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NEW POLAR ACID METABOLITES IN HUMAN URINE

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SUMMARY

A sequence of chromatographic methods (thin-layer chromatography, high-performance liquid chromatography and glass capillary gas chromatography) was used to separate the acid fraction of human urine. The power of this method to separate and detect previously unknown compounds and the elucidation of their final structure with mass spectrometry is exemplified by the identification of N-acetyl-2-aminooctanoic acid as a metabolic compound in the urine of healthy individuals.

In addition, the conjugate of glycine with indolepropionic acid, N-formylanthranilic acid, succinoylphenylalanine, δ-hydroxyvaleric acid, δ-hydroxycapric acid, 3-hydroxyadipic acid, and higher homologues were detected in a polar fraction of human urine.

INTRODUCTION

The acid fraction of urine is of extreme complexity. A perfect separation — following appropriate derivatisation — is not possible even with glass capillary gas chromatography (GC). Therefore, we recently combined two different chromatographic systems — thin-layer chromatography (TLC) and glass capillary GC — for separation [1]. This enabled us to identify a number of previously unknown compounds [2, 3].

The separation power was then enlarged by applying a third, different technique—high-performance liquid chromatography (HPLC). The combination of these three methods in a sequence allowed the determination even of trace compounds which are usually hidden under large peaks in the GC runs of unfractionated samples. The power of this technique is described in this paper dealing with the structure elucidation of previously unknown γ- and δ-hydroxy mono- and dicarboxylic acids, as well as amino acid conjugates, obtained from a polar fraction of urinary acid compounds.

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MATERIALS AND METHODS

Isolation, derivatisation and chromatography

Urine work-up. One litre of urine, acidified to pH 1, was extracted three times with 300 ml of ethyl acetate; the combined extracts were dried over sodium sulfate and evaporated in vacuo. The residue was dissolved in methanol and methylated by adding diazomethane in diethyl ether. This solution was concentrated in a nitrogen atmosphere.

Thin-layer chromatography. About half of the solution of the methylated compounds, corresponding to 500 ml of urine, was chromatographed on twenty thick-layer plates (1 mm, 20 × 20 cm), made from silica gel 60 HF_{254} (E. Merck, Darmstadt, G.F.R.) using a mixture of diethyl ether—cyclohexane (5 : 3) as mobile phase. Eight zones were marked under UV light (254 nm) and then scraped off. The R_F values for the zones were as follows: zone 1, 0.64—1.00; zone 2, 0.54—0.64; zone 3, 0.48—0.54; zone 4, 0.42—0.48; zone 5, 0.24—0.42; zone 6, 0.20—0.24; zone 7, 0.12—0.20; zone 8, 0.00—0.12.

High-performance liquid chromatography. The four most polar fractions (zones 5—8) were subjected to HPLC. They were chromatographed on a Waters Assoc. (Milford, MA, U.S.A.) μBondapak C_{18} (10 μm) column (300 mm × 3.9 mm I.D.) using a Spectra-Physics liquid chromatograph Model 3500 B and a Model 230 UV detector. A linear gradient of methanol—water starting with a percentage ratio of 30:70 up to 99:1, with a flow-rate of 1.2 ml/min, was used. Fractions were cut according to signals on the UV detector (254 nm).

Thirty-seven fractions were obtained. The solvent was removed under reduced pressure and the residue dissolved in a few drops of methanol.

Gas chromatography—mass spectrometry. Each of the 37 fractions was investigated by GC—MS using the following conditions: LKB 2091 mass spectrometer combined with a Pye-Unicam gas chromatograph; WCOT capillary column, 25 m long, filled with OV-101; temperature, 100—300°C, programmed at 2°C/min; data system, PDP 11, LKB 2130.

High-resolution mass spectrometry. High-resolution data were obtained by peak-matching with a Varian Model 312 mass spectrometer combined to a MAT SS 200 data system.

Synthesis of comparison compounds

N-Acetyl-2-aminooctanoic acid (1a) [4]

2-Bromocaprylic acid. Bromine (8 g) was added to a mixture of 7.2 g of caprylic acid and 450 mg of red phosphorus under stirring. Within the next 30 min, an additional amount of 8 g of bromine was added. The mixture was heated for 48 h on a water-bath; 900 mg of water were then added and the mixture heated to 150°C for 10 min.

2-Aminooctanoic acid. The crude 2-bromocaprylic acid was added to a solution of 45 g of ammonium carbonate in 7 ml of water and 20 ml of concentrated ammonia and heated for 24 h to 50°C. The mixture was poured into a porcelain vessel and concentrated over a burner until the temperature reached 110°C. After cooling to 60°C, the residue was dissolved in methanol and precipitated in the refrigerator (4.1 g).

2-Aminooctanoic acid methyl ester. A 1.1-g portion of the 2-aminooctanoic acid was suspended in 20 ml of methanol; 1 g of concentrated sulfuric acid and 20 ml of tetrachloromethane were added. This mixture was refluxed for 8 h using a water separator, then concentrated in vacuo and poured into 100 ml of a saturated solution of potassium carbonate. The solution was extracted three times with 50 ml of diethyl ether; the ethereal extract was dried over sodium sulfate and concentrated in vacuo; 0.7 g of an oily residue remained.

