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Note

Determination of the absolute configuration of some biologically important urinary 2-hydroxydicarboxylic acids by capillary gas—liquid chromatography

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The analysis of urinary organic acids has become an integral part of the screening program for inborn errors of metabolism in the paediatric clinical laboratory. Especially, the widespread availability of gas chromatography—mass

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spectrometry (GC—MS) systems has led to the discovery of a variety of "new" metabolic disorders [1].

Many of the organic acids studied are chiral molecules. Because different enantiomers will be metabolized by separate pathways, the need for methods for the assignment of the absolute configurations of these chiral acids became evident. In our laboratories methods were developed for the separation of DL-hydroxymonocarboxylic acids, including capillary gas—liquid chromatography (GLC) of diastereomers obtained by esterification of the enantiomers with (—)-menthol [2]. Application of this technique has led to the discovery of a patient with D-glyceric acidemia [3] and a patient with permanent D-lactic aciduria [4]. Furthermore, it was demonstrated that urinary 2-hydroxybutyrate has the L-configuration [5]. The absolute configuration of urinary 3-hydroxybutyric acid in patients with ketosis and lactic acidosis has been verified to be D [5]; the excretion of L-glyceric acid in patients with hyperoxaluria type II was also checked (unpublished results). However, this menthylation method was not suited for all groups of chiral hydroxy acids and therefore other procedures had to be developed.

In this paper the separation of the diastereomers of the O-acetylated di-(—)-2-butyl esters of DL-2-hydroxysuccinic acid (DL-malic acid), DL-2-hydroxyglutaric acid and DL-2-hydroxyadipic acid on a non-chiral phase will be described, together with some applications.

EXPERIMENTAL

Chemicals

DL-2-Hydroxysuccinic acid and zinc DL-2-hydroxyglutarate were obtained from J.T. Baker Chemicals (Deventer, The Netherlands) and Sigma (St. Louis, MO, U.S.A.), respectively. Sodium L-2-hydroxyglutarate and L-2-hydroxysuccinic acid were purchased from Fluka (Buchs, Switzerland). DL-2-Hydroxyadipic acid was synthesized from adipic acid [6], whereas L-2-hydroxyadipic acid was prepared from L-2-aminoadipic acid (Calbiochem, San Diego, CA, U.S.A.) [7]. Salts were converted into the corresponding acids by treatment with Dowex 50 X-8 (H^+) in water.

(—)-2-Butanolic 1 *M* HCl was prepared by bubbling dry HCl gas through (—)-2-butanol (Fluka; (—)-antipode about 94% [8]) and stored at —18°C in a desiccator. The same procedure was followed for (±)-2-butanolic 1 *M* HCl.

Preparation of O-acetylated di-2-butyl esters of 2-hydroxydicarboxylic acids

To an ampoule containing 5 mg of a 2-hydroxydicarboxylic acid, 0.5 ml of 2-butanolic 1 *M* HCl was added. After heating for 2 h at 100°C the solvent was evaporated under reduced pressure. The residue was acetylated in 1 ml of pyridine—acetic anhydride (1:1, v/v) for 30 min at 100°C. After evaporation in the presence of toluene the residue was dissolved in chloroform and analysed by capillary GLC.

Analysis of urinary 2-hydroxydicarboxylic acids

Organic acids, including 2-hydroxydicarboxylic acids, were extracted from urine with ethyl acetate and analysed quantitatively by GC of the correspond-

ing pertrimethylsilyl derivatives as reported earlier [2]. For the determination of the absolute configuration of 2-hydroxydicarboxylic acids the extracts were derivatized as described above (O-acetylated di-(—)-2-butyl esters). Additional purification procedures were not carried out.

Capillary gas—liquid chromatography

The O-acetylated di-2-butyl esters of 2-hydroxydicarboxylic acids and the urinary extracts treated with (—)-2-butanolic 1 *M* HCl and acetic anhydride were analysed by capillary GLC on SP-1000 as non-chiral stationary phase at 140°C (2-hydroxysuccinic acid) or 160°C (2-hydroxyglutaric acid and 2-hydroxyadipic acid) (carrier gas nitrogen flow-rate 1 ml/min; make-up gas nitrogen flow-rate 30 ml/min [2]).

