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Derivatization and gas chromatographic determination of hydroxycarboxylic acids treated with chloroformates[a]

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ABSTRACT

Hydroxymonocarboxylic acids with a hydroxyl group in position 2, 3 or 4 can be converted into derivatives amenable to capillary gas chromatography by treatment with alkyl chloroformates instantaneously. The carboxylic group is esterified to the corresponding alkyl ester, and the hydroxyl group adjacent to the carboxylic group is converted to the alkoxycarbonyl ether. Hydroxyl groups in other positions remain free. The yield of 2-hydroxyl group alkylation proved to be strongly influenced by the presence of the corresponding alkyl alcohol in the reaction medium and optimization of the reaction conditions was necessary.

INTRODUCTION

Hydroxymonocarboxylic acids (HA) and dicarboxylic acids are the predominant excretion products among the urinary aliphatic organic acids. They originate mainly from ketogenesis and from the metabolism of branched-chain aliphatic amino acids [1] and are mostly determined together with other urinary organic acids to scan metabolic profiling. Because of the polyfunctional nature of the pattern, reagents of general use are required to prepare the sample for high-resolution capillary gas chromatographic (cGC) analysis. Almost exclusively silylating reagents are the choice, as they are able to convert groups with active hydrogen into trimethylsilyl esters, ethers and amides at room or elevated temperature in 30–60 min [2–5]. This chemical approach is preferred even for the treatment of HA as a group or individual components [6–9]. As an alternative, methylation of the acids with diazomethane is carried out [1,10,11], which is easy to perform but results in various problems such as double peaks and side-product formation. Two-step procedures, using an alcohol (or diazomethane) for esterification of the carboxylic groups and another reagent for treatment of the hydroxyl group, have been elaborated, *e.g.*, for analysis of optical isomers in form of isopropylurethane isopropyl esters [12] or for purposes of electron-capture detection after converting HA into pentafluorobenzoyl [13] or heptafluorobutyryl methyl esters [14].

[a] Dedicated to Luboslav Stárka, Head of the Institute of Endocrinology, on the occasion of his 60th birthday.

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High-performance liquid chromatography (HPLC) has also been applied to the determination of HA [15], involving precolumn derivatization of the carboxylic group with 2-nitrophenylhydrazine to form a highly absorbing compound, with a detection limit down to picomoles in the visible range of the spectrum. This procedure was used successfully for monitoring HA and dicarboxylic acids in urine samples after two-step extraction of the derivatives with diethyl ether. The derivatization step, however, required heating of the sample at 60°C for 30 min so that no time gain in comparison with silylation procedure and cGC, which gives a more efficient separation, was achieved.

In this work we examined the possibility of whether the reaction conditions used for the esterification of carboxylic groups with chloroformates [16] would be suitable even for the derivatization of HA. It was found that optimization of the reaction conditions was a complex problem, as the derivatization of the hydroxyl group was far from smooth, being markedly influenced by the necessary presence of alcohol in the reaction medium. The results of this study show that carboxylic acids with hydroxyl groups in the aliphatic chain can be determined satisfactorily and instantaneously by cGC after a chloroformate treatment.

EXPERIMENTAL

Apparatus

A Hewlett-Packard HP 5890 gas chromatograph with a flame ionization detector and a Model 3392A integrator was employed. The injector and detector temperatures were 200 and 250°C, respectively. The analysis was carried out on a 10 m × 0.25 mm I.D. CP-Sil 19 CB fused-silica capillary column with a 0.2-μm thick layer of OV-1701 (Chrompack, Middelburg, Netherlands) in the temperature range 50–200°C programmed at 15°C/min. Helium was used as the carrier gas with a head pressure of 70 kPa.

Chemicals

Methyl and ethyl chloroformate (MCF, ECF) and the organic solvents, *i.e.*, pyridine, acetonitrile, chloroform, methanol and ethanol, were obtained from Fluka (Buchs, Switzerland). HA were obtained from Sigma (St. Louis, MO, USA) and an equimolar mixture of (1) 2-hydroxyacetic (glycolic), (2) 2-hydroxypropionic (lactic), (3) 2-hydroxybutyric (HB), (4) 2-hydroxyisovaleric (HIV), (5) 2-hydroxyvaleric (HV), (6) 2-hydroxyisocaproic (HIC), (7) 2-hydroxycaproic (HC), (8) 3-hydroxybutyric (3-HB) and (9) 4-hydroxybutyric acid (4-HB) was prepared in water (25 μmol/ml).