N-Acetyl-2-aminooctanoic acid (1a). To 0.7 g of 2-aminooctanoic acid were added 5 ml of acetic anhydride and left for 24 h at room temperature. The excess acetic anhydride was distilled off; 0.8 g of 1a remained.

3,3'-Indolepropionylglycine methyl ester (2a)

3,3'-Indolepropionic acid (95 mg), prepared from gramine and malonic acid diethyl ester [5], and 62 mg of glycine methyl ester hydrochloride were suspended in acetonitrile; 110 mg of dicyclohexylcarbodiimide and a few drops of pyridine were added. The mixture was stirred for 2 h. The solution was filtered off from dicyclohexyl urea, and the filtrate evaporated to dryness. The residue was dissolved in diethyl ether and washed with dilute hydrochloric acid and a saturated aqueous solution of sodium hydrogen carbonate. After drying (sodium sulfate) and evaporation, 100 mg of 2a in the form of a viscous oil remained.

N-Formylanthranilic acid (3a)

Anthranilic acid (1.4 g, 0.01 mol) was added to 20 ml of formic acid and refluxed for 1 h. The solution was evaporated in vacuo. The solid residue (3a) was crystallized from dilute ethanol. Yield 1.5 g; m.p. 164°C (167°C [6]).

(N-Succinoylmonomethyl ester)-phenylalanine methyl ester (5b)

Phenylalanine methyl ester hydrochloride (2.15 g) was dissolved in 20 ml of pyridine to which 1.5 g of succinic acid monomethyl ester monochloride were added under stirring. After a few minutes the warm solution was poured into a mixture of ice and hydrochloric acid. The solution was extracted three times with ethyl acetate. The combined organic extracts were washed with a 1 *N* sodium carbonate solution and dried with sodium sulfate. After evaporation a residue of 2.2 g of 5b remained which was recrystallized from diethyl ether; m.p. 76—80°C.

N-2(3-Phenylpropionic acid methyl ester)-succinimide (4a)

Phenylalanine methyl ester hydrochloride (1 g) was mixed with well-powdered succinic anhydride. This mixture was heated in a test-tube above the melting point until the development of gaseous HCl and water was finished. The cooled residue was dissolved in 1 *N* sodium carbonate which was extracted three times with diethyl ether. After evaporation, 0.6 g of residue remained; 4a was recrystallized from ether, m.p. 80°C (needles).

5-Oxotetrahydrofuryl-2-acetic acid (11a) [7]

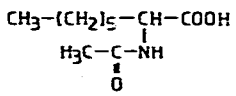
3-Hexenedioic acid (1 g) was dissolved in half-concentrated sulfuric acid and heated for 3 h to 110°C. After cooling the solution was extracted with diethyl

ether. The extract was evaporated. A residue, containing a mixture of 5-oxotetrahydrofuryl-2-acetic acid and 3-hexenedioic acid, remained.

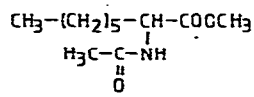
RESULTS AND DISCUSSION

Characterization of N-acetyl-2-aminooctanoic acid

The power of the applied combined separation methods will be exemplified by the detection and final characterization of N-acetyl-2-aminooctanoic acid (1a).



1a



1b

Fig. 1 shows the profile of the methylated acid fraction using glass capillary GC alone. N-Acetyl-2-aminooctanoic acid methyl ester (1b) was not detectable. The region where it is buried under the other compounds is marked by an arrow.

