ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 759–765

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Liquid extraction and ion-pair HPLC for determination of hydrophilic 3-hydroxypyridin-4-one iron chelators

Ding Y. Liu, Zu D. Liu, Shu L. Lu, Robert C. Hider *

Department of Pharmacy, King's College London, University of London, Manresa Road, Chelsea, London SW3 6LX, UK

Received 20 January 1999; received in revised form 21 June 1999; accepted 28 June 1999

Abstract

Hydrophilic 3-hydroxypyridin-4-ones (HPOs), such as 1-(2′-carboxyethyl)-2-methyl-3-hydroxypyridin-4-one (CP38), 1-(3′-hydroxypropyl)-2-methyl-3-hydroxypyridin-4-one (CP41) and 1-(2′-hydroxyethyl)-2-ethyl-3-hydroxypyridin-4-one (CP102), are orally active iron chelators and ester prodrugs of these molecules are currently under investigation. A liquid extraction method using acetonitrile and 2-propanol (80:20 v/v) under acidic and NaCl-saturated conditions has been developed in order to efficiently extract these HPOs from various matrices. The extracted HPOs were determined using a reversed phase polymer HPLC column (PLRP-S 100 Å) and the gradient ion-pair mobile phase containing tetrabutylammonium chloride (5 mM) and EDTA (0.5 mM). The extraction recovery of these chelators in phosphate buffer, rat blood and liver homogenate varied from 85 to 94%. The coefficients of variation (C.V.) for within-day determination were in the range of 1.4–3.3% at 1 mM and 2.0–4.7% at 0.1 mM. High accuracy of determination was also achieved.

Keywords: Liquid extraction; Ion-pair HPLC; Iron chelator; 3-Hydroxypyridin-4-ones; Ester prodrug

1. Introduction

The clear benefit of an orally active, non-toxic, selective iron chelator for transfusion-dependent patients, such as those suffering from β-thalassaemia, has stimulated research effort into the design of new iron chelators during the past two decades [1–4]. Amongst the main candidates of the orally active iron chelators, 3-hydroxypyridin-4-ones (HPOs) have been extensively investigated both in iron overloaded animal models and in thalassaemic patients [5]. Several HPO ligands have shown significant enhancement of excretion of iron via both the urinary and biliary routes

Abbreviations: HPOs, 3-hydroxypyridin-4-ones; CP20, 1,2-dimethyl-3-hydroxypirydin-4-one; CP38, 1-(2′-carboxyethyl)-2-methyl-3-hydroxypyridin-4-one; CP41, 1-(3′-hydroxypropyl)-2-methyl-3-hydroxypyridin-4-one; CP102, 1-(2′-hydroxyethyl)-2-ethyl-3-hydroxypyridin-4-one; CP94, 1,2-diethyl-3-hydroxypyridin-4-one; CP179, phenethyl 3-[2-methyl-3-hydroxypyridin-4(1H)-one-1-yl]-propionate; CP183, 2-[2-ethyl-3-hydroxypyridin-4(1H)-one-1-yl]-ethyl benzoate; CP283, 3-[2-methyl-3-hydroxypyridin-4(1H)-one-1-yl]-propyl benzoate.

* Corresponding author. Tel.: +44-207-848-4646/4882; fax: +44-207-8484195.

E-mail address: robert.hider@kcl.ac.uk (R.C. Hider)

0731-7085/99/$ - see front matter
PII: S0731-7085(99)00211-3

such as 1-(3′-hydroxypropyl)-2-methyl-3-hydroxypyridin-4-one (CP41) and 1-(3′-hydroxyethyl)-2-ethyl-3-hydroxypyridin-4-one (CP102) [6,7]. These candidate chelators possess superior iron scavenging properties to other closely associated HPOs (e.g. CP20 and CP94) as they do not undergo extensive conjugative metabolism which leads to the inactivation of the chelators [8–11]. However, the clinical potential of these chelators is shadowed by their relatively high polarity which is the main reason responsible for their poor oral absorption and insufficient extraction by the liver (the major iron storage organ) [12]. Therefore, an ester prodrug strategy of these chelators is currently under investigation, in order to improve both their gastrointestinal absorption and liver extraction [13,14].

