Journal of Chromatography, 338 (1985) 25—32
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2407

GAS CHROMATOGRAPHIC PROFILING OF PHENOLIC ACIDS IN URINE OF PATIENTS WITH CIRRHOSIS OF THE LIVER

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(Received August 17th, 1984)

SUMMARY

Phenolic acids are analysed within the profile of the organic acids in urine of patients with cirrhosis. For the following constituents an increased urinary excretion is observed: 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxyhippuric acid, vanillic acid, homovanillic acid, 4-hydroxymandelic acid, 4-hydroxy-3-methoxyphenylpropionic acid and *p*-cresol.

The phenols are metabolites of tyrosine and are produced in the liver, in extrahepatic tissues and by intestinal microorganisms. They are suggested as biochemical control parameters for the metabolizing function of the liver, for the effect of therapy and for the existence of portal-systemic venous collaterals.

INTRODUCTION

Tyrosine metabolism is the source for a number of phenolic acids, especially 4-hydroxy compounds. The acids are produced in the liver, in extrahepatic tissues and by intestinal microorganisms. Livercirrhosis is accompanied by increased levels of phenolic substances in serum and urine [1, 2]. It is assumed that the phenols, or the metabolic situation leading to these components, contribute to the development of hepatic encephalopathy or coma [2]. Since the detoxifying function of the cirrhotic liver is limited, therapeutic measures are taken to reduce the production of bacterial metabolites. The therapy is aimed, in particular, at ammonia, but also at that part of the phenols which is formed in the intestine. Reduced dietary proteins, neomycin and lactulose are chosen for therapy.

Because patients in coma respond poorly to all forms of therapy, early recognition and treatment of hepatic encephalopathy are essential. The phenols appear to be useful biochemical parameters to control the metabolic situation

0378-4347/85/$03.30

of patients with cirrhosis in order to reduce the risk of hazards, and to follow the effect of therapy.

On the basis of a diazo method, phenols were determined by a colorimetric group reaction [3]. Separated phenolic compounds in serum and urine were investigated by paper, thin-layer (TLC) and gas chromatographic (GC) methods [1, 2, 4]. The efficiency of the separations obtained was not satisfactory. This report describes a gas chromatographic profiling method which allows shifts to be detected in the relative concentrations of the separated phenolic acids within the pattern of the urinary organic acids.

EXPERIMENTAL

Patients and specimens

Urine samples (24 h) were collected from seven patients with liver cirrhosis and three normal controls. Five of the seven patients were treated conservatively with low-protein diet, neomycin and lactulose; two patients had undergone surgery and obtained a splenorenal and portacaval shunt, respectively. Aliquots of 60 ml of urine were kept frozen at −20° C until they were analysed.

Sample preparation

The sample preparation procedure, which has been described in detail before [5], included the O-methyloximation of urinary oxocarboxylic acids, the extraction of the organic acids by an anion-exchange procedure, the methylation of the acids with diazomethane and the pre-fractionation of the derivatives by preparative TLC. Instead of using 10-ml samples, volumes of 30 ml and 60 ml were employed in this study. The amounts of isopropanol and O-methylhydroxylamine hydrochloride were increased proportionally. The TLC plates used had a silica gel thickness of 2 mm. Fraction 2 of the TLC pre-fractionation was divided into two equally broad subfractions 2a and 2b.

Gas chromatographic and mass spectrometric analysis

Fractions 2a, 2b and 3 were analysed by GC. The identity of the substances labelled in the chromatograms was confirmed by gas chromatography—mass spectrometry (GC—MS). For the GC separations a Model 3700 gas chromatograph with flame-ionization detector (Varian, Darmstadt, F.R.G.) was used. The operating conditions were as follows: 25 m × 0.2 mm I.D. glass capillary column, coated with OV-17 (Perkin-Elmer, Überlingen, F.R.G.); carrier gas, nitrogen at 4 ml/min; column temperature, 40° C for 10 min, then programmed to 230° C at 2° C/min; injector block temperature, 250° C; sample size, 1 μl at a splitting ratio of 1:20.

