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ABSOLUTE CONFIGURATION DETERMINATION OF CHIRAL ORGANIC ACIDS IN PHYSIOLOGICAL FLUIDS BY CAPILLARY GAS-LIQUID CHROMATOGRAPHY

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INTRODUCTION

Many inborn errors of metabolism are detected by the analysis of characteristic low-molecular-weight metabolites. A variety of chromatographic techniques for the determination of these metabolites are used, including thin-layer chromatography, column liquid chromatography, gas-liquid chromatography (GLC), and GLC combined with mass spectrometry (GLC-MS). The latter technique has proved particularly useful for the study of patients with inherited organic acidurias, who excrete abnormal amounts of typical carboxylic acids.

The application of GLC-MS has led to the discovery of many "new" defects. New information about metabolic pathways, both normal and abnormal, has been gained by using this technique. Reviews on the developments in this field have been published recently[1-4].

In case we have to deal with organic acids having chiral centres, the absolute configuration of these compounds should be determined, as different stereoisomers may represent different metabolic routes.

During the last 20 years significant progress has been made on the application of GLC for the determination of the absolute configuration of biologically interesting chiral substances[5,6]. For the GLC separation of racemic mixtures

(D- and L-enantiomers) two approaches are in use, namely, separation of the enantiomers (if necessary, after protection of polar groups) on a chiral stationary phase and conversion of the enantiomers into diastereoisomers (a specific derivatization) using a chiral reagent, followed by separation of the diastereoisomers on a non-chiral stationary phase. After assignment of the GLC peaks by co-chromatography with enantiopure substances, both methods are applicable for chirality determinations.

GLC methods for the determination of the absolute configuration have several advantages. They are highly sensitive and are generally applicable. Preliminary purification of the compounds under investigation to a high extent is not necessary, which facilitates the analysis at the microgram level in extracts from physiological fluids. Also mixtures of different chiral substances can be analysed without prior separation. Furthermore, the enantiopurity of chiral compounds can be determined easily. Finally, GLC-MS for identification and verification is possible.

During the last 5 years we have, in relation to screening for inborn errors of metabolism, investigated several chiral hydroxy acids as diastereoisomeric derivatives using non-chiral stationary phases. A number of typical results will be given in this presentation. These examples show that urinary metabolite profiles may be more complicated than is reflected by ordinary GLC analyses.

EXPERIMENTAL

Quantitative analysis of urinary organic acids

To 5 ml of urine are added 5 ml of a saturated NaCl solution and 0.5 mg of 2-phenylbutyric acid (internal standard). The mixture is acidified with conc. HCl to pH 1-2 and, after the addition of 10 mg vitamin C (anti-oxidant), extracted twice with 20 ml of ethyl acetate. The total organic phase is dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure at 40^oC. Finally, the residue is trimethylsilylated with 0.5 ml of N,O-bis(trimethylsilyl)acetamide in chloroform (1:3, v/v) for 30 min at 37^oC. The trimethylsilylated derivatives are analysed by GLC on 5% GESE-52[19].

Derivatization procedures for the conversion of chiral hydroxy acid enantiomers into diastereoisomeric derivatives

Menthylation of carboxyl groups. (-)-Menthol (∿300 mg) is added to 1-5 mg of dry hydroxy acid. The esterification is carried out for 2 h at 110^oC by bubbling dry HCl gas through the solution. Subsequently, the excess of HCl and menthol is removed by a gentle stream of nitrogen at 110^oC. The residue is acetylated with 1 ml of acetic anhydride in pyridine (1:1, v/v) for 30 min at 100^oC.

Finally, the solvent is evaporated off in the presence of toluene, and the residue is dissolved in chloroform[8].

2-Butylation of carboxyl groups. To an ampoule containing 1-5 mg of dry hydroxy acid, 0.5 ml of 1 *M* (-)-2-butanolic HCl is added. After heating for 2 h at 100°C, the solvent is evaporated off under reduced pressure. The residue is acetylated and worked-up as described above[16].

