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HIPPURIC ACID AND 3-CARBOXY-4-METHYL-5-PROPYL-2-FURANPROPIONIC ACID IN SERUM AND URINE

ANALYTICAL APPROACHES AND CLINICAL RELEVANCE IN KIDNEY DISEASES

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SUMMARY

Hippuric acid (HA) and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (FA) were determined in serum, plasma, ultrafiltrate and urine by gas chromatography (GC), high-performance liquid chromatography and GC with mass-selective detection, and the methods were compared. As determined by affinity chromatography and analysis of serum and ultrafiltrate, 0.5% of FA in serum occurs free and 99.5% is bound to albumin. In haemodialysed patients with chronic renal failure, the plasma levels of HA and FA are elevated in comparison with normal controls and hospital patients without kidney diseases: HA, 11.1 ± 5.7 mg/dl (n = 86); FA, 1.9 ± 1.2 mg/dl (n = 86). Gradual increases in HA in serum, depending on the creatinine concentrations, are found in non-dialysed patients with chronic renal failure. By haemodialysis and haemofiltration the HA levels are lowered (53–66 and 30–36%, respectively), whereas FA is not dialysable.

INTRODUCTION

In patients with chronic renal failure, a variety of substances are retained in blood which are normally excreted by the kidneys. Their accumulation leads to uraemia, in the course of which some of the retained constituents, the so-called uraemic toxins, exert toxic effects on many organs and functions of the body. They must be therapeutically eliminated by blood purification procedures such as haemodialysis, haemofiltration, haemoperfusion or plasma exchange. Despite adequate treatment, certain clinical features of the uraemic syndrome persist. So far, the toxicity of uraemic plasma has not been attributed to a single substance or group of substances. Among the organic acids which accumulate in blood as a result of renal failure, the two major components are hippuric aid (HA) and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (furancarboxylic acid, FA). Both substances appear to be important parameters in the investigation of the renal excretion function in kidney diseases.

HA, the glycine conjugate of benzoic acid which is primarily formed from

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aromatic amino acids by the metabolism of the gastrointestinal flora, has long been known as a major urinary excretion product. Its concentration in blood plasma of healthy individuals is low, 0.2 ± 0.1 mg/dl[1]. Recent studies indicate that the elevated concentration of HA in uraemic serum is an independent parameter, which correlates better with the residual renal function than the conventional retention parameters creatinine and urea[2]. FA has been identified to be a regular urinary excretion product[3,4] and to occur in low concentrations in serum of normal subjects[5]. The origin of FA is not well known. Furanoid fatty acids have been found in the livers and testes of several fish species[6–8], in corals[9] and in natural rubber latex[10] as constituents of lipids and have been demonstrated to be catabolized to FA and other metabolites in the rat[11]. FA and the furanoid fatty acids differ in structure and molecular weight from another group of known urinary furan compounds, *e.g.*, furan-2,5-dicarboxylic acid, probably originating from carbohydrates[12,13].

Several gas (GC) and high-performance liquid chromatographic (HPLC) procedures have been reported for the determination of HA[1,2,14–17] and FA[14,18]. As complementary information can be derived from the levels of HA and FA, methods for the simultaneous determination of the two substances are required. They must be applicable to serum and urine and suitable for extended clinical investigations with large numbers of samples.

EXPERIMENTAL

Samples

Blood samples were obtained from healthy volunteers, healthy blood donors, non-dialysed patients with chronic renal failure, haemodialysed patients with chronic renal failure, haemodialysed patients with acute renal failure, patients with chronic renal failure treated by haemofiltration and control patients suffering from hypercholesterolaemia, hypertriglyceridaemia, hyperuricaemia, hyperbilirubinaemia, diabetes mellitus and liver cirrhosis. Blood was collected by venipuncture and the serum was obtained by centrifugation of the blood at 1500 *g* for 10 min. As patients received heparin during dialytic treatment, plasma was obtained during and after dialysis.

Twenty four-hour urine samples were collected from healthy individuals and haemodialyzed patients with chronic renal failure and residual urinary excretion. All samples were stored at −20°C prior to analysis.

Chemicals

All solvents were of the highest grade available. HA was purchased from Aldrich (Steinheim, F.R.G.) and FA was synthesized according to a method generally described for furanoid acids[5]. The following chemicals were used as reagents for the synthesis of FA or as internal standards: 2-hexanone, azobis(isobutyronitrile), 3-phenoxypropionic acid (Aldrich), 3-oxoadipic acid, sulphuryl chloride, phenoxyacetic acid and 2-acetylaminobenzoic acid (Fluka, Neu-Ulm, F.R.G.), 3-hydroxy-2-phenylpropionic acid (Serva, Heidelberg, F.R.G.) and 4-acetyl-3,5-dimethyl-2-pyrrolecarboxylic acid (EGA-Chemie, Steinheim, F.R.G.).