For the GC—MS analyses a combination of a Model 2700 gas chromatograph, CH 5 mass spectrometer and Spectrosystem 100 MS computer (Varian-MAT, Bremen, F.R.G.) was used employing an open coupling system between the gas chromatograph and the mass spectrometer. By automatic repetitive scanning, the mass spectra were recorded over the mass range *m/e* 15—300 and stored on magnetic tape. The GC conditions were the same as described for the GC separations, except that helium was used as the carrier gas. The MS conditions were as follows: ionization by electron impact; ionization energy, 70 eV;

accelerating voltage, 3 kV; multiplier voltage, 2.25 kV; emission current, 300 μA; resolution, 600.

RESULTS AND DISCUSSION

Aspects of the method

The phenolic acids are analysed within the profile of other organic acids. Figs. 1, 3 and 5 show the chromatograms of the fractions 2a, 2b and 3 of the organic acids in urine of a patient with cirrhosis. The corresponding profiles of a control person are depicted in Figs. 2, 4 and 6. In comparison with previous methods for phenolic compounds [1—3], the profile concept offers the

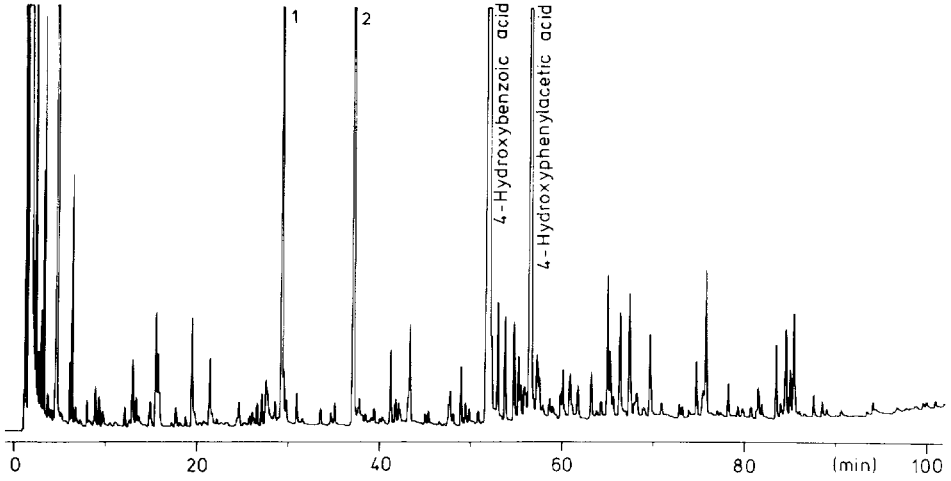

Fig. 1. Gas chromatogram of fraction 2a of the organic acids in urine of a patient with cirrhosis. Numbered peaks: 1 = benzoic acid, 2 = phenylacetic acid. All phenolic acids in Figs. 1—6 are methylated at the carboxyl group and at the —OH group.

