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DETERMINATION OF HIPPURIC ACID AND FURANIC ACID IN SERUM OF DIALYSIS PATIENTS AND CONTROL PERSONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (furanic acid) and hippuric acid in human serum is described Quantitative data were obtained from 20 blood donors, 26 non-dialysis patients and 41 dialysis patients In healthy persons hippuric acid ranged from 0 2 to 0 6 mg/dl, furanic acid from 0 13 to 0 53 mg/dl In dialysis patients the mean concentration of hippuric acid was elevated to 17 2 mg/dl (range 1 7–50 8 mg/dl) and the mean concentration of furanic acid was elevated to 1 89 mg/dl (range 0 17–6 45 mg/dl) In patients without renal insufficiency the concentrations were not elevated These data are in accordance with previous data obtained by gas chromatographic methods Preliminary results indicate that hippuric acid and furanic acid may be more specific parameters than other uremic retention products, and better indicators for the need for dialysis treatment than urea or creatinine

INTRODUCTION

In the blood serum of uremic patients treated by hemodialysis various metabolites accumulate as a result of insufficient elimination These uremic retention products have a wide range of molecular masses To control the effectiveness of hemodialysis, the serum concentrations of creatinine, urea, potassium and inorganic phosphorus are regularly measured in clinical chemistry Differences between individual patients can be explained by the residual renal functions and the kind of dialysis applied Different dialysis techniques vary in their elimination capacity for large-, small- and medium-size molecules Medium-molecular-mass substances, which are insufficiently elimi-

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nated by all techniques, may be specific uremic toxins However, efforts to assess this toxicity to chemically distinct substances have not been successful so far [1] Among the low-molecular-mass substances, amines [2], sugars [3] and organic acids [4] have been found in elevated concentrations in uremic serum By gas chromatography–mass spectrometry (GC–MS) it has been demonstrated, that, among the organic acids, two substances in particular, 3-carboxy-4-methyl-5-propyl-2-furanpropionic (furanic acid), a normal constituent in urine [5], and hippuric acid, occur in characteristically elevated concentrations in uremic serum [6] Hippuric acid has been analysed by GC [6, 7] and high-performance liquid chromatography (HPLC) [8–11] in uremic serum Furanic acid in serum has been quantitated only by GC [7] with identification by MS

In the clinical course of dialysis, the two organic acids exhibit different behaviour For hippuric acid the elimination rate through conventional hemodialysis is similar to that for creatinine and urea [7, 9]. Furanic acid cannot be removed by hemodialysis, so its concentration remains constant during dialysis [7], or may even rise Renal transplantation leads to a rapid decrease of hippuric acid, whereas the concentration of furanic acid remains elevated for several weeks after transplantation [7]

The sample work-up procedure renders the GC method time-consuming, thereby hampering extended clinical investigations with large numbers of samples This paper describes a rapid method for the quantitative determination of hippuric acid and furanic acid in serum by HPLC using reversed-phase chromatography and ion-pair reagents

EXPERIMENTAL

Samples

Twenty serum samples from healthy blood donors, 26 serum samples from non-dialysis patients and 41 serum samples from dialysis patients were obtained by venous puncture and centrifugation within 1 h Renal insufficiency in the group of the blood donors was excluded by normal values of creatinine and urea The non-dialysis group was composed of 8 patients with renal insufficiency (creatinine range 1 4–5 5 mg/dl) and 18 with various pathological deviations hypercholesterolemia, hypertriglyceridemia, hyperuricemia and hyperbilirubinemia Creatinine, urea, total protein, cholesterol, triglycerides, uric acid, bilirubin and potassium were analysed the same day Serum for the analysis of furanic acid and hippuric acid were stored at -20°C and thawed batchwise directly prior to the analysis

Clinical chemical routine parameters

Creatinine was analysed by the Jaffé method, uric acid by oxidation through phosphotungstate, serum total protein by the biuret reaction, potassium by

flame photometry and total bilirubin by the azosulphanilic acid method. Cholesterol, triglycerides and urea where analysed by enzymic methods. The analyses were performed on a Parallel analyser (American Monitor, Bornheim, F.R.G.).

