ABSTRACT:
This study compares the biliary and urinary metabolic profiles of 1,2-diethyl-3-hydroxypyridin-4-one (CP94), an orally active iron chelator, in the normal rat. Surprisingly, CP94 was found to form two phase II metabolites, the 3-O- and 4-O-glucuronides. These glucuronides accounted for 38 and 28% of the administered CP94 dose, in bile and urine, respectively. Unchanged CP94 accounted for 5% of the CP94 dose in both bile and urine. The 2-(1'-hydroxy) metabolite of CP94 was found to be the dominant metabolite in urine. In addition, an unstable metabolite was detected in the bile although its structure remains unknown at the present stage. The excretion of iron in bile, after administration of CP94, was found to parallel the biliary elimination of CP94 together with its hydroxylated derivatives, indicating the importance of metabolites in iron excretion.

Regular blood transfusions will inevitably lead to heavy iron overload in patients suffering from hemoglobinopathic disorders such as β-thalassemia major. Fortunately, the complications associated with elevated iron levels can be largely prevented by the use of iron-specific chelating agents (Hershko et al., 1998). 3-Hydroxy-2-ethyl-3-hydroxypyridin-4-one (HPO) forms one class of molecule with potential as an orally active iron chelator (Tilbrook and Hider, 1998) and specifically 1,2-diethyl-3-hydroxypyridin-4-one (CP94) previously has been investigated for its clinical potential as a chelator (Porter et al., 1994). CP94 can efficiently mobilize iron in various animal models, such as mouse (Porter et al., 1990), rat (Bergeron et al., 1992; Florence et al., 1992; Porter et al., 1993), and Cebus monkey (Bergeron et al., 1992), in either the normal or iron-overloaded state. In contrast, the iron mobilization promoted by CP94 in guinea pig (Porter et al., 1993) and humans (Porter et al., 1994; Alrefaie et al., 1995) is much less effective. Studies also demonstrated severe toxicity after 2 weeks in noniron-overloaded rats at CP94 doses of 300 mg/kg/24 h, whereas no significant toxicological effects were observed in guinea pigs (Porter et al., 1993). These species differences can be readily accounted for by the existence of different metabolic routes. The metabolic profiles of CP94 in rats, guinea pigs, and thalassemic patients have been investigated, and three major metabolites have been identified (Epemolu et al., 1992; Singh et al., 1992; Porter et al., 1993; Epemolu et al., 1994).

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1 Abbreviations used are: HPOs, 3-hydroxy-2-ethyl-3-hydroxypyridin-4-one; CP94, 1,2-diethyl-3-hydroxypyridin-4-one; CP102, 1-(2'-hydroxyethyl)-2-ethyl-3-hydroxypyridin-4-one; CP365, 1-ethyl-2-(1'-hydroxyethyl)-3-hydroxypyridin-4-one; UDPGA, 5'-diphosphoglucuronic acid; LC-MS, liquid chromatography-mass spectrometry; MOPS, 3-(N-morpholino)propanesulfonic acid.

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One metabolite, the 3-O-glucuronide, is a nonchelating form of CP94, and accounts for approximately 14, >99, and >90% of the administered CP94 dose in the rats, guinea pigs, and thalassemic patients, respectively (Singh et al., 1992; Porter et al., 1993, 1994). Efficient glucuronidation markedly reduces the iron-scavenging efficacy of CP94 in both guinea pigs and humans.

CP94 is also extensively hydroxylated at the 2-ethyl position to yield the 2-(1'-hydroxyethyl) metabolite (CP365), which in the rat accounts for up to 40% of the administered dose (Singh et al., 1992). A 6-hydroxylated metabolite also has been reported to be formed in the rat, although more supporting evidence is required. Unlike glucuronidation, hydroxylation generates metabolites that are effective iron chelators; consequently, CP94 is a more effective iron scavenger in the rat than in either humans or guinea pigs (Epemolu et al., 1992, 1994; Singh et al., 1992; Porter et al., 1993, 1994; Alrefaie et al., 1995).

These studies demonstrate that metabolism is an important factor influencing the iron-scavenging efficacy of HPOs. Clearly, a comprehensive understanding of HPO metabolism is essential for the development of HPOs with clinical potential. As previous studies have been limited to CP94 metabolic profiles in the urine and blood, and as bile is a major pathway for iron excretion, we have determined the biliary metabolic profile of CP94 and its relationship with iron excretion.