2-Phenylpropionylation of hydroxyl groups. Dry hydroxy acid (25 μmoles) is dissolved in 1 ml of ethyl acetate and esterified with diazomethane in diethyl ether. After removal of the excess of diazomethane by evaporation to dryness, the residue is treated with 100 μmoles of D-2-phenylpropionyl chloride in 200 μl of dry benzene and 50 μl of dry pyridine. The mixture is kept for 2 h at room temperature. Subsequently, 2 ml of benzene is added and the organic phase is extracted twice with 1 ml of 1 *M* $NaHCO_3$, followed by washing twice with 1 ml of water. The benzene layer is dried over Na_2SO_4, and, after evaporation, the residue is dissolved in ethyl acetate.

Extracts of physiological fluids. Extracts can be treated in the same ways as mentioned above for pure hydroxy acids. If necessary, a partial purification can be carried out, before derivatization.

RESULTS AND DISCUSSION

Lactic acid

Lactic acid is excreted normally in small amounts, but the level is elevated in many primary and secondary conditions. Generally, this acid has the L-configuration being produced from endogenous pyruvate, mediated by L-lactate dehydrogenase.

In malabsorptive diseases D-lactic acid is produced from non-absorbed carbohydrates by the intestinal bacterial flora. Being an inert metabolite, it passes through the human body and is excreted unchanged into the urine. There is one example of life-long D-lactic aciduria in a now ten-year-old boy. However, this is most probably due to an endogenous metabolic abnormality[7].

The enantiomers of lactic acid can be separated on an SP-1000 capillary column using the O-acetylated (-)-menthyl ester diastereoisomeric derivatives[8]. Fig. 1 shows the resolution of DL-lactic acid and the occurrence of high levels of D-lactic acid in an ethyl acetate extract of the urine from the above-mentioned ten-year-old boy. In this case D-lactic acid was also found to occur in the blood and cerebrospinal fluid.