Procedures

A 2-μl volume of the aqueous HA solution was covered with 100 μl of a medium composed of acetonitrile, alcohol (methanol or ethanol), water and pyridine in various proportions and 5 μl of the corresponding chloroformate were added. Subsequently, 100 μl of chloroform were added and, after brief shaking the tube by striking it against a pad for about 5 s, an aliquot of the chloroform layer was injected into the capillary column.

Optimum compositions of the reaction media were as follows: (a) for the formation of O-methoxycarbonyl (MOC) methyl esters, acetonitrile–pyridine–methanol

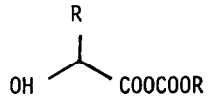
(22:2:1 or, in the presence of 10% of water, 7:1:1), 100 μl of solvent treated with 5 μl of MCF; (b) for the formation of O-ethoxycarbonyl (EOC) ethyl esters, acetonitrile–ethanol–water–pyridine (5:2:2:1 or 4:3:2:1), 100 μl of solvent treated with 7 μl of ECF; and (c) for the formation of O-MOC (EOC) ethers MOC (EOC) esters ("mixed anhydrides"), acetone–acetonitrile–water (6:3:1, containing 4% of pyridine in), 100 μl of solvent treated with 5 μl of the corresponding chloroformate.

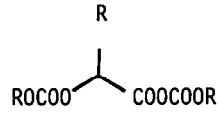
RESULTS AND DISCUSSION

Earlier studies with amino acids [17] and biogenic amines treated with chloroformates [18] and our recent examinations with the same kind of compounds [19–20] revealed that hydroxyl groups not neighbouring the carboxylic group are not derivatized by the action of the reagents mentioned. In a preceding paper [16], conditions for the rapid esterification of carboxylic groups with alkyl chloroformates were given. In view of these findings, the treatment of HA with this kind of reagent can represent a complex problem.

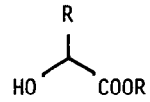
Our first experiments with the derivatization of HA under conditions close to those for carboxylic acids showed that four possible derivatives with 2-hydroxy acids are formed:

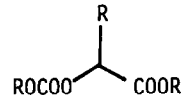
Alkoxycarbonyl esters:





alkyl esters:





Because with a well deactivated capillary column all of the presented forms can be eluted, one will obtain a blend of peaks and a complicated baseline.

The aim of the subsequent experiments was to find reaction conditions under which the formation of esterified alkyl esters, the prevailing and desired form, is promoted.

The experiments revealed that the presence of an alcohol in the reaction medium, necessary for esterification of the carboxylic group, prevented the effective esterification of the 2-hydroxyl group. This is apparent from Fig. 1, where the treatment with chloroformates was done in alcohol alone. The amount of side-products with the shortest retention, *i.e.*, alkyl esters with a free hydroxyl group, is highest under these conditions and higher with methanol than with ethanol. With ethanol, on the other hand, the decarboxylation of the alkoxycarbonyl esters (mixed anhydrides) does not proceed smoothly and this results in a complicated baseline at the rear of the chromatogram.

In a second series of experiments, the alcohol was partially replaced with acetonitrile, water or a mixture of the two (Fig. 2). Comparing the chromatograms, it can be seen that the methanol–MCF and ethanol–ECF systems behave differently. In the former system the use of water in combination with methanol is clearly disadvanta-