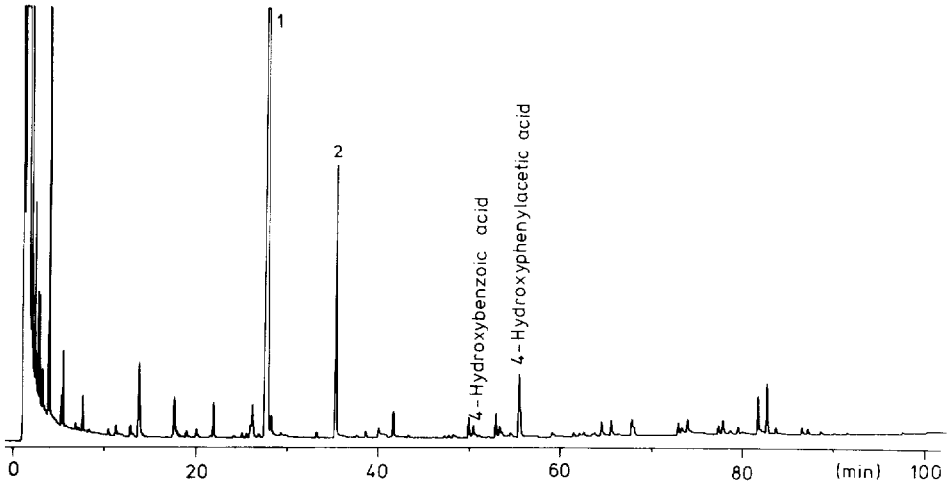

Fig. 2. Gas chromatogram of fraction 2a of the organic acids in urine of a healthy individual. Numbered peaks as in Fig. 1.

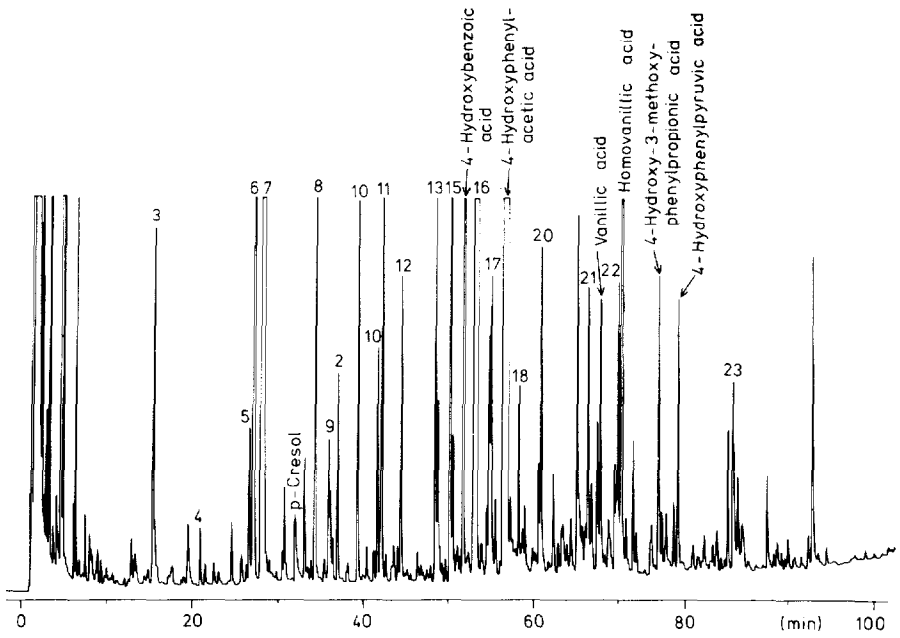

Fig. 3. Gas chromatogram of fraction 2b of the organic acids in urine of a patient with cirrhosis. Numbered peaks: 2 = phenylacetic acid, 3 = pyruvic acid, 4 = methylmalonic acid, 5 = ethylmalonic acid, 6 = succinic acid, 7 = 5-methylfurancarboxylic acid, 8 = glutaric acid, 9 = 3-methylgutaric acid, 10 = 3-methylglutaconic acid, 11 = adipic acid, 12 = 3-methyladipic acid, 13 = 3,4-methyleneadipic acid, 15 = anthranilic acid, 16 = 2-oxoglutaric acid, 17 = suberic acid, 18 = 2-oxoadipic acid, 20 = azelaic acid, 21 = sebacic acid, 22 = 5,6-decynedioic acid, 23 = indoleacetic acid. All oxocarboxylic acids in Figs. 3 and 4 are O-methyloximated at the carbonyl group.