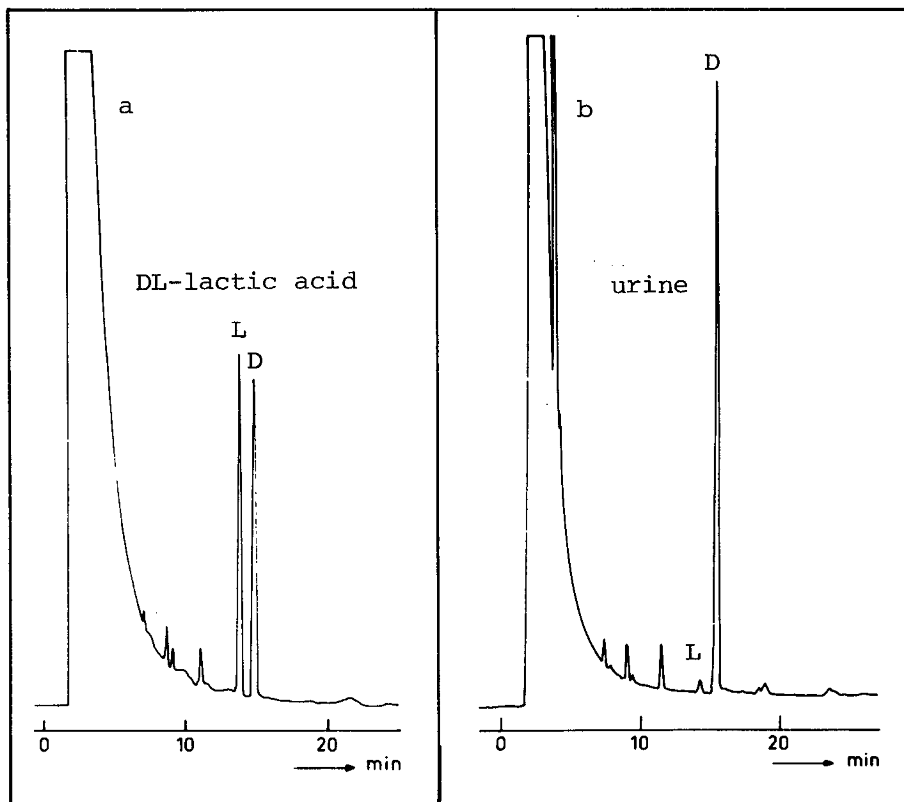

Fig. 1. (a) Gas chromatographic resolution of the O-acetylated (-)-menthyl ester derivatives of D- and L-lactic acid. (b) Urinary excretion of D-lactic acid in a patient described in ref. 7. For the analyses, an SP-1000 WCOT glass capillary column (25 m x 0.3 mm I.D.) was used. Oven temperature: 150°C; N_2-flow: 1 ml/min.

Glyceric acid

Both enantiomers of glyceric acid can be present in the urine of patients with metabolic disorders. L-Glyceric acid and oxalic acid accumulate in a condition called hyperoxaluria type II, clinically presenting as nephrolithiasis[9]. Up to now D-glyceric aciduria has been found in four patients with different clinical symptoms. Also other chemical parameters are different, suggesting basic biochemical heterogeneity. Heredity has not yet been demonstrated[4,10-12].

As is evident from Fig. 2, the enantiomers of glyceric acid can be separated on an SP-1000 capillary column using the O-acetylated (-)-menthyl ester diastereoisomeric derivatives[8]. Also an example[11] of the absolute configuration determination of glyceric acid in a urinary ethyl acetate extract is included.

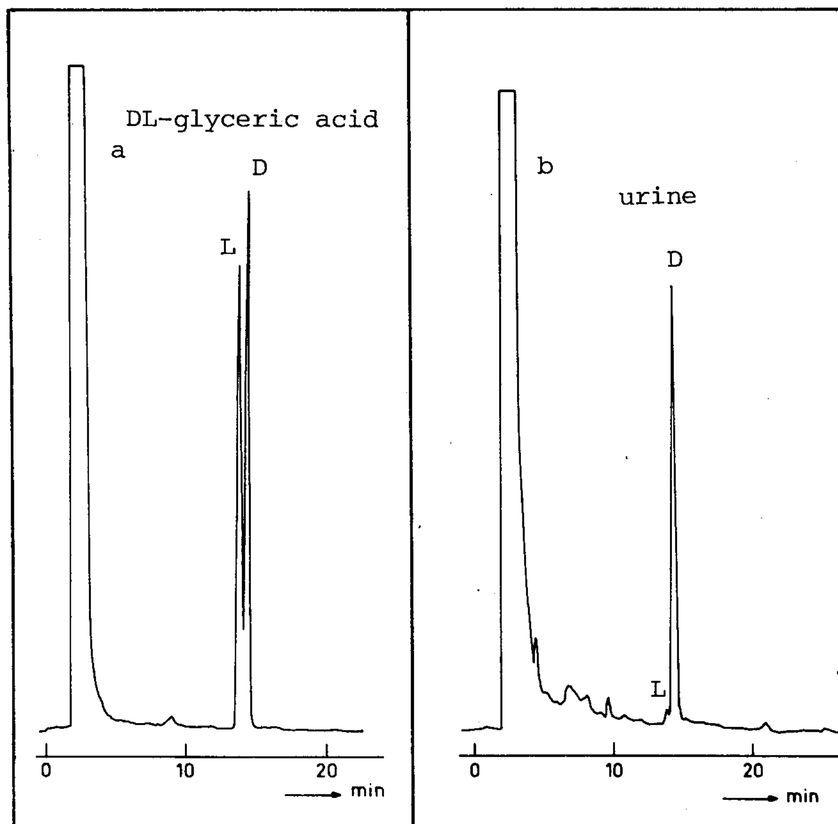

Fig. 2. (a) Gas chromatographic resolution of the O-acetylated (-)-menthyl ester derivatives of D- and L-glyceric acid. (b) Urinary excretion of D-glyceric acid in a patient described in ref. 11. For the column material and N_2-flow, see Fig. 1. Oven temperature: 200°C.

