Identification by Nuclear Magnetic Resonance and Mass Spectrometry of a Glucuronic Acid Conjugate of \( \alpha \)-Hydroxybenzoic Acid in Normal Urine and Uremic Plasma

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An endogenous compound (included in the fraction of uremic toxins often called the "uremic middle molecules") was separated from plasma of uremic patients and urine from normal persons. As elucidated by mass spectrometry, enzymatic hydrolysis, and \( ^1 \)H, \( ^13 \)C nuclear magnetic resonance, it appears to be a conjugate of \( \alpha \)-hydroxybenzoic acid with glucuronic acid. Its presence in urine of healthy subjects indicates its physiological character.

**Additional Keyphrases:** uremic middle molecules · uremic toxins · organic acids

In recent years, it has been demonstrated that solutes with molecular mass in the range 300–2000 Da accumulate in the plasma of uremic patients. These compounds, often referred to as the "uremic middle molecules" (UMM), are probably responsible for numerous toxic effects (1, 2). At present, only a few of them have been isolated in pure form (3–5). We have elucidated the structure of one of these, the one represented by peak 2-5-10 (6). Moreover, we have shown that these UMM can be found in the urine of healthy subjects (7). Here we report the identification by nuclear magnetic resonance and mass spectrometry of another such compound: that represented by peak 2-5-7 (7).

**Materials and Methods**

**Biological Fluids**

UMM were obtained from urine of three healthy subjects, members of the laboratory staff who had received no medical treatment for one month: C, male, 45 years old; M, female, 25; G, male, 30. UMM were also obtained from three uremic patients: B, male, 20 years old; D, female, 34; C, female, 40.

**Isolation of the 2-5-7 Compound**

We described the isolation technique in full detail elsewhere (6, 7). Briefly, the different steps of this technique are as follows. Gel-permeation chromatography of plasma ultrafiltrate or urine yields crude UMM (fraction 2). Anion-exchange chromatography allows separation of fraction 2 into seven components, 2-1 through 2-7. Finally, fraction 2-5 is resolved into 10 subfractions (2-5-1 through 2-5-10) by means of "high-performance" liquid chromatography.

**Identification of the 2-5-7 Subfraction**

**Mass spectrometry.** The trimethylsilyl (TMS) derivative of the 2-5-7 compound was prepared according to Larsen and Bakke (8). The spectra were recorded with a Hewlett-Packard 5985 gas chromatograph–mass spectrometer.

For electron impact, the mass spectrometer conditions were: direct insertion probe; maximal temperature 250 °C; ion-source temperature 200 °C; emission current 300 μA; electron energy 70 eV.

For chemical ionization, the experimental conditions were: capillary column WSCOT (12 m × 0.32 mm i.d.) with polydimethylsiloxane CP-Sil 5 (Chrompack, France) as stationary phase; carrier gas, helium (column head pressure 8 × 10⁻² Pa), temperature program, 100 to 280 °C at 15 °C/min; ion-source temperature, 200 °C; solid injector temperature, 200 °C; emission current, 300 μA; electron energy, 230 eV; and reagent gas, methane.

Enzymatic hydrolysis. For hydrolysis of compound 2-5-7 we used \( \beta \)-glucuronidase (EC 3.2.1.31) according to the method of Greenblatt et al. (9).

**NMR spectrometry.** \( ^1 \)H and \( ^13 \)C spectra were recorded at 200.13 and 50.32 MHz, respectively, in the pulsed Fourier transform mode with a Bruker AM 200 spectrometer at the Service Interuniversitaire de RMN, Marseille, France. Typical experimental conditions were as specified previously (6). The number of scans were 100–500 for \(^{1} \)H NMR and 80 000–300 000 for \(^{13} \)C NMR because of the low quantity of 2-5-7 compound obtained (0.5 to 1.0 mg). Proton resonance assignments were performed by spectrum integration, homonuclear double irradiation, and comparison with free compounds previously recorded under the same conditions: \( \alpha \)-hydroxybenzoic acid and \( \alpha \)-glucuronic acid (both Sigma Chemical Co. products). Carbon resonances were determined by comparison with those of the free compounds.

**Results and Discussion**

In previous studies we reported that UMM peak 2-5 has the same qualitative composition regardless of its origin, uremic plasma or normal urine (6, 7). Our present results corroborate this. Indeed, the 2-5-7 substance isolated from plasma of uremics and from urine of healthy persons has the same spectral characteristics, which eliminates the possibility of its being an artifact caused by drugs taken by uremic patients (10).