Gas chromatography—mass spectrometry

The 75-eV mass spectra of the derivatized dicarboxylic acids were recorded on a Jeol JGC-20 KP/JMS-D100/W-JMA combination using 3% OV-225 on Chromosorb W HP, 100—120 mesh, as stationary phase (ion-source temperature 150°C; accelerating voltage 3 kV; ionizing current 300 μA). The oven temperature was dependent on the sample under investigation.

RESULTS

The gas chromatograms of the O-acetylated di-(—)-2-butyl esters of DL-2-hydroxysuccinic acid, DL-2-hydroxyglutaric acid and DL-2-hydroxyadipic acid on SP-1000 as non-chiral stationary phase are presented in Fig. 1a—c, respectively. In each case the two peaks, related to the D- and L-enantiomers, were identified by co-chromatography with the O-acetylated di-(—)-2-butyl esters of the corresponding L-2-hydroxydicarboxylic acids (Fig. 1d—f). The diastereomeric derivatives of DL-2-hydroxysuccinic acid elute at about 17 min at 140°C (resolution factor R_D/R_L = 1.02), whereas those of DL-2-hydroxyglutaric acid (R_D/R_L = 1.02) and DL-2-hydroxyadipic acid (R_D/R_L = 1.02) show retention times of about 14 min and 18 min, respectively, at 160°C. In all cases the peak derived from the L-enantiomer has the shortest retention time. For 2-hydroxyglutaric acid, the applied esterification procedure gives rise to lactone formation. The identity of the various peaks was verified by GC—MS using packed columns of OV-225 as stationary phase. The mass spectra of the various derivatives are depicted in Fig. 2.

Reaction of the L-2-hydroxydicarboxylic acids with (±)-2-butanol gives rise to four diastereomers, namely (L; +,+), (L; —,—), (L; +,—) and (L; —,+)*. However, capillary GLC of the O-acetylated samples on SP-1000 showed only two peaks for each dicarboxylic acid. The pictures are exactly the same as those given in Fig. 1a—c. On non-chiral stationary phases like SP-1000, (L; +,+) and (D; —,—) forms are eluted together, as are (L; —,—) and (D; +,+), (L; +,—) and (D; —,+), and (L; —,+) and (D; +,—). Using the data mentioned above, it can be

*(L; —,+) means L-2-hydroxydicarboxylic acid with a (—)-2-butyl group at the carboxyl function connected directly to the chiral centre of the acid and a (+)-2-butyl group at the additional carboxyl function, etc.

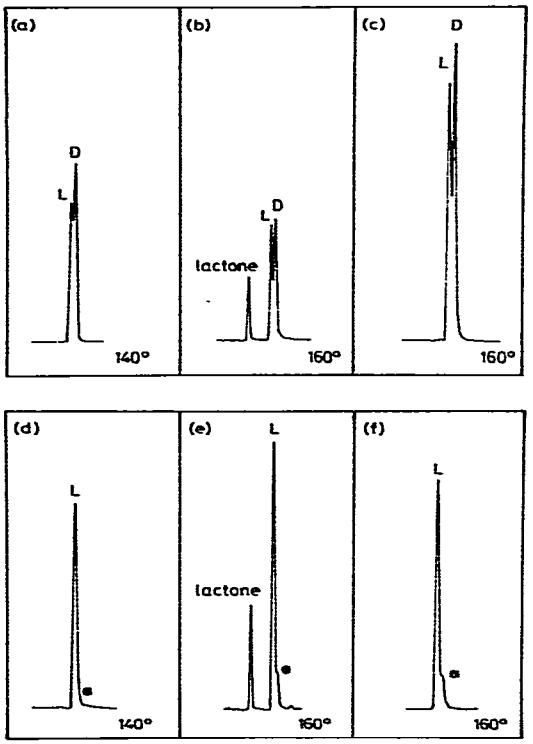

Fig. 1. Partial gas chromatograms on a SP-1000 WCOT capillary column (25 m × 0.3 mm I.D.) of the O-acetylated di-(—)-2-butyl ester derivatives of (a) DL-malic acid, (b) DL-2-hydroxyglutaric acid, (c) DL-2-hydroxyadipic acid, (d) L-malic acid, (e) L-2-hydroxyglutaric acid, and (f) L-2-hydroxyadipic acid (* indicates the shoulder obtained from the contaminating (+)-2-butanol in the (—)-isomer).

concluded that the first peaks correspond with the (L; —,—) diastereomers and the second ones with the (L; +,+) diastereomers. In view of the two peaks obtained instead of four, it is highly probable that the chiral ester function not directly attached at the chiral centre of the acid does not influence the chromatographic behaviour of the diastereomeric derivatives, so that the (L; —,+) form will coincide with the (L; —, —) form, and the (L; +, —) form with the (L; +, +) form.