2-Hydroxy- and 3-hydroxybutyric acids

Patients with lactic acidosis and ketosis regularly show urinary excretion of increased amounts of 2-hydroxybutyric acid. The absolute configuration of this acid can be determined using the O-acetylated (-)-menthyl ester derivatives on an SE-30 capillary column. This phase can also be used for the analysis of the O-acetylated (-)-menthyl ester derivatives of lactic acid and 3-hydroxy-butyric acid. For 2-hydroxybutyric acid and lactic acid the L-configuration was found, while for 3-hydroxybutyric acid the D-configuration was found[13].

Branched-chain hydroxy acids excreted in maple syrup urine disease (MSUD)

In patients with MSUD, due to branched-chain ketoacid dehydrogenase deficien-

cy, the branched-chain amino acids valine, leucine, isoleucine and *allo*-isoleucine and the corresponding oxo and hydroxy acids accumulate in all the body fluids[3,4].

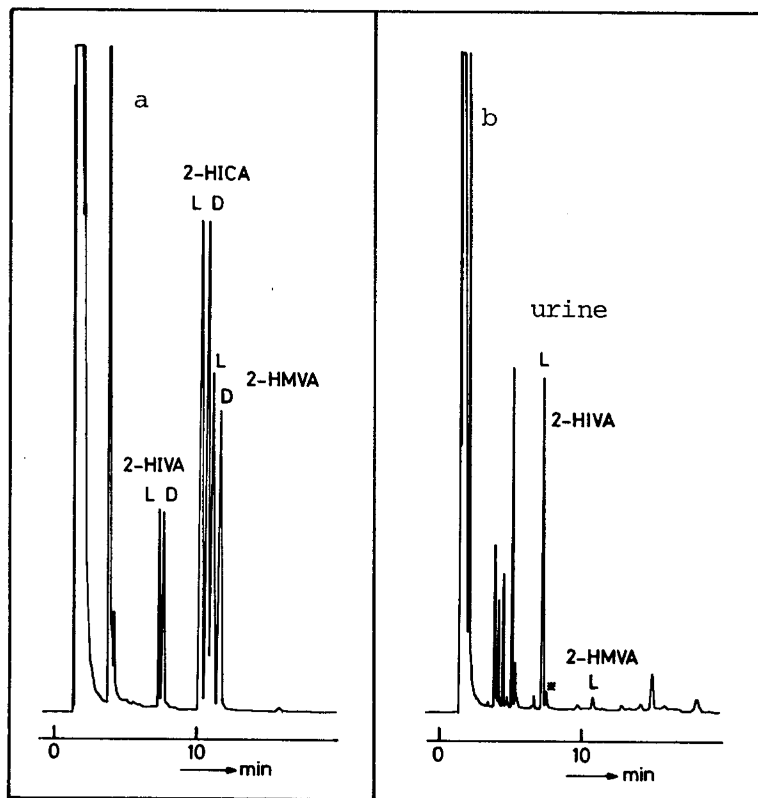

Fig. 3. (a) Gas chromatographic resolution of the O-acetylated (-)-2-butyl ester derivatives of D- and L-2-hydroxyisovaleric acid (2-HIVA), D- and L-2-hydroxyisocaproic acid (2-HICA), and D- and L-2-hydroxy-3-methylvaleric acid (2-HMVA). (b) Urinary excretion of L-2-HIVA and L-2-HMVA in a patient with maple syrup urine disease (* indicates the small peak obtained from the contaminating (+)-2-butanol in the commercial (-)-2-butanol sample). For the column material and N_2-flow, see Fig. 1. Oven temperature: 100°C.

The enantiomers of the branched-chain hydroxy acids 2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid and 2-hydroxy-3-methylvaleric acid can be resolved on an SP-1000 capillary column as the corresponding O-acetylated (-)-2-butyl ester derivatives (Fig. 3). The observed resolution of the diastereoisomeric derivatives of the latter hydroxy acid was only influenced by the absolute configuration of the substituents at carbon-2. Analysis of the urine of a patient with

MSUD shows the presence of relatively large amounts of L-2-hydroxyisovaleric acid and smaller amounts of L-2-hydroxy-3-methylvaleric acid (Fig.3). The investigated urine sample contained only very small amounts of 2-hydroxyisocaproic acid. Therefore, the absolute configuration of this acid could not be determined directly in the ethyl acetate extract. In this case an enrichment of the product has to be carried out prior to analysis.