Chemicals

Chemicals and solutes for HPLC and for the synthesis of furanic acid were of the highest quality available. For HPLC the following reagents were used: 2-propanol, 1 *M* sodium hydroxide, *n*-hexane, 25% hydrochloric acid, diethyl ether (E. Merck, Darmstadt, F.R.G.); tetrabutylammonium hydroxide, tetrabutylammonium phosphate, 2-phenoxypropionic acid, 3-phenoxypropionic acid and hippuric acid (Aldrich, Steinheim, F.R.G.).

Synthesis of furanic acid

The synthesis was performed according to a method generally described by Pfordt et al. [12]. 2-Hexanone (100 g; Aldrich) was chlorinated with sulphuryl chloride (Fluka, Neu-Ulm, F.R.G.) and azobis(isobutyronitrile) (Aldrich) to form 3-chloro-2-hexanone (I), which was hydrolysed with sodium carbonate (Merck) to form 3-hydroxy-2-hexanone (II). Condensation with 6 g of 3-oxoadipic acid (Fluka) and zinc chloride (Merck) gave 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid dimethyl ester (III), which was hydrolysed with methanol–35% potassium hydroxide (4:1, v/v) to form free furanic acid. The final product was purified by preparative thin-layer chromatography (Kieselgel F 254, layer thickness 2 mm, E. Merck). The plates were developed in chloroform–ethanol–acetic acid (93:5:2, v/v). The *R* value of furanic acid was 0.5. The substance was localized by fluorescence quenching on the plate and after extraction from the silicagel with methanol, it was purified by crystallization from chloroform. The yield of the pure product was 300 mg (5% on the basis of 3-oxoadipic acid as starting material). The purity of the product was checked by HPLC and GC–MS after remethylation with diazomethane, and found to be better than 95%.

Sample preparation for HPLC analysis

A 1-ml serum sample was pipetted into a glass centrifuge tube. After addition of 50 μl of internal standard (100 mg/dl 3-phenoxypropionic acid in acetonitrile) the proteins were precipitated with 2 ml of 2-propanol. The samples were vortexed vigorously and centrifuged, and the supernatant was decanted into another 10-ml tube with a ground glass joint. Then 1 ml of 1 *M* sodium hydroxide was added, and the 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. Next, 2 ml of diethyl ether were added, and the tube was stoppered and vortex-mixed. Phase separation was achieved by short centrifugation, and the upper phase was sampled. The extraction with diethyl ether was repeated

once To the combined organic phases, 100 μl of tetrabutylammonium phosphate–tetrabutylammonium hydroxide (TBA) (1 5 1, v/v) (pH 8 0) were added The diethyl ether was removed by placing the tubes into a desiccator connected to a vacuum pump The residue (organic acids in a mixture of ca 300 μl of TBA–2-propanol–water) was filtered with a syringe through a low-dead volume filter (Millipore HV, 0 45 μm, Eschborn, F R G), and the filtrate was placed in microvials for the autosampler

HPLC analysis

The mobile phase for HPLC was a mixture of acetonitrile (A) and water with 5 m*M* tetrabutylammonium phosphate (B) HPLC was performed with a Merck-Hitachi apparatus consisting of a ternary gradient pump (Model L 6200), an autosampler (Model 655A-40), a column oven (Model 655A-52) set at 40°C, a photodiode array detector (Model L 3000), and a two-channel integrator (Model D2000) with storage of raw data for recalculation Separation was done on a 12 5 cm×0 5 cm I D C_8 reversed-phase column (LiChrocart RP 8, 5 μm, E Merck). The mobile phase for gradient elution was initially 10% A+90% B held 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 reequilibrated for 5 min The flow-rate was 1 5 ml/min Detection was achieved at two wavelengths simultaneously (214 nm and 250 nm) using an autozero command at the beginning of each analysis. Samples of 30 μl were analysed by automatic injection with the autosampler The analysis time was 30 min All chromatograms were recorded in duplicate and independent series The mean value of the two peak areas was used for calculation

