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GAS CHROMATOGRAPHIC AND GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF ORGANIC ACIDS IN PLASMA OF PATIENTS WITH CHRONIC RENAL FAILURE

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SUMMARY

Apart from increased concentrations of aliphatic dicarboxylic acids and phenolic aromatic acids in plasma from patients with chronic renal failure, there is large elevation of a furanoid acid, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, and of hippuric acid. The levels of 3-hydroxy- and 4-hydroxyhippuric acid are also raised. The quantitative results are as follows: furanoid acid in hemodialysis patients, 1.4 ± 0.6 mg/dl; in healthy individuals, 0.2 ± 0.1 mg/dl; hippuric acid in hemodialysis patients, 9.8 ± 6.5 mg/dl; in healthy individuals, 0.4 ± 0.5 mg/dl. Both compounds are dialysable, but less effectively than creatinine and urea. The mean elimination rate of the furanoid acid is only 21%.

INTRODUCTION

Patients with chronic renal failure exhibit, besides the retention of creatinine and urea, an accumulation of a number of other metabolites, which can be eliminated to various degrees by hemodialysis. Which of these substances contribute as toxins in the development of the uremic syndrome has long been a topic of discussion.

In addition to constituents of medium molecular weight, *ca.* 500–3500, which are mainly peptides[1], several low-molecular-weight substances have been found in blood or hemodialysates from chronic dialysis patients and connected with the uremic symptoms. They include guanidines[2], amines[3], myoinositol[4,5], polyols[5,6], phenols[7–9], phenolic aromatic acids[9–11], aliphatic dicarboxylic acids[12] and glycine conjugates[13]. It is obvious that many of the above low-molecular-weight substances which are considered to contribute to the uremic syndrome belong to the class of organic acids.

In the present investigation of the profiles of organic acids in plasma from chronic dialysis patients, the amounts of two acids, in particular, were elevated, hippuric acid and a furanoid acid. The latter compound had not previously been described in patients with renal failure.

EXPERIMENTAL

Patients and control persons

The study included eleven patients under chronic hemodialysis, four of whom

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were males and seven females, aged 23–60, and an 18-year-old patient who had to undergo acute hemodialysis after nephrectomy. In each case the plasma samples were collected before dialysis. From four patients, three males and one female, aged 31–60, plasma samples were taken before and after dialysis.

The controls were a group of seven healthy people, three males and four females, aged 20–61, and a group of five hospital patients without renal diseases, four males and one female, aged 31–69. From the controls, serum samples were used. All samples were deep-frozen until they were analyzed.

Profiles

The investigation comprised the comparison of profiles of the organic acids in samples from dialysis patients and controls as well as the determination of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid and hippuric acid.

The method for the profiles has been described elsewhere, as applied to oxocarboxylic acids[14]. It includes the deproteinization of the serum or plasma by isopropanol, the reaction of the sample with O-methylhydroxylamine hydrochloride to stabilize labile oxocarboxylic acids as O-methyloximes for further analysis, the isolation of the organic acids by anion exchange on Amberlyst A-26, the reaction of the isolated acids with diazomethane to form the methyl esters, the pre-fractionation of the methyl esters into four fractions by preparative thin-layer chromatography (TLC) and the gas chromatographic (GC) and mass spectrometric (MS) analyses[15] of fractions 2 and 3 which are relevant in the case of dialysis patients. The GC column was a 25-m fused-silica column, coated with OV-1701 (SGE, Weiterstadt, F.R.G.), which was temperature programmed from 60°C to 230°C at 2°C/min.

Quantifications

For the quantifications of the furanoid acid and of hippuric acid the same analytical procedure was used as for the profiles, except that internal standards were applied. Phenoxyacetic acid was used for the furanoid acid which appears in fraction 2, and tropic acid (3-hydroxy-2-phenylpropionic acid) for hippuric acid which appears in fraction 3. To 5 ml of serum or plasma, 100 μl of an aqueous solution of phenoxyacetic acid (100 mg/dl) and tropic acid (400 mg/dl) were added, resulting in final concentrations in the sample of 2 mg/dl phenoxyacetic acid and 8 mg/dl tropic acid. When the sample volumes were smaller, the same concentrations were achieved by reducing the volume of the internal standard solution. The calculation of the concentrations of the furanoid acid and of hippuric acid was based on the ratio of the peak areas of each acid and the internal standard.