Hydroxydicarboxylic acids

Malic acid and 2-hydroxyadipic acid have been detected in the urine of patients with inborn errors. Also 2-hydroxyglutaric aciduria has been described recently[14,15].

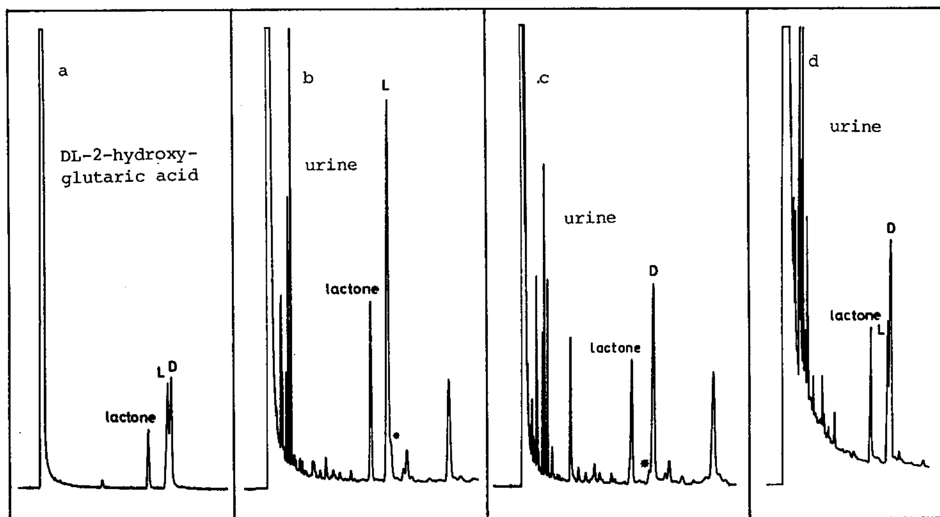

Fig. 4. (a) Gas chromatographic resolution of the O-acetylated di-(-)-2-butyl ester derivatives of D- and L-2-hydroxyglutaric acid. (b) Urinary excretion of L-2-hydroxyglutaric acid in a patient described in ref. 14. (c) Urinary excretion of D-2-hydroxyglutaric acid in a patient described in ref. 15. (d) Urinary excretion of D- and L-2-hydroxyglutaric acid in a patient described in ref. 16. For the column material and N_2-flow, see Fig. 1. Oven temperature: 160°C. For explanation of asterisks, see Fig. 3.

Determination of the absolute configuration of these acids can be carried out on an SP-1000 capillary column after derivatization with (-)-2-butanolic HCl and O-acetylation[16]. As an example, Fig. 4 shows the resolution of the diastereoisomeric derivatives of 2-hydroxyglutaric acid. Esterification of this acid yields considerable amounts of the lactone form. For the hydroxydicarboxylic acid derivatives it was found that only the (-)-2-butyl ester group vicinal to

the chiral carbon-2 centre influences the separation of the enantiomers on SP-1000[16].

Two patients with lactic acidemia (one with a proven deficiency of pyruvate carboxylase in the liver) excreted L-malic acid[16]. Two patients with 2-keto-(amino)-adipic aciduria, with a proven reduced degradation of 2-oxo-adipate to glutaryl-CoA in the fibroblasts, excreted L-2-hydroxyadipic acid[16]. The patients excreting 2-hydroxyglutaric acid showed different results (see Fig. 4). One of these patients, a mentally retarded five-year-old boy, excreted exclusively the L-form[14]. However, D-2-hydroxyglutaric acid was detected in two other patients. One of them had hereditary fructose intolerance (fructose-1-phosphate aldolase deficiency); the other had egg allergy and protein-losing gastroenteropathy. For the latter patient D-2-hydroxyglutarate dehydrogenase was proposed as the underlying defect[15]. Finally, a patient with severe neurological abnormalities, convulsions, failure to thrive, intermittent hyperammonemia, uraciluria and necrosis of both white and gray matter, showed simultaneous occurrence of both enantiomers[16].