Calculation

Substances were determined quantitatively according to their peak-area response at 214 and 250 nm on the basis of the internal standard Response factors were established by adding different amounts of the authentic reference substances (hippuric acid and furanic acid) to a serum pool with low concentrations of furanic and hippuric acid For furanic acid (214 nm) the concentrations were calculated according to

$$\frac{14\ 76\ A_F}{A_{IS}}\text{mg/dl} = C_F\ \text{mg/dl} \qquad (1)$$

For hippuric acid (250 nm) the concentrations were calculated according to:

$$\frac{1\ 19 A_H}{A_{IS}}\text{mg/dl} = C_H\ \text{mg/dl} \qquad (2)$$

where A_{IS} = peak area of internal standard, A_F = peak area of furanic acid,

A_H=peak area of hippuric acid, C_F=concentration of furanic acid, C_H=concentration of hippuric acid The peak ratio 214/250 nm was taken as an additional criterion of substance identity

RESULTS

Analytical aspects

Precipitation of the proteins by alcohol in the sample preparation procedure was necessary because hippuric acid and especially furanic acid are considerably protein-bound [13] During precipitation, hippuric acid and furanic acid are liberated from the proteins Extraction of the neutral lipids with hexane is recommended to reduce the deterioration of the column Baseline instabilities resulting from the gradient elution system are overcome using an autozero command at the beginning of each analysis Furanic and hippuric acid were localized within the chromatograms according to their retention times relative

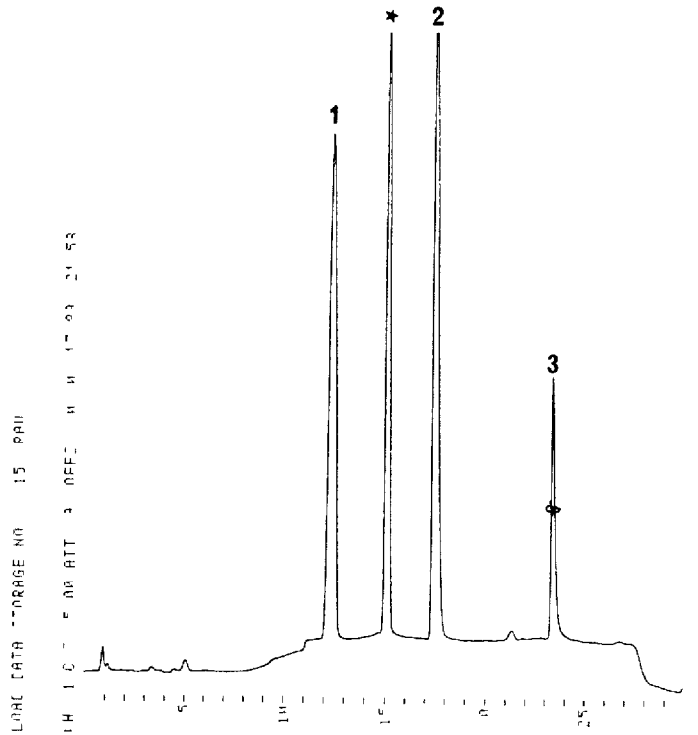

Fig 1 Chromatogram of a standard solution of 10 mg/dl hippuric acid (1), 20 mg/dl 2-phenoxypropionic acid (★), 20 mg/dl 3-phenoxypropionic acid (2) and 20 mg/dl furanic acid (3), with detection at 214 nm The x-axis is calibrated in minutes and the y-axis is E=0 001×512 (full scale)

