CHARACTERIZATION OF A DOUBLE CONJUGATE IN UREMIC BODY FLUIDS

Glucuronidated *o*-hydroxybenzoylglycine

Lena ZIMMERMAN, Hans JÖRNVALL, Jonas BERGSTRÖM, Peter FÜRST and Jan SJÖVALL
Department of Physiological Chemistry, Karolinska Institutet, S-104 01 Stockholm 60, Metabolic Research Laboratory, S:t Eriks sjukhus and *Department of Renal Medicine, Huddinge University Hospital, Stockholm, Sweden*

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1. Introduction

Different compounds accumulate in body fluids of patients with uremia. Those of 350–2000 M_r are called middle molecules [1,2]. They are assumed to exert toxic effects [3]. Many UV-absorbing middle molecule fractions can be recovered by gel-filtration followed by ion-exchange chromatography [4]. Some of these fractions are heterogeneous [5] requiring further separation into individual components.

One middle molecule fraction, usually very prominent in severe uremia (peak 7c in [4]) has been isolated and shown to be a new glycine-containing compound [6]. We now report the complete structure of this middle molecule, showing it to be a double conjugate of *o*-hydroxybenzoic acid with glycine and glucuronic acid. Glucuronides and glycine conjugates are constituents of normal urine and of dialysis fluid from uremic patients [7–9] but they are presently also shown to be combined in a middle molecule.

2. Materials and methods

Pre-dialysis ultrafiltrate of plasma from a uremic patient on intermittent hemodialysis (urea 29 mmol/l, creatinine 1000 μmol/l) was obtained by applying negative pressure in the dialysate compartment of a Gambro Lundia dialyzer with 13.5 μm cuprophane membranes, while no dialysis fluid was running through the dialyzer [10].

Fractionation on Sephadex G-15 in 0.03 M ammonium acetate followed by ion-exchange chromatography on DEAE A-25 with increasing ammonium acetate concentrations yielded middle molecule fraction 7c [4,5]. Analytical isotachophoresis [5] was performed on an LKB 2127 Tachophor [11], and high-performance liquid chromatography (Hewlett Packard 1084 B) using a C_{18} column, in 5 mM sodium acetate (pH 4.0) with a gradient of acetonitrile.

For solvolysis, the sample was dissolved in 1 vol. ethanol and 9 vol. ethyl acetate equilibrated with 2 M aqueous sulphuric acid and kept at 39°C overnight. Alkaline hydrolysis was made with 15% (w/v) NaOH in 50% aqueous ethanol for 10 h at 110°C in closed steel tubes, partial acid hydrolysis in 10 M HCl for 18 h at room temperature and enzymatic hydrolysis in 5 ml 0.2 M sodium acetate buffer (pH 4.5) with 10 000 units of β-glucuronidase (Sigma, type B-10, from bovine liver) at 37°C for 24 h. Elemental analysis was made on a Carlo Erba Elemental Analyzer [13–15].

Gas chromatography–mass spectrometry (GC/MS), was carried out on a modified LKB 9000 instrument [15]. Direct inlet probe mass spectra were recorded using a modified LKB 2091 mass spectrometer. Samples were methylated with diazomethane in diethyl ether/methanol (9:1, v/v). Trimethylsilyl (TMS) ethers were then prepared in pyridine/hexamethyldisilazane/trimethylchlorosilane (3:2:1, by vol.) at room temperature for 3–60 min. Reagents were removed under nitrogen and the samples were immediately dissolved in hexane.

Starting with *o*-, *m*- and *p*-hydroxybenzoic acids the corresponding hydroxyhippuric acids were synthesized by an active ester method using *N*-hydroxysuccinimide and dicyclohexylcarbodiimide. UV spectra were recorded with a Beckman Model 24 spectrophotometer. Melting points were determined with an Olympus instrument.

3. Results and discussion

3.1. *Initial characterization*

The compound was isolated from a uremic patient and had the known structure A–B–Gly [6]. Partial acid hydrolysis had been found to cleave the bond between A and B but not to liberate glycine. As a further test for homogeneity the substance was analyzed by high-performance liquid chromatography before and after methylation. As seen in fig.1 the compound appeared as a single peak after derivatization.

