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Characterization of two isomeric β-D-glucosiduronic acids derived from 1,2-diethyl-3-hydroxypyridin-4-one (CP94) in rat liver homogenate incubates

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Abstract

1,2-Diethyl-3-hydroxypyridin-4-one (CP94) is an orally active iron chelator with potential for use in photodynamic therapy. This investigation reports the formation and characterization of two isomeric glucuronides of CP94 in rat liver homogenate incubates. To assign the glucuronidation sites in the CP94 molecule, two *O*-methylated derivatives of CP94 have been synthesized. By comparing the spectral characteristics of the CP94 3-*O*- and 4-*O*-methyl derivatives with CP94 and the CP94 glucuronides formed during incubation, evidence was obtained which enabled the assignment of these two isomeric glucuronides to the 3-*O*-glucuronide and 4-*O*-glucuronide of CP94. It was found that the 3-*O*-glucuronide was the dominant CP94 metabolite under in-vitro conditions. In an attempt to understand the potential influence of structural variation on the glucuronidation of CP94 analogues, the 1- and 2-monoethyl derivatives of CP94 were investigated. The 2-monoethyl derivative of CP94 yielded only the 3-*O*-glucuronide in rat liver homogenate incubate, while no glucuronide was formed from the 1-monoethyl derivative. In addition, no glucuronide from the 3-*O*-methyl or 4-*O*-methyl derivatives of CP94 could be detected. The relevance of these findings to the development of new 3-hydroxypyridin-4-one iron chelators is discussed.

Introduction

Regular blood transfusion remains the most effective treatment for patients suffering from haemoglobinopathic disorders such as β-thalassaemia major. As man is unable to excrete excess iron, progressive iron overload, which is associated with blood transfusion, is unavoidable. Iron overload leads to tissue damage, organ failure and eventually death (Brittenham 1991). Complications related to elevated iron levels can be diminished by the use of iron-specific chelating agents and in particular desferrioxamine (Hershko et al 1998). Unfortunately, desferrioxamine lacks oral activity and has to be administered parentally, which is not only expensive but also leads to poor patient compliance.

In an attempt to overcome the disadvantages of desferrioxamine, the design of an orally active, non-toxic, selective iron chelator has been a goal for medicinal chemists over the last two decades. Among a wide range of iron chelating compounds, 3-hydroxypyridin-4-ones (HPOs) form an important candidate class for the development of chelators which are able to selectively promote the excretion of iron (Tilbrook & Hider 1998). Of this class of chelators, 1,2-diethyl-3-hydroxypyridin-4-one (CP94) has been investigated in both animals and thalassaemic patients for its clinical potential (Bergeron et al 1992; Florence et al 1992; Porter et al 1994). Unfortunately, though CP94 possesses a high efficacy for iron mobilization in rat, it is much less effective in man (Porter et al 1994), although it has potential for photodynamic therapy (Curnow et al 1998, 1999).

The metabolic profiles of CP94 in the urine and blood of rats have been previously investigated (Epemolu et al 1992, 1994; Singh et al 1992) and it has been established that 2-(1′-alkyl)-hydroxylation, 6-hydroxylation and 3-*O*-glucuronidation are the major

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pathways of CP94 metabolism in several animal species, including man. Although the hydroxylated metabolites are effective iron chelators, the 3-*O*-glucuronide is inactive. In guinea-pig and man, glucuronidation was found to be the dominant metabolic route, accounting for >99% and >90% of the administered dose, respectively (Singh et al 1992; Porter et al 1993, 1994). The corresponding figure for the rat is 14% (Singh et al 1992). By virtue of the extremely fast rate of this glucuronidation step, the iron scavenging ability of CP94 in man is much lower than that in the rat. In previous investigations, only the 3-*O*-glucuronide was identified as a metabolite (Epemolu et al 1992, 1994; Singh et al 1992).