The extraction of highly hydrophilic compounds, such as CP38 which possesses a low distribution coefficient ($D_{7.4} < 0.001$), from aqueous media presents an analytical challenge. Previously a methyl esterification method was developed by Singh et al. [10] in order to extract and analyse CP38 in biological samples. Unfortunately, the method is unsatisfactory, not only because of lack of efficiency for sample preparation, but also because of the marked analytical variation introduced by the derivative method. In the present study, a simple and reproducible extraction method is described, which is potentially useful for the extraction of most known HPO iron chelators. The application of this extraction method to the study of the hydrolysis and metabolism of ester prodrugs of CP38, CP41 and CP102 is reported.

2. Materials and methods

2.1. Chemicals

CP38, CP41 and CP102 were prepared using the methodology reported by Dobbin et al. [15]. The ester prodrugs (CP179, CP183 and CP283) were prepared using the method described by Liu et al. [13] and Rai et al. [14]. The chemical struc-

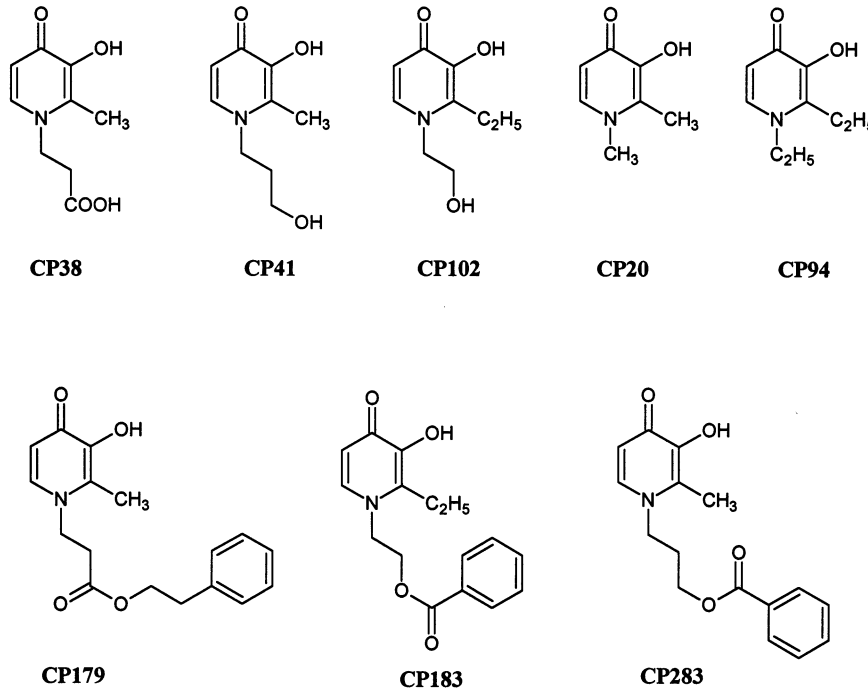

Fig. 1. Structures of some 3-hydroxypyridin-4-ones and ester prodrugs.

tures of these compounds are shown in Fig. 1. Tetrabutylammonium chloride and EDTA were the products of Aldrich (Gillingham, Dorset, UK).

2.2. HPLC system

A Hewlett-Packard model 1090M Series HPLC system with 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 μm, Polymer Laboratories, Church Stretton, Shropshire, UK) was used in the present study. The gradient ion-pair mobile phase system (2–10% MeCN in 9 min, 10–35% MeCN from 9 to 10 min and hold to 15 or 20 min) contains tetrabutylammonium chloride (5 mM) and EDTA (0.5 mM). CP102 was used as the internal standard for determination of CP38 and CP41, whilst CP41 was used as the internal standard for determination of CP102. The flow rate was 1 ml/min and the analytes were monitored at 285 nm.

2.3. Determination of the extraction recovery

Stock solutions containing CP38, CP41 and CP102 (1 or 0.1 μmol of each compound in 100 μl water) were added to water (1 ml), rat blood (0.5 ml blood and 0.5 ml phosphate buffer, pH 7.4, 0.139 M) or rat liver homogenate (1 ml, containing 2 mg fresh liver tissue per ml, see below for detail), followed by the addition of the internal standard solution (0.4 μmol in 100 μl water), HCl solution (100 μl, 2 M), solid sodium chloride (0.5 g per sample) and extraction solvent (5 ml, acetonitrile/2-propanol = 80:20 v/v). The samples were then mixed for 15 min and centrifuged at 3000 rev./min for 10 min. The upper organic layer was separated and evaporated to dryness under a nitrogen stream at 50°C. The residues were reconstituted in 0.3 ml of acetonitrile solution (30% v/v in water) and 30 μl injected into the HPLC system for determination. The peak areas were compared with those non-extracted samples in order to calculate the extraction recovery. In addition, the within-day coefficient of variation (C.V.) and the accuracy of the determination were also determined.