3.2. o-*Hydroxybenzoic acid as the non-terminal constituent (B)*

The initial characterization indicated that the compound was a double conjugate, partly analogous to a sulphated glycine conjugated bile acid. Therefore, a method to deconjugate bile acids, involving solvolysis and alkaline hydrolysis was employed [15]. After extraction, methylation and silylation, the derivative was analyzed by GC/MS. The result indicated the presence of *o*-hydroxyhippuric acid, a conjugate of *o*-hydroxybenzoic acid and glycine (fig.2A). Thus, the bond between A and B (B being hydroxybenzoic acid) in the formula A–B–Gly was cleaved but not that between hydroxybenzoic acid and glycine.

The identification of hydroxyhippuric acid was unequivocal but the *ortho*-position of the hydroxyl, suggested as the most likely alternative was not positively established. The *o*-, *m*- and *p*-hydroxyhippuric acids were therefore synthetized. Spectral properties and melting points of the 3 isomers are summarized in table 1, and show that the aromatic hydroxyl of the investigated middle molecule compound is in the *ortho*-position. This result was confirmed by isotachophoretic analysis after partial acid hydrolysis. Synthetic *o*-hydroxyhippuric acid was similarly analyzed alone and mixed with the partially hydrolyzed middle molecule fraction. One of the two zones derived from the biological material was identical with the single zone from the synthetic *o*-hydroxyhippuric acid (fig.2B). These experiments establish that the product released upon partial hydrolysis is identical with *o*-hydroxyhippuric acid, in agreement with the result of the GC/MS analysis after alkaline hydrolysis. Consequently, B in the initial structure is equal to *o*-hydroxybenzoic acid.

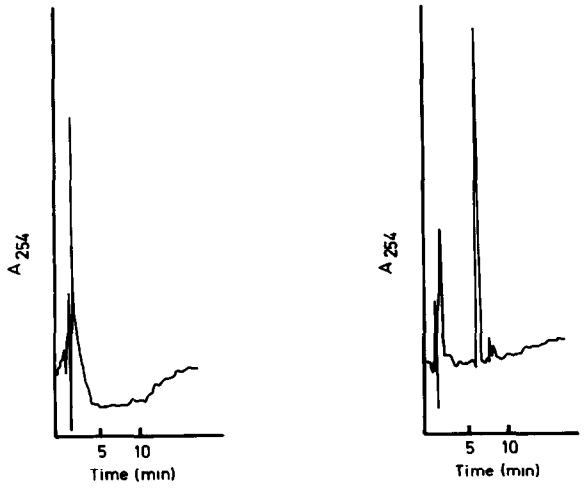

Fig.1. High-performance liquid chromatography of fraction 7c underivatized (left) and methylated (right). The early peak complex at ~2 min is derived from injection artifacts. The underivatized sample gives no resolved peaks.

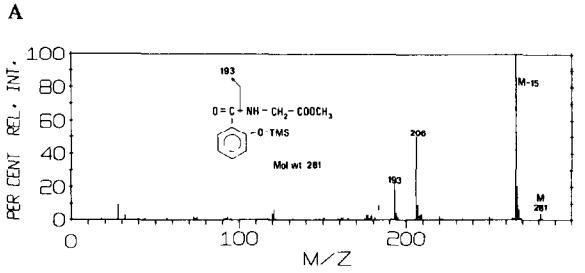

Fig.2A. Mass spectrum of the methyl ester TMS ether derivative of fraction 7c after solvolysis and alkaline hydrolysis.

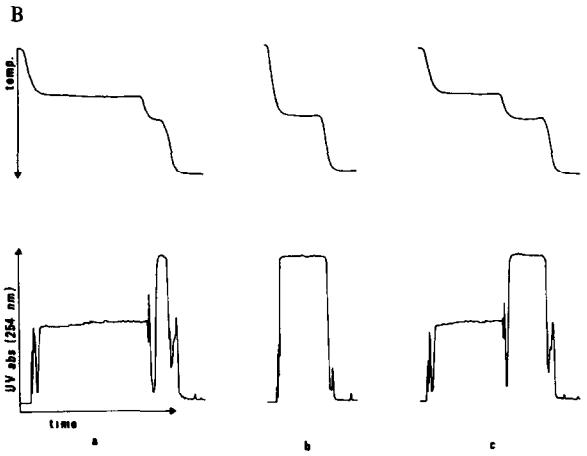

Fig.2B. Isotachophoretic separation of: (a) fraction 7c after partial hydrolysis; (b) synthetic *o*-hydroxyhippuric acid; (c) mixture of (a) and (b).