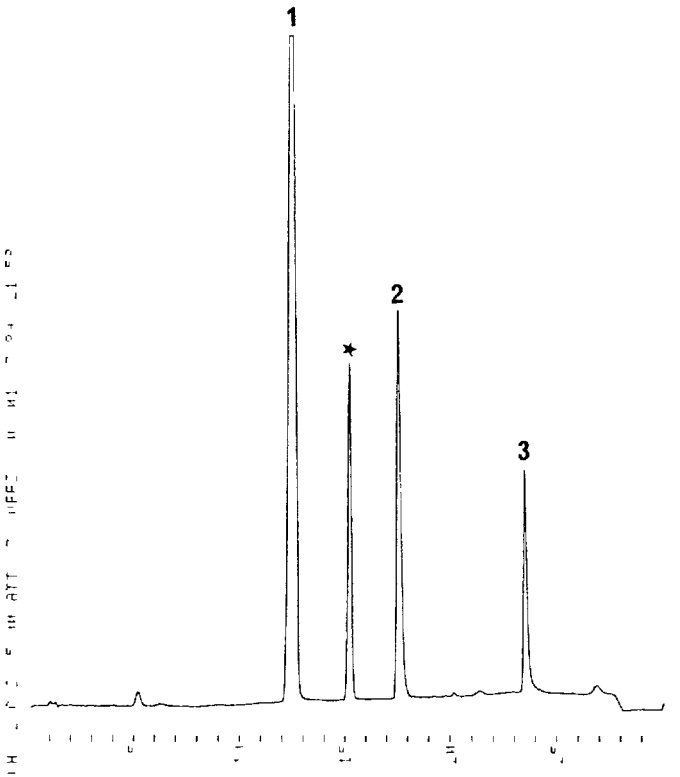

Fig 2 Chromatogram of a standard solution of 10 mg/dl hippuric acid (1), 20 mg/dl 2-phenoxypropionic acid (★), 20 mg/dl 3-phenoxypropionic acid (2) and 20 mg/dl furanic acid (3), with detection at 250 nm The x-axis is calibrated in minutes and the y-axis is $E=0\,001\times128$ (full scale)

to the internal standard, which elutes between the two substances Either of two acids can be used as internal standard (2-phenoxypropionic acid and 3-phenoxypropionic acid), both elute between the analytes and do not occur naturally in human serum 3-Phenoxypropionic acid was preferred because of its better separation from the hippuric acid peak. To minimize variations in the retention times caused by shifts in the column temperature, the separation was done at 40 °C By placing a standard sample (consisting of hippuric acid, 2-phenoxypropionic acid, 3-phenoxypropionic acid and furanic acid) between two serum samples, minor shifts in the retention times were recognized

Figs 1 and 2 show the separation of a standard solution of furanic acid, hippuric acid and the internal standards Figs 3 and 4 are chromatograms obtained from a serum sample from a dialysis patient

Precision of the method

For quality control, a serum pool from healthy persons was made up and controlled for normal values of creatinine, urea, electrolytes, enzymes, lipids