Materials and Methods

Chemicals. CP94 and 1-(2'-hydroxyethyl)-2-ethyl-3-hydroxypyridin-4-one (CP102) were prepared using the method reported by Dobbin et al. (1993), whereas 1-ethyl-2-(1'-hydroxyethyl)-3-hydroxypyridin-4-one (CP365) was prepared according to the method described by Hider et al. (1998) (Fig. 1). β-Glucuronidase type IX-A (from Escherichia coli), thiglycolic acid sodium salt, and uridine 5'-diphosphoglucuronic acid (UDPGA, trisodium salt) were purchased from Sigma (Poole, Dorset, UK). EDTA, nitrilotriacetic acid, bathophenanthrolone disulfonic acid disodium salt hydrate, 2-mercaptoethanol, and Triton X-100 were obtained from Aldrich (Gillingham, Dorset, UK). Hypnorm, a mixture of fentanyl citrate and fluanisone, was produced by Janssen-Cilag Ltd. (High Wycombe, Buckinghamshire, UK). Hypnovel (midazolam)
was the product of F. Hoffmann-La Roche Limited (Grenzacherstrasse, Basel, Switzerland). Other reagents and solvents were either analytical grade or HPLC grade from various commercial sources.

**Rat Bile Duct Cannulation.** Male Wistar rats (180–200g) were purchased (local breed) from A. Tuck and Son (Battlesbridge, Essex SS1, UK) and housed in the Biological Service Unit, King’s College London. The animals were maintained at a temperature between 20–23°C, with food and water ad libitum. All animal experiments carried out have been specified in project license PPL 70/4561, which was authorized by the Secretary of State (England) under Animals Act 1986. The animals were fasted overnight before the start of experimentation but had free access to water. The animals were anesthetized with combined i.p. application of Hypnorm and Hypnovel, and anesthesia conditions were maintained during the entire period of experimentation using the same anesthetic. The cannulation of bile duct was undertaken followed by exteriorization of cannulas. Animals were treated with CP94 (dissolved in water, 450 μmol/kg of b.wt.) by gavage. Bile samples were collected hourly for 10 h. During the experimental period, animals were fed with glucose solution (0.5 ml/h, 40% v/w) and NaCl solution (0.5 ml/h, 0.9% w/v) alternately using a syringe feeder to avoid dehydration. Meanwhile, another rat group, which were not bile-cannulated, were orally administered with the same CP94 dose and located in metabolism cages to collect urine for 10 h.

**In Vitro Glucuronidation.** Wistar rats (male, 200-g) were fasted overnight before the start of experimentation but had access to water ad libitum. After the animals were sacrificed by cervical dislocation, livers were rapidly removed and immersed in ice-cold Tris-HCl buffer (100 mM, pH 7.4) (1 g of liver tissue was homogenized in Tris-HCl buffer (100 mM, pH7.4) using a glass homogenizer. The homogenate was centrifuged at 500 rpm for 10 min using a refrigerated centrifuge at 0 – 4°C. The supernatants were separated and filtered using syringe filters (0.2-
m pore size, 13-mm GD/X disposable Filter Device; Whatman) and directly injected onto the HPLC system for analysis. The incubations were complete, the incubates were added to trichloroacetic acid (TCA) (100 μl, 10% w/v in water), mixed, and centrifuged (3000 rpm for 10 min). The supernatants were separated and filtered using syringe filters (0.2-μm pore size, 13-mm GD/X disposable Filter Device; Whatman), and 30 μl of the filtrates were injected on an HPLC column for analysis.

**HPLC Method.** Bile and urine samples were diluted (1:4, v/v) in HPLC buffer, filtered using syringe filters (0.2-μm pore size, 13-mm GD/X disposable Filter Device; Whatman) and directly injected onto the HPLC system for analysis. The HPLC system (Hewlett Packard 1090 M Series) with an auto-injector, an autosampler, a diode-array detector, and a reversed phase polymer HPLC column (PLRP-S 100 A, 15 × 0.46 cm i.d., 5 μm; Polymer Laboratories Ltd, Shropshire, UK) was used in the study. The analyses were monitored at 285 nm.

A gradient mobile phase system (System 1) was applied for separation with a flow rate of 1 ml/min. The mobile phase A was acetonitrile (CH₃CN) and mobile phase B was phosphate buffer (10 mM, pH 2.9) containing 2 mM EDTA. The gradients were 0.01 to 9.00 min (98–84% of buffer B) and 9 to 22 min (84–76% of buffer B).

**Determination of Glucuronide.** To determine the glucuronidation metabolites, bile and urine samples were incubated in the absence (controls) or presence of β-glucuronidase in 60 mM MOPS buffer, pH 7.4 (the final concentration of 2000 U/ml) for 16 h at 37°C. After filtration, the unchanged chelators were determined as above and the difference between the control and the β-glucuronidase-treated sample was accounted as the amount of glucuronidated metabolite.