2.4. In vivo metabolism of the CP41 ester: CP283

Wistar rats (200 g, male) were anaesthetised by the combined i.p. application of Hypnorm® (a mixture of fentanyl citrate and fluanisone) and Hypnovel® (midazolam). Cannulation of the bile duct was followed by exteriorisation of cannulae. After CP283 was administered (450 μmol/kg body wt) by gavage, bile samples were collected hourly over an 8-h period. CP102 solution (IS, 100 μl, 0.4 μmol per sample) was added to bile samples (200 μl), diluted with water (800 μl). The resulting solutions were filtered using syringe filters (13 mm GD/X disposable filter device, 0.2 μm pore size) and 100 μl of the filtrates was injected for HPLC analysis.

2.5. In vitro hydrolysis of ester prodrugs

In order to evaluate the clinical potential of the HPO ester prodrugs as orally active iron chelators, the in vitro hydrolytic rates of the selected ester prodrugs in phosphate buffer (100 mM, pH 7.4), rat whole blood and liver homogenate were determined using the procedures described by Liu et al. [13]. After incubation, the samples were extracted and analyzed as described above.

3. Results and discussion

It was previously found that the chromatography of the 3-hydroxypyridin-4-ones is difficult on normal ODS columns as the analytes are often characterised by broad asymmetrical and sometimes multiple peaks [16]. The use of silica columns with high carbon loading and of non-silica columns, such as Ultracarb, Polymer PLRP and Hypercarb PGC columns, were found to dramatically improve the peak shape and symmetry [16]. HPLC procedures based on the use of these columns have been successfully applied to metabolic and pharmacokinetic studies of a series of HPOs including CP102 [10,11]. In order to prevent the formation of iron complexes of the HPOs with ferric ions present in the sample and HPLC system, the mobile phase is normally adjusted to pH 3.0 and EDTA (2 mM) is included in

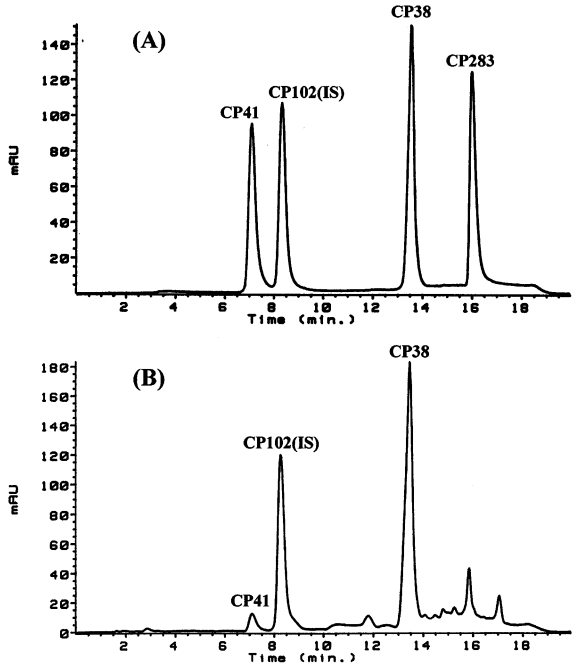

Fig. 2. HPLC chromatograms: (A) authentic CP38, CP41, CP102 and CP283 samples; (B) rat bile sample after oral administration of CP283 (450 μmol/kg body wt). A polymer HPLC column (PLRP-S 100 Å, 15 × 0.46 cm i.d., 5 μm) was used and a gradient ion-pair mobile phase system (2–10% MeCN in 9 min, 10–35% MeCN from 9 to 10 min and hold to 15 min), containing tetrabutylammonium chloride (5 mM) and EDTA (0.5 mM), was applied with 1 ml/min flow rate. The analytes were monitored at 285 nm.

the mobile phase. However, these HPLC procedures are not suitable for the investigation of the hydrolysis of HPO ester prodrugs, as the hydrolytic products (aromatic acids or alcohols) may interfere with the HPO determination. Furthermore, these procedures were found to be unsuitable for the separation of CP38, the major metabolite of CP41, from the parent drug CP41, as the two analytes possess close retention times. Therefore, a gradient ion-pair mobile phase using tetrabutylammonium chloride in combination with EDTA was developed resulting in satisfactory separation of hydrophilic HPOs. An example is presented in Fig. 2 a rat bile sample obtained after oral administration of an ester prodrug of CP41, i.e. CP283. The ester prodrug CP283 was not detected in bile, indicating its rapid hydrolysis in vivo. As previously reported [6], CP38 was found to be the major excretion form in bile whilst CP41 was only present in a minor amount.