For hippuric acid a calibration factor, f, was determined as a measure of the differences in its recovery rates and those of the added internal standard, and as a measure of the differences in the GC responses. For this purpose, 500 μl of an aqueous solution of hippuric acid (80 mg/dl) were added to a 5-ml aliquot of a pooled serum, corresponding to an addition of 8 mg/dl hippuric acid to the serum. The initial serum and the serum spiked with hippuric acid were analyzed by the described procedure. The difference in the areas of the hippuric acid peaks was determined, and from the ratio between this value and the peak area of the internal standard a calibration factor, $f = 0.91$, was established. In the case of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid this procedure could not be applied, since the synthetic

compound was not available. For the calculation a calibration factor, $f = 1$, was assumed.

The following formula was used for the calculations:

$$C_{\text{substance}}\ (\text{mg/dl}) = \frac{\text{peak area of substance}}{\text{peak area of int.stand.}} \times f \times C_{\text{int.stand.}}\ (\text{mg/dl})$$

Apparatus

For the profiles and the quantifications a Model 3700 gas chromatograph with flame ionization detector, connected to a CDS 111 integrator (Varian, Darmstadt, F.R.G.), was used. The mass spectrometric identifications were performed on a Model 2700 gas chromatograph, CH 5 mass spectrometer and Spectrosystem 100 MS computer (Varian, Bremen, F.R.G.). The gas chromatograph and the mass spectrometer were interfaced by an open coupling system.