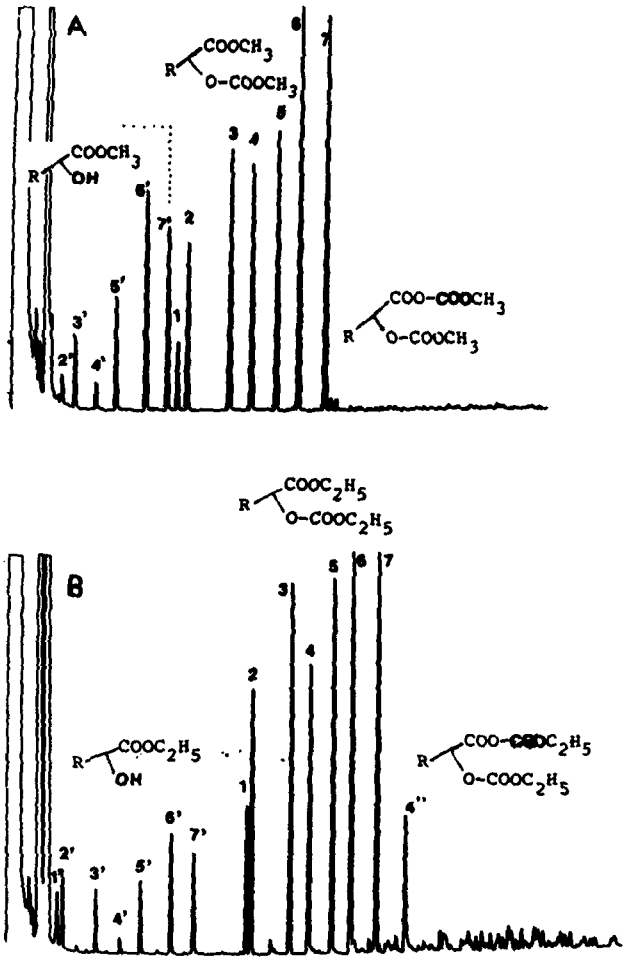


Fig. 1. HA 1–7 analysed after treatment with (A) MCF in methanol and (B) ECF in ethanol in the presence of 8% (v/v) of pyridine in the medium. Primed numbers represent side-products with a free hydroxyl group and the double-primed number (and baseline impurities) are non-decarboxylated side-products, *i.e.*, alkoxycarbonyl esters.

geous: the amount of the volatile side-products does not decline (dotted line) and the dashed line shows a decrease in yield of straight-chain HA in comparison with the branched HIV acid, which reacts differently to the others. The same phenomenon, *i.e.*, decrease in the yield of unbranched HA, can be observed to a lesser extent with ECF in the ethanol–water system; however, the difference between water and acetonitrile is not as pronounced as in the former instance; this concerns even the formation of the side-products with a free hydroxyl group.

In order to suppress the formation of products with a free 2-hydroxyl group, it was clear that the amount of alcohol (methanol more than ethanol) should be held as low as possible. Moreover, in accordance with the previous findings [17–20], hydroxyl