Apparatus and instrumental conditions

Determinations by GC were performed on a Model 3700 gas chromatograph

(Varian, Darmstadt, F.R.G.) and a Vega 6130 gas chromatograph (Carlo Erba, Hofheim, F.R.G.) with flame ionization detectors and equipped with 25 m × 0.25 mm I.D. fused-silica columns, coated with OV-1701 (Macherey-Nagel, Düren, F.R.G). The conditions were as follows: carrier gas, nitrogen at 4 ml/min; column temperature, 60°C for 10 min, then programmed at 2°C/min to 230°C and held at this temperature for 30 min; injector block temperature, 250°C; and sample size, 1 μl with a splitting ratio of 1:20.

For quantitation by GC combined with mass-selective detection (MSD) a Model 5890 gas chromatograph interfaced with a 5971 A mass-selective detector and equipped with a Model 7673 A automatic injector (Hewlett-Packard, Böblingen, F.R.G.) was employed under the following conditions: column, HP-1 (dimethylpolysiloxane), 25 m × 0.2 mm I.D. fused-silica (Hewlett-Packard); carrier gas, helium; column temperature, programmed from 130 to 180°C at 2°C/min, then to 240°C at 40°C/min between each injection; injector block temperature, 230°C; interface temperature, 280°C; and ion source temperature, 170°C.

Determinations by HPLC were performed on a Merck-Hitachi apparatus consisting of a Model L 6200 ternary gradient pump, a Model 655 A-40 autosampler, a Model 655 A-52 column oven, a Model L 3000 photodiode-array detector and a Model D 2000 two-channel integrator (Merck, Darmstadt, F.R.G.). The column oven temperature was set at 40°C. The columns used were 125 × 5 mm I.D. LiChroCART RP-8, 5 μm (Merck) and 125 × 4 mm I.D. Hypersil MUS, 5 μm (Grom, Herrenberg, F.R.G.). The mobile phase was composed of acetonitrile (A) and water with 5 m*M* tetrabutylammonium phosphate (B) and was applied under the following conditions: flow-rate, 1.5 ml/min; gradient elution with initially 10% A + 90% B for 5 min, then programmed linearly within 15 min to 30% A + 70% B, which was held for another 5 min, thereafter it was switched back to the initial conditions and re-equilibrated for 5 min. Detection was achieved at two wavelengths simultaneously (214 and 250 mm); the sample size was 30 μl.

For ultrafiltration of serum pool samples (45 ml), a Model 8010 stirred ultrafiltration cell with a Diaflo YM10 ultrafiltration membrane (Amicon, Witten, F.R.G.) was used, applying nitrogen at a pressure of 4.5 bar.

Affinity chromatography of the serum proteins was performed on a Super Rac fraction collector and a Uvicord S II UV monitor (LKB, Gräfelfing, F.R.G.). Protein separation was achieved using anti-human albumin antibodies from rabbits (Dakopatts, Hamburg, F.R.G) fixed on cyanogen bromide-activated Sepharose R 4B (Pharmacia, Freiburg, F.R.G); detection was effected at 280 nm. Desorption of albumin was performed with 2.5 *M* ammonium thiocyanate in phosphate buffer. The eluates were concentrated using a Model 8 MC ultrafiltration system (Amicon) with a YM10 membrane. The purity of the albumin fraction was controlled by polyacrylamide gel electrophoresis using a Model GE-2/4 electrophoresis system (Pharmacia).

For haemodialysis, Cuprophane D 3, polysulphone F 60 (Fresenius, Bad Homburg, F.R.G.) and polyacrylonitrile AN 69 (Hospal, Nürnberg, F.R.G.) membranes were used. Haemofiltration was performed with Filtral 16 (Hospal, Nürnberg, F.R.G.), PAN-250 NOVA (Diamed, Cologne, F.R.G.), Hemoflow HF 80 (Fresenius) and Hemofilter FH 88H (Gambro, Munich, F.R.G.) filters.

Sample preparation

The sample preparation for the GC method, including deproteinization with 2-propanol, extraction by anion-exchange chromatography, methylation with diazomethane and prefractionation by thin-layer chromatography, has been described previously[14].