RESULTS AND DISCUSSION

Profiles

The described procedure permits the complete removal in the pre-fractionation

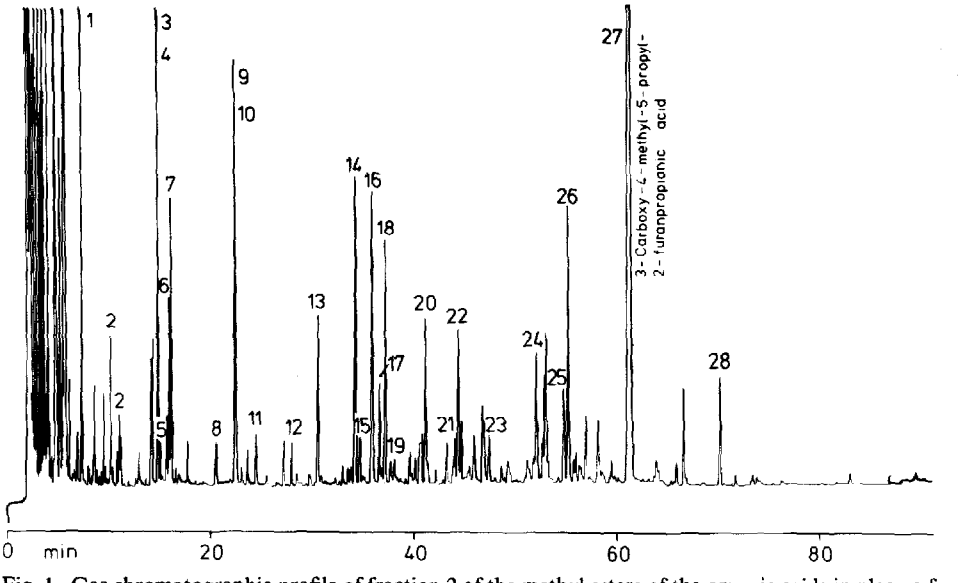

Fig. 1. Gas chromatographic profile of fraction 2 of the methyl esters of the organic acids in plasma from a chronic hemodialysis patient. Peaks: 1 = pyruvic acid; 2 = 2-oxoisovaleric acid (two *syn–anti* isomers of the methyloxime derivatives); 3 = succinic acid; 4 = 3-methyl-2-oxovaleric acid; 5 = 5-methylfuran-2-carboxylic acid; 6 = methylsuccinic acid; 7 = 2-oxoisocaproic acid; 8 = gluraric acid; 9 = 3-methylglutaric acid; 10 = phenylacetic acid; 11 = 3-methylglutaconic acid; 12 = adipic acid; 13 = 3-methyladipic acid; 14 = 3,4-methyleneadipic acid; 15 = pimelic acid; 16 = anthranilic acid; 17 = 4-hydroxybenzoic acid; 18 = 2-oxoglutaric acid; 19 = 2-hydroxyphenylacetic acid; 20 = 4-hydroxyphenylacetic acid, 21 = 3-methylsuberic acid; 22 = phthalic acid; 23 = azelaic acid; 24 = vanillic acid; 25 = homovanillic acid; 26 = 5-decynedioic acid; 27 = 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid; 28 = 3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid. The phenolic aromatic acids are present as methoxy-derivatives, because the phenolic OH groups are methylated by diazomethane.

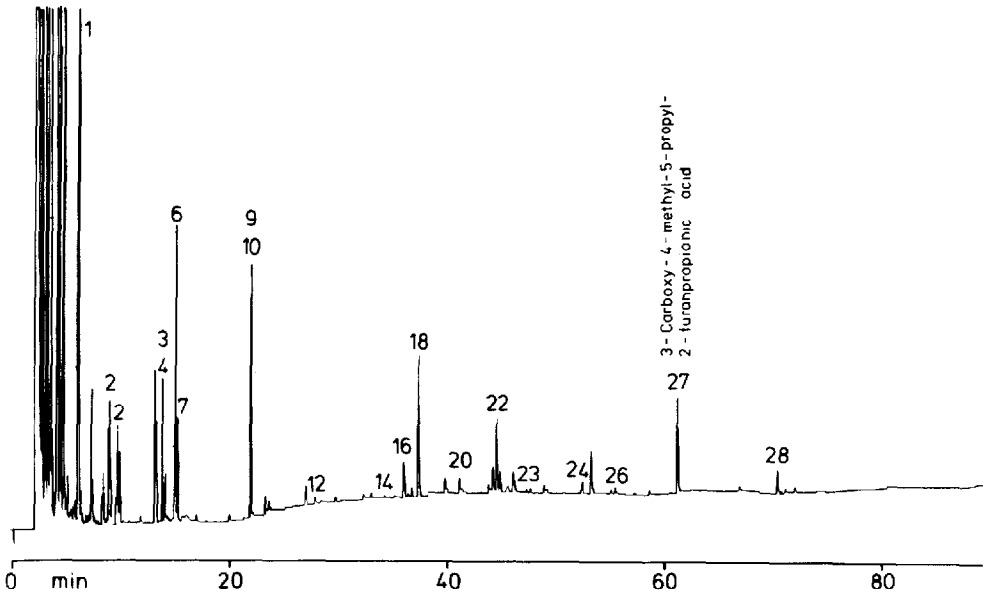

Fig. 2. Gas chromatographic profile of fraction 2 of the methyl esters of the organic acids in serum from a control person. Peak numbers as in Fig. 1.

step of the fatty acids whose high concentrations in serum and plasma would disturb the analysis of the organic acids. The fatty acids appear in fraction 1 of the TLC separation and were not investigated in detail. Fraction 2 contains mainly aliphatic oxocarboxylic acids, aliphatic dicarboxylic acids, phenolic aromatic acids and furanoid acids.

A comparison of the profiles of fraction 2 of the organic acids in samples from chronic hemodialysis patients (Fig. 1) and controls (Fig. 2) reveals that, in dialysis patients, besides the previously mentioned elevated concentrations of succinic acid, adipic acid, 3-methyladipic acid, pimelic acid and azelaic acid[12], the concentrations of glutaric acid, 3-methylglutaric acid, 3-methylsuberic acid, 3-methylglutaconic acid, 3,4-methyleneadipic acid and 5-decynedioic acid are also increased. Among the phenolic aromatic acids, increases in concentrations are observed for 4-hydroxybenzoic acid, 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, vanillic acid and homovanillic acid. The most striking change in the profile of the organic acids in fraction 2 is the pronounced elevation in the concentration of a furanoid acid (peak 27 in Figs. 1 and 2). The chemical structure of this acid, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, is shown in Fig. 3. The high concentration of this substance in plasma from dialysis patients had been overlooked by other workers, possibly because in those investigations the serum or plasma was deproteinized by ul-

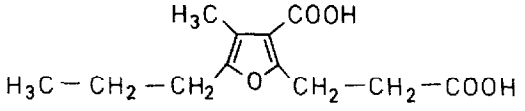

Fig. 3. Chemical structure of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid.

