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IDENTIFICATION OF 3-OXODICARBOXYLIC ACIDS IN HUMAN URINE

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SUMMARY

Gas chromatography–mass spectrometry has been used to detect a series of 3-oxodicarboxylic acids in human urine as their corresponding dimethyl ester trimethylsilyl enol ethers. 3-Oxohexanedioic acid, 3-oxooctanedioic acid and 3-oxodecanedioic acid were demonstrated to be normal urine constituents. Increased amounts of these acids were excreted after ingestion of decanedioic acid. These findings support the hypothesis that dicarboxylic acids are degraded by ordinary β-oxidation.

INTRODUCTION

It has been known for several decades that dicarboxylic acids administered to human or animals may be degraded and excreted into the urine as shorter dicarboxylic acids [1–4]. It is suggested that the acids are degraded by the normal fatty acid β-oxidation system [5]. There is, however, a remarkable lack of literature concerning the β-oxidation of dicarboxylic acids, compared with the wealth of information available concerning their formation by ω-oxidation of fatty acids. What seems clear is that dicarboxylic acids follow the same reaction path as fatty acids in regard to activation by coenzyme A (CoA) [6–8], as well as transportation into the mitochondria [6], utilizing the same enzymes as the fatty acids do. Furthermore, once transported into the mitochondria, it is generally assumed that the enzymes of the normal fatty acid β-oxidation start to convert the dicarboxylic acids into the well known intermediates in the process, first by making an α,β-unsaturation, followed by a hydration to the L-(*S*)-3-hydroxydicarboxylic acid, then oxidation to the 3-oxo derivative and ending by a thiolytic cleavage into a shortened dicarboxylic acid and acetyl-CoA.

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It is evident from several studies on inborn errors of metabolism that CoA thioesters of organic acids may be hydrolysed, and the corresponding acids are excreted into the urine [9,10]. Several 3-hydroxydicarboxylic acids have been detected in the urine from a patient suffering from ketoacidosis [11] and in the urine from healthy human following the ingestion of dodecanedioic acid [12,13]. Furthermore, it has been shown that the urinary 3-hydroxyhexanedioic acid exhibits the expected L-configuration [14]. Apart from tentative identification of 3-oxohexanedioic acid in rats following the administration of hexanedioic acid [15], no 3-oxo intermediates in the metabolism of saturated, straight-chain dicarboxylic acids have been detected.

In the present work some 3-oxodicarboxylic acids were identified as normal constituents of human urine, and excretion of increased amounts was demonstrated following ingestion of a longer dicarboxylic acid. These findings support the hypothesis that dicarboxylic acids are degraded by normal β-oxidation.

EXPERIMENTAL

Chemicals

N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), dimethyl 3-oxohexanedioate, nonanedioic acid and decanedioic acid were all purchased from Fluka (Buchs, Switzerland). All other chemicals used were commercial products of high purity.

Synthesis of reference compounds

Dimethyl 3-oxoheptanedioate, dimethyl 3-oxooctanedioate, dimethyl 3-oxononanedioate and dimethyl 3-oxodecanedioate were prepared by pyridinium chlorochromate oxidation of the corresponding dimethyl 3-hydroxyalkanedioates [16,17]. The dimethyl 3-oxoalkanedioates were converted into the corresponding trimethylsilyl enol ether derivatives by treating the diesters with a ten-fold excess of BSTFA–TMCS (99:1) for 20 min at 120°C. Unchanged silylating reagent was removed under a stream of dry nitrogen, before analysis by gas chromatography–mass spectrometry (GC–MS).

Preparation of urine extracts

Two healthy male volunteers, J.E.W. (41 years, 68 kg) and J.S.S. (26 years, 79 kg), were examined in the fed state (liberal amounts of carbohydrate) and after ingestion of 20 mmol of nonanedioic and decanedioic acid, respectively. Urine was collected quantitatively for 12 h. The urine was diluted to 1000 ml and an aliquot (100 ml) of the urine was cooled to 4°C, carefully acidified to pH 1 with 1 *M* hydrochloric acid and saturated with sodium chloride. This solution was extracted with three 100-ml portions of diethyl ether at 4°C, dried over magnesium sulphate and evaporated under reduced pressure until a light brown viscous oil was obtained. The residue was dissolved in a small volume of diethyl ether and treated with an ethereal solution of diazomethane until the reaction ceased. The solution was evaporated under a stream of dry nitrogen and silylated with 500 μl of BSTFA–TMCS (99:1) for 20 min at 120°C and evaporated under dry nitrogen.

The resulting viscous solution was diluted with 1 ml of chloroform prior to GC–MS analysis.