Mass-spectral analysis (Figures 1 and 2) shows fragment ions of a TMS derivative of a glucuronide (Table 1). Indeed, concerning the glucuronic moiety of molecule 2-5-7, the observation of fragment ions \( m/z \) 465, 375, 333, 217, and 204 is in good agreement with the previously described (11) mass-spectral characteristics of glucuronic acid. Moreover, the presence of fragment ions \( m/z \) 285 (\( m/z \) 375 – TMS-OH) and \( m/z \) 257 (\( m/z \) 375 – HCOO-TMS) confirms this.

As for the aglycone moiety, the ion fragment \( m/z \) 267 (\( m/z \) 282 – CH₃) originates in a di-TMS-hydroxybenzoic acid (12). In our case, this ion is obtained after a classical transfer of the TMS group (11). Fragment ions \( m/z \) 324, due to the cleavage of the glucuronic ring (Figure 3), \( m/z \) 193 (Figure 3), and \( m/z \) 211 (Figure 4) confirm the nature of the aglycone part. Finally, in the chemical ionization mass spectrum, the ion fragment \( m/z \) 659 is \( M – 15 \), the molecular ion classically recovered after the loss of a methyl group (11).
These data allow us to assert that 2-5-7 compound is a glucuronidate of hydroxybenzoic acid (ortho, meta, or para).

The enzymic hydrolysis by means of β-glucuronidase which catalyzes the hydrolysis of O-ether, O-ester, and S-ether bonds (13), confirms this glucurono conjugation and permits us to state that the aglycone moiety of compound 2-5-7 is o-hydroxybenzoic acid. Indeed, the 1H NMR spectra for compound 2-5-7 (aromatic region) after enzymatic hydrolysis and for the free model molecule, o-hydroxybenzoic acid, are shown in Figure 2.
acid, show the same coupling features and analogous chemical shifts (maximum deviation 0.08 ppm) (Figure 1).

How are the two components joined? Fragment ion m/z 193 (m/z 324 = C6H2O-TMS) (Figure 3) corresponds to the cleavage of either the aromatic ester linkage or the aromatic ether linkage. Classically, the former is easier than the latter (14, 15); thus the glucuronic acid probably is bonded via the carboxyl group (Figure 5).

Although the 13C NMR spectra show the classic resonances of salicylic acid and p-glucuronic acid in 2-5-7 compound (Table 2), the quaternary carbons that could confirm this supposed ester bond are not visible in NMR, even though we performed a considerable number of scans, because of the low quantity of compound 2-5-7.

Nevertheless, the 1H NMR results permit us to establish the linkage of p-glucuronic acid via the carboxyl group of o-hydroxybenzoic acid. Indeed, with respect to the aglycone moiety, we previously observed H1 (+0.02 ppm) and H3 (+0.02 ppm) deshielding and H2 (−0.03 ppm) and H4 (−0.09 ppm) shielding when the carboxyl group is substituted as in hippuric acid (6). In contrast, in substitution via the salicylic acid hydroxyl group, we observed a shielding for all the protons: H1 +0.38 ppm; H2 +0.09 ppm; H3 +0.26 ppm; H4 +0.03 ppm (6). By comparison, data for compound 2-5-7 (Table 3) show deshielding of H1 (+0.24 ppm) and H3 (+0.23 ppm), and shielding of H2 (−0.14 ppm) and H4 (−0.46 ppm), as is observed when the carboxyl group of salicylic acid is substituted. The magnitude of shielding and deshielding values betweenhippuric acid and compound 2-5-7 is a function of the nature of the substituent group—glycine in hippuric acid and p-glucuronic acid in compound 2-5-7.

Lastly, concerning the p-glucuronic acid moiety, the de-shielding observed about protons H1 (+0.38 ppm) and H2 (+0.37 ppm) in compound 2-5-7, in comparison with the free compound, is due to ring current caused by the nearby o-hydroxybenzoic acid (Table 3). We emphasize that H1 and H2 are differently deshielded (+0.54 ppm and +0.51 ppm, respectively) when p-glucuronic acid is bonded via the hydroxyl group of salicylic acid (6).

Thus, p-glucuronic acid is bonded via the salicylic acid carboxyl in compound 2-5-7. Moreover, the high values for the 3J coupling constants of the glucuronic moiety in compound 2-5-7 (7.2, 9.4, 9.2, and 9.7 Hz in Table 3) proves the predominance of the axial form of p-glucuronic acid in this compound (16). Lastly, the integration measurements of 1H NMR spectra show that 2-5-7 substance is composed of one molecule of each of the free model components.

Although this glucuronidate of o-hydroxybenzoic acid is reported as a drug metabolite (17), its presence in urine of healthy subjects who were taking no drugs proves the physiological character of this molecule. The structural similarities between the aglycone moieties of compound 2-5-7 and compound 2-5-10, previously reported (6), suggest that these products may originate in the same secondary metabolic pathway (18, 19).

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References