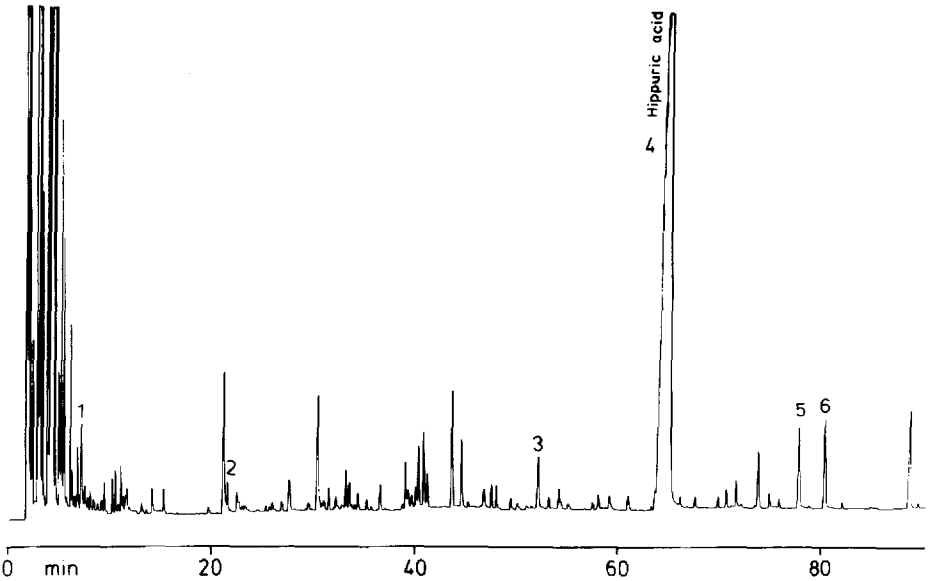

Fig. 4. Gas chromatographic profile of fraction 3 of the methyl esters of the organic acids in plasma from a chronic hemodialysis patient. Peaks: 1 = 3-hydroxybutyric acid; 2 = phenylacetic acid; 3 = furoylglycine; 4 = hippuric acid; 5 = 3-hydroxyhippuric acid; 6 = 4-hydroxyhippuric acid. The hydroxyhippuric acids are present as methoxy derivatives.

trafiltration and the furanoid acid was partially lost as a result of a strong protein binding. In addition to this main component, the homologous compound 3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid (peak 28 in Figs. 1 and 2) is found, but its increase in concentration is less pronounced.