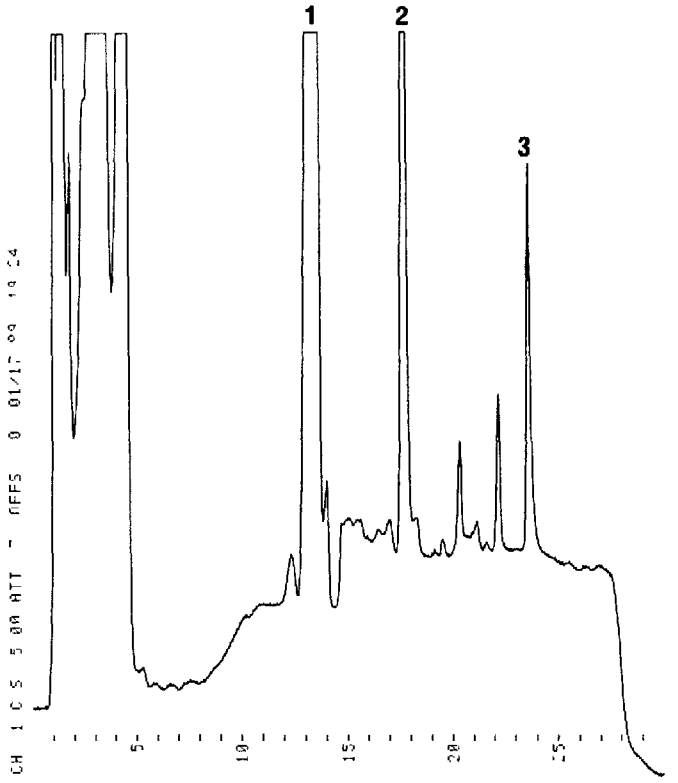

Fig 3 Chromatogram of a serum sample from a dialysis patient with 12 2 mg/dl hippuric acid (1), 5 mg/dl 30-phenoxypropionic acid (internal standard, 2) and 6 45 mg/dl furanic acid (3), with detection at 214 nm The x-axis is calibrated in minutes and the y-axis is $E=0\,001\times128$ (full scale)

and bilirubin The pool was spiked with additional amounts of 2 5 mg/dl hippuric acid and 1.25 mg/dl furanic acid, and divided in 1-ml portions into 40 tubes. The samples were immediately frozen and stored at $-20°C$ For eight quality control samples analysed in one series the mean value for furanic acid was 0 99 mg/dl, with an S D of 0 19 [coefficient of variation (C V) 19% within-series] For hippuric acid the mean value was 3 3 mg/dl with an S D of 0 6 (C V 18% within-series) The between-assay variation was estimated by comparing the values of the quality control samples in five analytical series The mean for furanic acid was 1 17 mg/dl with an S D of 0 28 (C V 24% between-series) The mean value for hippuric acid was 3.6 mg/dl with an S D of 0 2 (C V 7% between-series)

Concentrations of furanic acid and hippuric acid

In the sera from the blood donors, the concentration of furanic acid was 0.30 mg/dl (mean) with an S D of 0 12 mg/dl, and a range of 0 13–0 53 mg/dl The

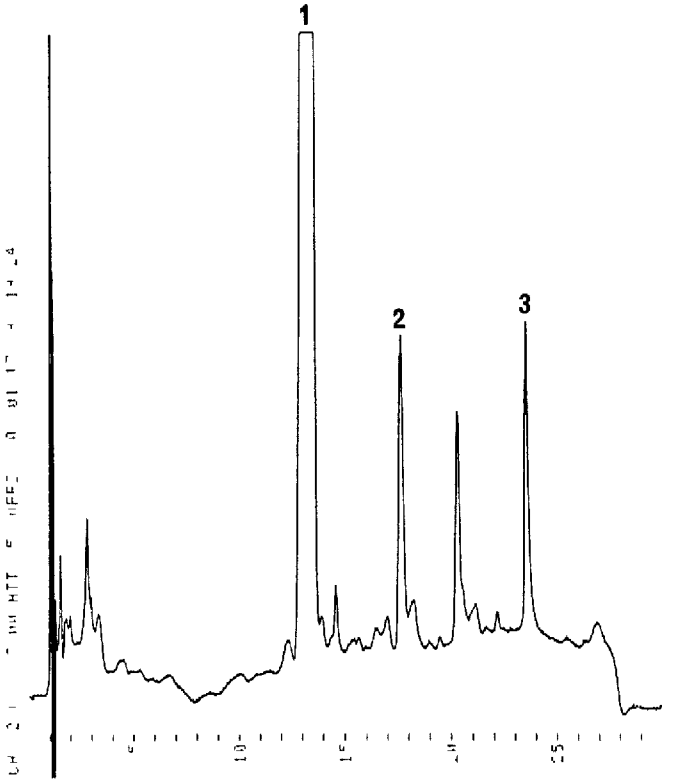

Fig 4 Chromatogram of a serum sample from a dialysis patient with 12 2 mg/dl hippuric acid (1), 5 mg/dl 3-phenoxypropionic acid (internal standard, 2) and 6 45 mg/dl furanic acid (3), with detection at 250 nm The x-axis is calibrated in minutes and the y-axis is $E=0\ 001\times32$ (full scale)