For GC–MSD analysis, to 2 ml of serum, plasma, haemofiltrate or urine 200 μl of internal standard solution containing 50 mg of 2-acetylaminobenzoic acid and 5 mg of 4-acetyl-3,5-dimethyl-2-pyrrolecarboxylic acid in 100 ml of water were added. The mixture was deproteinized with 2 ml of 1 *M* perchloric acid and centrifuged. The supernatant was subjected to solid-phase extraction using Bond Elut C_{18} and Bond Elut 2 OH cartridges in series and a Vac Elut SPS 24 vacuum system (ICT, Frankfurt, F.R.G.). Elution was achieved with 2 ml of methanol–2-propanol (1:1, v/v) and the acids in the eluate were methylated with diazomethane.

For HPLC analysis, 50 μl of internal standard containing 100 mg of 3-phenoxypropionic acid in 100 ml of acetonitrile were added to 1 ml of serum or plasma (for haemofiltrate, 2 ml of sample were used). The proteins were precipitated with 2 ml of 2-propanol. After centrifugation the supernatant was decanted into a 10-ml tube with a ground-glass joint and 1 ml of 1 *M* sodium hydroxide solution was added. This mixture was extracted twice with 4 ml of *n*-hexane, then the aqueous sample was adjusted to pH < 1 with 200 μl of 25% hydrochloric acid and extracted twice with diethyl ether. To the combined organic phases 100 μl of tetrabutylammonium phosphate–tetrabutylammonium hydroxide (1.5:1, v/v; pH 8.0) were added. The diethyl ether was removed under vacuum and the residue was passed through a 0.45-μm low-dead-volume filter (Millipore, Eschborn, F.R.G.) and subjected to HPLC analysis.

Identification

The identities of HA and FA in the chromatograms and the peak compositions were verified on the basis of the retention data of the substances and reference compounds and mass spectrometrically with the GC–MSD system.

Quantification

Quantifications were achieved on the basis of the internal standards. Response factors were established by adding different amounts of the reference compounds to a serum pool with low concentrations of HA and FA. The ions of *m/z* 193 and 134 were used for the GC–MSD determinations of HA and *m/z* 179 and 148 for the determination of FA.

Clinical chemical parameters

Creatinine was measured by the Jaffé reaction, urea by reaction with *o*-phthalaldehyde, uric acid according to the phosphotungstate method, total protein by the biuret reaction, total bilirubin according to a modified Jendrassik–Grof reaction and cholesterol and triglycerides by enzymatic methods. The analyses were performed on a PARALLEL analyser[19] (American Monitor, Bornheim, F.R.G.).

RESULTS AND DISCUSSION

Free and protein-bound fractions

The major part of FA is bound to serum proteins. Its free fraction has been estimated to be *ca.* 0.5%[14] and 1%[20]. As for fatty acids and other carboxylic acids, serum albumin was assumed to be the most likely binding protein for FA. The assumption was confirmed by fractionating the serum proteins by affinity chromatography and by analysing the fractions. FA was detected only in the albumin fraction.

The mean percentage of the free fraction of FA as determined by analysing the ultrafiltrates and the corresponding serum samples of seven pools prepared from serum samples from haemodialysed patients (range of FA concentrations in the serum pools, 0.35–2.0 mg/dl) was 0.49% with a range of 0.32–0.63%.

Analytical aspects

Because of its binding to serum albumin, the determination of FA in serum, plasma and albumin-containing urine requires its displacement from the proteins. This procedure is also necessary for the determination of HA as HA is also partially protein-linked[14,21]. The methods applied for displacing the substances and precipitating the proteins include boiling the sample at 100°C[22], addition of acetonitrile[23], hydrochloric acid, acetic acid and ethyl acetate[21] and, in this investigation, either 2-propanol or 1 *M* perchloric acid.

The GC procedure is applicable to all types of samples: serum, plasma, ultrafiltrate and urine. As a result of the elaborate sample preparation procedure, in particular the pre-fractionation, complete separation of HA, FA and the internal standards from other endogenous and eventual exogenous substances is usually