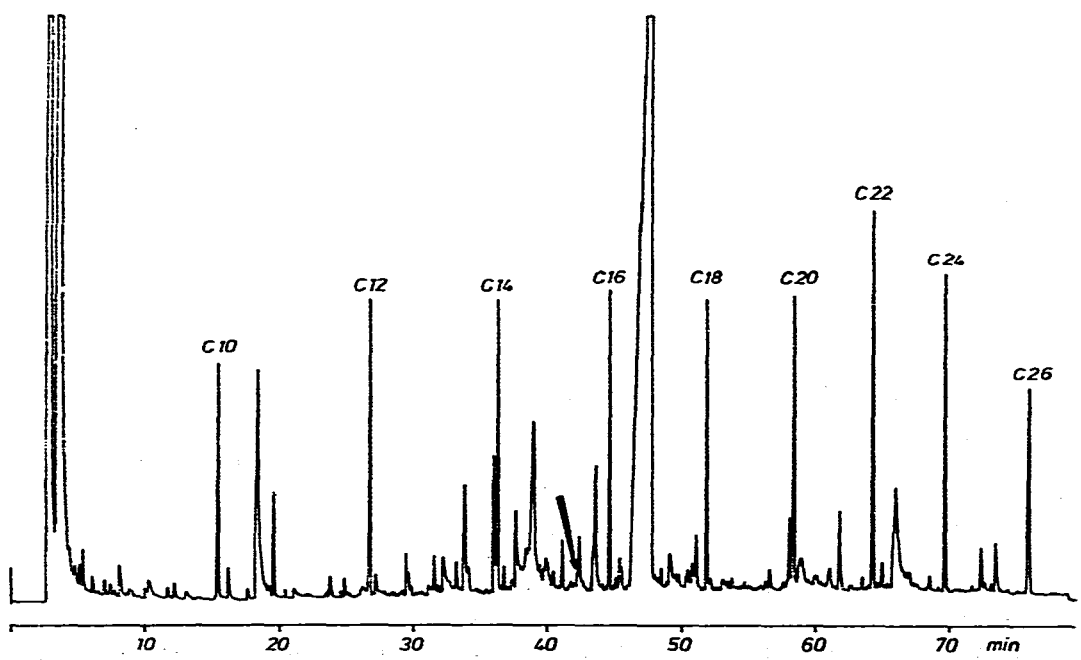


Fig. 1. Glass capillary gas chromatogram of the methylated acid fraction of human urine.

After TLC separation 1b was detected in fraction 7 (Fig. 2) by glass capillary GC—MS. Its mass spectrum is represented in Fig. 3. The mass spectrum is characterized by key ions of mass 156 and 114, produced by α-cleavage followed by loss of ketene and further degradation to an ion of mass 30.

Another α-cleavage reaction produces the ion of mass 88. The ion of mass 43

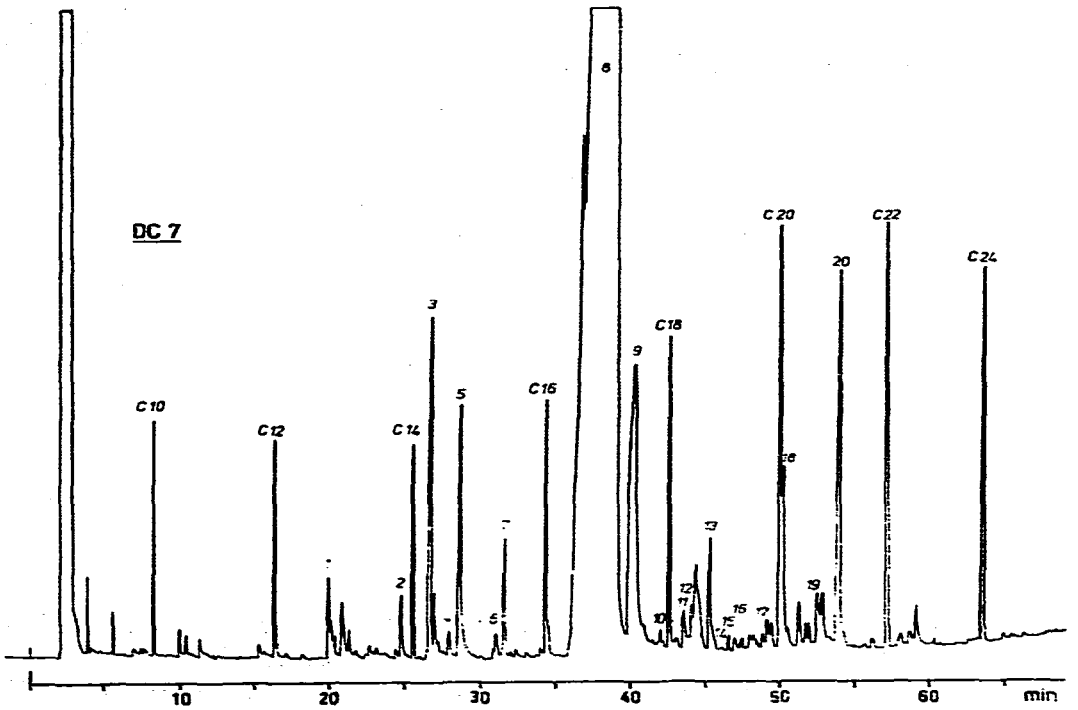


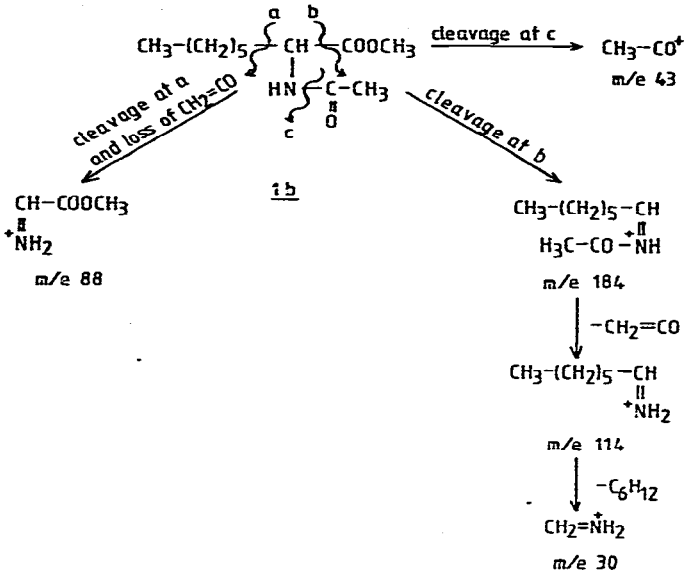
Fig. 2. Glass capillary gas chromatogram of fraction 7 of the thin-layer separation of the urinary acids. The numbers above the peaks correspond to the compounds listed in Table I.