The peaks of the O-acetylated di-(—)-2-butyl esters of the L-2-hydroxydicarboxylic acids (Fig. 1d—f) show a shoulder with a higher retention time, owing to the presence of a small amount of the (+)-enantiomer in the (—)-2-butanol sample [(—)/(+) = 94:6]. As expected (see above), the shoulders coincide with the O-acetylated di-(—)-2-butyl esters of the corresponding D-2-hydroxydicarboxylic acids.

BIOMEDICAL APPLICATIONS

The separation method developed has been applied to the determination of the absolute configuration of 2-hydroxydicarboxylic acids present in the urine of patients with various metabolic disorders.

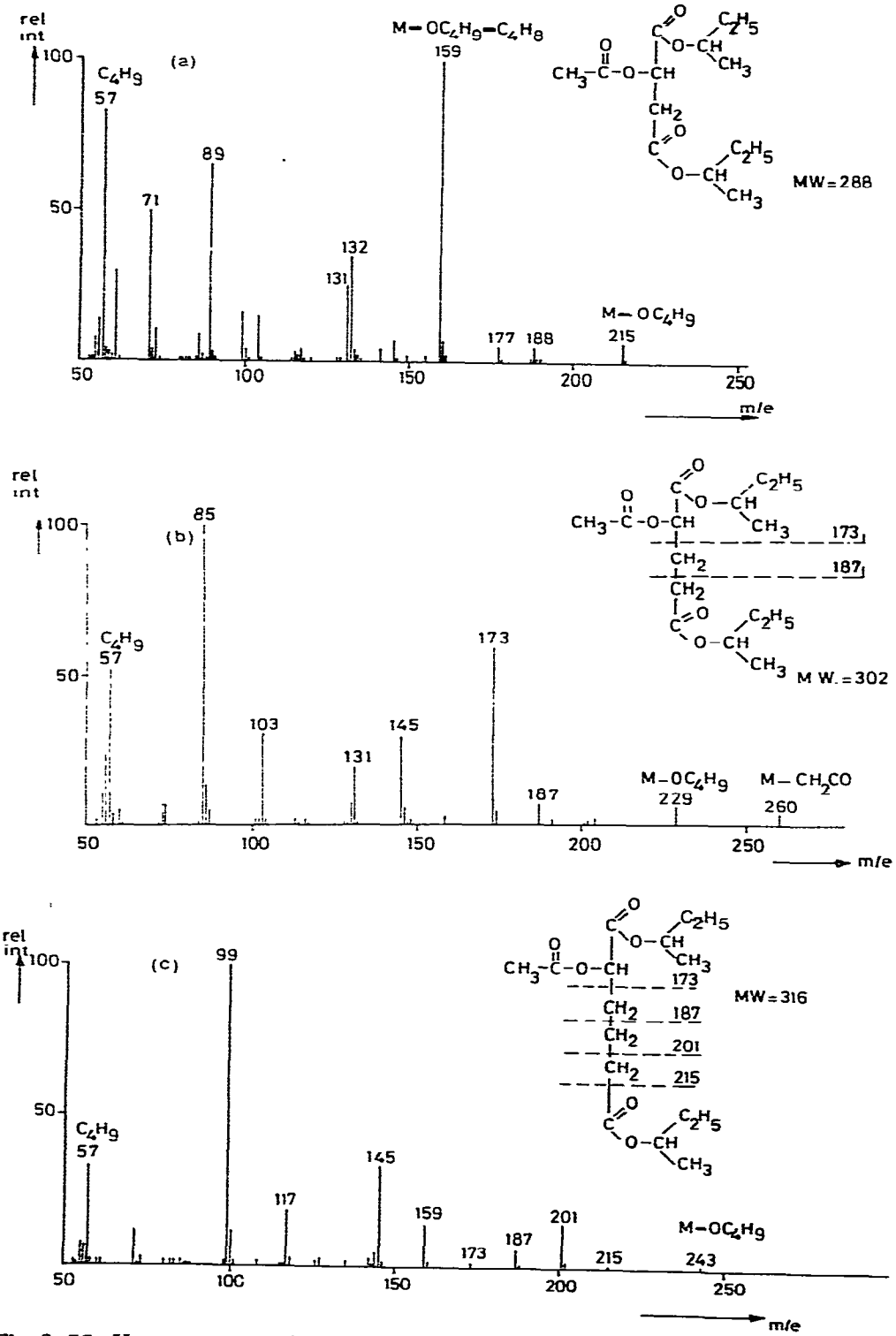

Fig. 2. 75-eV mass spectra of the O-acetylated di-(−)-2-butyl ester derivatives of (a) DL-malic acid, (b) DL-2-hydroxyglutaric acid, and (c) DL-2-hydroxyadipic acid.

2-Hydroxysuccinic acid (malic acid)

The profile of urinary organic acids was determined in two patients (aged 9 and 18 months) with lactic acidemia. In one of them a deficiency of pyruvate carboxylase was established in the liver. In addition to large amounts of lactic acid, substantial excretions of succinic, fumaric, 2-hydroxysuccinic, 2-oxoglutaric and 3-hydroxybutyric acids, and to a lesser degree of glutaric, adipic and 2-hydroxyglutaric acids were found. The urinary 2-hydroxysuccinate excretion was about 0.6 mmol/l in both patients. Capillary GC of the urinary extract treated with (—)-2-butanol