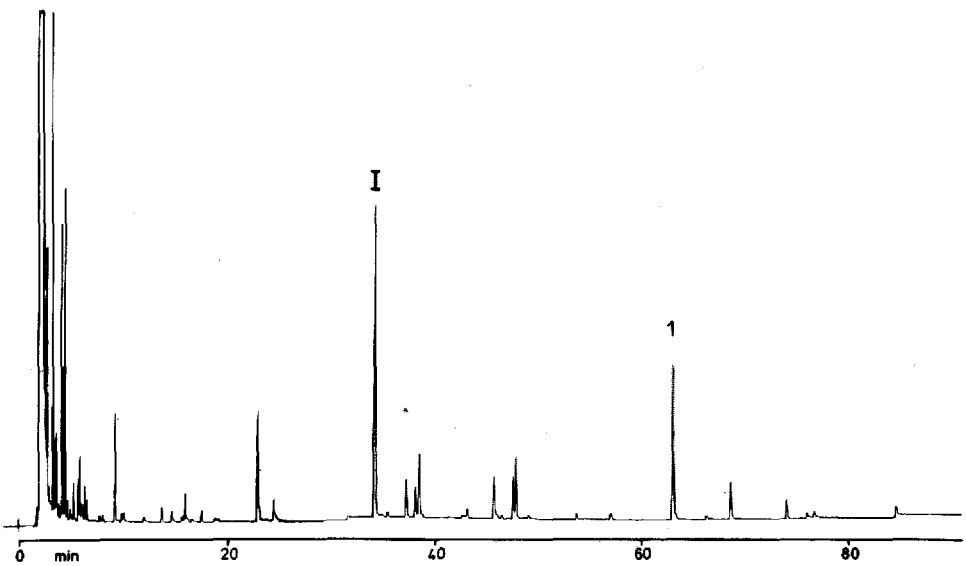

Fig. 1. Gas chromatogram of the organic acid fraction 2 in plasma of a haemodialysed patient. Peaks: I = internal standard (phenoxyacetic acid); 1 = FA.

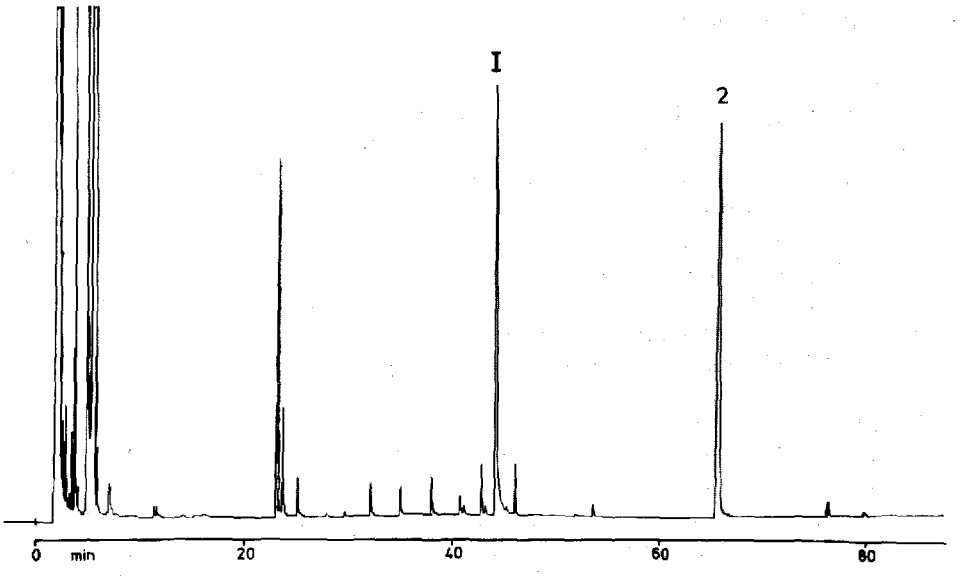

Fig. 2. Gas chromatogram of the organic acid fraction 3 in plasma of a haemodialysed patient. Peaks: I = internal standard (3-hydroxy-2-phenylpropionic acid); 2 = HA.

achieved (Figs. 1 and 2). Additional metabolites can also be detected and quantified. The major limitation of the method is its complexity, its long analysis time of 2 days and the large sample size of 3–5 ml. The lengthy sample preparation is responsible for only a moderate precision of the method, with coefficients of variation of 8% for HA and FA.