Recently, using a modified procedure based on the previously adopted HPLC method (Epemolu et al 1990) and using LC-MS technique, Lu et al (2000) identified two isomeric CP94 glucuronides in both rat bile and urine, following oral administration of CP94. However, as CP94 glucuronide standards were not available, characterization of the two glucuronides required further investigation. This report assigns structures for the two isomeric b-D-glucuronic acids of CP94 and investigates the glucuronidation of CP94 together with its analogues, 1-ethyl-3-hydroxypyridin-4-one (CP61) and 2-ethyl-3-hydroxypyridin-4-one (CP99), in rat liver homogenate incubates.

Materials and Methods

Chemicals

1,2-Diethyl-3-hydroxypyridin-4-one (CP94), 2-ethyl-3-hydroxypyridin-4-one (CP99) and 1-ethyl-3-hydroxypyridin-4-one (CP61) (Figure 1) were prepared according to the procedures described by Dobbin et al (1993). Uridine 5′-diphosphoglucuronic acid (UDPGA) and Triton X-100 (reduced) were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). The synthetic routes and physicochemical characteristics of the 3-*O*- and 4-*O*-methylated derivatives of CP94 are described below.

1,2-Diethyl-3-methoxypyridin-4-one hydrochloride (CP94-3-OMe)

CP94-3-OMe was conveniently prepared using the route outlined in Figure 2. The 3-hydroxy function of ethyl maltol was methylated using methyl iodide in aqueous sodium hydroxide. The resulting pyranone was then converted to the corresponding *N*-ethyl pyridinone by reaction with aqueous ethylamine, the final product being isolated as a hydrochloride salt. mp 162–164°C. ^{1}H NMR (400 MHz, DMSO-d_6) d: 1.22 (t, 3H, 2-$CH_2\mathbf{CH_3}$, J = 7.5 Hz), 1.44 (t, 3H, N-$CH_2\mathbf{CH_3}$, J = 7.2 Hz), 2.97 (q, 2H,

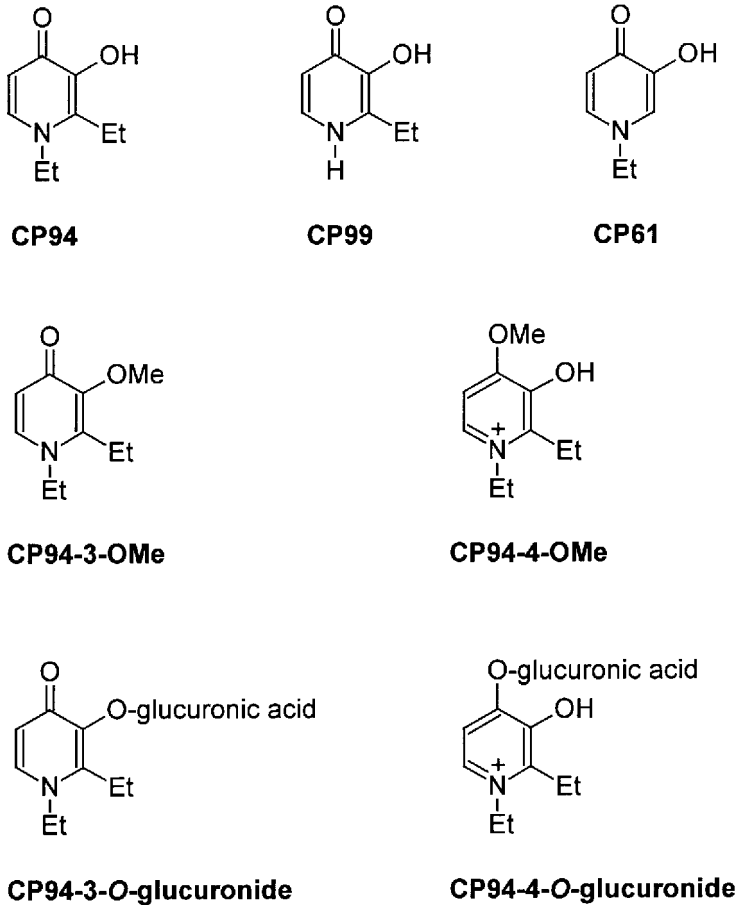

Figure 1 The structure of 1,2-diethyl-3-hydroxypyridin-4-one (CP94), its analogues and metabolites.