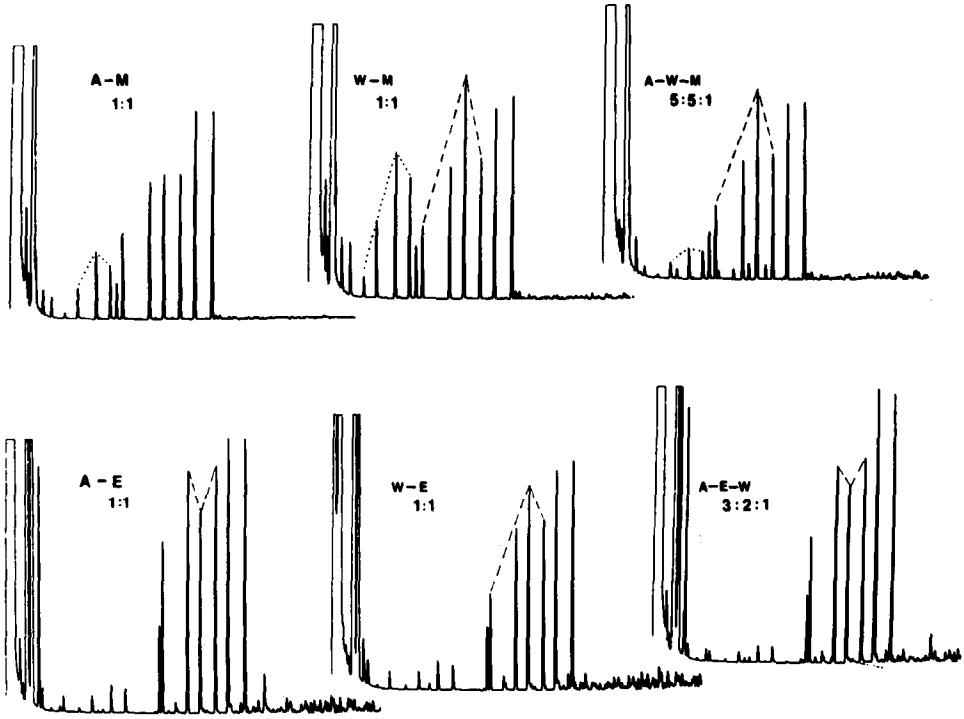


Fig. 2. Influence of composition of the reaction medium on the derivatization of HA 1–7 with (top) MCF and (bottom) ECF. Pyridine (P) (8 vol.%) was added to each medium. A = Acetonitrile; M = methanol; E = ethanol; W = water. Side-products with a free hydroxyl group are marked with the dotted line and the decline of unbranched HA in comparison with HIV acid is marked by the dashed line.

groups in positions 3 and 4 are not derivatized, *i.e.*, 3-HB and 4-HB acids are eluted first and do not form side products. They were therefore used as "internal standards" in a further study dealing with influence of water on the derivatization yields (Figs. 3 and 4).

From both figures it appears that an increase in the water content in the medium has a deteriorating effect on the yield of straight-chain HA; the decline is marked by the dotted line and even by the dashed line when compared with HIV acid. At the same time, the degradation of the baseline, *i.e.*, the amount of non-decarboxylated products, increases. The composition of the medium designated D is close to that used for esterification of fatty acids under aqueous conditions [16]; being optimum for fatty acids is far from optimum for HA. In all these studies, 100 μl of the medium (containing 8% pyridine) were treated with 5 μl of MCF or ECF.

Finally, as a result of numerous experiments, the most favourable reaction conditions were found under which the formation of O-alkoxycarbonyl alkyl esters is promoted and the amount of side-products is suppressed. The optimum reaction conditions are given under Experimental and the corresponding chromatograms are shown in Fig. 5. With MCF, exclusion of water from the medium afforded the best results, but a water content up to 10 vol% was found to be acceptable. With ECF the

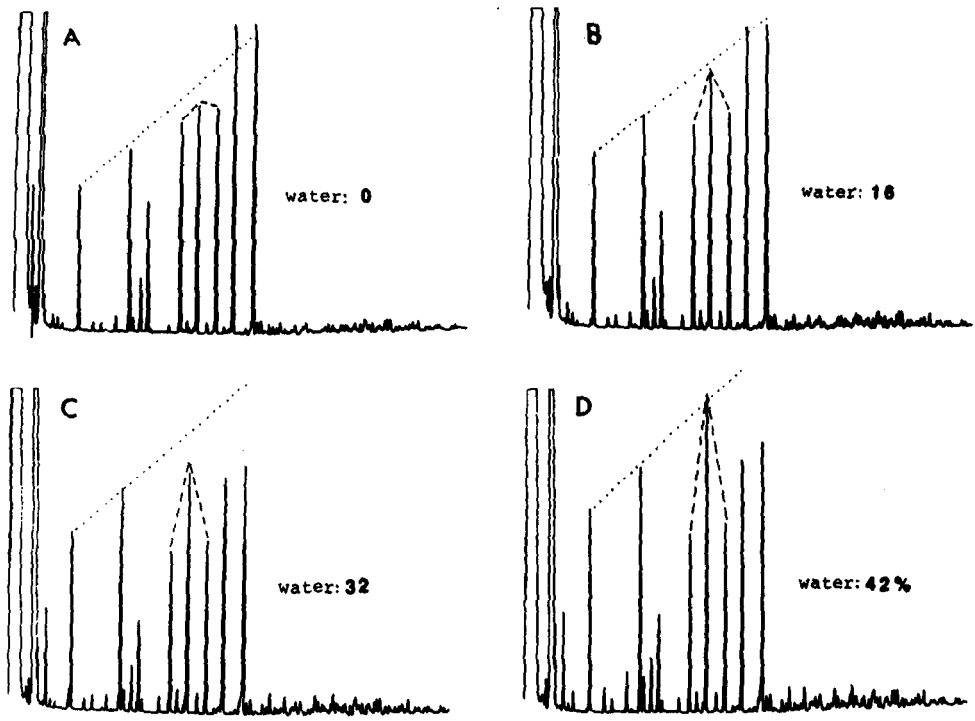


Fig. 3. Influence of the presence of water in the reaction medium on HA 1–9 derivative formation with MCF. Acetonitrile in the reaction medium (with 8% of pyridine and 4% of methanol) was partially replaced with water as indicated.

