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HPLC determination of 1,2-diethyl-3-hydroxypyridin-4-one (CP94), its iron complex [Fe(III) $(CP94)_3$] and glucuronide conjugate [CP94-GLUC] in serum and urine of thalassaemic patients

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Abstract: Sensitive and selective high performance liquid chromatographic (HPLC) methods for the quantification of 1,2-diethyl-3-hydroxypyridin-4-one (CP94), its iron complex [Fe(III) $(CP94)_3$] and glucuronide metabolite (CP94-GLUC) in urine and serum of thalassaemic patients are described. Three separate analyses are involved. The first assay quantifies both CP94 and its iron complex. This procedure requires the conversion of the iron complex to the free ligand and is carried out using diethylenetriaminepentaacetic acid (DTPA). CP94 and the internal standard, 1-propyl-2-ethyl-3-hydroxypyridin-4-one (CP95) present in either serum or urine are then extracted at pH 7.0 with dichloromethane. Extraction efficiency is 96.0 ± 5.6% and 100 ± 7.1% for CP94 and CP95, respectively, and 31.2 ± 2.1% at 30 μM and 53.2 ± 4.2% at 300 μM for the corresponding iron complex. In the second assay, samples are incubated (16 h) with β-glucuronidase and processed as before. In this assay, the drug, its iron complex and glucuronide conjugate are measured. In the third assay the iron complex of CP94, [Fe(III) $(CP94)_3$] is quantified. From the three separate analyses it is possible to calculate the individual concentrations of the three separate components present in serum and urine of thalassaemic patients. Calibration for both components, i.e. CP94 (assays 1 and 2) and its iron complex (assay 3) are linear with correlation coefficients >0.99 and are reproducible over the required concentration range of 0–500 μM for the free ligand and 0–100 μM for the iron complex. The minimum quantifiable level is 0.5 μM for the free ligand and 1.0 μM for the iron complex.

Keywords: *Iron chelator; thalassaemia; analysis; 3-hydroxypyridin.*

Introduction

Patients who suffer from haemoglobinopathic disorders such as β-thalassaemia major are heavily iron-overloaded due to their dependency on frequent blood transfusions for survival [1–2]. This arises largely due to the inability of man to excrete appreciable quantities of iron. If not treated by chelation therapy, the iron levels gradually build up to levels which are toxic to organs such as the liver, heart and adrenal glands, eventually leading to death. The only widely used drug available to treat this disorder is desferrioxamine. Desferrioxamine, although a relatively safe and effective drug, when used with care, is only poorly absorbed by the oral route and is therefore administered parenterally. This inevitably leads to poor patient compliance. Over the past 20 years a wide range of compounds including catecholates, aminocarboxylates, substituted phenolates and hydroxypyridinones have been investigated as orally active iron-chelating alternatives to desferrioxamine. Many of these compounds have been rejected due to insufficient oral activity, poor iron mobilisation or unacceptable toxicity. However, the 3-hydroxypyridin-4-ones (Fig. 1) are currently one of the main candidates for the development of an orally active alternative to desferrioxamine [3].

In order to study the pharmacokinetics and

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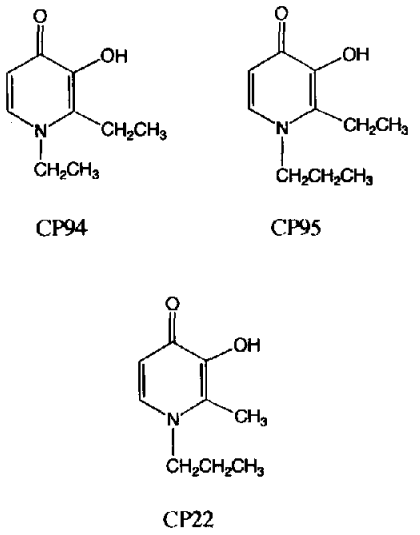

Figure 1
Chemical structures of CP94, CP95 (internal standard) and CP22.

metabolism of the 3-hydroxypyridin-4-ones in iron overloaded patients, methods for analysis of the drug, both as free ligand, iron complex and metabolites are required. One of the main problems associated with these analytical techniques is the inherent ability of chelating agents to bind iron impurities present in most chromatographic systems. The 3-hydroxypyridin-4-ones also can interact extensively with the silanol groups on silica based reversed-phase columns. Methods designed to overcome the above difficulties previously have been described in detail [4]. In addition, it is important to ensure sample integrity and minimal *ex-vivo* interconversion during sample preparation and analysis. With bidentate ligands including the 3-hydroxypyridin-4-ones, this is a critical problem due to the inherent kinetic lability of their corresponding iron complexes [5]. These problems are not restricted to the hydroxypyridinones and also have been reported for other chelating agents such as disulphiram [6].