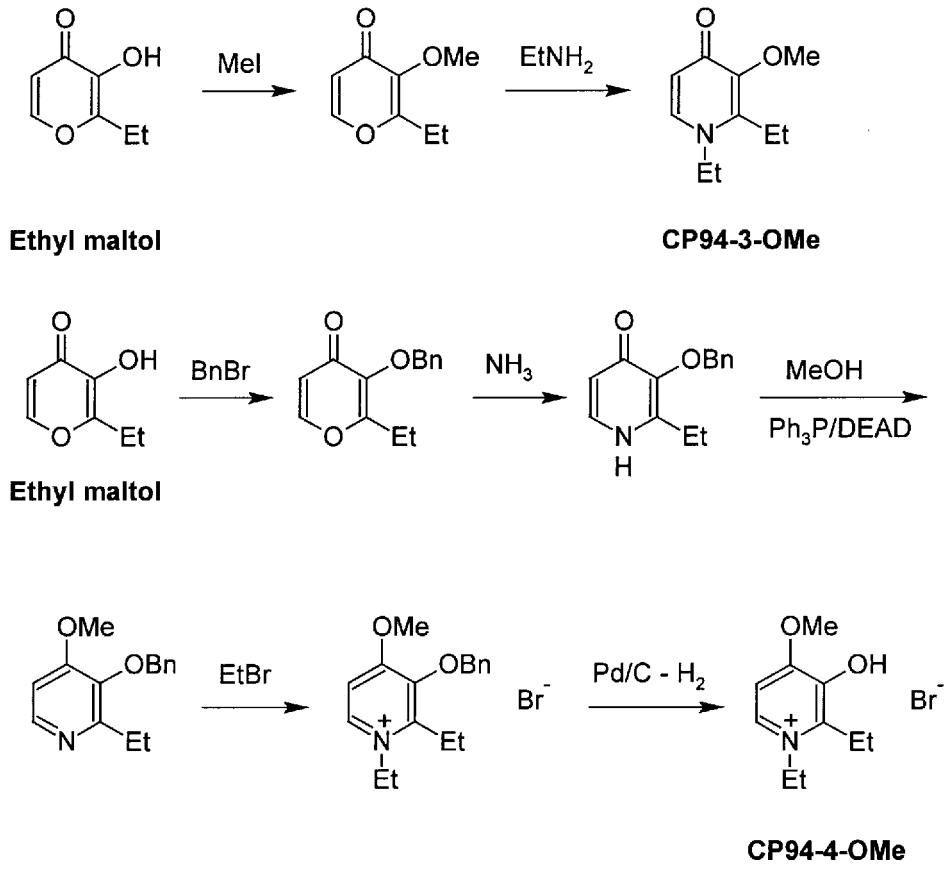

Figure 2 Routes to the synthesis of 3- and 4-methoxy substituted CP94.

2-$\mathbf{CH_2}CH_3$, J = 7.5 Hz), 3.91 (s, 3H, 3-O$\mathbf{CH_3}$), 4.42 (q, 2H, N-$\mathbf{CH_2}CH_3$, J = 7.2 Hz), 7.64 (d, 1H, **5-H** (pyridinone), J = 7.0 Hz), 8.57 (d, 1H, **6-H** (pyridinone), J = 7.0 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) d: 164.1, 153.2, 144.3, 141.9, 113.4, 61.0, 51.2, 20.2, 16.7, 13.2 (excluding solvent); MS (FAB): m/z, 182 [(M-Cl)$^{+\cdot}$]; Anal. Calcd. for $C_{10}H_{16}NO_2Cl$: C, 55.17; H, 7.41; N, 6.43; Cl, 16.29%. Found: C, 55.23; H, 7.26; N, 6.40; Cl, 16.13%.