Gas chromatography–mass spectrometry

The measurements were performed with a VG Analytical (Manchester, U.K.) MicroMass 7070 double-focusing mass spectrometer equipped with a VG 2050 data system (PDP 8a computer), a Hewlett-Packard 5710 gas chromatograph and a digital selected-ion recording (SIR) unit controlled by the data system. The gas chromatograph was equipped with a CP-Sil 5 CB Chrompack (Middelburg, The Netherlands) chemically bonded fused-silica capillary column (25 m × 0.22 mm I.D.). Injection port temperature was 250 °C and the helium flow-rate was 2.0 ml/min. The samples were injected by the splitless technique and the oven temperature was programmed from 120 to 240 °C at 6 °C/min after a start delay of 2 min. The GC–MS interface was a direct inlet jet heated to 250 °C with the column protruding directly into the ion source. Ionizing and accelerating potentials were 70 eV and 4 kV (standard, otherwise controlled by the SIR unit), respectively, and the temperature in the ion source was 220 °C. The SIR recordings were performed on nine characteristic ions from each of the compounds. The channel time was 50 ms.

RESULTS AND DISCUSSION

The urinary acidic extracts are very complex matrices, shown by combined by GC–MS to contain more than 500 different constituents [10], of which ca. 200 compounds have been identified by interpretation of their mass spectra [18]. However, many minor compounds cannot be identified by this method, mainly due to the fact that urine is so complex that most GC peaks remain unresolved even on capillary columns, and thus mixed mass spectra are obtained. Another grave obstacle is that in many cases definitive structural assignment cannot be derived from the mass spectrum alone.

One of the most widely used approaches to circumvent these problems is to make different derivatives of the same compound and thus obtain some additional structural information. This technique normally requires that the compounds of interest be relatively abundant and that the urine be prechromatographed in some way, simplifying the GC separation in order to give pure mass spectra. A completely different approach in the identification of unknown urinary constituents is based on the synthesis of reference compounds and subsequent comparison of the results obtained for the urine samples with those for the synthetic compounds. Furthermore, the technique allows a semiquantitative determination of the compounds by standard addition methods. This approach has been employed in the present study for the identification of 3-oxodicarboxylic acids in human urine. These metabolites have been detected using the SIR technique. By sampling a relatively large number of characteristic ions we achieved a selectivity almost as great as that obtained in full scanning mode.

The 3-oxodicarboxylic acids are very labile compounds, and great care has to be taken in order to avoid decarboxylation during the work-up. This undesirable

reaction was, however, minimized by keeping the pH above 1 and by carrying out the extraction at 4 °C.

When oxo acids are derivatized, several derivatives may be formed. Easily enolizable keto groups, as in the 3-oxodicarboxylic acids, can be derivatized by enol ether formation. In the present study, a double derivatization method involving methyl esterification of the acid groups followed by trimethylsilylation of the keto group has been used. The resulting dimethyl 3-trimethylsilyloxy-2-alkenedioates, which exist as both *E* and *Z* isomers, have excellent GC properties and mass spectra ideal for analytical purposes with many high-mass ions with high intensity [17]. Only quantitative differences were observed in the mass spectra between the isomers, and the derivatives are therefore suitable for quantitative SIR analysis using the same fragment ions for both isomers. In the present study, four ions specific for each compound were chosen for SIR (Table I). In addition, five fragments common to all investigated compounds were also used, viz. *m/z* 201 ($C_9H_{17}O_3Si$), *m/z* 188 ($C_8H_{16}O_3Si$), *m/z* 185 ($C_8H_{13}O_3Si$), *m/z* 173 ($C_7H_{13}O_3Si$), and *m/z* 169 ($C_8H_{13}O_2Si$).

A five-channel SIR on the M−15 ions showed the GC behaviour for the different 3-oxodicarboxylic acids as dimethyl ester trimethylsilyl enol ether derivatives (Fig. 1). For all derivatives the *E* isomer had the shorter retention time of the two isomers. The assignment of the *E* and *Z* forms was based on quantitative differences between the mass spectra of the isomers [17].

Fig. 2 shows the three-channel SIR obtained when a human urine sample following ingestion of decanedioic acid was analysed with respect to dimethyl 3-trimethylsilyloxy-2-hexenedioate. The ions chosen are characteristic for this compound (Table I) and give the same profiles as the synthetic compound. Addition of the synthetic reference compound did not produce any extra peaks in the SIR chromatogram: the only effect was an increased intensity of existing peaks. Furthermore, this standard addition technique made it possible to estimate the approximate amount of the excreted acids. Analyses of the same urine samples with respect to the other compounds included in Table I gave three-channel SIRs almost identical with those shown in Fig. 2 (the selected ions were of course different for each compound).

Table II shows the results from an examination of urine samples from two normal persons on an ordinary diet and following the intake of 20 mmol of decanedioic and nonanedioic acid, respectively. As can be seen, 3-oxohexanedioic,

TABLE I

CHARACTERISTIC IONS FROM A SERIES OF 3-OXODICARBOXYLIC ACIDS AS THEIR CORRESPONDING DIMETHYL ESTER TRIMETHYLSILYL ENOL ETHER DERIVATIVES

3-Oxodicarboxylic acid	$M^{+\cdot}$	$(M-CH_3)^+$	$(M-CH_3O)^+$	$(M-CH_3OH)^{+\cdot}$
3-Oxohexanedioic acid	260	245	229	228
3-Oxoheptanedioic acid	274	259	243	242
3-Oxooctanedioic acid	288	273	257	256
3-Oxononanedioic acid	302	287	271	270
3-Oxodecanedioic acid	316	301	285	284