and acetic anhydride showed 2-hydroxysuccinic acid to possess exclusively the L-configuration (Fig. 3a). The concentrations of 2-hydroxyglutaric acid in these samples were too low to allow determination of their absolute configuration directly.

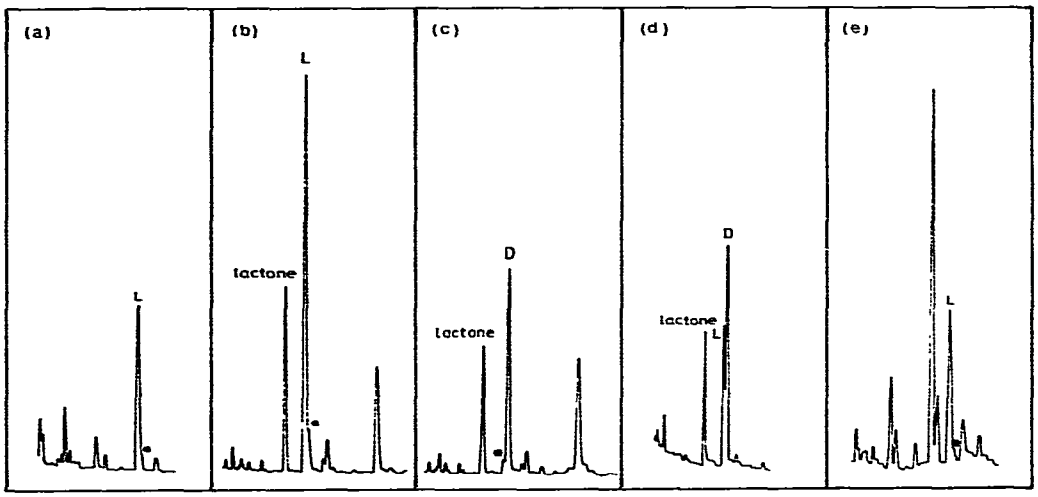

Fig. 3. Partial gas chromatograms of urinary extracts from different patients, derivatized with (—)-2-butanol and acetic anhydride. (a) L-Malic acid; (b) L-2-hydroxyglutaric acid; (c) D-2-hydroxyglutaric acid; (d) D- + L-2-hydroxyglutaric acid; (e) L-2-hydroxyadipic acid.

2-Hydroxyglutaric acid

Permanent excretion of increased amounts of 2-hydroxyglutaric acid (6 mmol/l) without the keto-analog was observed in a mentally retarded five-year-old boy. The capillary GC tracing of the derivatized urinary organic acids showed the presence of the O-acetylated di-(—)-2-butyl ester of L-2-hydroxyglutaric acid (Fig. 3b). There is no clue to the over-excretion of L-2-hydroxyglutarate in this patient [9].

By capillary GC the excessive excretion of D-2-hydroxyglutaric acid was demonstrated in two patients: a 13-month-old boy with hereditary fructose intolerance (fructose-1-phosphate aldolase deficiency) who excreted 0.6 mmol/l; and a 13-year-old boy suffering from egg allergy and protein-losing gastroenteropathy (5 mmol/l) (Fig. 3c). It has been proposed that the latter patient suffered from a defect of D-2-hydroxyglutarate dehydrogenase [10].