A method for quantifying CP94 in the rat has previously been reported by the authors [7]. This method was specifically developed to study the pharmacokinetics of CP94 in non-iron overloaded rodents, where negligible amounts of the corresponding iron complex is present due to incompletely saturated transferrin. In contrast, the present study is concerned with iron overloaded thalassaemic patients with fully saturated transferrin and therefore, requires a method which is capable of quantifying both the free ligand and the iron complex. Due to the rapid and extensive glucuronidation of CP94 by man [8], it is also important to measure the glucuronide conjugate levels. In order to allow determination of the individual components present in serum and urine, it was necessary to develop three separate assays. The first assay determines the free ligand and iron complex ($[CP94]_t$), the second measures total drug including the glucuronide conjugate ($[CP94]_T$), and finally the third assay measures the iron complex in serum of thalassaemic patients.

Materials and Methods

The 3-hydroxypyridin-4-ones were synthesized as previously described [9]. Morpholinopropanesulphonic acid (MOPS) buffer (sodium salt), diethylenetriaminepentaacetic acid (DTPA) and β-glucuronidase (Type 1X-A from *E. coli*) were purchased from Sigma Chemical Company (Poole, Dorset, UK). EDTA (trisodium salt, Convol) was obtained from BDH (Poole, Dorset, UK). All the solvents and reagents used were either of HPLC or analytical grade and were purchased from Fisons Scientific Apparatus Ltd. (FSA, Loughborough, Leics, UK).

The pellicular guard column packing material (Co-Pell ODS) was purchased from Whatman International Ltd (Maidstone, Kent, UK). The Hypercarb PGC column (10 × 0.46 cm i.d.) packed porous graphitized carbon was purchased from Shandon Scientific Ltd (Runcorn, Cheshire, UK) and the Chrom-Sphere, 3-ODS glass column (10 × 0.3 cm i.d.) was from Chrompak (Holland). The MOPS buffer (60 mM) was prepared by dissolving the appropriate amount in 100 ml distilled water; the pH was adjusted to 7.0 with 1 M NaOH. The NaH_2PO_4 solution (10 mM) was prepared by dissolving the appropriate weight of reagent in 1 l of water; 2 ml of 1 M EDTA Tris–sodium was added and the pH adjusted to 3.0 with orthophosphoric acid, before being made up to volume. Similarly, the phosphate buffer (5 mM) for the assay of the iron complex [Fe(III) $(CP94)_3$] was prepared by dissolving the appropriate weight in water, the required amount of CP22 (2 mM) added and pH adjusted to 7.0 with NaOH before making up to 1.0 l with water. All buffers were prepared using milli-Q water.

Preparation of standard solutions

Standard solutions of CP94 (6 mM) and the internal standard (CP95, 1-propyl-2-ethyl-3-hydroxypyridin-4-one, 6 mM) were prepared in Milli-Q water and 50 mM DTPA, respectively. Standard solutions containing 1 mM $Fe(III)(CP94)_3$ were prepared by weighing 3.05 mg of CP94-HCl, adding 50 μl of 100 mM $FeCl_3$ solution in 0.01 M HCl and the pH adjusted to 7.0 with NaOH before making up to 5.0 ml in a volumetric flask with water. This solution gave 1:3 complex of iron to ligand. These solutions were stored at 4°C for use in subsequent procedures such as extractability studies and preparation of calibration curves. All samples were stable for several months under these storage conditions.

Preparation of transferrin saturated serum

From a stock solution of atomic absorption standard iron (1020 μg Fe per ml) a 33 μl aliquot was spiked into 10.0 ml of normal serum. The addition of excess iron (60 μM) leads to saturation of transferrin. The total iron binding capacity (TIBC) of transferrin is approximately 60 μM in normal individuals and is only approximately 30% saturated with iron. Saturation of transferrin is essential to ensure that iron bound to CP94 is not donated to apo-transferrin. This iron saturated serum was used for constructing the serum calibration curves.