TABLE I

SUBSTANCES CORRESPONDING TO THE NUMBERS ON THE GLASS CAPILLARY GAS CHROMATOGRAM OF FIG. 2

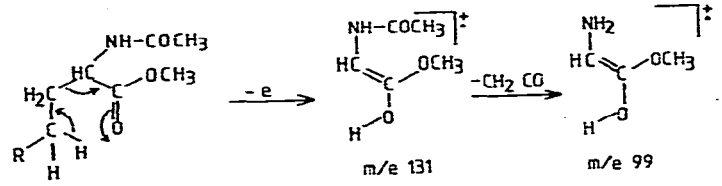
No.	RI*	Mol. wt.	Substance
1	1280	158	5-Oxotetrahydrofuryl-2-acetic acid
2	1383	171	3-Methylcrotonylglycine
3	1424	234	Citric acid
4	1452	234	Isocitric acid
5	1471	183	2-Furoylglycine
6	1523	186	4-(5-Oxotetrahydrofuryl-2)-butyric acid
7	1537	215	N-Acetylaminooctanoic acid
8	1650	193	Hippuric acid
9	1745	196	3-(3-Hydroxyphenyl)-3-hydroxypropionic acid
10	1784	207	3-Methylhippuric acid
11	1825	209	Hydroxyhippuric acid
12	1838	208	Ferulic acid
13	1873	241	5-Methoxycarbonylfuran-2-carbonyl-glycine
14	1892	261	N-2-(3-Phenylpropionic acid)-succinimide
15	1905	223	3-Methoxyhippuric acid
16	1930	223	4-Methoxyhippuric acid
17	1976	219	3-Indolyllactic acid
18	2006	219	Cinnamoylglycine
19	2065	205	5-Hydroxy-3-indolylacetic acid
20	2110	293	N-Phenylacetylglutamic acid

*RI = Kováts' index.



indicates the presence of the acetyl group, that of mass 60 a —NH—$COCH_3$ group connected to an alkyl chain with several carbon atoms [8].

References to the chain length of R were obtained by the peaks of mass 131 and 99, resulting possibly from loss of C_6H_{12} followed by loss of ketene:



To confirm these assumptions high-resolution data were needed. This, however, demanded the accumulation of enough sample to carry out peak matching. Knowledge of the mass spectrum (Fig. 3) made a control of further enrichment possible by running mass spectra of fractions obtained by HPLC separation (Fig. 4). It must be emphasized that the peaks indicated in this chromatogram do not correlate with the compounds found in the GC runs, since UV-inactive

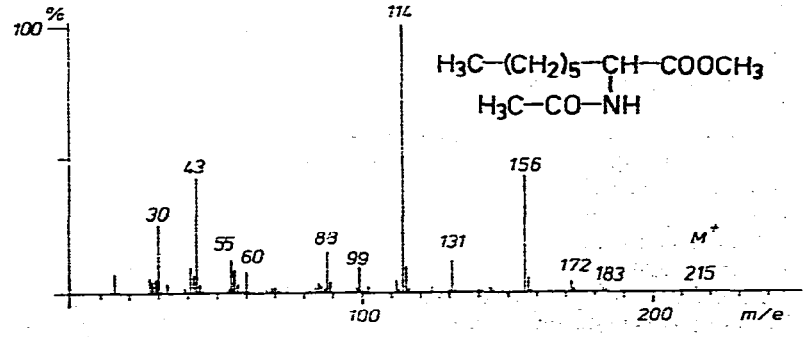


Fig. 3. Mass spectrum of N-acetyl-2-aminooctanoic acid methyl ester (1b).

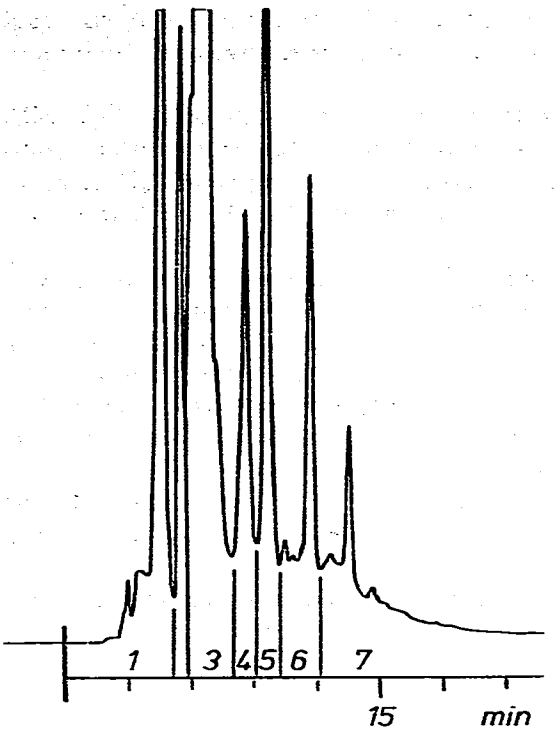


Fig. 4. HPLC separation of the thin-layer fraction 7.