Analysis of the 2-hydroxyglutaric acid excreted by a five-month-old girl, admitted because of severe neurological abnormalities, convulsions, and failure to thrive, showed the simultaneous occurrence of both enantiomers. The

excretion of 2-hydroxyglutarate was variable, as was the ratio of the D- and L-enantiomers (Fig. 3d). Extended screening for metabolic disorders revealed that the girl excreted large amounts of uracil. In a brain biopsy specimen necrosis of both white and gray matter was demonstrated.

2-Hydroxyadipic acid

Two patients with 2-aminoadipic aciduria were studied: a girl of 4 [11] and a boy of 3. A reduced degradation of 2-oxo-adipate to glutaryl-CoA was demonstrated in one of the patient's fibroblasts. In addition to 2-aminoadipic acid, considerable amounts of 2-oxo-adipic acid and 2-hydroxyadipic acid were excreted. The latter compound proved to have the L-configuration in both cases (Fig. 3e). Because of the presence of interfering substances in one of the samples, the hydroxy acid was isolated from the urine by means of one-dimensional paper chromatography using 1-butanol—acetic acid—water (4:1:1, v/v) as solvent.

DISCUSSION

As has been demonstrated in this study, the optical isomers of the mono-hydroxylated dicarboxylic acids 2-hydroxysuccinic acid (malic acid), 2-hydroxyglutaric acid and 2-hydroxyadipic acid can be separated on the non-chiral stationary phase SP-1000 using the corresponding O-acetylated di-(—)-2-butyl ester derivatives. The resolution of the malic acid enantiomers has been reported earlier by Pollock and Jermany [12] after derivatization with 2-butanol, 3-methyl-2-butanol or 3,3-dimethyl-2-butanol and O-acetylation. Absolute configuration studies of malic and 2-hydroxyadipic acid using O-(—)-menthyloxycarbonyl methyl ester derivatives were reported in relation to the absolute configuration of unsaturated hydroperoxy fatty acids formed from certain unsaturated fatty acids by incubation with different lipoxygenases [13, 14]. For the determination of the absolute configuration of urinary chiral organic acids it is important that extensive purifications can be omitted. The possibility of using simple ethyl acetate extracts makes the approach more generally applicable in clinical chemistry. By GC both enantiomers of a compound can be analyzed simultaneously. The same holds for the analysis of series of chiral organic acids. The use of 2-butanol as an esterifying agent instead of the previously used menthol has the advantage of higher volatility of the derivatives, enabling the study of larger chiral organic acids. A minor drawback of the use of (—)-2-butanol is the presence of about 6% of (+)-2-butanol in all commercially available preparations of this reagent; on the other hand, the specific peak pattern so obtained can give direct information about the purity of the main GC peak.

The absolute configurations of the hydroxylated dicarboxylic acids in the patients' urine samples give rise to the following comments.

The absolute configuration of the urinary malic acid is the expected one. The reactions of the citric acid cycle are highly stereospecific and it has been known for a long time that the fumarase-catalyzed hydration of fumaric acid leads exclusively to L-malic acid. Hence it can be concluded that the excessive excretion of L-malic acid by patients with, for example, pyruvate carboxylase

deficiency is due to excessive production and/or loss of citric acid cycle intermediates.

The two forms of 2-hydroxyglutaric acid are undoubtedly produced via different metabolic pathways and it may even be questioned whether both compounds are of endogenous origin. It is tempting to relate the 2-hydroxyglutarate production to the availability of excess 2-oxo-glutarate, but only the last patient excreted substantial amounts of 2-oxo-glutarate and there appeared to be no correlation between the excretory levels of these two acids.

The urinary L-2-hydroxyadipic acid in patients with 2-aminoadipic aciduria is probably produced from 2-oxo-adipic acid by a non-specific enzyme. The finding that synthetic L-2-hydroxyadipate does not react with L-lactate dehydrogenase makes it improbable that this enzyme would mediate the in vivo formation of L-2-hydroxyadipate from 2-oxo-adipate.

Finally, it is evident from this study that a search for the enzymes responsible for the formation of 2-hydroxycarboxylic acids would be worthwhile for a better understanding of normal and especially of abnormal organic acid metabolism.

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