The efficient extraction of polar compounds from biological matrices is frequently found to be a serious challenge. In many situations liquid extraction is not an efficient technique for the extraction of highly hydrophilic compounds. Although solid-phase extraction is useful for some polar compounds [17,18], it leads to poor extraction recoveries for hydrophilic HPOs such as CP38. An extraction method utilising acetonitrile has recently been successfully applied to the extraction of cotinine metabolites from the NaCl-saturated microsomal incubates [19]. In the present study, a similar extraction system was developed for HPO extraction. Acetonitrile is a polar organic solvent which is capable of mixing with water in any ratio, but such mixtures can be separated into two phases when saturated by NaCl, thus permitting analytes distributed in the acetonitrile layer to be separated from the aqueous layer. In order to inhibit the dissociation of carboxylate-containing HPOs and to minimise the formation of metal complexes, which may modulate the extraction behaviour of HPOs, the samples were acidified with HCl. The use of 2-propanol further increased the distribution of HPOs in the organic phase.

The extraction recoveries, the within-day coefficients of variation (C.V.) of determination and the accuracy of determination for CP38, CP41 and CP102 are presented in Table 1. The extraction recoveries of hydrophilic HPOs in phosphate buffer and liver homogenate fall in the range 89–94% while the extraction recoveries from blood are slightly lower (85–88%). The extraction recoveries are highly reproducible. The precision and accuracy of the extraction procedure and chromatographic method were established by analyses of the two standard solutions containing CP38, CP41 and CP102 at concentrations of 1 or 0.1 mM. The within-day C.V. (intra-day precision) and accuracy were checked by replicate analysis ($n = 10$) on the same day. The assay is highly reproducible with low variation, although the variation in blood is slightly higher than that in phosphate buffer and liver homogenate (Table

1). The accuracy of the method with blood samples is also slightly lower than that observed for phosphate buffer and liver homogenate. The accuracy values for all three analytes fall in the range of 97–102% for the concentration of 1 mM and 96–105% for the concentration of 0.1 mM. Calibration plots of CP38, CP41 and CP102 over the concentration range 10 μM–1 mM in all three matrices were measured by spiking a known amount of analytes with blank matrices and analysing as described above. The correlation coefficient for each calibration graph exceeded 0.99, an indication of high linearity of analysis. The limit of detection (LOD) was measured based on a signal to noise ratio of three and the values of LOD shown in Table 1 indicate that the analytes can be determined in all three matrices down to the 1–5 μM range using the present analytical method.

The present chromatographic method is based on the formation of ion-pair interaction between HPO molecules and the ion-pair reagent, tetrabutylammonium chloride. Therefore, the variation of tetrabutylammonium chloride concentration in the mobile phase buffer will lead to the corresponding change of HPO retention time. In addition, the presence of EDTA will also affect the formation of ion-pair thus modulating the HPO retention time. However, it was found that the quantitative determination of the HPOs was not markedly affected by the slight change of HPO retention time, provided such changes permitted adequate resolution of the analytes (results not shown).

The in vitro hydrolysis of CP179, an ester of CP38, was investigated using this novel extraction method for sample preparation and the hydrolytic profiles of CP179 in phosphate buffer, in rat blood and liver homogenate are shown in Fig. 3. This method has been used in the in vitro hydrolysis investigations of over 20 ester prodrugs of CP41, CP102 and CP38, and some examples are presented in Table 2.