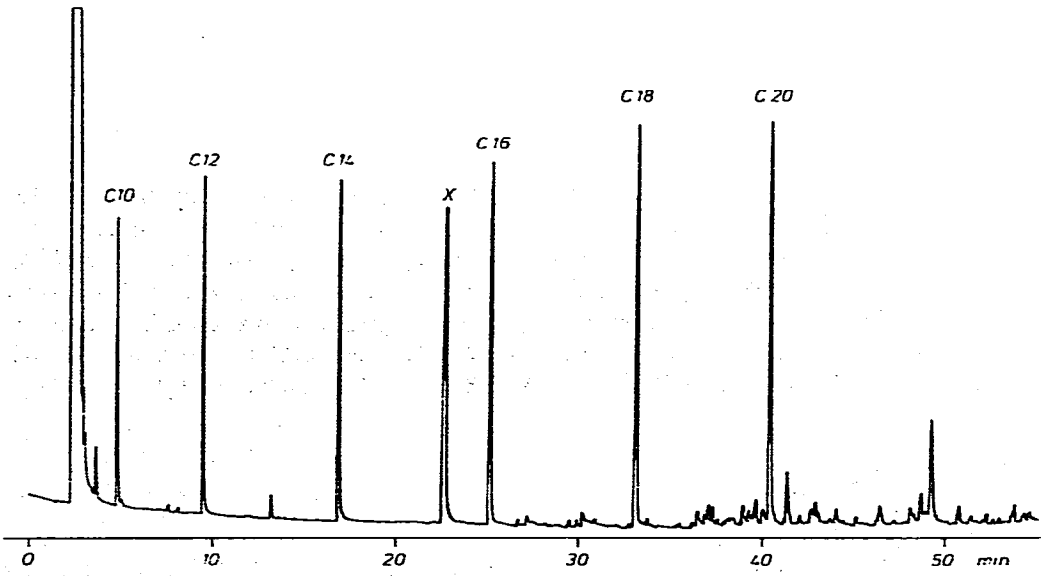


Fig. 5. Glass capillary gas chromatogram of the HPLC fraction 7, shown in Fig. 4. The peak of the enriched compound 1b is indicated by X.

compounds are not registered. Nevertheless, the peaks in the HPLC chromatogram allowed the same distinct section in subsequent runs, always to be collected.

Each fraction of the HPLC chromatogram was again investigated by GC—MS. HPLC fraction 7 contained the unknown compound (Fig. 5) in nearly pure form. By direct insertion of this HPLC fraction into the high-resolution mass spectrometer, the molecular formulae of the key ions listed in Table II were obtained by peak matching.

The high-resolution data confirmed that the chain corresponded to C_6H_{13}. The mass spectrum and the high-resolution data did not allow any conclusion about the arrangement of the six carbon atoms. To clarify this point, the most probable isomer, the compound with a straight C_6 chain, was synthesized.

To confirm the identity of the natural product with the synthetic sample the mass spectra of both components and their Kovats' indices were compared and found to be identical.

N-Acetyl-2-aminooctanoic acid was found to occur in human milk [9] and in *Aspergillus atypique* [10]. Our investigations revealed that it is obviously also a normal metabolite in healthy individuals.

In a similar way the structures of the other so far unknown compounds were determined.

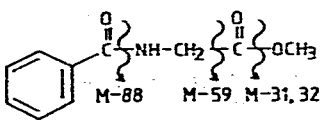
TABLE II

DATA OF THE UNKNOWN COMPOUND OF FIG. 5, OBTAINED BY HIGH-RESOLUTION MASS SPECTROMETRY

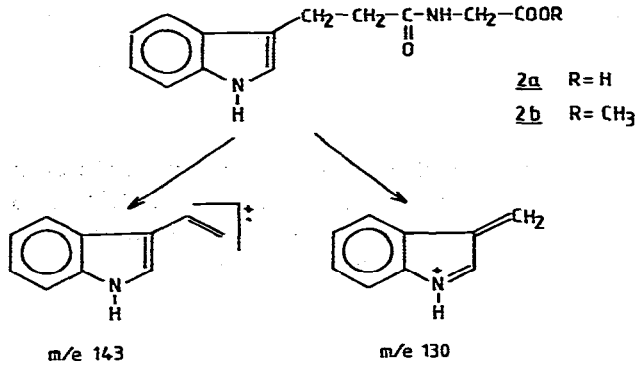
Mass	Molecular formula
215	$C_{11}H_{21}NO_3$
156	$C_9H_{18}NO$
114	$C_7H_{16}N$

Amino acid conjugates

Amino acid derivatives, especially conjugates of glycine, are common metabolic products [11]. Besides hippuric acid, the main component in the acid fraction of urine, 3- and 4-hydroxy-hippuric acid, α-picolinuric acid as well as 5-carboxy-furan-2-carbonyl-glycine [3] are known to occur in normal urine in low amounts. These methyl esters of glycine conjugates are usually easy to detect by the presence of key ions at M—31 (M—OCH_3) and/or M—32 (M—CH_3OH), M—59 (M—$COOCH_3$) and M—88 (M—$NHCH_2COOCH_3$).