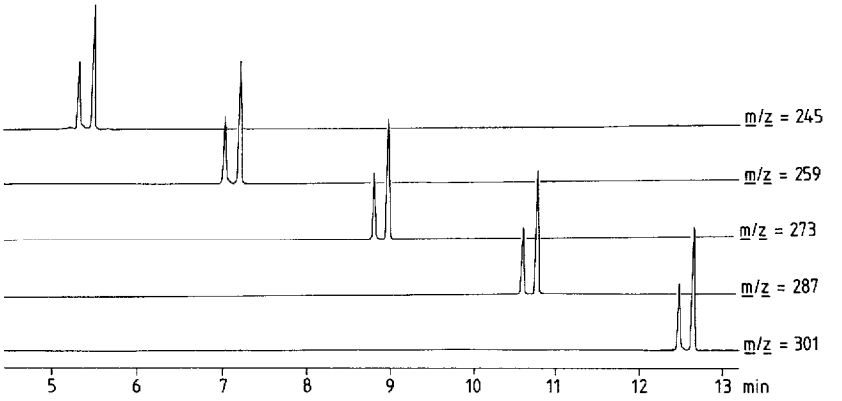

Fig. 1. SIR chromatograms of the M−15 ions from dimethyl 3-trimethylsilyloxy-2-hexenedioate (*m/z* 245), dimethyl 3-trimethylsilyloxy-2-heptendioate (*m/z* 259), dimethyl 3-trimethylsilyloxy-2-octenedioate (*m/z* 273), dimethyl 3-trimethylsilyloxy-2-nonedioate (*m/z* 287) and dimethyl 3-trimethylsilyloxy-2-decenedioate (*m/z* 301).

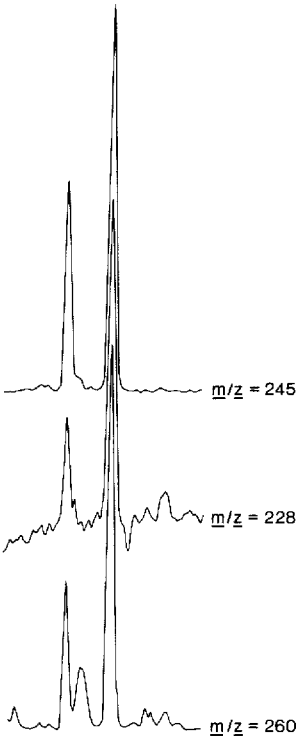

Fig. 2. SIR chromatograms of three selected ions from dimethyl 3-trimethylsilyloxy-2-hexenedioate in a urine sample following ingestion of decanedioic acid.

3-oxooctanedioic and 3-oxodecanedioic acid are excreted in small amounts as normal constituents of human urine, whereas the odd-carbon acids 3-oxoheptanedioic and 3-oxononanedioic acid could not be detected. Ingestion of decanedioic acid increased the excretion of even-carbon 3-oxodicarboxylic acids,

TABLE II

DETECTION OF 3-OXODICARBOXYLIC ACIDS IN HUMAN URINE

3-Oxoheptanedioic acid was not detected in any of the urine samples. N.D. means not detected. The asterisks refer to the relative amounts of the excreted acids: one asterisk describes an excretion of less than 0.5 μg acid per l urine, three mean more than 5.0 μg acid per l urine, and two mean an intermediate value.

Subject	Compound ingested	3-Oxo-hexanedioic	3-Oxo-octanedioic	3-Oxo-nonanedioic	3-Oxo-decanedioic
J.S.S.	Normal diet	**	*	N.D.	*
J.E.W.	Normal diet	**	*	N.D.	*
J.S.S.	Decanedioic acid	***	**	N.D.	***
J.E.W.	Decanedioic acid	***	**	N.D.	***
J.S.S.	Nonanedioic acid	**	*	**	**
J.E.W.	Nonanedioic acid	**	*	***	**

without significantly affecting the excretion of odd-carbon acids. When nonanedioic acid was ingested, 3-oxononanedioic acid was excreted. In addition, there was a small increase of 3-oxodecanedioic acid excretion; this was probably not due to a chain-lengthening process but may be explained by a hindrance of the normal degradation of endogenously formed dicarboxylic acids by the load of nonanedioic acid.

CONCLUSION

Our investigation shows that 3-oxohexanedioic acid, 3-oxooctanedioic acid and 3-oxodecanedioic acid are normal urine constituents, and ingestion of decanedioic acid yielded increased excretion on these acids. Ingestion of nonanedioic acid increased as expected the excretion of 3-oxononanedioic acid, together with a simultaneous increase in the 3-oxodecanedioic acid excretion. The demonstration of increased amounts of the 3-oxo intermediates when dicarboxylic acids were ingested supports the hypothesis that dicarboxylic acids are degraded by β-oxidation processes.

As for the ordinary fatty acids, the degradation of dicarboxylic acids may take place either in the mitochondria or in the peroxisomers [19]. Although the β-oxidation enzymes in the two organelles differ [20–24], the intermediates are identical. Thus, our results give no clue as to where dicarboxylic acid metabolism takes place.

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