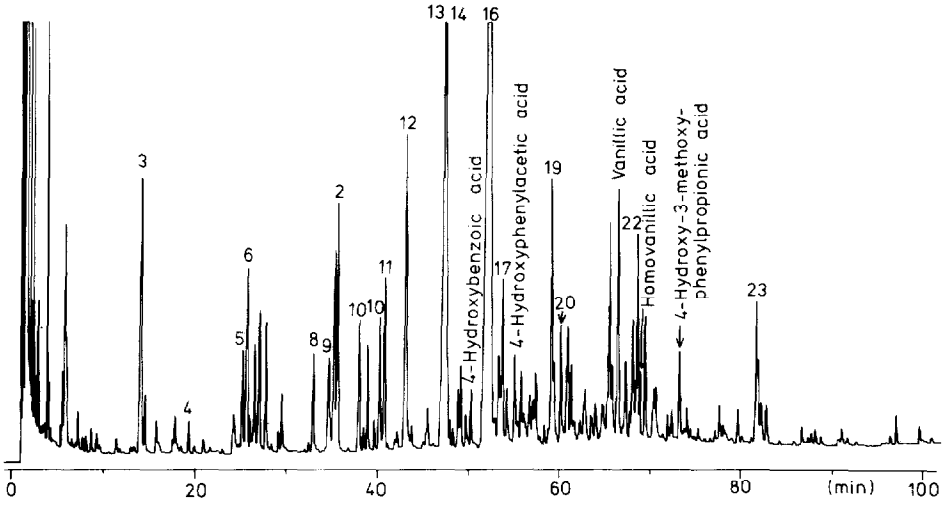

Fig. 4. Gas chromatogram of fraction 2b of the organic acids in urine of a healthy individual. Numbered peaks as in Fig. 3; additional peaks: 14 = pimelic acid, 19 = 3,4-methylenesuberic acid.

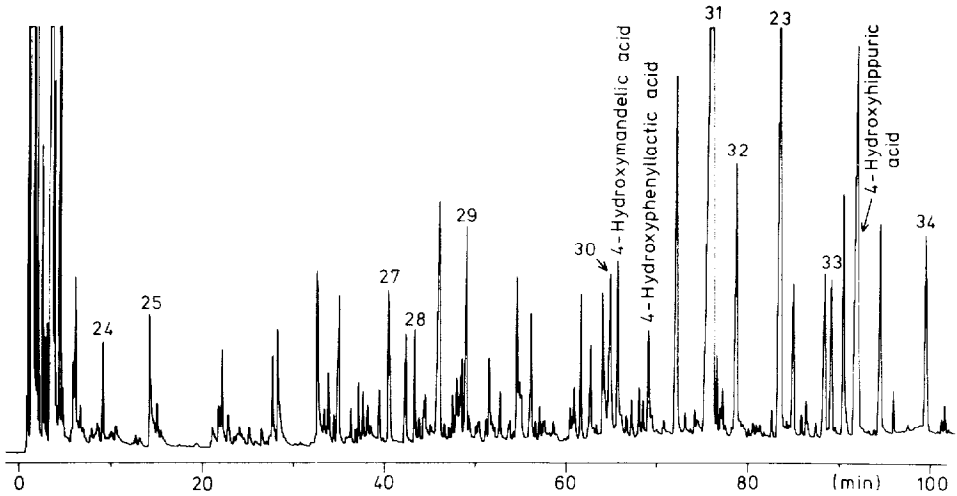

Fig. 5. Gas chromatogram of fraction 3 of the organic acids in urine of a patient with cirrhosis. Numbered peaks: 23 = indoleacetic acid, 24 = 3-hydroxyisovaleric acid, 25 = 3-hydroxyisobutyric acid, 27= 3-hydroxy-3-methylglutaric acid, 28 = 2-isopropyl-2-hydroxysuccinic acid, 29 = mandelic acid, 30 = furoylglycine, 31 = hippuric acid, 32 = vanillylmandelic acid, 33 = 3-hydroxyhippuric acid, 34 = phenylacetylglutamic acid.