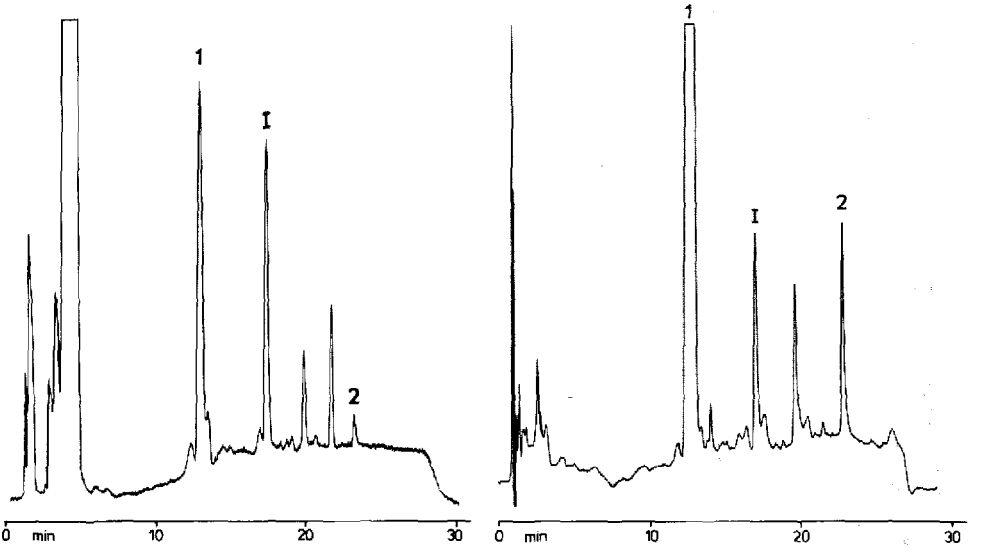

Fig. 3. Liquid chromatograms of a serum sample from a haemodialysed patient. Peaks: I = internal standard (3-phenoxypropionic acid); 1 = HA; 2 = FA. Detection at 214 nm (left) and 250 nm (right).

The HPLC method can be used for serum, plasma and ultrafiltrate samples. It is not suitable for urine, because varying interferences occur from endogenous constituents in urine. On the other hand, it has a number of advantages over the GC method. Sample preparation is short (1–2 h) and simple to perform, series of 20 samples or more can be analysed in parallel, the sample size is small (0.5–2 ml) and the separation can be automated. The coefficients of variation within series were determined to be 4.2% for HA and 7.8% for FA. The extraction of the neutral lipids from the serum with *n*-hexane is recommended to reduce the deterioration of the column. To minimize variances in the retention times due to shifts in the column temperature, the separation is done at 40°C. Fig. 3 shows an example of a serum sample from a haemodialysed patient with detection at 214 and 250 nm.

The GC–MSD method combines the advantages of the other two methods. It is applicable to all types of samples, interferences from endogenous and exogenous substances are not observed, the analysis time is short (1–2 h), the sample size is small (1–2 ml), longer series (20–40 samples) can be analysed and the separation is automated. The analytical specificity is higher than those of the GC and HPLC methods and mass spectrometric controls can be run. For HA, which is analysed as its methyl ester, *m/z* 134 and 193 are chosen as selected masses even though their relative abundances are only 15% and 9%, respectively. The main fragment ion of *m/z* 105 is not specific for HA and is formed from many other aromatic compounds. The base peak of the methyl ester of FA is at *m/z* 179, which is a specific ion for the furan derivative. When used as a selected mass it allows a specific and sensitive determination of FA. The internal standard 2-acetylaminobenzoic acid is an isomer of HA. Its molecular ion of *m/z* 193 with a relative abundance of 30% (Fig. 4) is suitable as a selected mass for the determination of HA. 4-Acetyl-3,5-dimethyl-2-pyrrolecarboxylic acid with its base peak *m/z* 148 (Fig. 5) as the selected mass is used as an internal standard for FA. It has a different molecular mass, but exhibits similar polarity to FA. The MSD traces for the measurement of HA (Fig. 6) and FA (Fig. 7) in serum samples demonstrate the absence of interferring peaks. The same applies for HA (Fig. 8) and FA (Fig. 9) in urine. The asymmetric peak form in the MSD trace for HA in urine is caused by

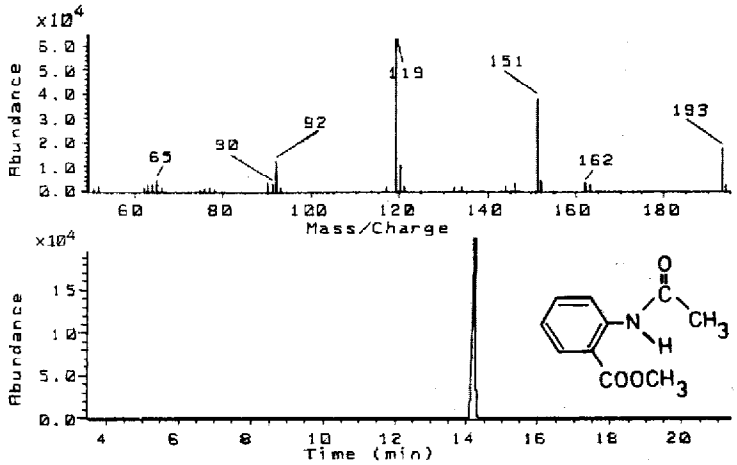

Fig. 4. Mass spectrum and total ion current trace of 2-acetylaminobenzoic acid (methyl ester).