This is not so in the case of the methyl ester of the conjugate of glycine with indolylpropionic acid (2b), detected in the HPLC fraction 6 of TLC zone 8, the mass spectrum of which is represented in Fig. 6. The main peaks correspond to the indolyl part of the molecule:



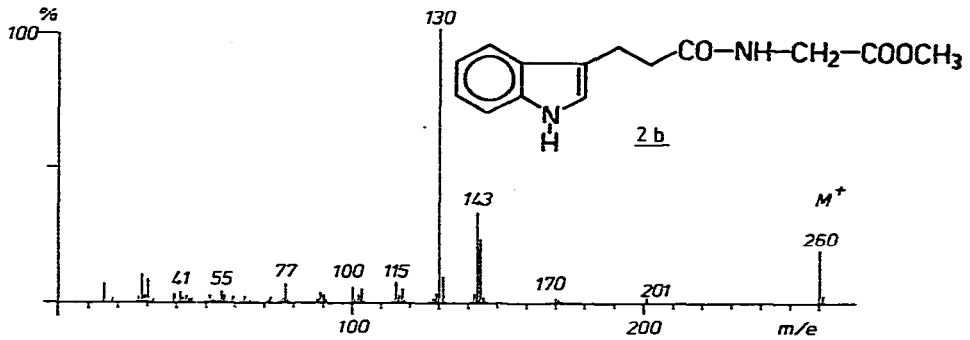


Fig. 6. Mass spectrum of the conjugate of indolylpropionic acid methyl ester with glycine (2b).

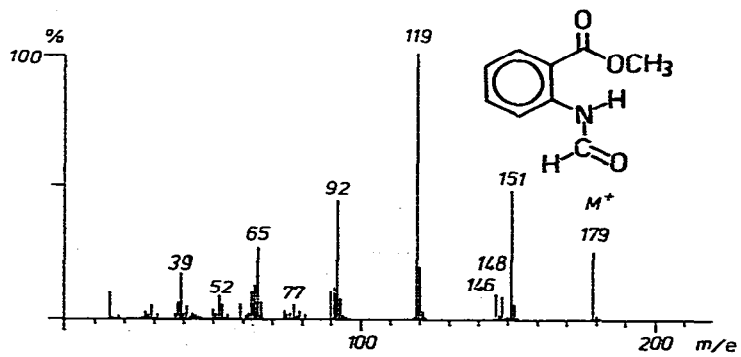
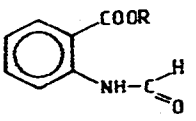


Fig. 7. Mass spectrum of N-formyl-anthranilic acid methyl ester (3b).

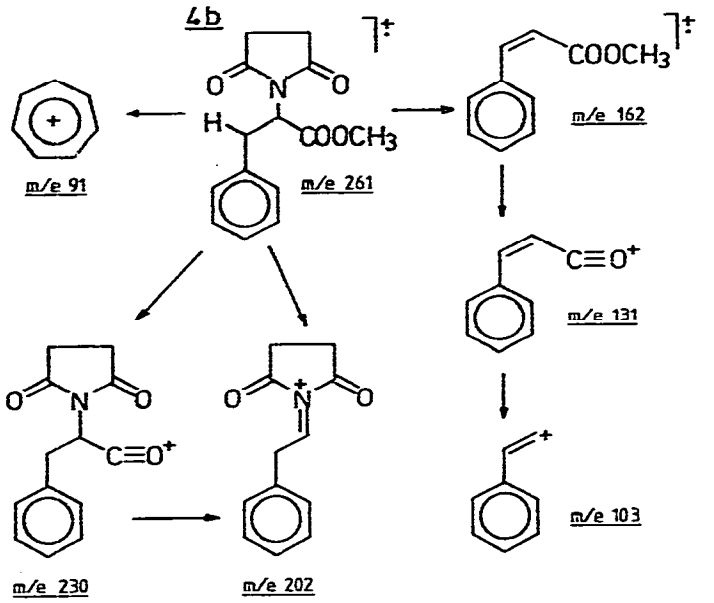
The presence of 2 was not known previously although it is well known that indolepropionic acid occurs in urine [12]. Anthranilic acid is a long-known urinary metabolite [13]. Its N-formyl derivative 3a has now been detected in human urine in the form of its methyl ester 3b in TLC zone 5. The mass spectrometric degradation of its methyl ester (3b) (Fig. 7) starts with the loss of the formyl group in the form of CO, thus producing the molecular ion of anthranilic acid which is degraded in the same way as described for this compound [14].