Table 1
Assay validation for CP38, CP41 and CP102 quantification ($n = 10$)

Parameter	Matrix	[ligand] (mM)	CP41	CP38	CP102
Extraction recovery (%) (mean ± S.D.)	PB	1	90.2 ± 3.9	91.0 ± 3.7	91.9 ± 4.0
		0.1	88.6 ± 3.2	88.9 ± 2.3	93.5 ± 3.1
	Blood	1	86.7 ± 2.2	85.1 ± 4.6	87.9 ± 2.5
		0.1	87.4 ± 3.6	86.4 ± 3.3	85.6 ± 2.8
	Liver homogenate	1	91.1 ± 3.3	90.6 ± 2.3	93.3 ± 2.8
		0.1	87.9 ± 3.8	89.2 ± 3.6	90.1 ± 4.1
Within-day C.V. (%)	PB	1	1.4	2.1	3.3
		0.1	4.7	2.4	2.0
	Blood	1	3.3	4.1	3.8
		0.1	5.4	5.7	4.7
	Liver homogenate	1	1.0	2.7	2.5
		0.1	2.2	3.9	2.0
Accuracy (%)	PB	1	98.0	99.1	96.9
		0.1	97.6	96.7	97.7
	Blood	1	101.3	102.3	100.2
		0.1	104.5	95.5	105.2
	Liver homogenate	1	101.7	99.5	102.0
		0.1	99.8	97.8	104.3
Limit of detection (μM)	PB		2.5	1.2	1.8
	Blood		4.8	3.7	4.2
	Liver homogenate		2.7	1.5	3.0

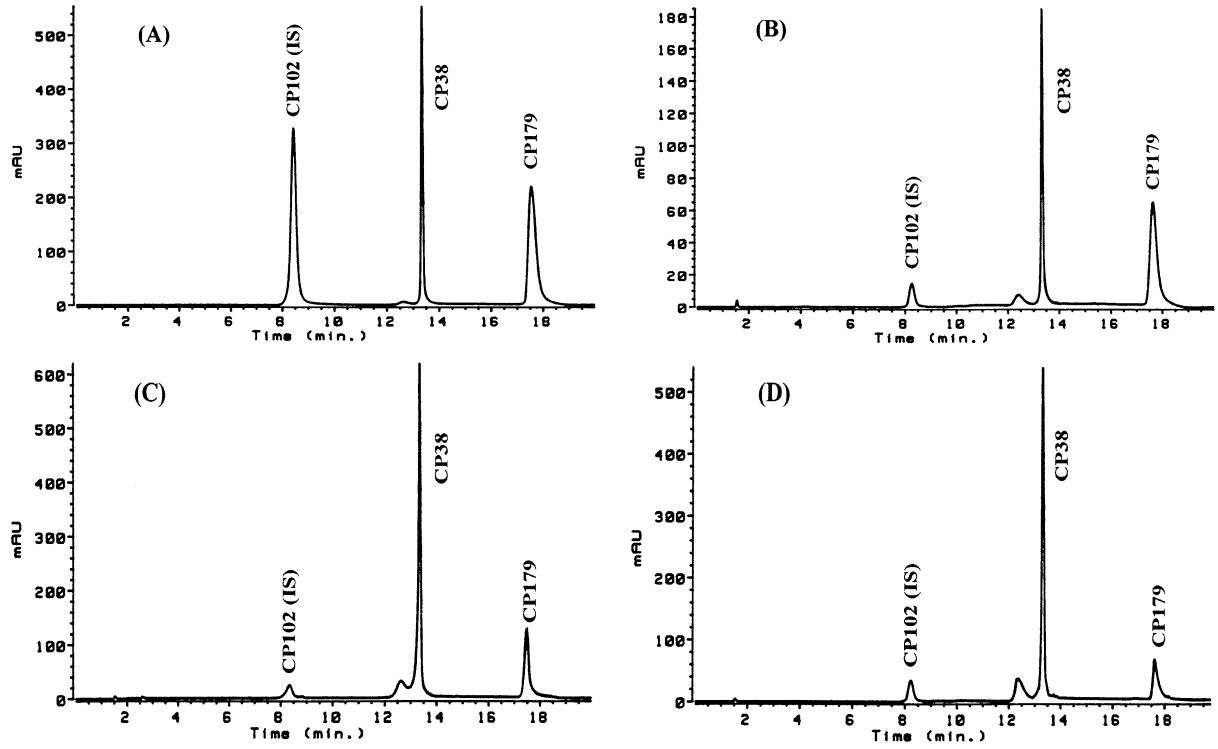

Fig. 3. HPLC chromatograms: (A) authentic CP179, CP38 and CP102 (IS) samples; and the hydrolytic profiles of CP179 (B) in phosphate buffer (0.1 M, pH 7.4), (C) in rat blood, and (D) in rat homogenate. A polymer HPLC column (PLRP-S 100 Å, 15 × 0.46 cm i.d., 5 μm) was used and a gradient ion-pair mobile phase system (2–10% MeCN in 9 min, 10–35% MeCN from 9 to 10 min and hold to 15 min), containing tetrabutylammonium chloride (5 mM) and EDTA (0.5 mM), was applied with 1 ml/min flow rate. The analytes were monitored at 285 nm.