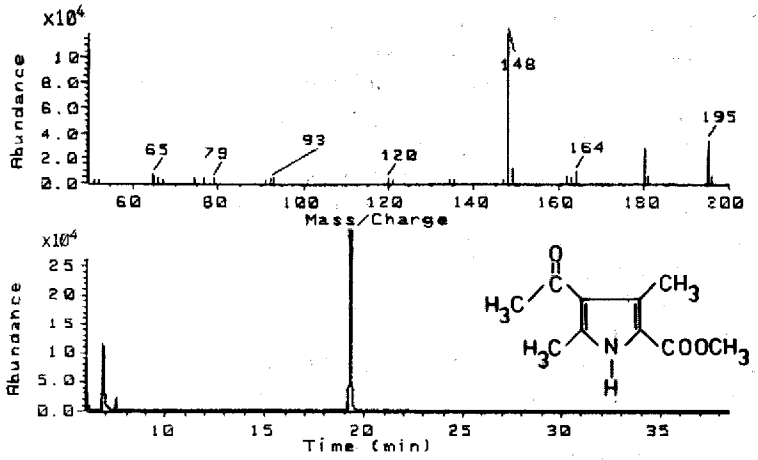

Fig. 5. Mass spectrum and total ion trace of 4-acetyl-3,5-dimethyl-2-pyrrolecarboxylic acid (methyl ester).

overloading the column with the large amounts of urinary HA, a problem that is easily overcome by diluting the sample or using a splitting ratio of 1:40.

Clinical studies

In the clinical studies performed so far, the GC and HPLC procedures have been used. These two methods give comparable quantitative data, which are in agreement with the data obtained from the GC–MSD method. The following normal values [mean value ($\bar{x}$) ± standard deviation (s)] have been determined in samples from healthy volunteers and blood donors. HA in serum, 0.2 ± 0.15 mg/dl (n = 34); FA in serum, 0.25 ± 0.15 mg/dl (n = 34); HA in urine, 410 ± 240 mg/24 h (n = 7); and FA in urine, 0.8 ± 0.5 mg/24 h (n = 7).

Normal values for HA and FA have been found in hospital patients with other than kidney diseases and syndromes, including patients with diabetes mellitus (n =

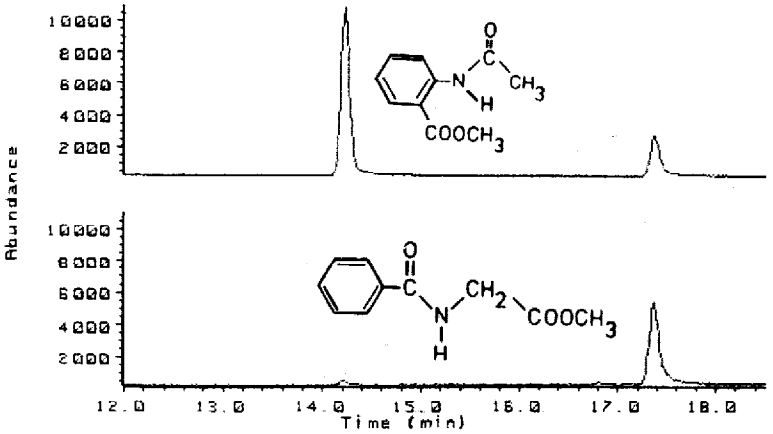

Fig. 6. MSD trace for the determination of HA in serum. Selected masses m/z 193 (top) and m/z 134 (bottom).

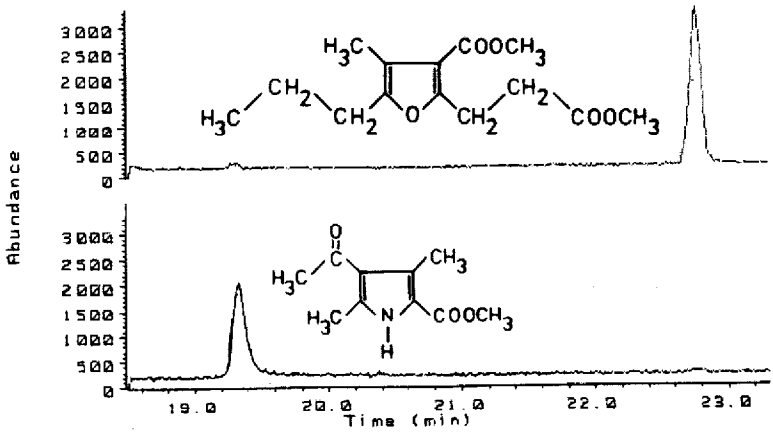

Fig. 7. MSD trace for the determination of FA in serum. Selected masses *m/z* 179 (top) and *m/z* 148 (bottom).