Preparation of β-glucuronidase solutions

An appropriate amount of the lyophilized enzyme preparation was dissolved in MOPS buffer (pH 7.0, 60 mM) to give a concentration of 10,000 U ml^{-1}. Final concentrations of 500 U ml^{-1} and 2000 U ml^{-1} were employed for the serum and urine analyses, respectively. These concentrations of β-glucuronidase were used to account for differing amounts of the CP94 glucuronide conjugate present in serum and urine and to ensure complete hydrolysis to the parent drug.

Extractability of compounds from serum

The extractabilities of CP94 and the internal standard were assessed by spiking 20 μg of each compound into tubes containing 1.0 ml of urine or iron saturated serum and 0.5 ml of MOPS buffer (60 mM, pH 7.0). This was followed by extraction with 1 × 10 ml of dichloromethane (DCM). The organic extracts were evaporated to dryness in a water bath at 40°C and the residues reconstituted in 1.5 ml of MOPS. Aliquots (20 μl) of these samples were injected onto the HPLC column. The extractabilities were calculated by comparing peak areas obtained for 10 ml DCM samples directly spiked with 20 μg of each component and processed as above. This procedure also was repeated in order to estimate the extractability of $Fe(III)(CP94)_3$.

Assay of 1,2-diethyl-3-hydroxypyridin-4-one, the corresponding glucuronide conjugate and iron complex in the serum and urine of thalassaemic patients

To 1 ml of serum was added 0.5 ml of 60 mM MOPS buffer pH 7.0 and 50 μg (300 μM) of the internal standard, (CP95, in 50 mM DTPA), the samples were extracted with 1 × 10 ml dichloromethane (DCM), evaporated to dryness at 40°C, reconstituted in the mobile phase (100 μl) and analysed by HPLC-system 1. The first assay (Assay 1) measures both free CP94 and its corresponding iron complex ($[CP94]_t$). To a second set of serum samples (1.0 ml) was added 0.5 ml of β-glucuronidase solution (1500 U ml^{-1}) and incubated at 37°C for 16 h. These samples were then processed as for Assay 1. Assay 2 measures free CP94, the iron complex and glucuronide conjugate ($[CP94]_T$). The difference in the levels of CP94 in the presence (Assay 2) and absence of β-glucuronidase (Assay 1) corresponds to the concentration of CP94 glucuronide conjugate. For urine samples, 0.25 ml aliquots of urine were added to 0.75 ml aliquots of MOPS buffer in the presence and absence of β-glucuronidase. 50 μl (300 μM) of the internal standard was added and the samples processed as described for the serum samples. Assay 3 was developed for the quantification of the iron-complex; 1.0 ml samples of serum were extracted with 11.0 ml of DCM. Exactly 8.0 ml of the DCM layer was then evaporated to dryness, reconstituted with 100 μl of 5 mM phosphate buffer pH 7.0 and analysed by HPLC-system 2. The same procedure was employed for the analysis of urine samples except that 0.25 ml of phosphate buffer and 0.75 ml of urine were used. The phosphate buffer (5 mM, pH 7.0) was included to maintain the urine pH at 7.0.

An internal standard was not included in the iron complex assay, because of rapid ligand exchange which occurs both in solution and on the chromatographic column due to the kinetic

lability of the bidentate hydroxypyridin-4-ones iron complexes. The addition of an internal standard would lead to the formation of mixed complexes, indistinguishable from those already present in the serum.

Preparation of calibration standards

In order to facilitate the accurate measurement of CP94 during the latter phase post-drug administration, when drug levels are expected to be low, it was necessary to construct both high- and low-level calibration curves. The low calibration for the late time points ranged from 0 to 10 μM and the high calibration curve relevant to the early time points ranged from 10 to 600 μM. Calibration curves were constructed by spiking known amounts of CP94 into extraction tubes as previously described. The internal standard (50 μl of 6 mM CP95 in 50 mM DTPA solution) was added to each tube before extraction and subsequent analysis as described above for Assays 1 and 2.

Known amounts (0–50 μg ml^{-1}) of Fe(III) $(CP94)_3$ were spiked into tubes containing 1.0 ml of iron saturated serum. These samples were then extracted with exactly 11.0 ml of DCM and 8.0 ml extracts processed as described above for Assay 3.

The urine calibrations were set up in a similar manner to that of the serum.