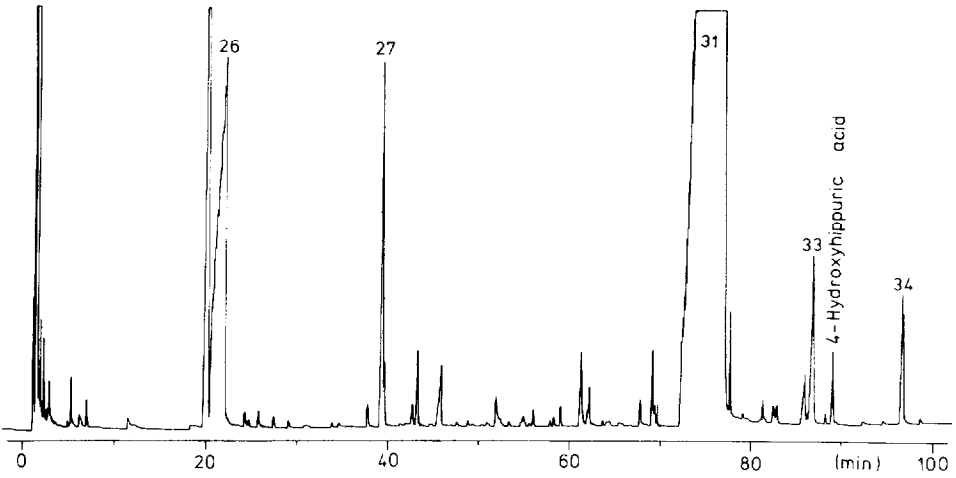

Fig. 6. Gas chromatogram of fraction 3 of the organic acids in urine of a healthy individual. Numbered peaks as in Fig. 5; additional peak: 26 = phosphoric acid (as trimethyl ester).

advantage that several phenolic acids can be analysed simultaneously and that besides the phenolic substances other metabolites can also be examined.

In cirrhosis and other liver diseases the conjugation capacity of the liver can be observed. The glycine conjugates furoylglycine, hippuric acid, 3-hydroxyhippuric acid and 4-hydroxyhippuric acid and the glutamic acid conjugate phenylacetylglutamic acid appear in fraction 3. In the cases studied so far, it is observed that in liver cirrhosis the conjugation with glycine and glutamic acid is functioning, except that the amount of hippuric acid excreted in urine is lower than in normal subjects. Because of their low volatility, the conjugates with glucuronic acid and sulphate (e.g. of phenol and *p*-cresol)

cannot be directly examined by this method. However, they can be indirectly analysed after hydrolysis of the urine with β-glucuronidase—arylsulphatase.

Other groups of non-phenolic metabolites that can be studied by the profiling technique in conjunction with the neomycin therapy of patients with liver cirrhosis, are methyl-branched dicarboxylic acids and acids with a cyclopropane ring. These substances are presumably formed by ω- and β-oxidation from bacterial fatty acids [6]. From the few cases followed so far, there is an indication that with the neomycin therapy applied the excretion of these acids (e.g. 3-methyladipic acid, 3,4-methyleneadipic acid and 3,4-methylenesuberic acid) is decreased compared to unbranched dicarboxylic acids. This can be explained by lower production of bacterial fatty acids and reduced bacterial metabolism.

A disadvantage of the described profiling technique is that primarily it is not an exact quantitative method. It allows comparative studies and the recognition of pronounced changes in the relative concentrations of different components in the profile, such as the high urinary excretion of phenolic acids in patients with liver cirrhosis as compared to normals. Quantitative data would require suitable internal standards and several calibration standards for each component to be quantified.