6), liver cirrhosis ($n = 6$), hypercholesterolaemia ($n = 9$), hypertriglyceridaemia ($n = 9$) and hyperbilirubinaemia ($n = 7$). Only HA has been found to be slightly elevated in patients with diabetes mellitus (0.40 ± 0.27 mg/dl) and liver cirrhosis (0.52 ± 0.20 mg/dl).

Non-dialysed patients with chronic renal failure

In chronic renal failure, the plasma levels of HA rise. The correlation between HA and creatinine and urea is moderately close. Dividing the non-dialysed patients into four groups according to the creatinine concentrations, HA concentrations are increased in all groups, even in patients with only slightly elevated creatinine values (Table I). Statistical evaluation by the Wilcoxon test showed significant differences in

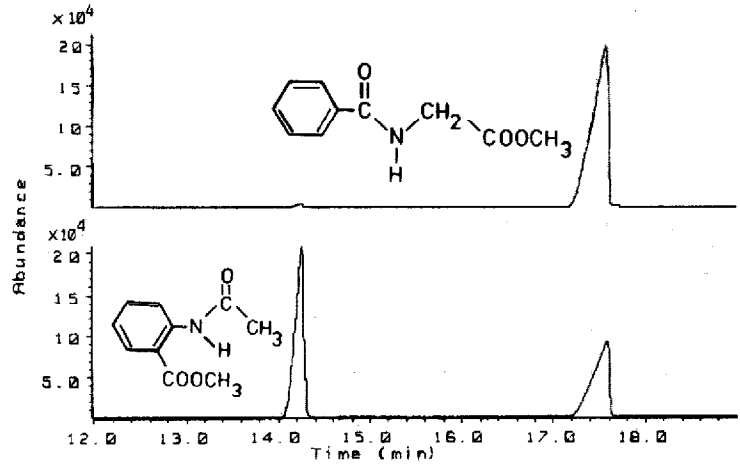

Fig. 8. MSD trace for the determination of HA in urine. Selected masses *m/z* 134 (top) and *m/z* 193 (bottom).

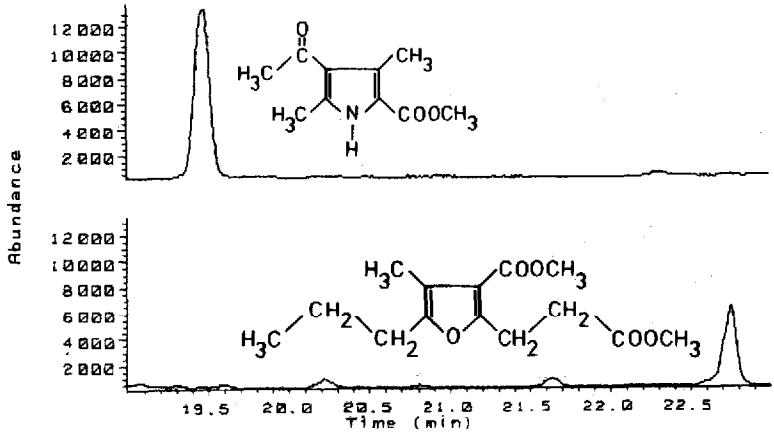

Fig. 9. MSD trace for the determination of FA in urine. Selected masses *m/z* 148 (top) and *m/z* 179 (bottom).

all four groups as compared with normal controls ($p < 0.01$). On the other hand, no statistically significant differences were found for the FA concentrations. In conjunction with the findings that FA is elevated in plasma of haemodialysed patients with chronic renal failure, the results suggest that the accumulation of FA is a gradual process taking place more slowly than the accumulation of HA.

Haemodialysed patients with acute renal failure

In acute renal failure, no statistically significant increase in the concentrations of HA and FA is found in spite of elevated levels of creatinine (8.7 ± 5.2 mg/dl, $n = 6$) and urea (159 ± 68 mg/dl, $n = 6$), showing that in this group of patients there is no correlation with creatinine and urea.