Isolation of partially purified CP94-glucuronide from human urine

CP94-glucuronide was isolated from human urine. The procedure employed capitalizes on the poor extractability of the glucuronide conjugate into DCM in contrast to the parent compound which has an extraction efficiency of 96%. Excess DCM (10:1) was added to 24 h urine sample in a separating funnel. The DCM layer, which contained CP94, was decanted off, leaving the glucuronide conjugate in the aqueous layer. This process was repeated three times to ensure complete extraction. The purity of the isolated glucuronide was confirmed by HPLC and enzyme hydrolysis using β-glucuronidase.

Chromatography

The HPLC system was from LDC Ltd (Stones, Staffordshire, UK) and consisted of a Consta Metric 3000 pump, a SpectroMonitor model 3100 variable wavelength UV detector and a model CI-4000 integrator. Samples were introduced *via* a Rheodyne injector fitted with a 20 μl sample loop.

HPLC of CP94 — System 1. HPLC was carried out on a Hypercarb, PGC column (10 × 0.46 cm i.d.) with a precolumn (5 × 0.25 cm i.d.) packed with pellicular ODS. The mobile phase consisted of 14:86 (% v/v) acetonitrile: NaH_2PO_4 buffer (10 mM, containing 2 mM EDTA, pH 3.0 adjusted with phosphoric acid). The eluent was monitored at 280 nm and the flow rate was 1.0 ml min^{-1}.

HPLC of Fe(III) $(CP94)_3$ — System 2. The HPLC of the iron complex was performed using Chrom-Sphere 3 ODS, (10 × 0.3 cm i.d.) (Chrompack, Holland). The mobile phase was 20:80 (% v/v) acetonitrile: phosphate buffer (5 mM), containing 3 mM of 1-propyl-2-methyl-3-hydroxypyridin-4-one (CP22), pH adjusted to 7.0 with NaOH. The eluent was monitored at 450 nm at a flow rate of 1.0 ml min^{-1} [5].

Calculation of results

Assay 1. Measures both the free ligand (CP94) and the corresponding iron complex {Fe(III)$(CP94)_3$}. $[CP94]_t$ can be expressed as in equation (1).

$$[CP94]_t = [CP94] + 3.[Fe(III)(CP94)_3]. \quad (1)$$

Assay 2. Measures the total drug recovered in the urine and can be expressed as in equation (2).

$$[CP94]_T = [CP94] + 3\,[Fe(III)(CP94)_3] + [CP94\text{-}GLUC] \quad (2)$$

Assay 3. Measures Fe(III)$(CP94)_3$. Therefore,

$$[CP94] = [CP94]_t - 3.\,[Fe(III)(CP94)_3] \quad (3)$$

and

$$[CP94\text{-}GLUC] = [CP94]_T - [CP94]_t \quad (4)$$

Assay validation

The accuracy and precision of the assay was determined by spiking both low and high concentrations of CP94, Fe(III) $(CP94)_3$ and CP94-GLUC into extraction tubes containing 1.0 ml of serum and either 0.5 ml of MOPS or MOPS with β-glucuronidase as stated pre-

Table 1
Precision and accuracy of the HPLC method for the quantitation of 1,2-diethyl-3-hydroxypyridin-4-one, its iron complex and its glucuronide conjugate in thalassaemic serum

	(Amount spiked)			Before deconjugation			After deconjugation		
	CP94 (μM)	Fe(III)-(CP94)$_3$ (μM)	CP94-Gluc (μM)	Expected	Observed	RSD	Expected	Observed	RSD
4	166.04	34.8	120.8	201.2	201.2 ± 1.3	0.64	322.0	324.6 ± 2.4	0.74
4	0	198.3	11.3	198.3	186.9 ± 0.5	0.27	209.6	207.2 ± 1.8	0.87
4	15.2	198.2	5.4	213.2	13.4 ± 0.5	0.25	218.8	213.8 ± 1.2	0.56

*During the sample preparation, the spiked iron complex dissociates to yield three equivalents of CP94, i.e. 11.6 μM [Fe(III)-(CP94)$_3$] gives 34.8 μM CP94 free ligand.

viously. The study was performed on four separate occasions. The validation also was carried out for each component separately. Assay precision and accuracy were indicated by the relative standard deviation (RSD) and mean percentage difference (M%D), respectively. The minimum quantifiable level (MQL) was taken as the concentration affording a signal three times above that of the background signal [10]. The urine validation had a RSD and M%D of less than 2%, respectively (Table 1).