3a R=H

3b R=CH3

In HPLC fraction 6 of TLC zone 7 we detected another previously unknown acid compound. The mass spectrum (Fig. 8) showed ions at *m/e* 162, 131, 103 and 77, reminiscent of the ions found in the mass spectrum of cinnamic acid methylate, indicating (together with the ion of mass 91) the presence of a substituted phenylpropionic acid derivative:



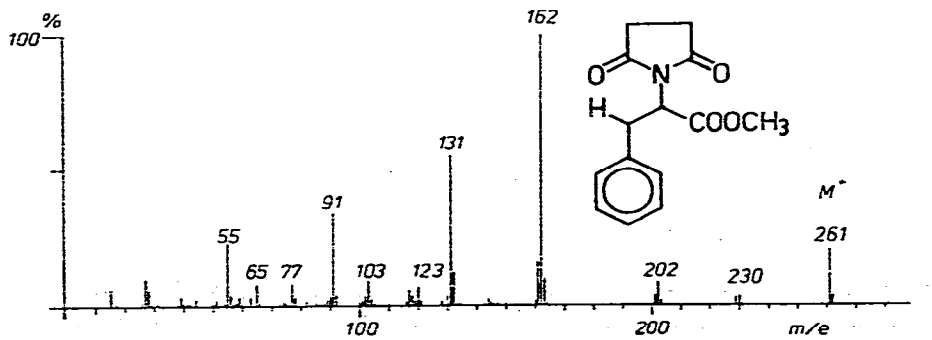


Fig. 8. Mass spectrum of N-(3-phenylpropionic acid methyl ester)-succinimide (4b).

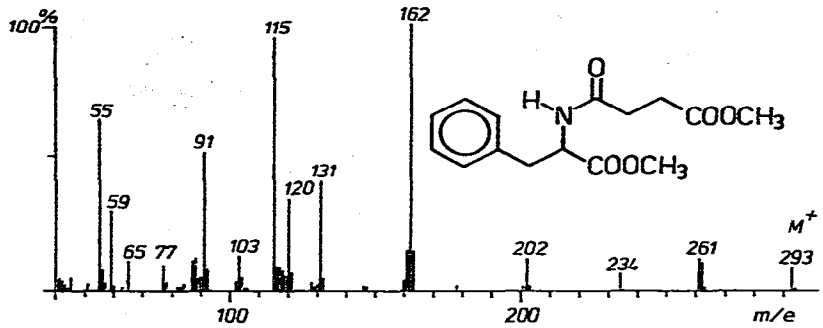
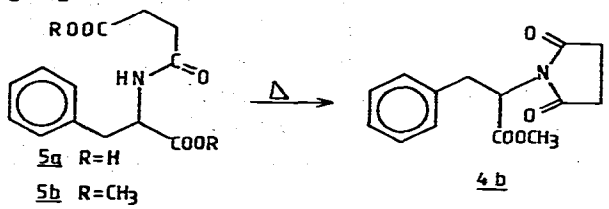


Fig. 9. Mass spectrum of the dimethyl ester of N-succinoylphenylalanine (5b).

Peak matching of the molecular ion gave values in agreement with the molecular formula $C_{14}H_{15}NO_4$. The ion at *m/e* 162 was determined to be $C_{10}H_{10}O_2$. Thus the substituent should be $C_4H_5O_2N$. Succinimide corresponds most likely with this formula. The combination of both parts allows the structure of 4b to be deduced. This assignment was proved by synthesis and comparison of the spectroscopic data and Kovats' indices. In the same fraction, but only in traces, a compound was detected, whose mass spectrum (Fig. 9) resembled strongly that of 4b but which showed a molecular ion 32 mass units higher than that of 4b. The compound was assumed to be the dimethyl ester of the succinoyl derivative of phenylalanine (5b). A synthesis proved this assumption. The imide 4b is probably an artefact of the dimethyl ester 5b, formed in the injecter of the gas chromatograph: 5b is partly cyclized to 4b in the gas chromatograph [15].



5a R=H
5b R=CH3

4b

γ- and δ-hydroxycarboxylic acids

In a previous paper we reported the detection of compounds in urine, that are characterized by intense ions of mass 85 and 99 in their mass spectra. We tentatively regarded these compounds to be γ- and δ-lactones [1]. The improved separation technique now allowed us to obtain spectra free of by-products, enabling us to recognize even small peaks free from background, important for the deduction of the structure of lactones. Thus, γ-valerolactone