Haemodialysed patients with chronic renal failure

The gradual increase in FA in the course of chronic renal failure leads to significantly elevated levels of FA in addition to HA in the group of the haemodialysed patients ($p < 0.001$ for both substances). The following concentrations were determined in serum: HA, 11.1 ± 5.7 mg/dl ($n = 86$); FA, 1.87 ± 1.2 mg/dl ($n = 86$). The

TABLE I

CONCENTRATIONS OF HA AND FA IN SERUM OF NON-DIALYSED PATIENTS WITH CHRONIC RENAL FAILURE

Creatinine range (mg/dl)	*No. of patients (n)*	$\bar{x}$ ± *S.D. (mg/dl)*	
		HA	*FA*
1.3–2.0	12	0.25 ± 0.14	0.35 ± 0.23
2.1–4.0	13	0.35 ± 0.11	0.31 ± 0.23
4.1–7.0	13	0.74 ± 0.79	0.20 ± 0.18
>7.0	12	2.3 ± 2.5	0.31 ± 0.20

TABLE II

ELIMINATION OF HA FROM PLASMA BY HAEMODIALYSIS

Mean values for eight patients; hydrogencarbonate used as buffer.

Membrane	*Haemodialysis time (min)*	*Mean value $\bar{x} \pm S.D.$ (mg/dl)*	*Elimination rate (%)*
Cuprophane D 3	0	10.6 ± 5.7	–
	110	7.3 ± 5.2	31
	200	4.9 ± 3.1	53
Polysulphone F 60	0	7.6 ± 3.7	–
	110	4.5 ± 2.3	40
	200	2.6 ± 1.4	66
Polyacrylonitrile AN 69	0	11.3 ± 5.5	–
	110	5.9 ± 3.2	48
	200	4.9 ± 1.9	57

values for HA are in close agreement with data from other workers[17,21]. The results for FA do not agree with data on a smaller group of patients showing higher concentrations[18]. In haemodialysed patients with residual urinary excretion, HA in urine is diminished in comparison with normals (167 ± 133 mg/dl, $n = 9$). The same applies for urinary FA (0.17 ± 0.13 mg/dl, $n = 9$). No statistically significant differences were proved for the HA and FA concentrations in plasma of haemodialysed patients with chronic renal failure without and with residual urinary excretion. Only a tendency was observed towards lower values in the latter group.

Elimination by haemodialysis

HA can be eliminated by haemodialysis. For the three membranes used, elimination rates after 200 min of haemodialysis were between 53% and 66% (Table II). No significant differences were observed between the different membranes. The results agree with the findings of other groups[15]. For FA, no elimination was observed. The levels of FA appear to rise between 15 and 26% for the different membrane materials, which is due to the haemoconcentration connected with haemodialysis. The absence of elimination of FA by haemodialysis is caused by its strong binding to serum albumin (99.5% albumin-bound fraction).

Elimination by haemofiltration

HA is less effectively removed from the plasma by haemofiltration than by haemodialysis (Table III). After a 3-h treatment the elimination rate is between 30% and 36% for the different membranes. No significant difference is observed between the materials. As with haemodialysis, the levels of FA are not reduced by haemofiltration. They rise with the albumin during haemoconcentration.

As with many other strongly protein-bound substances in serum, FA can be more effectively removed by two other blood purification procedures, haemoperfusion and plasma exchange, for which removal rates of 16% and 52% have been reported[20].

TABLE III

ELIMINATION OF HA FROM PLASMA BY HAEMOFILTRATION

Mean values of eighteen haemofiltration treatments with each membrane.

Membrane	*Haemofiltration time (h)*	*Elimination rate (%)*
Filtral 16	0	–
	1	11
	2	26
	3	30
PAN-250 NOVA	0	–
	1	14
	2	25
	3	36
Hemoflow HF 80	0	–
	1	11
	2	24
	3	36
Hemofilter FH 88H	0	–
	1	13
	2	26
	3	32

As another result of its binding to albumin, the elimination of FA from plasma after successful renal transplantation requires 2–4 months[14].

CONCLUSIONS

Neither HA nor FA correlate well with the conventional retention parameters creatinine and urea. Therefore, additional information on the residual function of the kidneys in chronic renal failure may be derived from measuring HA and FA. In particular, a number of effects of the non-dialysable FA have been observed, suggesting that FA is a potent biochemical agent in uraemic serum. In addition to its inhibition of drug binding due to its strong affinity to serum protein[22–24], an inhibitory effect has been described on hepatic glutathione S-transferase[25]. The role of FA as a potential uraemic toxin must therefore be investigated further.

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