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Determination of a new oral iron chelator, ICL670, and its iron complex in plasma by high-performance liquid chromatography and ultraviolet detection

M.C. Rouan[a,*], F. Marfil[a], P. Mangoni[a], R. Séchaud[b], H. Humbert[a], G. Maurer[a]

[a]*Drug Metabolism and Pharmacokinetics, Novartis Pharma SA, 2–4 rue Lionel Terray, 92500, Rueil-Malmaison, France*
[b]*Clinical Pharmacology, Novartis Pharma AG, 4002 Basel, Switzerland*

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Abstract

ICL670 is a representative of a new class of orally active tridentate selective iron chelators. Two molecules of ICL670 are required to form a complete hexacoordinate chelate Fe–$[ICL670]_2$ with one ferric iron. A simple and rapid HPLC–UV method for the separate determination of ICL670 and Fe–$[ICL670]_2$ in the plasma of iron-overloaded patients is described. Plasma samples were prepared as rapidly as possible, the tubes being kept at 4°C. Plasma proteins were precipitated with methanol. The supernatant was diluted with water and placed on the refrigerated sample rack of an autosampler before injection. The chromatographic separations were achieved on an Alltima C_{18} column using 0.05 *M* Na_2HPO_4 and 0.01 *M* tetrabutylammonium hydrogen sulfate–acetonitrile–methanol (41:9:50, v/v/v) as mobile phase. The analytes were detected at 295 nm. Calibration and quality control samples were prepared in normal human plasma. The mean accuracy ($n=6$) over the entire investigated concentration range 0.25–20 μg/ml ranged from 91 to 109% with a coefficient of variation (C.V.) from 4 to 8% for ICL670, and from 95 to 105% with a C.V. from 2 to 20% for the iron complex. The dissociation of the complex during analysis was shown to be marginal. The iron removal from plasma of iron-overloaded patients by free ICL670 during analysis was low. The in vitro iron transfer from the iron pools of iron-overloaded plasma onto ICL670 was shown to be a slow process.

Keywords: ICL670; Iron chelators

1. Introduction

Iron accumulates in the body as a result of repeated transfusions, e.g. in β-thalassemia major, or due to excessive dietary iron uptake in anemias and hereditary hemochromatosis. Man is unable to actively eliminate iron from the body once it has been acquired. Iron chelation therapy has been shown to reduce iron-related morbidity and to improve quality of life in patients with β-thalassemia. Desferal® (desferrioxamine) is currently the only widely adopted drug available to mobilize iron deposits. But the poor oral bioavailability and the short plasma half-life of desferrioxamine necessitate its application as slow subcutaneous or intravenous infusions, thereby limiting the acceptance of long-term therapy by patients. Difficulties in separating the pharmacological effect from toxic effects have hampered for a long time the development of iron chelators which

*Corresponding author. Tel.: +33-4-9395-4798; fax: +33-4-9395-7071.

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can be given orally. Currently, deferiprone (L1; a bidentate ligand) is the orally active iron chelator with the broadest clinical experience. Treatment with deferiprone may involve some complications and requires close monitoring [1]. ICL670 is a representative of a new class of orally active tridentate selective iron chelators. Two molecules of ICL670 are required to form a complete hexacoordinate chelate Fe–$[ICL670]_2$ with one ferric iron (Fig. 1).

In order to establish relationships between the pharmacological effect of an iron chelator and its plasma kinetics, the separate determination of the ligand and its iron complex in plasma is required. Many attempts to determine separately the free ligand and the iron complex in plasma have been made with desferrioxamine. Most of the described methods involve high-pressure liquid chromatography (HPLC). One of the major difficulties encountered was due to the high chelating property of desferrioxamine which binds labile iron from the HPLC devices. In order to avoid conversion of the ligand to the iron complex during chromatography, the proposed methods involve either the use of iron-free HPLC devices [2], or addition of a chelating agent to the mobile phase such as nitrilotriacetic acid [3] or ethylenediaminetetraacetic acid (EDTA) [4,5]. The addition of radioactive iron has been used in another HPLC method [6] for conversion of unbound drug and metabolites to radio-iron bound species in order to overcome stability problems in frozen samples. Dual detection (UV–Vis absorption and radioactive substance measurement) was applied.