TABLE I

FURANIC ACID AND HIPPURIC ACID CONCENTRATIONS IN SERUM

Group	n	Furanic acid (mg/dl)			Hippuric acid (mg/dl)		
		Mean	S D	Range	Mean	S D	Range
Blood donors	20	0 30	0 12	0 13–0 53	0 3	0 1	0 2– 0 6
Dialysis patients	41	1 89	1 54	0 17–6 45	17 2	11 1	1 7–50 8
Uremic patients (not dialysed)	8	0 61	0 51	0 17–2 00	0 6	0 6	0 1– 1 8
Other patients[a]	18						
Uric acid > 5 7 mg/dl	9	0 34	0 28	0 05–0 81	0 3	0 2	0 1– 0 7
Cholesterol > 250 mg/dl	9	0 38	0 24	0 05–0 81	0 3	0 2	0 1– 0 7
Triglycerides > 172 mg/dl	11	0 41	0 25	0 05–0 81	0 3	0 2	0 1– 0 7
Bilirubin > 0 5 mg/dl	7	0 28	0 25	0 05–0 72	0 3	0 2	0 1– 0 7

[a]These patients may appear in more than one subgroup

normal range of furanic acid, calculated from these data, was 0–0 66 mg/dl. The mean value for hippuric acid was 0 3 mg/dl with an S D of 0.1 mg/dl and a range of 0 2–0 6 mg/dl, resulting in a normal range of 0–0 7 mg/dl (Table I) In the sera from dialysis patients the mean concentration of furanic acid was 1.78 mg/dl with an S.D of 1.54 mg/dl and a range of 0.17–6.45 mg/dl. The mean concentration of hippuric acid was 16.3 mg/dl with an S D of 11.1 mg/dl and a range of 1 7–50.8 mg/dl (Table I). In the sera of patients with renal insufficiency but not undergoing dialysis treatment, the mean furanic acid concentration was 0.60 mg/dl and the mean hippuric acid concentration was 0 6 mg/dl (Table I) In the serum samples of 24 patients with various pathological conditions, furanic acid and hippuric acid levels were not elevated (Table I)

DISCUSSION

The HPLC method described has a number of advantages compared with the GC method. Sample preparation is simple, and series of twenty samples or more can be analysed in parallel A sample of 1 ml is sufficient, and in dialysis patients with high concentrations of the analytes the volume may be reduced to 0.5 ml The HPLC method can be easily automated The HPLC method has been developed for serum, which is characterized by a relatively constant pattern of constituents In sera of patients with extremely abnormal values of routine parameters (i e. triglycerides of 3140 mg/dl, cholesterol of 1640 mg/dl or bilirubin of 24.8 mg/dl) no interferences were observed. The method is not applicable to urine samples owing to the high complexity of endogenous compounds in urine and the resulting interferences in the HPLC separation. The determination of urinary furanic acid and hippuric acid requires the previously described GC methods [6, 7]

The precisions within analytical series and between analytical series are acceptable in the high concentration ranges that occur in dialysis patients They are not satisfactory for slight concentration differences within the normal range For this range the amount of serum should be doubled to 2 ml.

The concentrations of furanic acid and hippuric acid in healthy individuals and dialysis patients determined by the described HPLC method are in good agreement with the values obtained by the GC method [6, 7] and, in the case of hippuric acid, with the values from other HPLC methods [8–11].

The clinical interpretation of furanic acid and hippuric acid remains to be established. Recent data reported by Schoots et al. [9] indicate that the concentration of hippuric acid in uremic serum is an independent parameter, which correlates better with the residual renal function than creatinine or urea In this study, among the patients with hippuric acid levels greater than 0 6 mg/dl, 41 out of 46 cases belonged to the dialysis patient group, indicating a sensitivity of 100% and a specificity of 84% for discriminating dialysis patients in a group of hospital patients on the basis of hippuric acid. Among the patients

with furanic acid levels greater than 0.66 mg/dl, 32 out of 38 cases belonged to the dialysis patient group, indicating a sensitivity of 78% and a specificity of 81% for furanic acid This certainly does not mean that hippuric acid or furanic acid are indicators for the need to begin a dialysis treatment in patients with renal insufficiency. However, the data may be interpreted in the sense that furanic acid and hippuric acid are more dialysis-specific uremic retention products than creatinine or urea.

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