**Liquid Chromatography-Mass Spectrometry (LC-MS) Method.** Bile and urine samples after administration of CP94 were subjected to LC-MS analysis to further identify the major metabolites of CP94. The same HPLC polymer column was used for the separation, and the isotropic mobile phase (system 2) consisted of acetonitrile (5% v/v) and formic acid (0.05% v/v) in water with a flow rate of 0.3 ml/min. HPLC was performed with an ABI 140B gradient micro HPLC system (ABI, Warrington, UK) coupled to a Platform I mass spectrometer (Micromass, Manchester, UK) operating in positive ion electrospray mode.

**Determination of Iron.** The amount of iron excreted in bile and urine after oral administration of CP94 was determined using a colorimetric method developed by Gorrivatana et al. (1999). Briefly, bile samples were diluted 10 times with MOPS (5 mM, pH 7.4) before determination and 680 μl was added to 120 μl of 800 mM nitrolotriacetic acid and allowed to stand for 30 min at room temperature. The sample was then added to 100 μl of 120 mM thioglycolic acid and 100 μl of 60 mM bathophenanthroline disulfonic acid, disodium salt. The absorbance of each sample was measured at 537 nm after 30 min.

**Results and Discussion**

The use of the HPLC conditions described in this work leads to improved resolution of bile and urine hydroxypropyridone metabolites over those previously reported by Singh et al. (1992). The HPLC chromatogram of the rat bile shows six major components (Fig. 2B) compared with the control (Fig. 2A). Unchanged CP94, which was identified in bile using authentic CP94, was excreted only at a low level (Fig. 2B). Two peaks (metabolites A and C) were found to be quantitatively dominant in bile. To identify the glucuronide derived from CP94, bile samples were incubated in the presence/absence of β-glucuronidase at 37°C for 16 h. It was found that two peaks (metabolites A and C) disappeared after incubation in the presence of β-glucuronidase, while resulting in a concomitant increase in CP94 peak (Fig. 2D compared with Fig. 2B). The spectra of these two metabolites and CP94 are shown in Fig. 3A. Obviously, the structures of these metabolites are quite different as one (metabolite A) showed a slight red-shift, whereas the other (metabolite C) showed a violet-shift, as compared with CP94.

Singh et al. (1992) have reported that CP365, an iron-chelatable HPO, was identified as a major metabolite of CP94 in urine by using fast atom bombardment-mass spectrometry and H1-NMR. As authentic CP365 was available in this study, this hydroxylated metabolite was assigned to metabolite B in bile on the basis of HPLC retention time and spectral analyses (Fig. 2, B and H). Figure 3C compares the spectra of metabolically formed CP365 and an authentic synthetic sample. This finding is important because CP365 has been recently reported to be a promising high-efficacy iron chelator (Hider et al., 1998; Liu et al., 1999). The finding that CP365 presented in bile at a relatively high level as compared with unchanged CP94 (Fig. 2B) probably accounts for the powerful iron-scavenging ability of CP94 in rats.

Metabolite D is a major metabolite of CP94 which is present in bile (Fig. 2B), but not in urine (Fig. 2F). This metabolite was found to disappear after incubation at 37°C for 16 h (Fig. 2C). Metabolite D is not a glucuronide, however, as it disappears in the absence of β-glucuronidase. Further investigation of metabolite D was undertaken by collecting the bile samples and processing them under nitrogen to minimize possible autoxidation. It was found that metabolite D re-

![Fig. 1. Structure of HPOs.](https://example.com/fig1.png)
Fig. 2. HPLC chromatograms of bile (A–D) and urine samples (E–H).

A, control bile; B, bile after oral administration of CP94; C, bile sample incubated at 37°C for 16 h in the absence of β-glucuronidase; D, bile sample incubated with β-glucuronidase at 37°C for 16 h; E, control urine; F, urine sample after oral administration of CP94; G, urine sample incubated with β-glucuronidase at 37°C for 16 h; and H, authentic CP102, CP365, and CP94.
Fig. 3. Spectral characteristics of CP94 and its metabolites.

mained detectable after incubation at 37°C for 16 h under nitrogen even in the presence of β-glucuronidase (Fig. 2D). Clearly, this metabolite is oxygen-sensitive. The spectroscopic properties of metabolite D are consistent with the incorporation of an electron-releasing species such as a hydroxyl group as a ring substituent as the spectra shows a red-shift (Fig. 3B). Additional investigations centered on this unstable metabolite are currently in progress.

