3$ shows some advantage over triethylamine (Fig. 2). Under mild reaction conditions palmitic acid reacts quantitatively with CMI under crown ether catalysis in less than 10 min. Method A is suitable for the determination of small amounts of carboxylic acids. Smaller amounts of by-products are formed because of the short reaction time. Method B can be used for the preparation of greater amounts of derivatives.

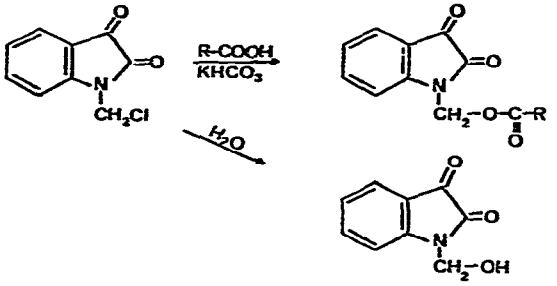

Fig. 1. Scheme of the reaction of CMI with carboxylic acids.

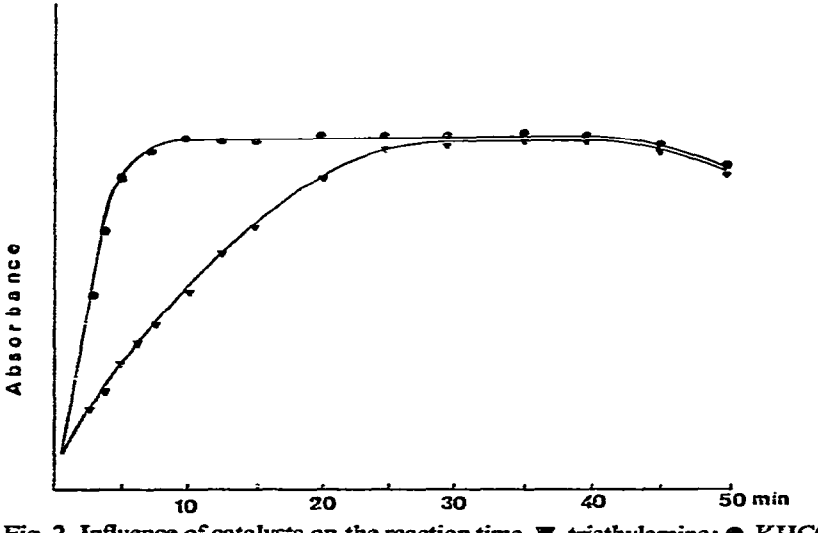

Fig. 2. Influence of catalysts on the reaction time. ▼, triethylamine; ●, $KHCO_3$–dibenzo-18-crown-6.

Excess CMI is converted into HMI by treatment of the reaction mixture with water. Extraction with chloroform is necessary because derivatives of higher molecular weight fatty acids are insoluble in the reaction mixture. The extraction, however, is quantitative, as comparative experiments with authentic samples have shown. Furthermore, the extraction results in a cleaning up process and an increase in sensitivity, if the extract is concentrated.

The derivatives were separated on silica gel and reversed-phase columns: Fig. 3 shows the separation of caproic, caprylic and capric acid derivatives. However, better separation of the isatinylmethyl esters was obtained on RP-8 columns. Using a linear water–methanol gradient (1% methanol/min), starting with 50% methanol, complete separation of 13 saturated aliphatic fatty acids between C_1 and C_{18} was achieved in less than 1 h (Fig. 4). All isatinylmethyl esters are well separated from HMI. By-products formed by the decomposition of CMI interfere only with the

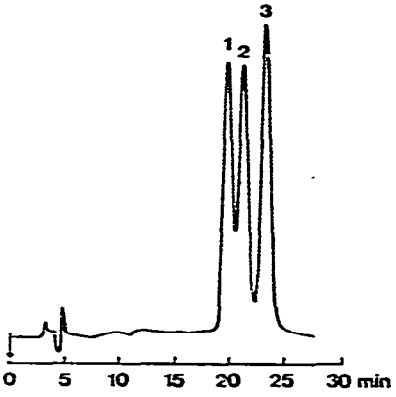

Fig. 3. Separation of three IM esters. Column, Hibar LiChrosorb Si 60, 25 cm × 4 mm I.D.; mobile phase, hexane–chloroform (80:20); flow-rate, 0.5 ml/min; detection wavelength, 240 nm. 1 = Caproic acid; 2 = caprylic acid; 3 = capric acid.

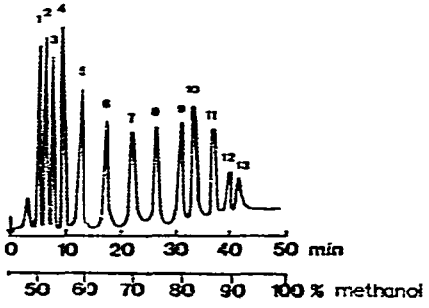

Fig. 4. Mixture of authentic IM esters. Column, Hibar RP-8, 25 cm × 4 mm I.D.; mobile phase, methanol–water gradient, starting with 50% methanol, 5 min isocratic, then linear increase of methanol (1%/min). Flow-rate, 1 ml/min; detection wavelength, 240 nm. 1 = Formic acid; 2 = acetic acid; 3 = propionic acid; 4 = *n*-butyric acid; 5 = *n*-valeric acid; 6 = caproic acid; 7 = heptanoic acid; 8 = caprylic acid; 9 = capric acid; 10 = lauric acid; 11 = myristic acid; 12 = palmitic acid; 13 = stearic acid.

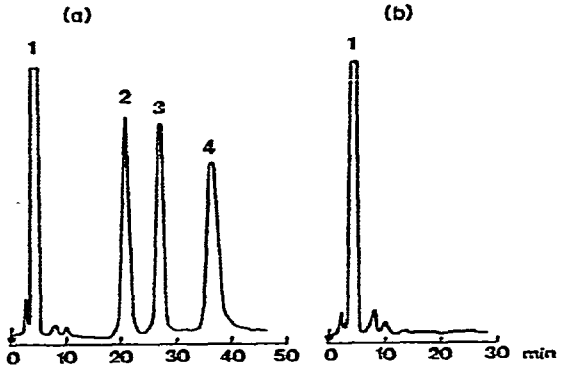

Fig. 5. (a) Separation of a mixture of unsaturated acids after reaction with CMI. Column, Hibar RP-8, 25 cm × 4 mm I.D.; mobile phase, methanol–water (70:30), isocratic; flow-rate, 1 ml/min; detection wavelength, 240 nm. 1 = 1-Hydroxymethylisatin; 2 = linolenic acid; 3 = linoleic acid; 4 = Oleic acid. (b) Blank reaction mixture.

quantitative determination of small amounts of short-chain acids up to C_4. Fig. 5 shows the separation of some unsaturated fatty acids under isocratic conditions. The calibration curves show a good linearity ($r = 0.996$–0.999). The relative standard deviation determined by reaction of 200 nmol of palmitic acid ($n = 6$) was 1.8%. If the reaction volume is reduced to 10 μl the derivatization can be carried out at the nanomole level. According to the high molar absorptivity of the esters (13,000–15,000 l mole^{-1} cm^{-1} in methanol) the detection limits are between 1 and 10 ng.

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