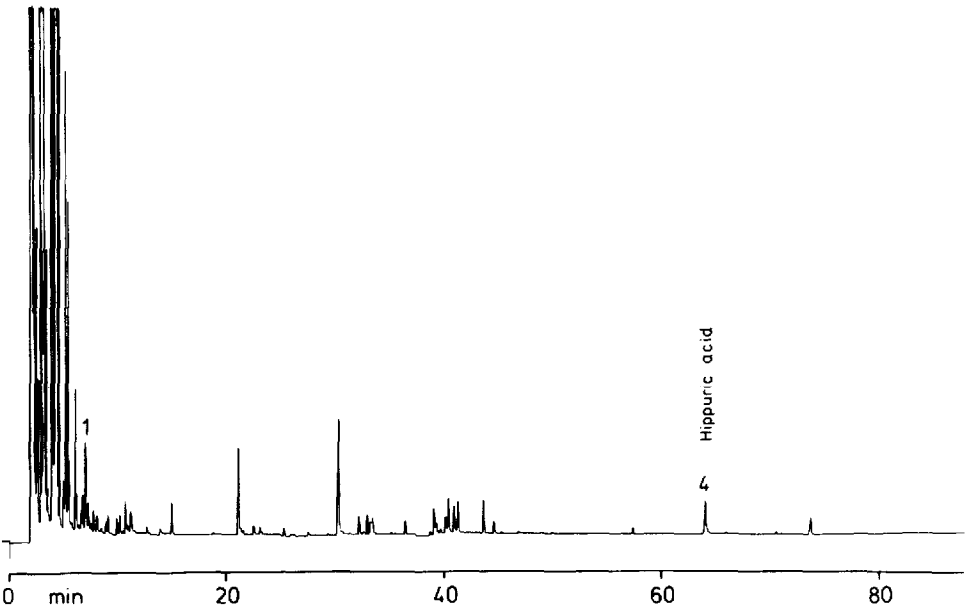

Fig. 5. Gas chromatographic profile of fraction 3 of the methyl esters of the organic acids in serum from a control person. Peak numbers as in Fig. 4.

Both furanoid acids were first identified by MS as normal excretion products in urine and their mass spectra have been published[16]. Later they were described in blood[17]. The biosynthesis of these substances is unknown. Furans with similar chemical structures have been found only as acid components in triglycerides and cholestery esters of several fish species[18,19].

The other large rise in the concentration of an organic acid in dialysis patients is observed for hippuric acid in fraction 3 (peak 4 in Figs. 4 and 5). Hippuric acid is the glycine conjugate of benzoic acid, which on the one hand stems from nutrition, on the other hand from the degradation of aromatic amino acids by intestinal microorganisms. Apart from hippuric acid, the glycine conjugates of other acids are elevated in plasma from hemodialysis patients, *e.g.*, furoylglycine (peak 3 in Fig. 4) and especially 3-hydroxyhippuric acid and 4-hydroxyhippuric acid (peaks 5 and 6 in Fig. 4) as conjugates of phenolic aromatic acids.

Quantification of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid and hippuric acid

For the quantifications the same sample preparation procedure as for the profile is recommended, when the profiles are studied at the same time. When the investigation is limited to the above two components, the derivatization with O-methylhydroxylamine hydrochloride can be omitted and the sample size reduced. The removal of the fatty acids remains essential. The chosen internal standards, phenoxyacetic acid and tropic acid, meet the requirements that they do not occur in biological material, do not interfere with plasmatic acids in the GC profile and have similar chemical properties, *e.g.*, comparable R_F values in the TLC pre-fractionation, as the components to be determined. Tropic acid was used by other authors[20] as internal standard for the analysis of organic acids.

Figs. 6 and 7 give examples of chromatograms for the quantitation of the furanoid acid and of hippuric acid. The results of the measurements are summarized in Table I and complemented by the values for creatinine and urea. The mean level

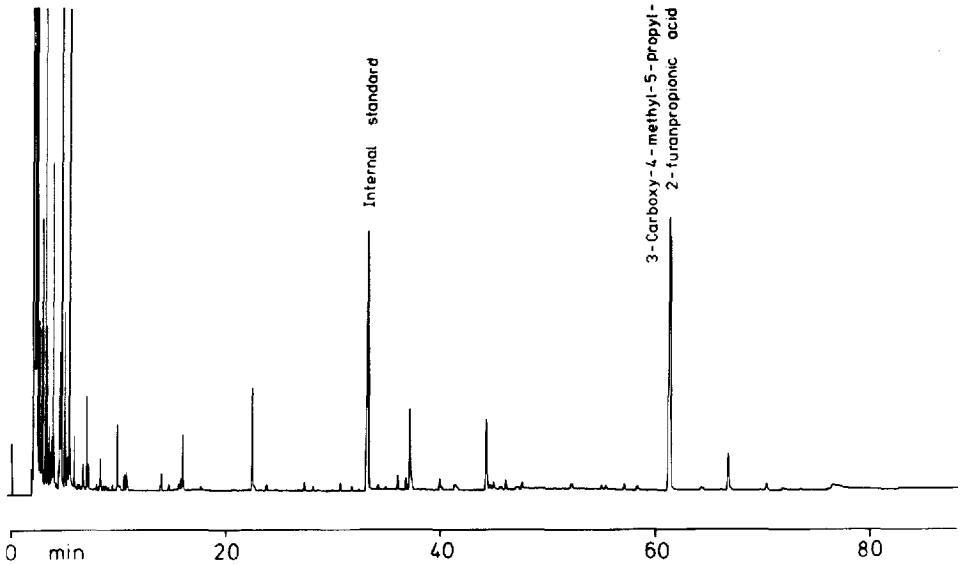

Fig. 6. Gas chromatogram of fraction 2 for the quantitation of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid. Internal standard: phenoxyacetic acid.