1,2-Diethyl-3-hydroxy-4-methoxypyridinium bromide (CP94-4-OMe)

The route adopted for the synthesis of CP94-4-OMe is summarized in Figure 2. The benzylated ethyl maltol was first converted to the corresponding pyridinone by reaction with aqueous ammonia. The 4-oxo function was then methylated with methanol in the presence of diethyl azodicarboxylate (DEAD) and triphenylphosphine. 1,2-Diethyl-4-methoxypyridinium salt was finally synthesized by alkylation at nitrogen with ethyl bromide, followed by hydrogenation to remove the benzyl group. mp 131–133°C. ^{1}H NMR (400 MHz, DMSO-d_6) d: 1.18 (t, 3H, 2-$CH_2\mathbf{CH_3}$, J = 7.5 Hz), 1.42 (t, 3H, N-$CH_2\mathbf{CH_3}$, J = 7.0 Hz), 2.99 (q, 2H, 2-$\mathbf{CH_2}CH_3$, J = 7.5 Hz), 4.17 (s, 3H, 4-O$\mathbf{CH_3}$), 4.41 (q, 2H, N-$\mathbf{CH_2}CH_3$, J = 7.0 Hz), 7.55 (d, 1H, **5-H** (pyridinone), J = 7.0 Hz), 8.51 (d, 1H, **6-H** (pyridinone), J = 7.0 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) d: 157.8, 146.2, 143.1, 139.1, 107.9, 66.3, 43.8, 19.8, 13.9, 10.8 (excluding solvent); MS (FAB): m/z, 182 [(M-Br)$^+$]; Anal. Calcd. for $C_{10}H_{16}NO_2Br$: C, 45.82; H, 6.15; N, 5.34; Br, 30.48%. Found: C, 46.13; H, 6.37; N, 5.15; Br, 30.17%.

Preparation of hepatic microsomes

Male Wistar rats (200–220 g) were purchased (local breed) from A. Tuck & Son (Battlesbridge, Essex SS1, UK) and housed in the Biological Service Unit, King's College London. Rats were fasted overnight with free access to water. After the rats were killed by neck dislocation, livers were rapidly removed and immersed in ice-cold Tris-HCl buffer (100 mM, pH 7.4). The livers were perfused using the same buffer and were blotted to dryness using filter paper. The livers were weighed, cut into small pieces and homogenized in Tris-HCl buffer (100 mM, pH 7.4) (3 mL of buffer per g of liver) using a glass homogenizer. The homogenate was centrifuged at 500 rev min^{-1} for 10 min at 0–4°C. The supernatant was separated and stored at −80°C and used within one week of preparation.

Incubation procedure for in-vitro glucuronidation

In-vitro glucuronidation was undertaken using the method developed by Pacifici et al (1988) with minor modifications. The incubates consisted of substrate (1 l mol in 50 l L

water), $MgCl_2$ (2.5 l mol in 50 l L water), UDPGA (2 l mol in 50 l L water) and liver homogenate (350 l L, equivalent to about 90 mg fresh liver tissue). The liver homogenate was added with Triton X-100 (10 l L per 6 mL homogenate) at 0–4°C and gently mixed immediately before incubation (Yu & Shen 1996). The final volume was adjusted to 0.5 mL with Tris-HCl buffer (100 mM, pH 7.4). The incubations were carried out at 37°C for 30 min unless otherwise stated. The incubations were stopped by the addition of trichloroacetic acid (100 l L, 25% w/v in water), mixed and centrifuged (3000 rev min^{-1} for 10 min). The supernatants were separated and filtered using syringe filters (0.2 l m pore size, 13 mm GD/X disposable Filter Device, Whatman). The filtrates (30 l L) were injected directly on the HPLC column.