The pre-fractionation step is useful to improve the separation of the compounds and thereby the specificity. Phenolic acids of higher polarity, such as 4-hydroxymandelic acid, 4-hydroxyphenyllactic acid and 4-hydroxyhippuric acid are constituents of fraction 3 (Fig. 5) and are well separated from other components. 4-Hydroxy-3-methoxyphenylpropionic acid and 4-hydroxyphenylpyruvic acid, which appear in fraction 2b (Fig. 3), would be covered by large amounts of hippuric acid, a component of fraction 3, if pre-fractionation were not performed. The subfractionation into 2a and 2b which leads to a splitting of the total amounts of 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid into two fractions can be omitted when only phenolic acids are being studied. It should be carried out, however, when branched-chain oxocarboxylic acids are being studied as well. They are enriched in fraction 2a. In accordance with decreased concentrations of branched-chain amino acids in the blood of cirrhotic patients, the urinary excretion of their metabolites, i.e. the branched-chain oxocarboxylic acids, is low.

In the reaction of phenolic acids with diazomethane, the carboxyl group as well as the phenolic hydroxyl group are methylated. For this reason it is not possible to differentiate by MS between $-OCH_3$ groups that are originally present in the molecule and those that are formed from —OH groups in the methylation step. To establish the original structure of the substances, in one analysis deuterated diazomethane was used in the sample preparation, making MS differentiation possible.

Phenolic acids in liver cirrhosis

As demonstrated in Figs. 1—6, the urinary excretion of the following phenolic substances is increased in patients with liver cirrhosis as compared to normal controls: 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxyhippuric acid, vanillic acid, homovanillic acid, 4-hydroxymandelic acid, 4-hydroxy-3-

methoxyphenylpropionic acid, and the non-carboxylic compound *p*-cresol. Between the seven cirrhotic patients studied, a considerable variance in the elevation of each single component was observed, the increases of 4-hydroxyphenylacetic acid and 4-hydroxybenzoic acid usually being the most pronounced.

The common structural feature of all the substances is the 4-hydroxyl group, which is explained by their common precursor, the amino acid tyrosine. It has been known for a long time that the metabolism of tyrosine is abnormal in cirrhosis. In the basal state and after oral administration of tyrosine, the plasma tyrosine levels [7, 8] and the excretion of the metabolite 4-hydroxyphenylpyruvic acid [8] were elevated. Increased excretion was also observed for 4-hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid [1, 2].

The degradation of tyrosine is accomplished in the liver, in extrahepatic tissues and by microorganisms of the intestine. In liver cirrhosis the hepatic homogentisic acid pathway is partially blocked by defects of the enzymes tyrosine transaminase, 4-hydroxyphenylpyruvic acid oxidase and homogentisic acid oxidase [8]. In particular, as a consequence of the second enzyme defect, the accumulation of 4-hydroxyphenylpyruvic acid and its metabolites 4-hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid is explained. All three phenolic acids can also be produced from tyrosine by intestinal bacteria [9]. Probably a pure bacterial metabolite is an oxidation product of 4-hydroxyphenylacetic acid, i.e. 4-hydroxybenzoic acid, which is partially conjugated with glycine to form 4-hydroxyhippuric acid. The intestinal origin of 4-hydroxybenzoic acid is supported by our findings that a cirrhotic patient who received parenteral nutrition after portacaval shunt surgery, excreted very low amounts of this acid. The excretion reached a high level when a normal diet was resumed. A further purely bacterial product is *p*-cresol [9, 10]. In experiments with rats it was shown that its urinary excretion is reduced when the intestinal flora is suppressed by oral neomycin administration [11]. Vanillic acid, homovanillic acid and 4-hydroxymandelic acid are metabolites formed in extrahepatic tissues. 4-Hydroxy-3-methoxyphenylpropionic acid may originate from the bacterial 4-hydroxyphenylpropionic acid by further extrahepatic metabolization.

Using the described profiling method, changes in the excretion of all these phenolic constituents can be observed. On the basis of their different origins, the phenols would supply information on the metabolizing function of the liver of cirrhotic patients and on the effect of therapy with neomycin, lactulose and reduced dietary proteins. Since the bacterial products of tyrosine accumulate when they do not reach the liver for further metabolization, these intestinal phenols are biochemical indicators for portal-systemic venous collaterals. In contrast to previous techniques, the method presented appears suitable for detailed and systematic studies.

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