5-Hydroxycaproic acid

This hydroxy acid is excreted together with dicarboxylic acids and related compounds in patients with an increased ω- and (ω-1)-oxidation. To obtain more detailed information, the absolute configuration of 5-hydroxycaproic acid was investigated. A separation of the enantiomers with the diastereoisomeric derivatives mentioned above could not be achieved. However, these enantiomers were separated on an SE-30 capillary column using the D-2-phenylpropionate derivatives of the methyl 5-hydroxycaproates (Fig. 5). Analysis of the urinary extracts of four patients has led to the conclusion that in all cases both enantiomeric forms occur, the L-enantiomer being the dominating isomer (ratio ca. 70:30). A typical example is also included in Fig. 5. These results are in accordance with reported non-stereospecific microsomal (ω-1)-oxidations of lauric acid[17] and decanoic acid[18].

Aromatic hydroxy acids

Patients with phenylketonuria and tyrosinemia show urinary excretion of relatively high amounts of 3-phenyllactic acid and 3-(4-hydroxyphenyl)lactic acid, respectively. The enantiomers of these acids can be separated on an SP-1000 capillary column using the corresponding O-acetylated (-)-2-butyl ester diastereoisomeric derivatives. Analysis of the urinary ethyl acetate extracts of the patients shows in both cases the presence of only the L-enantiomer. However, both L- and D-enantiomers of 3-phenyllactic acid were identified in the urine of a patient with coeliac disease. This patient excreted excessive amounts of metabolites produced by the intestinal flora from non-absorbed amino acids.