HPLC method

A gradient ion-pair system, which had been specifically designed for the chromatography of HPO iron chelators (Liu et al 1999), was used throughout this investigation. The analytes included CP94, its 3-*O*- and 4-*O*-methyl analogues, glucuronides and other CP94 derivatives studied. Briefly, the HPLC system (Hewlett-Packard model 1090M Series) consisted of an autoinjector, an autosampler, a diode-array detector and a reversed-phase polymer HPLC column (PLRP-S 100 Å, 15 × 0.46 cm i.d., 5 l m; Polymer Laboratories Ltd, Church Stretton, Shropshire, UK). The buffer of the ion-pair gradient system contained 1-heptanesulfonic acid (sodium salt) (5 mM) and was adjusted to pH 2.0 using hydrochloric acid. The linear gradient was 2–35% acetonitrile in 20 min and post-run was followed for 5 min using 98% buffer and 2% acetonitrile. The flow rate was 1 mL min^{-1} and the analytes were monitored using a diode array detector, which allowed using multiple wavelengths to record the chromatograms simultaneously.

Quantification of CP94 glucuronides

An indirect method was used, based on the spectral characteristics of CP94-3-OMe, CP94-4-OMe, CP94-3-OG and CP94-4-OG, to estimate the in-vitro formation rates of CP94-3-OG and CP94-4-OG.

The concentration of CP94 total glucuronides ($[G_{total}]$) formed during incubation was measured by subtracting the molar concentration of remaining CP94 after incubation from the initial concentration (molar concentration was used all through the experiments and the following calculations). In addition,

$$[G_{total}] = [3G] + [4G] \quad (1)$$

where [3G] and [4G] are the concentrations of CP94-3-OG and CP94-4-OG formed during incubation.

The k_{max} (wavelength with maximal absorbance) of CP94-3-OMe, CP94-4-OMe, CP94-3-OG and CP94-4-OG, was found to be 266 nm (k_{3Me}), 280 nm (k_{4Me}), 262 nm (k_{3G}) and 282 nm (k_{4G}), respectively.

$$\text{When } [4Me]/[3Me] = 1,\ A_{4Me(k_{max})}/A_{3Me(k_{max})} = 1.39 \quad (2)$$

where [4Me] and [3Me] are the concentrations of CP94-4-OMe and CP94-3-OMe, respectively, and $A_{4Me(kmax)}$ and $A_{3Me(kmax)}$ are their peak areas recorded simultaneously in HPLC chromatograms at k_{4Me} and k_{3Me}. The ratio of e_{4Me} and e_{3Me}, the extinction coefficients of CP94-4-OMe and CP94-3-OMe at k_{4Me} and k_{3Me}, respectively, is calculated as 1.39.

Thus if

$$e_{4G}/e_{3G} = e_{4Me}/e_{3Me} \quad (3)$$

then

$$[4G]/[3G] = A_{4G(k_{max})}/(1.39 \times A_{3G(k_{max})}) \quad (4)$$

where [4G] and [3G] are the concentrations of CP94-4-OG and CP94-3-OG respectively; A_{4G} and A_{3G} are the peak areas of CP94-4-OG and CP94-3-OG recorded in HPLC chromatograms at k_{4G} and k_{3G}.

Combining equations 1 and 4, it is possible to deduce that

$$[4G] = \frac{[G_{total}]}{\left\{1 + \frac{1.39 \times A_{3G(k_{max})}}{A_{4G(k_{max})}}\right\}} \quad (5)$$

and

$$[3G] = \frac{[G_{total}]}{\left\{1 + \frac{A_{4G(k_{max})}}{1.39 \times A_{3G(k_{max})}}\right\}} \quad (6)$$