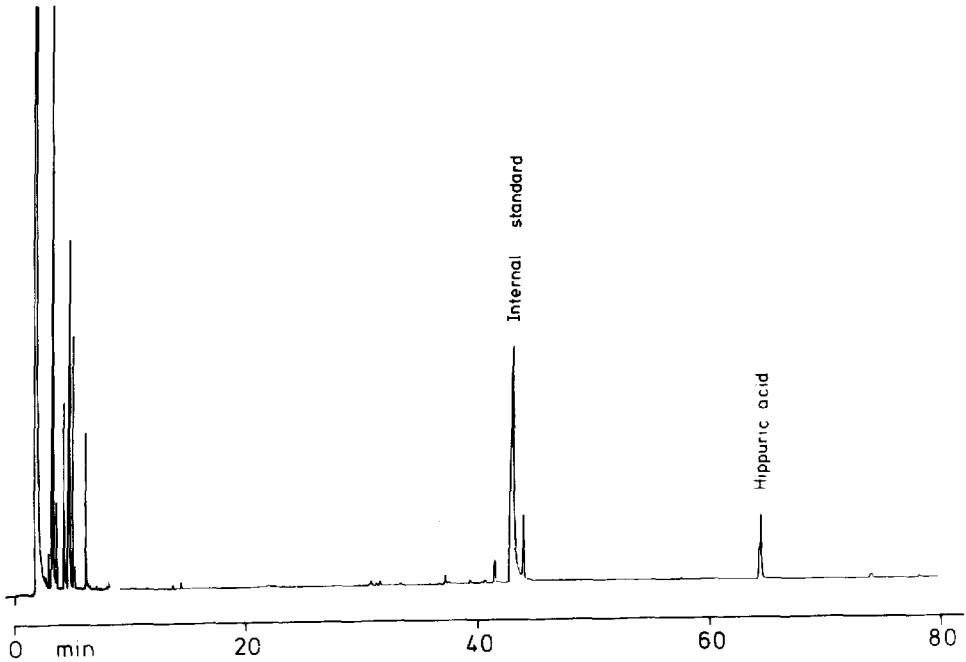

Fig. 7. Gas chromatogram of fraction 3 for the quantitation of hippuric acid. Internal standard: tropic acid.

of the furanoid acid in healthy individuals is 0.2 mg/dl. It is increased seven-fold to 1.4 mg/dl, in patients who have undergone chronic hemodialysis. For hippuric acid the elevation is even more pronounced. For healthy individuals a mean value of 0.4 mg/dl is found, for dialysis patients 9.8 mg/dl, which is a 25-fold rise. In the case of the patient treated by acute hemodialysis after nephrectomy the accumulation of the furanoid acid and of hippuric acid in blood is delayed as compared to the increase of creatinine and urea. Whereas creatinine has already reached a level of 13.0 mg/dl and urea of 158 mg/dl, normal values are still found for the furanoid acid and hippuric acid. From the present data it cannot be determined when the levels of these

TABLE I

FURANOID ACID AND HIPPURIC ACID IN PLASMA/SERUM FROM CHRONIC HEMODIALYSIS PATIENTS AND CONTROLS

Results are given as mean value, $\bar{x}$ (mg/dl), followed by standard deviation, s (mg/dl).

Group	*Furanoid acid*	*Hippuric acid*	*Creatinine*	*Urea*
Chronic hemodialyses patients (n = 11)	1.4 0.6	9.8 6.5	12.9 2.6	175 36
Healthy individuals (n = 7)	0.2 0.1	0.4 0.5	0.7 0.1	27 10
Hospital patients without renal diseases (n = 5)	0.1 0.1	0.2 0.2	0.8 0.1	31 13
Patient with acute hemodialysis after nephrectomy (n = 1)	<0.1	0.5	13.0	158

TABLE II

FURANOID ACID AND HIPPURIC ACID IN PLASMA OF CHRONIC HEMODIALYSIS PATIENTS BEFORE AND AFTER DIALYSIS

Results are given as mean value, $\bar{x}$ (mg/dl), followed by standard deviation, s (mg/dl).