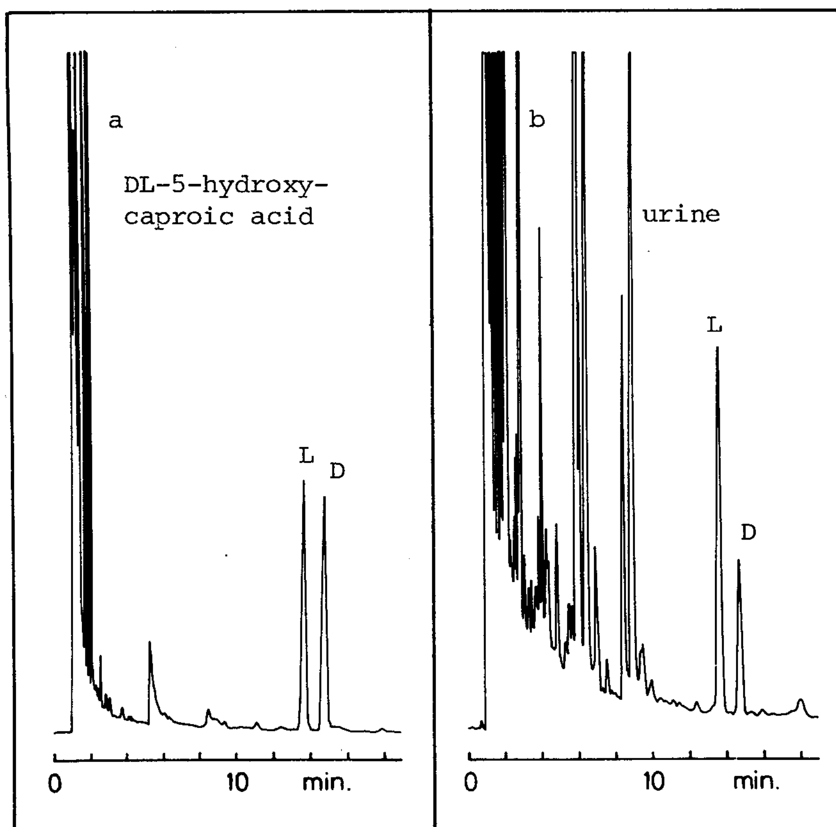

Fig. 5. (a) Gas chromatographic resolution of the D-2-phenylpropionate derivatives of the methyl esters of D- and L-5-hydroxycaproic acid. (b) Urinary excretion of D- and L-5-hydroxycaproic acid in a patient with non-ketotic dicarboxylic aciduria. For the analyses an SE-30 WCOT glass capillary column (25 m x 0.3 mm I.D.) was used. Oven temperature: 150°C; N_2-flow: 1.5 ml/min.

FINAL REMARKS

From the foregoing examples it is evident that the determination of the absolute configuration of urinary chiral organic acids is necessary for a reliable interpretation of metabolic profiles. Different enantiomers may reflect different endogenous metabolic pathways. The chirality determination may also be of use for the discrimination between endogenous and exogenous (bacterial) metabolic events.

REFERENCES

1 K. Tanaka, D.G. Hine, A. West-Dull and T.B. Lynn, Clin. Chem., 26 (1980) 1839.
2 K. Tanaka, A. West-Dull, D.G. Hine, T.B. Lynn and T. Lowe, Clin. Chem., 26 (1980) 1847.
3 M. Duran and S.K. Wadman, Recent Advan. Clin. Biochem., 2 (1981) 103.
4 S.K. Wadman, M. Duran and J.P. Kamerling, in CIBA Foundation Symposium No. 87, Metabolic Acidosis, 1982, p. 324.
5 E. Gil-Av and D. Nurok, Advan. Chromatogr., 10 (1974) 99.
6 P. Husek and K. Macek, J. Chromatogr., 113 (1975) 139.
7 M. Duran, J.P.G.M. Van Biervliet, J.P. Kamerling and S.K. Wadman, Clin. Chim. Acta, 74 (1977) 297.
8 J.P. Kamerling, G.J. Gerwig, J.F.G. Vliegenthart, M. Duran, D. Ketting and S.K. Wadman, J. Chromatogr., 143 (1977) 117.
9 H.E. Williams and L.H. Smith, N. Engl. J. Med., 278 (1968) 233.
10 N.J. Brandt, K. Rasmussen, S. Brandt, S. Kølvraa and F. Schønheyder, Acta Paediatr. Scand., 65 (1976) 17.
11 S.K. Wadman, M. Duran, D. Ketting, L. Bruinvis, P.K. de Bree, J.P. Kamerling, G.J. Gerwig, J.F.G. Vliegenthart, H. Przyrembel, K. Becker and H.J. Bremer, Clin. Chim. Acta, 71 (1976) 477.
12 D. Grandgeorge, A. Favier, M. Bost, P. Frappat, C. Boujet, S. Garrel and P. Stoebner, Arch. Fr. Pediatr., 37 (1980) 577.
13 J.P. Kamerling, G.J. Gerwig, M. Duran, D. Ketting and S.K. Wadman, Clin. Chim. Acta, 88 (1978) 183.
14 M. Duran, J.P. Kamerling, H.D. Bakker, A.H. van Gennip and S.K. Wadman, J. Inher. Metab. Dis., 3 (1980) 109.
15 R.A. Chalmers, A.M. Lawson, R.W.E. Watts, A.S. Tavill, J.P. Kamerling, E. Hey and D. Ogilvie, J. Inher. Metab. Dis., 3 (1980) 11.
16 J.P. Kamerling, M. Duran, G.J. Gerwig, D. Ketting, L. Bruinvis, J.F.G. Vliegenthart and S.K. Wadman, J. Chromatogr., 222 (1981) 276.
17 I. Björkhem and M. Hamberg, Biochem. Biophys. Res. Commun., 47 (1972) 333.
18 M. Hamberg and I. Björkhem, J. Biol. Chem., 246 (1971) 7411.
19 J.P. Kamerling, M. Brouwer, D. Ketting and S.K. Wadman, J. Chromatogr., 164 (1979) 217.