Results and Discussion

Glucuronidation of xenobiotics, catalysed by a family of UDP-glucuronosyltransferases, is a major detoxication and inactivation system in mammals (Kroemer & Klotz 1992; Mackenzie et al 1996, 1997). After glucuronidation, most substrates become more hydrophilic and are thus readily eliminated via biliary and urinary routes. Glucuronides of endogenous substances or xenobiotics can be identified by treating the samples with b-glucuronidase, an increase in substrate concentration following such treatment being taken as an indication of glucuronidation. Using this method, it was previously found that 3-*O*-glucuronidation was a major metabolic route for CP94 and other 3-hydroxypyridinones such as CP20 (Deferiprone, L1) (Singh et al 1992; Porter et al 1994). However, in contrast to previous reports, two CP94 glucuronides were recently found in the rat (Lu et al 2000). It was considered likely that the second CP94 glucuronide was the 4-*O*-glucuronide, as only the 3- and 4- positions of CP94 are available for glucuronidation. Conjugation of either positions with glucuronic acid will lead to inactivation of CP94.

In this investigation, when CP94 was incubated with rat hepatic microsomes, two metabolites were detected (Figure

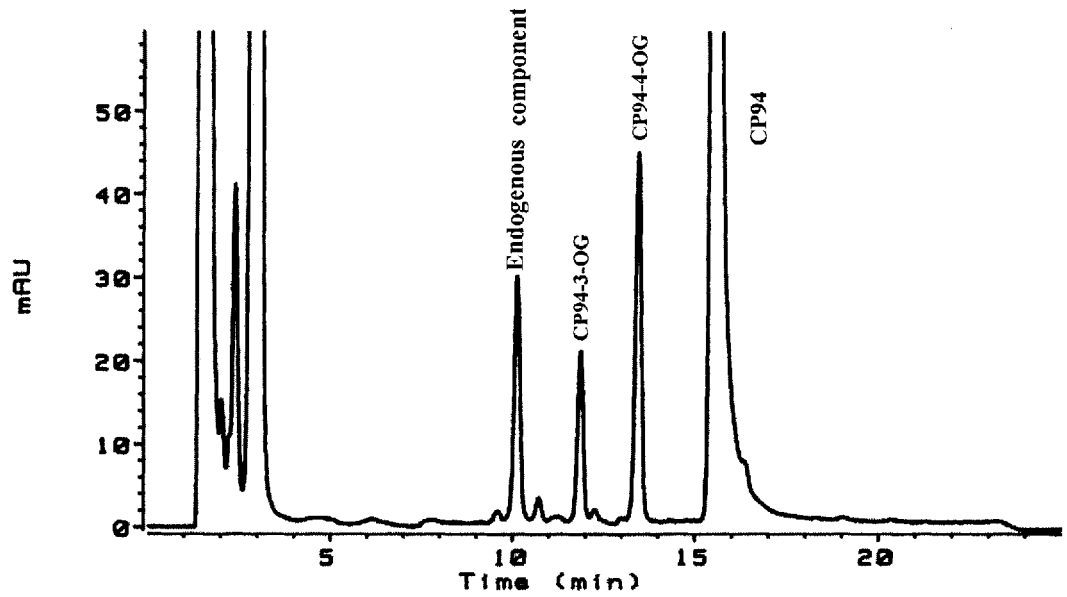

Figure 3 HPLC chromatogram of CP94 incubate in the presence of UDPGA.