Table 1
Properties of different hydroxyhippuric acid derivatives

	Melting point (H_2O)	UV max (nm)	
		CH_3OH	CH_3OH/OH^-
o-OH-hippuric acid (commercial)	168°C	237, 301	328
o-OH-hippuric acid (synthesized)	168°C	237, 301	328
m-OH-hippuric acid (synthesized)	191°C	292	317
p-OH-hippuric acid (synthesized)	240°C	254	291
Fraction 7c (after partial hydrolysis)		237, 301	

Table 2
Elemental analysis of fraction 7c

Element	Amounts (μg) recovered	Calculated amounts from formula (fig.3B; diammonium salt)
C	5.50	5.54
H	0.80	0.71
O	4.94	4.92
N	1.22	1.29
Total	12.46	12.46

3.3. *Glucuronic acid as the third constituent (A)*

To investigate if sulphate or phosphate were constituents of fraction 7c, elemental analyses were carried out. Sulphur and phosphorus were not found but considerable amounts of oxygen. This suggested the possibility of carbohydrate as a third constituent. The atomic ratios agreed reasonably well with those from the later derived formula. The comparison is shown in table 2, assuming that a diammonium salt was analyzed (which corresponds to the free acid plus about 8% (w/w) NH_3).

The nature of constituent A was further studied by mass spectrometry. Since the sample was pure by high-performance liquid chromatography (fig.1) it was introduced on a direct inlet probe after methylation and silylation. The mass spectrum (fig.3A) showed fragments typical for methyl ester TMS ether derivatives of glucuronic acid conjugates [16] and suggested glucuronic acid to be component A. This was confirmed by hydrolysis with β-glucuronidase, which also established the β-glycosidic nature of the linkage.

3.4. *Complete structure*

The structure of the compound is shown in fig.3B. The molecule is a double conjugate of *o*-hydroxybenzoic acid with glycine and glucuronic acid (M_r = 371).

A role of free and conjugated phenols as uremic toxins has been suggested [18,19], and some seem to exert a toxic effect on Na^+-transport [8]. UV-absorb-

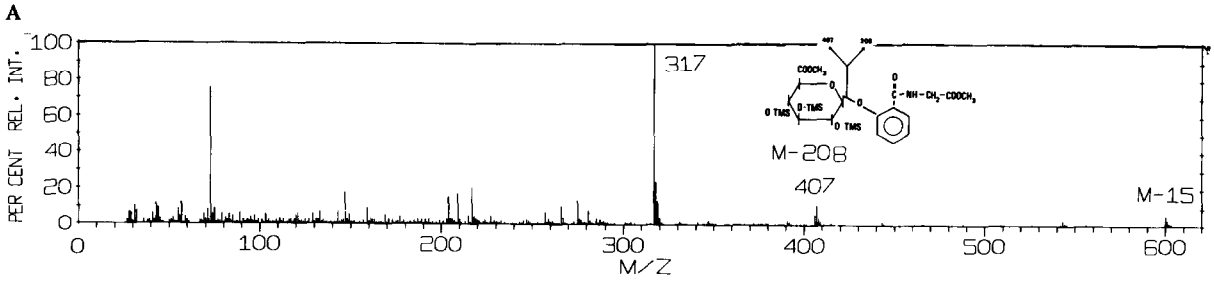

Fig.3A. Mass spectrum of the methyl ester TMS ether derivative of fraction 7c, obtained using the direct inlet probe.

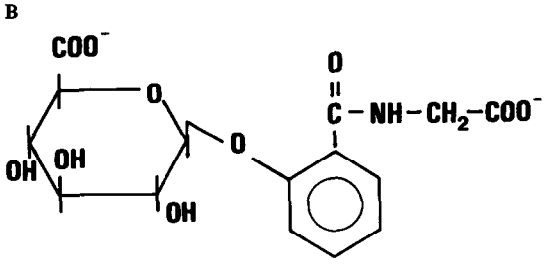

Fig.3B. Structure of compound 7c.

ing middle molecule fractions are released from homogenates and slices of liver but not from muscle, kidney and brain [20]. This fits with the assumption that the new compound is formed in the liver.

A prerequisite for the accumulation of middle molecule compounds seems to be substantially reduced glomerular filtration (<12 ml/min) [21]. Other factors also appear important since patients with the same degree of reduced renal function accumulate the compound in different amounts. Independent of these variations, however, fraction 7c, the major middle molecule in some patients, has now been shown to be a double conjugate.

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