6 7

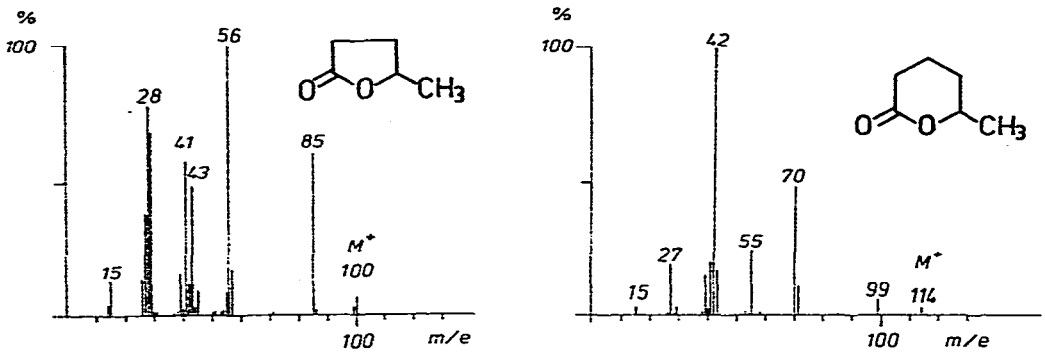
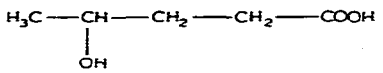


Fig. 10. Mass spectrum of γ-valerolactone.

Fig. 11. Mass spectrum of δ-caprolactone.

(6) (Fig. 10) and δ-caprolactone (7) (Fig. 11) could be identified by comparison with mass spectra already published [16, 17]. These lactones are probably not originally present in urine, but are produced from the corresponding hydroxy compounds 8 and 9 by acidifying the urine sample.



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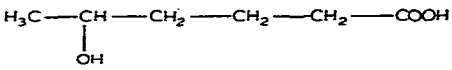

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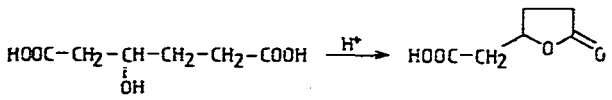
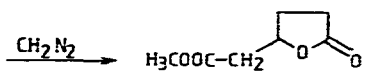
The occurrence of 5-hydroxyhexanoic acid has been reported in two cases of hypoglycaemia, but not in urine of healthy individuals [18].

Further compounds with the typical key ion of mass 85, indicating a γ-lactone, were found in the same fraction of the thin-layer chromatogram. The small amounts of sample prohibited recording spectra free of background. Nevertheless, we succeeded in identifying one of these compounds, 3-hydroxyadipic acid (10), which is converted obviously to 5-oxo-tetrahydrofuryl-acetic acid methyl ester (11b) first by treatment with acid to 11a and then by methylation with diazomethane:



10 11a



11b

The mass spectrum of 11b (Fig. 12) does not show a molecular ion; the ions of highest masses correspond to loss of H_2O and CO. The spectrum is further characterized by loss of $\cdot OCH_3$, CH_3OH and CO. The main ion is that of mass 85:

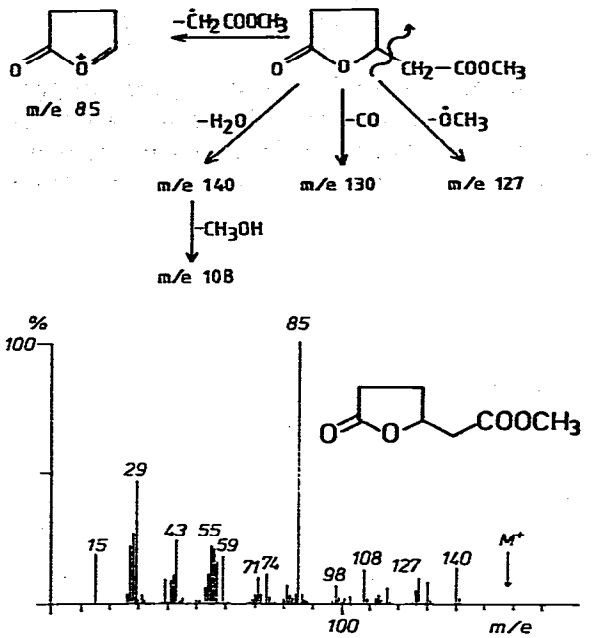


Fig. 12. Mass spectrum of 5-oxo-tetrahydrofurylacetic acid methyl ester (11b).

The validity of the structural assignment could be proved by synthesis. 4-Hydroxyoctanedioic acid, 4-hydroxyundecanedioic acid and 4-hydroxydodecanedioic acid were identified by mass spectra and Kováts' indices too (Table III).

TABLE III

KOVÁTS' INDICES OF SOME γ-LACTONES

No.	RI	Mol. wt.	Substance
1	1280	158	5-Oxotetrahydrofuryl-2-acetic acid methyl ester
2	1871	228	4-Hydroxyundecanedioic acid γ-lactone
3	1968	242	4-Hydroxydodecanedioic acid γ-lactone

GENERAL REMARKS

All the compounds described were found in the urine of healthy male and female adults, aged 20—35 years, living on a normal diet. Since we have not yet investigated profiles of acids in plants we are unable to distinguish between compounds introduced into the body by nutrition and which pass through the body unchanged and those that are produced in the body or are metabolised.

The identification of unknown compounds necessitates in most cases the use of the described combination of chromatographic and spectroscopic techniques. Then, after structure elucidation, identification should be possible by simple low-resolution GC—MS. In some cases even the measurement of the RI using glass capillary columns should allow an identification.

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