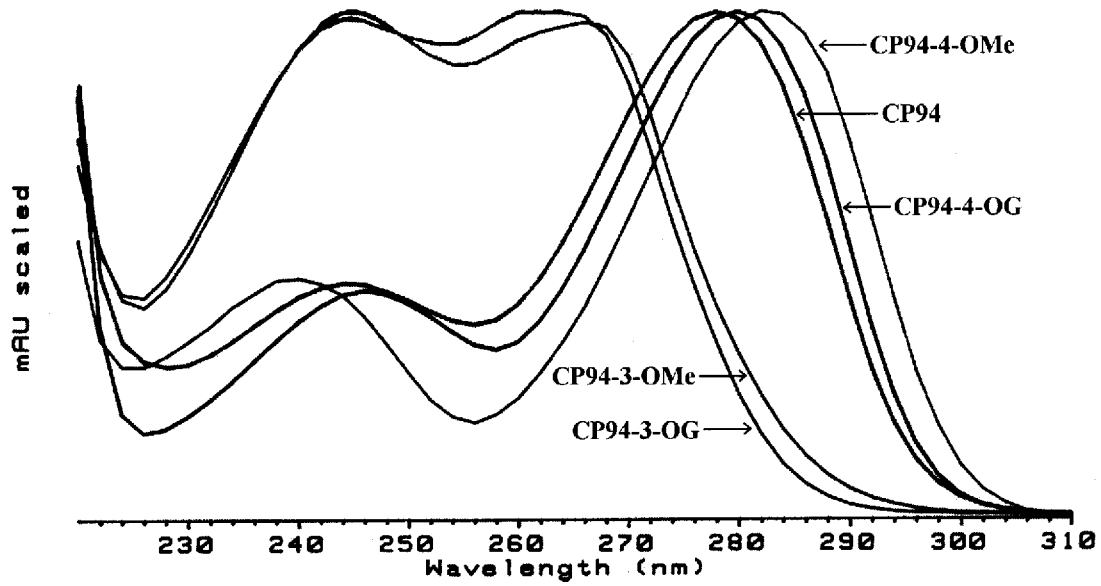

Figure 4 The spectra of authentic CP94, CP94-3-OMe, CP94-4-OMe and the two metabolically formed CP94 glucuronides.

3). These metabolites were clearly CP94 glucuronides as they only formed in the presence of UDPGA, the cofactor for glucuronidation. This is also consistent with previously reported in-vivo findings (Lu et al 2000).

Ideally, standards of the two CP94 glucuronides are required for further characterization of the two metabolites. However, in view of the anticipated difficulties in preparing these standards, it was decided to study the corresponding 3-*O*- and 4-*O*-methyl derivatives of CP94. When CP94 is glucuronidated at either the 3-*O*- or 4-*O*-positions, the glucuronic acid group induces different electronic effects on the aromatic ring, leading to changes in the UV spectral characteristics. In this study we have assumed that 3-*O*- and 4-*O*-methylation of CP94 induces analogous UV spectral differences. Thus, by comparing the UV spectra of CP94 *O*-methyl derivatives with the metabolically formed CP94 glucuronides, it was anticipated that it would be possible to distinguish the 3-*O* glucuronide from the 4-glucuronide of CP94. The 3-*O*- and 4-*O*-methyl derivatives of CP94 were prepared as described in Figure 2. CP94-4-OMe displayed a similar UV spectral pattern to that of CP94, with a slight red shift, whereas CP94-3-OMe showed a quite different spectral pattern with an appreciable significant violet shift (Figure 4).