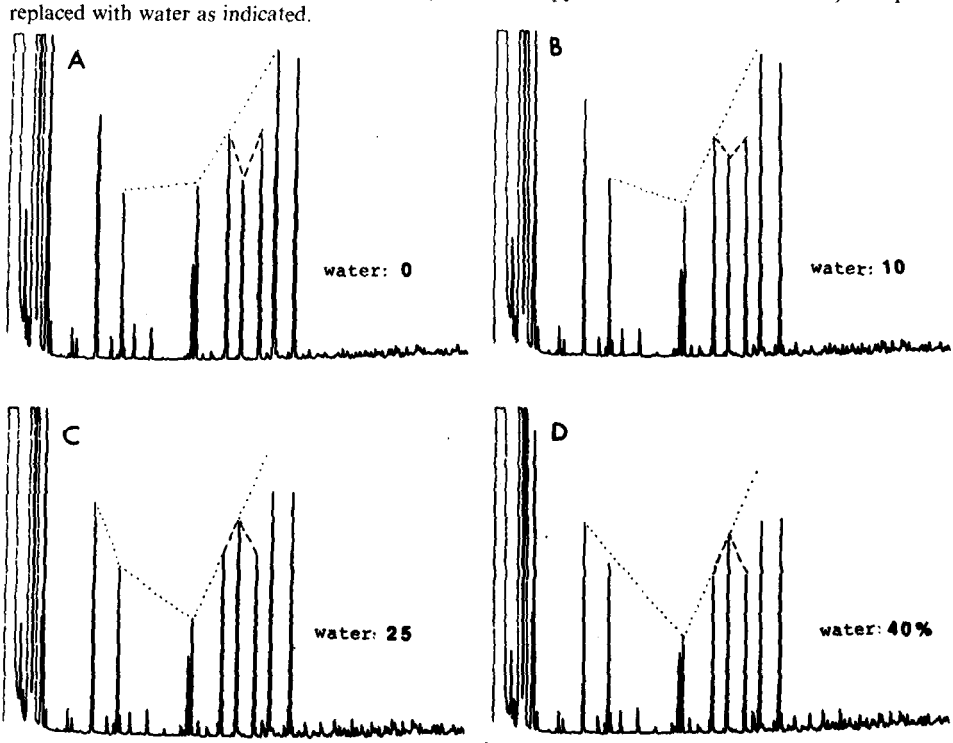


Fig. 4. Influence of the presence of water in the reaction medium on the HA 1–9 derivative formation with ECF. Acetonitrile in the reaction medium (1:1 with ethanol and containing 8% of pyridine) was partially replaced with water as indicated.