Table 2
Comparison of the hydrolytic rates of a CP38 ester (CP179), a CP41 ester (CP283) and a CP102 ester (CP183) in rat blood and liver homogenate[a]

Ester	Hydrolytic rate		
	In phosphate buffer (μmol/ml per h)	In rat blood (μmol/ml per h)	In rat liver homogenate (μmol/g tissue per h)
CP179	0.058 ± 0.005	4.42 ± 0.10	795.6 ± 5.5
CP283	0.006 ± 0.005	0.36 ± 0.12	230.1 ± 2.4
CP183	0.008 ± 0.003	0.59 ± 0.16	180.4 ± 5.7

[a] Values are mean ± S.D., $n = 3$.

Acknowledgements

The authors thank Apotex Research Inc. Canada and Biomed EC grant BMH4-CT97-2149 for financial support.

References

[1] G.S. Tilbrook, R.C. Hider, Iron Transport and Storage in Microorganisms, Plants and Animals, in: A. Sigel, H. Sigel (Eds.), Metal Ions in Biological Systems, vol. 35, Marcel Dekker, New York, 1998, pp. 691–730.

[2] C. Hershko, A.M. Konijn, G. Link, Br. J. Haematol. 101 (1998) 399–406.
[3] R.J. Bergeron, C.Z. Liu, J.S. McManis, M.X.B. Xia, S.E. Algee, J. Wiegand, J. Med. Chem. 37 (1994) 1411–1417.
[4] J.B. Porter, J. Morgan, K.P. Hoyes, L.C. Burke, E.R. Huehns, R.C. Hider, Blood 76 (1990) 2389–2396.
[5] J.B. Porter, Drug Safety 17 (1997) 407–421.
[6] G. Zanninelli, R. Choudury, O. Loreal, D. Guyader, G. Lescoat, J. Arnaud, R. Verna, B. Cosson, S. Singh, R.C. Hider, P. Brissot, J. Hepatol. 27 (1997) 176–184.
[7] B.L. Rai, L.S. Dekhordi, H. Khodr, Y. Jin, Z.D. Liu, R.C. Hider, J. Med. Chem. 41 (1998) 3347–3359.
[8] G.J. Kontoghiorghes, A.N. Bartlett, L. Sheppard, Clin. Pharmacol. Ther. 48 (1990) 255–261.
[9] J.B. Porter, R.D. Abeysinghe, K.P. Hoyes, C. Barra, E.R. Huehns, P.N. Brooks, M.P. Blackwell, M. Araneta, G. Brittenham, S. Singh, P. Dobbin, R.C. Hider, Br. J. Haematol. 85 (1993) 159–168.
[10] S. Singh, R. Choudhury, R.O. Epemolu, R.C. Hider, Eur. J. Drug Metab. Pharmacokinet. 21 (1996) 33–41.
[11] S. Singh, O. Epemolu, P.S. Dobbin, G.S. Tilbrook, B.L. Ellis, L.A. Damani, R.C. Hider, Drug Metab. Dispos. 20 (1992) 256–261.
[12] R.C. Hider, R. Choudhury, B.L. Rai, L.S. Dehkordi, S. Singh, Acta Haematol. 95 (1996) 6–12.
[13] Z.D. Liu, D.Y. Liu, S.L. Lu, R.C. Hider, J. Pharm. Pharmacol. 51 (1999) 555–564.
[14] B.L. Rai, Z.D. Liu, D.Y. Liu, S.L. Lu, R.C. Hider, Eur. J. Med. Chem. 34 (1999) 1–11.
[15] P.S. Dobbin, R.C. Hider, A.D. Hall, P.D. Taylor, P. Sarpong, J.B. Porter, G. Xiao, D. van der Helm, J. Med. Chem. 36 (1993) 2448–2458.
[16] R.O. Epemolu, S. Singh, R.C. Hider, L.A. Damani, J. Chromatogr. 519 (1990) 171–178.
[17] D.Y. Liu, J.W. Gorrod, J. Pharm. Biomed. Anal. 15 (1997) 1741–1750.
[18] International Union of Pure and Applied Chemistry, Pure Appl. Chem. 66 (1994) 278–304.
[19] Y. Li, J.W. Gorrod, Xenobiotica 24 (1994) 409–415.