The HPLC chromatogram of a urine sample collected after oral administration of CP94 shows four major peaks compared with a control urine sample (Fig. 2F compared with Fig. 2E). As with the
biliary profile of CP94, there was only a minor amount of unchanged CP94 detected in urine (Fig. 2F). Again, by analogy to the earlier report by Singh et al. (1992), CP365 was found to be a major metabolite of CP94 in urine. The possibility of ω-hydroxylation of the 1-ethyl substituent was also considered as a possible metabolic route, but the synthetic CP102 possessed a different HPLC retention time and spectrum to any of the observed metabolites (Fig. 2H). Metabolites A and C, the glucuronides, were also found to be major elimination forms of CP94 in the urine (Fig. 2G).

To further characterize the metabolites of CP94, both bile and urine samples were analyzed using a LC-MS method. Because the mobile phase for the conventional HPLC method, which contains EDTA and phosphate buffer, is unsuitable for LC-MS, a formic acid solution was adopted. Although the retention times of the analytes in bile and urine samples differ from those obtained with system 1, the elution sequence was found to be the same.

By monitoring selected ions, it was established that the two metabolites (A and C) in both bile and urine possess identical molecular ion weight (\((M+H)^+ = 344\)) (Fig. 4) and the same mass spectral pattern with those of the CP94 glucuronide. In addition to the molecular ion \((M+H)^+ = 344\), the major mass fragment \((M+H)^+ = 168\) derived from metabolites A and C corresponds with \((CP94+H)^+\). These data clearly show that metabolites A and C are both the glucuronidation products of CP94. When CP94 was incubated in an in vitro glucuronidation system with rat liver homogenate, two metabolites that possessed the same HPLC retention times to those of metabolites A and C (Fig. 5) formed. The conclusion that metabolites A and C are both conjugated metabolites of CP94 is additionally supported by the fact that these metabolites are only formed from CP94 in vitro in the presence of UDPGA, the cofactor for glucuronidation. One of these metabolites is certainly the 3-O-glucuronide, whereas the other is likely to be the 4-O-glucuronide, a novel compound.

Figure 4 also shows that metabolite B possesses the same mass, 184 Da, and an identical mass spectrum to that of the authentic compound CP365. Furthermore, a minor metabolite (metabolite E) was also present in bile as shown by LC-MS selective ion monitoring (Fig. 4). This may be a conjugated metabolite of a hydroxylated compound, as metabolite E possesses a molecular ion \((M+H)^+ = 360\). This metabolite is not detected in the urine and its precise structure requires

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Glucuronides</th>
<th>Unchanged CP94</th>
<th>Iron</th>
<th>Unchanged CP94/Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1901±356</td>
<td>132±77</td>
<td>123±98</td>
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<td>213±41</td>
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<td>2205±424</td>
<td>119±38</td>
<td>106±27</td>
<td>1.12</td>
</tr>
</tbody>
</table>

**TABLE 1**

*Amounts of the CP94 glucuronides, unchanged CP94, and iron excreted in bile after oral administration of CP94 (450 μmol/kg b.wt.)*

Values are mean ± S.D., n = 3.
additional elucidation. Unfortunately, metabolite D was not detected using LC-MS analysis, probably due to its instability under the conditions necessary for analysis.

In an attempt to relate the metabolic profile of CP94 with the efficacy of CP94 at promoting iron excretion, the amounts of the unchanged CP94, CP94 metabolites, and iron excreted via bile and urine were quantified after oral administration of CP94 (450 μmol/kg of b.wt.) to normal rats. During a 10-h period, only a small amount of CP94 (4.7 ± 1.1 μmol, mean ± S.D.) was found to be excreted in the bile, the majority of CP94 being eliminated from bile as glucuronidated metabolites (34.3 ± 2.9 μmol, mean ± S.D.) (Table 1). These figures correspond to 5.2 and 38.1% of the administered CP94 dose (n = 3). The excretion of iron in bile showed a parallel time distribution to the excretion of unchanged CP94 but in the ligand/iron molar ratio range of 0.9 to 1.2 (Table 1). As the iron is likely to be excreted as a 3:1 ligand/iron complex (Tilbrook and Hider, 1998), this range of values implies that the active metabolites of CP94, especially CP365, play an important role in iron excretion.

In urine, 4.8 ± 0.6 μmol (mean ± S.D.) of CP94 were found, whereas 25.1 ± 0.4 μmol (mean ± S.D.) of CP94 were excreted as glucuronides, which account for about 5.3 and 27.9% of the administered CP94 dose. In these experiments, which involve normal animals, iron was virtually undetectable in urine.

The metabolic pathways of CP94 in the rat are summarized in Fig. 6. Although it is well known that glucuronidation is one of the major pathways for CP94 metabolism leading to the loss of chelating capacity and the reduction of the efficacy, the production of two iso-

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