Storage and stability

The stability of CP94 in serum and urine was investigated by spiking known amounts of CP94, CP94-Gluc and {Fe(III)$(CP94)_3$} into serum and urine. Samples were stored at room temperature for 8.0 h and at −20°C for periods up to 2 months. Samples were spiked with internal standard prior to analysis and processed as previously described.

Results and Discussion

The analysis of bidentate chelating agents, unlike hexadentate ligands, is complicated by the fact that the former have a tendency to dissociate on chromatographic columns [4]. This, unlike the situation with hexadentate ligands, prevents simple analysis of both the free ligand and that of the intact bidentate metal–chelate complex.

In order to allow accurate and reliable quantification of the free ligand and iron complex of bidentate ligands ($[CP94]_t$), quantitative dissociation of the metal chelate is required prior to analysis. This can be achieved by the addition of a large excess of a high affinity ligand such as DTPA. Analysis of metabolites such as glucuronide conjugates can also be carried out as above, by pre-incubating serum samples with a sufficient excess of β-glucuronidase to ensure complete hydrolysis to reform the parent drug. Differences between the two analyses, in the presence ($[CP94]_T$) and the absence of β-glucuronidase ($[CP94]_t$) accounts for the glucuronide conjugate levels present in serum/urine (equation 4).

Direct analysis of iron complexes of bidentate compounds can be carried out under certain conditions. The dissociation of the iron complex can be prevented by the addition of excess ligand in the mobile phase [5]. The presence of large excess ligand in the mobile phase allows rapid ligand exchange to take place such that the iron complex elutes intact from the analytical column, indistinguishable from the original iron-complex injected.

The HPLC chromatograms of CP94 and the internal standard, CP95 (Fig. 2) show single symmetrical peaks with complete baseline resolution between components. The retention times of CP94 and the internal standard CP95 were 8 and 15 minutes, respectively. HPLC analysis of DCM extracts of control serum (Fig. 2(a)) revealed the absence of co-eluting peaks at the retention times of the compounds of interest, indicating that DCM was a suitable solvent for the extraction of these compounds. The extraction efficiencies of CP94 and the internal standard were 96.0 ± 5.6% and 100.0 ± 7.1%, respectively under the assay conditions described above, the calibration curves were linear over the concentration ranges 0–10 and 10–600 μM and correlation

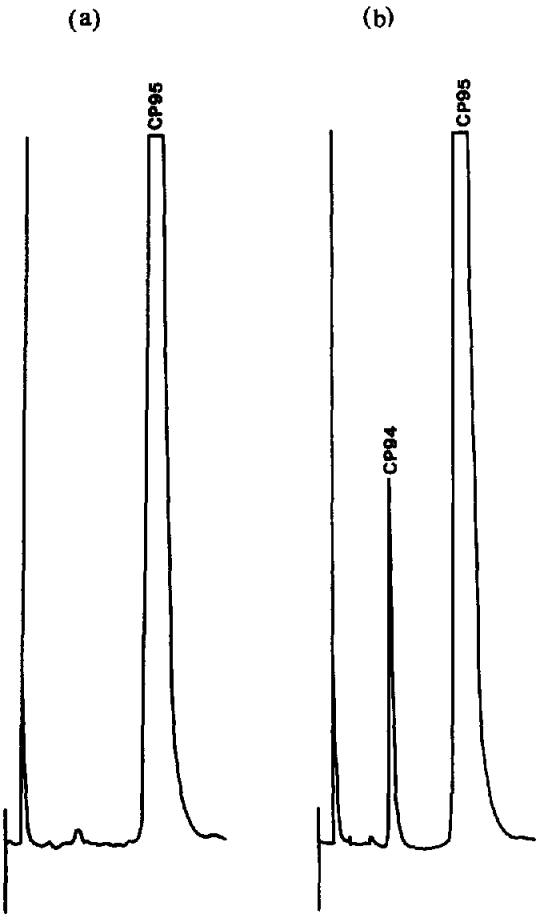

Figure 2
Typical HPLC chromatogram of (a) blank serum and (b) post CP94 (Rt = 8 min) and CP95, the internal standard (Rt = 15 min).