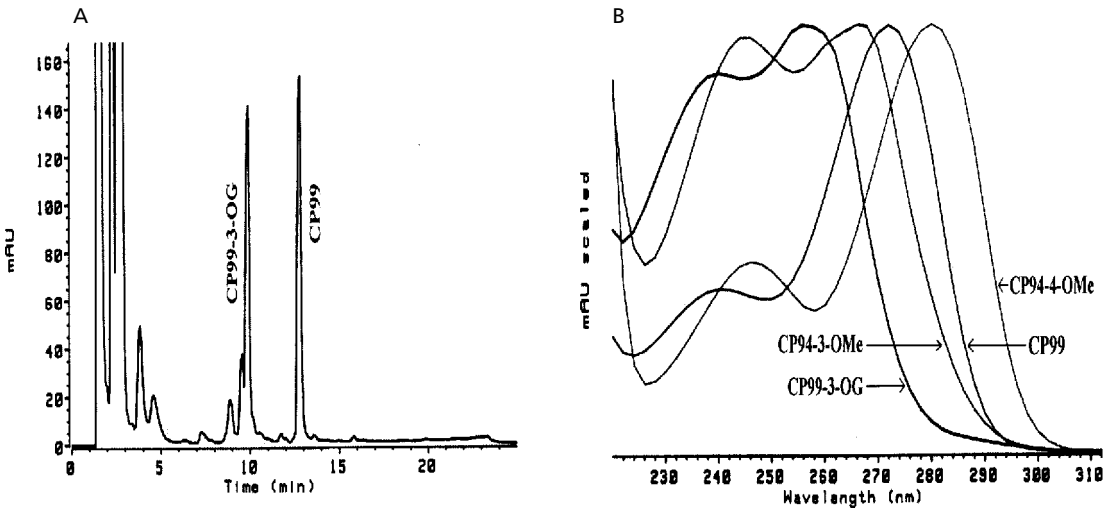

Figure 5 A. HPLC chromatogram of the CP99 incubate in the presence of UDPGA. B. The spectra of CP99, CP94-3-OMe, CP94-3-OMe and the CP99 metabolite.

When the UV spectra of the metabolically formed CP94 glucuronides were compared with those of CP94, CP94-3-OMe and CP94-4-OMe (Figure 4), the metabolite with an HPLC retention time of 12 min was found to possess a similar spectrum to that of CP94-3-OMe (Figure 3) and so was assigned to the CP94-3-*O*-glucuronide (CP94-3-OG). The second CP94 metabolite, with an HPLC retention time of 13 min (Figure 3), possessed similar spectral characteristics to that of CP94-4-OMe (Figure 4) and was therefore assigned as the CP94-4-*O*-glucuronide (CP94-4-OG). CP94-3-OG was found to be quantitatively the major glucuronide, as demonstrated by a 3-h in-vitro incubation with CP94 (2 mM) when the concentration of the two glucuronides reached 210 l M (3-*O*-glucuronide) and 65 l M (4-*O*-glucuronide).

To probe the structure–glucuronidation relationship of HPOs, CP94-4-OMe, CP94-3-OMe and the monoethyl analogues of CP94 (CP61 and CP99; Figure 1) were also incubated with rat liver homogenate. When CP94-4-OMe and CP94-3-OMe were incubated with rat liver homogenate in the presence of UDPGA, neither was found to form any detectable glucuronide. However, one glucuronide was formed from CP99, which was found to be the 3-*O*-glucuronide (Figure 5), while no glucuronide was found from the CP61 incubate. These results imply that the UDP-glucuronyltransferase(s) catalysing the glucuronidation of HPO chelators possesses a relatively high substrate specificity. Clearly modification of CP94 at either the 3- or 4-position greatly affects glucuronidation. However, guaiacol (2-methoxyphenol), which is structurally similar to CP94-4-OMe and CP94-3-OMe, was previously found to be readily glucuronidated at the phenol group (Ogata et al 1995). Therefore it is unlikely that blocking of the methoxy group in CP94-4-OMe and CP94-3-OMe prevents access to the enzyme-binding site. The location of the ethyl group at either the 1- or 2-position of CP94 also influences glucuronidation. These two isomers possess quite different physicochemical properties, however, the $D_{7.4}$ (octanol/water) values for CP99 and CP61 being 1.10 and 0.09, respectively. This provides a possible reason for CP99 being glucuronidated whilst CP61 is not, the lipophilicity of the HPO chelators being a major factor influencing glucuronidation (Lu et al 1998). Such information will be useful in the design of HPO chelators.

Conclusions

The 3-hydroxypyridin-4-one class of molecules possesses two conserved phenolic functions, both of which are involved in the chelation of iron – a property being utilised for the design of clinically useful iron chelators. The rapid metabolism of some of these chelators seriously limits their potential as therapeutic agents. The finding that both the 3- and 4-hydroxyl groups can be glucuronidated provides useful information for the design of hydroxypyridinone chelators.

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