	Furanoid acid	*Hippuric acid*	*Creatinine*	*Urea*
Before dialysis	1.9	9.9	10.2	150
(n = 4)	1.3	1.3	3.6	7
After dialysis	1.5	5.0	4.2	54
(n = 4)	1.1	1.3	1.0	7
Mean elimination rate (%)	21	50	59	64

two components begin to rise. The chronic dialysis patients studied had been dialysis dependent for 3–12 years. A correlation between the levels of the furanoid acid and of hippuric acid and the period of treatment is not observed.

The measurement of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid and hippuric acid before and after hemodialysis (Table II) shows that both substances are dialysable, however, with differences in the degree of elimination. Whereas for hippuric acid the mean elimination of 50% is only somewhat worse than those for creatinine and urea, the furanoid acid is eliminated less effectively by hemodialysis (mean elimination rate 21%). These results are in agreement with the water solubility of the constituents. Because of its chemical structure, the furanoid acid is more lipophilic than the other components. On this basis a stronger affinity for plasma proteins is expected, and a reduced elimination of the furanoid acid by dialysis.

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REFERENCES

1 J. Bergström and P. Fürst, *Clin. Nephrol.*, 5 (1976) 143.
2 R. Shainkin, Y. Berkenstadt, Y. Giatt and G. M. Berlyne, *Clin. Chim. Acta*, 60 (1975) 45.
3 M. L. Simenhoff, J. J. Saukkonen, J. F. Burke, L. G. Wesson and R. W. Schaedler, *Lancet*, ii (1976) 818.
4 R. S. Clements, Jr., P. V. De Jesus and A. I. Winegrad, *Lancet*, i (1973) 1137.
5 A. C. Schoots, F. E. P. Mikkers, C. A. M. G. Cramers and S. Ringoir, *J. Chromatogr.*, 164 (1979) 1.
6 F. W. Bultitude and S. J. Newham, *Clin. Chem.*, 21 (1975) 1329.
7 T. Niwa, K. Maeda, T. Ohki, A. Saito and K. Kobayashi, *Clin. Chim. Acta*, 110 (1981) 51.
8 E. N. Wardle and K. Wilkinson, *Clin. Nephrol.*, 6 (1976) 361.
9 G. A. Jutzler, H. J. Kramer, H. E. Keller, H. Kramer and F. Doenecke, *Arch. Klin. Med.*, 214 (1968) 214.
10 S. F. Rabiner and F. Molinas, *Amer. J. Med.*, 49 (1970) 346.
11 T. Niwa, T. Ohki, K. Maeda, A. Saito, K. Ohta and K. Kobayashi, *Clin. Chim. Acta*, 96 (1979) 247.
12 T. Niwa, T. Ohki, K. Maeda, A. Saito and K. Kobayashi, *Clin. Chim. Acta*, 99 (1979) 71.
13 B. Kramer, H. Seligson, H. Baltrush and D. Seligson, *Clin. Chim. Acta*, 11 (1965) 363.
14 H. M. Liebich, A. Pickert and J. Wöll, *J. Chromatogr.*, 217 (1981) 255.
15 H. M. Liebich, A. Pickert, U. Stierle and J. Wöll, *J. Chromatogr.*, 199 (1980) 181.
16 M. Spiteller, G. Spiteller and G.-A. Hoyer, *Chem. Ber.*, 113 (1980) 699.
17 J. Pfordt, H. Thoma and G. Spiteller, *Justus Liebigs Ann. Chem.*, (1981) 2298.
18 R. L. Glass, T. P. Krick, D. M. Sand, C. H. Rahn and H. Schlenk, *Lipids*, 10 (1975) 695.
19 R. L. Glass, T. P. Krick, D. L. Olson and R. L. Thorson, *Lipids*, 12 (1977) 828.
20 S. C. Gates, C. C. Sweeley, W. Krivit, D. De Witt and B. E. Blaisdell, *Clin. Chem.*, 24 (1978) 1680.