Table 2
Calibration values for low and high CP94 calibration in human plasma

CP94 (μM)	Peak area ratio
Low concentration range 0–12 μM	
0	0.0
0.3	0.07 ± 0.02
0.6	0.11 ± 0.02
3.0	0.47 ± 0.04
6.0	0.91 ± 0.06
10.0	1.61 ± 0.01
12.0	1.92 ± 0.19
High concentration range 10–600 μM	
0	0.000
10	0.038
18	0.067
30	0.110
60	0.220
150	0.560
300	1.097
600	2.035

coefficient values for both the low and high calibration plots were greater than 0.99 (Table 2). The extraction efficiency of the iron complex was 53.2 ± 4.2% at 300 μM and 31.2 ± 2.1% at 5 μM in serum. This was due to the increased binding of the iron complex to plasma protein at low concentrations. This problem was overcome by constructing the calibration curves for the low (<5 μM) and high (between 6–300 μM) concentrations of CP94. The calibration was linear over the range of 0–300 μM with a correlation coefficient >0.99. To accurately quantify $[CP94]_t$ and (free ligand and iron complex) in assay 1, the use of the high affinity hexadentate chelator DTPA was required to fully dissociate the hydroxypyridinone–iron complex to its free ligand. The assay precision (Table 1) as indicated by the RSDs and the accuracy as shown by the M%D were both lower than 1% for each of the spiked concentrations studied. The minimum quantifiable level (MQL) for CP94 assay was 0.5 μM and for the iron-complex it was 1 μM.

To ensure complete hydrolysis of the glucuronide conjugate, varying amounts of enzyme were used. The minimum concentrations which resulted in hydrolysis within the incubation period were 500 U ml^{-1} and 2000 U ml^{-1} for serum and urine, respectively. Inter-batch variation in enzyme preparation was monitored by using a solution with known concentration of CP94-glucuronide.

For the iron-complex calibrations, oversaturated transferrin was used in order to prevent donation of iron to transferrin. Oversaturation did not affect the calibration of $\{Fe(III)(CP94)_3\}$, as inorganic iron is not extractable. An internal standard was not included in this assay due to the possibility of forming mixed iron-complexes, which are not distinguishable from the iron complex originally present. The chromatogram of the iron complex in serum is shown in Fig. 3. The stability data (not shown) indicated that the concentration of the free ligand, the complex and the glucuronide showed no significant change during the storage period of 2 months at −20°C.

In conclusion a sensitive and selective assay has been developed for the quantitation of CP94, its glucuronide and iron complex in thalassaemic patient serum. The method developed is highly reproducible with minimal inter- and intra-assay variation (<5%). Currently the procedure is being used to study the pharmacokinetics of CP94 in the serum of thalassaemic patients.

Figure 4 shows a representative serum level versus time profile for each of the three

Figure 3
Representative HPLC chromatogram of CP94 iron-complex (RT = 3 min) in serum.

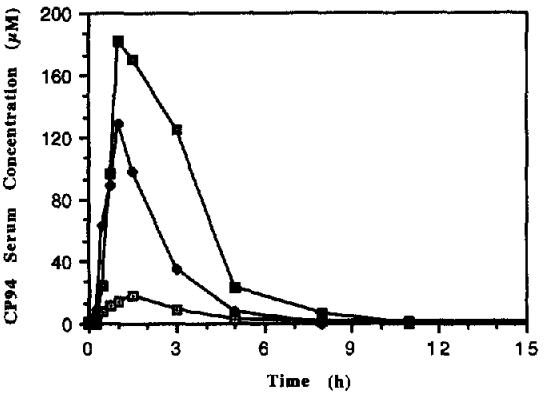

Figure 4
Representative serum level versus time profiles for CP94 (◆), CP94-glucuronide (■) and CP94-iron complex (□) after the administration of CP94 (50 mg kg^{-1}).

components found in thalassaemic patients. Preliminary data gathered to date suggests that CP94 has a serum half-life of 1.32 ± 0.28 h, clearance of 1.24 ± 0.20 $l.h^{-1}$ kg^{-1} and a t_{max} of 0.75 ± 0.15 h. The urinary recovery of CP94 as the free ligand varied from between 0 and 9% whilst that of the iron complex varied between 0.4 and 10% of the administered dose. The majority of drug recovered in the urine was as the glucuronide conjugate which accounted for between 40 and 95% of the dose. The large variability in urinary recovery is probably related to both the drug metabolizing capacity and plasma iron turnover in the individual patient.

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