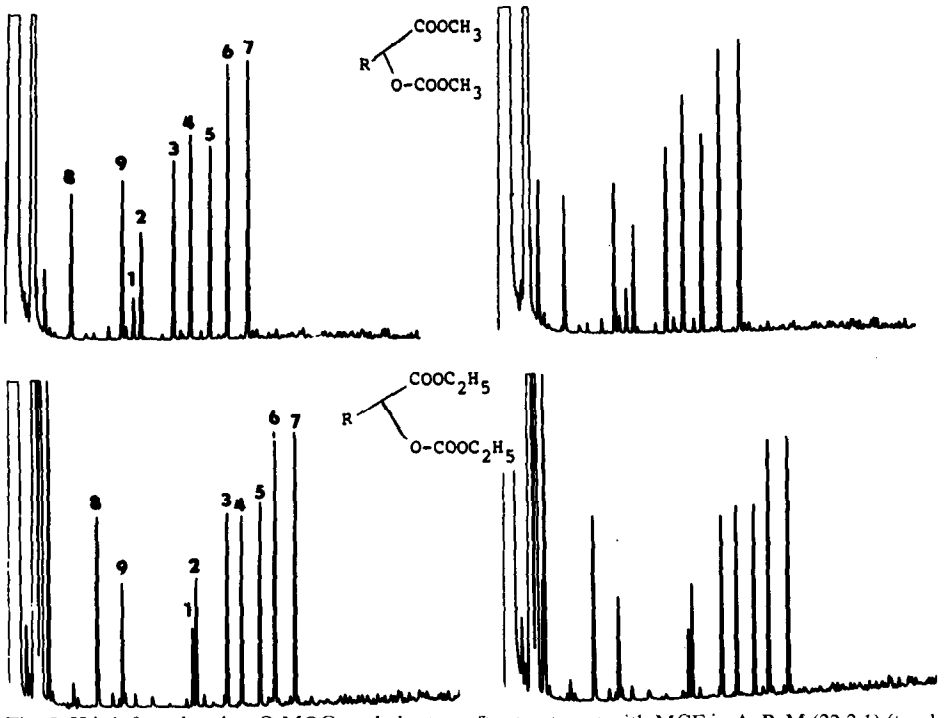


Fig. 5. HA 1–9 analysed as O-MOC methyl esters after treatment with MCF in A–P–M (22:2:1) (top left) or in A–P–M–W (7:1:1:1) (top right) and as O-EOC ethyl esters after treatment with ECF in A–E–W–P (5:2:2:1) (bottom left) or in the same solvent with ratios 4:3:2:1 (bottom right). For abbreviations, see Fig. 2.

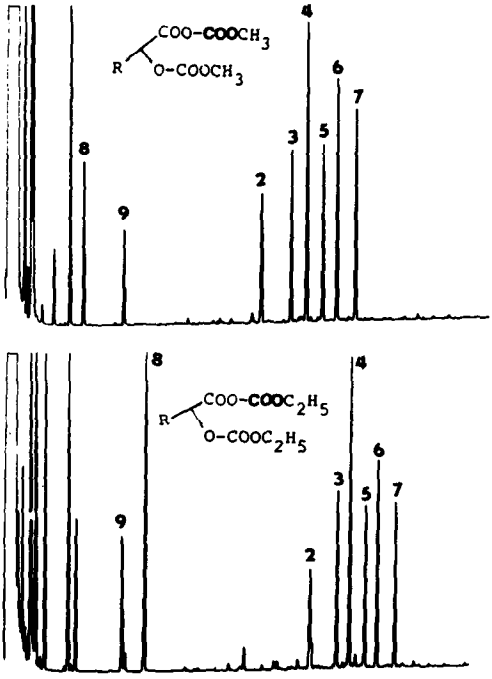


Fig. 6. HA 1–9 analysed as (top) O-MOC ethers MOC esters or (bottom) O-EOC ethers EOC esters after treatment with the corresponding chloroformate under optimum conditions for their formation. The 4-HB acid is analysed as the methyl or ethyl ester, respectively.

pyridine content in the medium was increased to 10% and the amount of reagent to 7 μl; under these conditions a water content of 20% was tolerable and two different mixing ratios gave virtually identical results.

Similarly to the formation of "mixed anhydrides" with fatty acids [16], it was possible to maintain and determine HA in the form of non-decarboxylated products, the alkoxycarbonyl ethers/esters (Fig. 6). For this purpose, the polarity of the medium was diminished by addition of acetone and the amount of pyridine was lowered below that of the reagent. However, the yield, compared with HIV acid, was not quantitative. As the 4-HB acid does not alter its retention time in comparison with the chromatogram in Fig. 5, it is measured as the alkyl ester, *i.e.*, it decarboxylates immediately even under reaction conditions convenient for the formation of the alkoxycarbonyl esters. Both 3-HB and 4-HB acids have the hydroxyl group underivatized.

In conclusion, if chloroformates are to be used as general-purpose reagents, *e.g.*, for metabolic profiling of urinary organic acids, the dissimilar reaction conditions for treatment with MCF and ECF on the one hand and for fatty acids and hydroxy acids on the other have to be taken in account. Attempts to unify the conditions in order to use the procedure in a broader range of applications are under study.

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