With deferiprone, the encountered difficulties in setting up an HPLC assay were peak tailing, conversion of the ligand to the complex and dissociation of the complex during chromatography. Goddard et al. [7] proposed an HPLC method based on protein precipitation and reversed-phase ion-pair chromatography at pH 2.2. Under these conditions, total deferiprone (sum of the ligand and the complex) is determined since the iron complex is dissociated and determined as deferiprone. Epemolu et al. [8] proposed another HPLC method involving liquid–liquid extraction into dichloromethane, chromatography of the ligand on a PGC Hypercarb column using a pH 3 mobile phase containing EDTA, and chromatography of the complex in a separate run at pH 7.

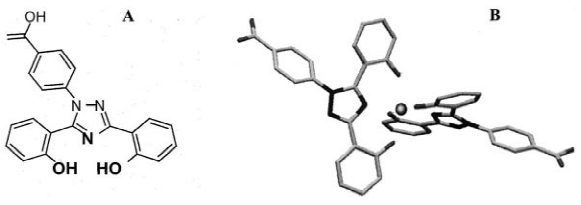

Fig. 1. Structure of ICL670 and its complex Fe–$[ICL670]_2$. (A) Structure of ICL670 with coordinating nitrogen and oxygens in bold; (B) CAMM view of Fe–$[ICL670]_2$.

The chromatographic behavior of an iron chelator and its chelate is dependent on its binding capacity. ICL670 has a lower binding affinity for iron than desferrioxamine but binds about one thousand-fold more tightly than deferiprone. The pM-value at pH 7.4 for desferrioxamine, ICL670 and deferiprone is 26.6, 22.5 and 19.5, respectively. Considering these differences in iron-binding affinity and the chromatographic behavior previously observed for desferrioxamine and deferiprone, an HPLC assay has been developed for the determination of ICL670 and Fe–$[ICL670]_2$ in plasma. This method is described herein.

2. Experimental

2.1. Chemicals and reagents

ICL670 and the iron complex Fe–$[ICL670]_2$ were synthesized at Novartis Pharma, Basle, Switzerland. Analytical grade methanol and acetonitrile were obtained from Carlo-Erba (Nanterre, France). Absolute ethanol was obtained from Prolabo (Fontenay sous Bois, France). Anhydrous di-sodium hydrogen phosphate and Titrisol pH 7 buffer were obtained from Merck (Nogent sur Marne, France), and tetrabutylammonium hydrogen sulfate from Aldrich (St Quentin Fallavier, France). Water was deionized, filtered and purified on a Milli-Q Reagent Grade Water System from Millipore (St Quentin en Yvelines, France).

2.2. Preparation of standard solutions

Primary stock solutions of ICL670 and Fe–$[ICL670]_2$ were prepared by dissolving 2 mg ICL670 in 10 ml ethanol and 2.65 mg Fe–$[ICL670]_2$ salt in

10 ml water. Appropriate dilutions of the stock solutions with either Titrisol pH 7 buffer for ICL670 or water for Fe–$[ICL670]_2$ were then made in order to prepare the spiking solutions at concentrations ranging from 2.5 to 200 μg/ml for both compounds. These solutions were used to spike the calibration plasma samples. Other stock solutions were prepared from a second weighing to prepare the quality control plasma samples. All the solutions were prepared in glass flasks and stored at 4°C.

2.3. Sample preparation

Plasma samples were prepared as rapidly as possible, the tubes being kept in a rack refrigerated at 4°C. A 10-μl aliquot of either the appropriate spiking solutions for calibration/control samples or drug-free solvent solutions for actual samples, 100 μl plasma and 300 μl methanol were successively added in an extraction tube. The tube was vortexed for a few seconds and centrifuged for 4 min at 3000 rpm at 4°C. The whole volume of the supernatant was transferred into another tube and 0.6 ml water was added. The tube was vortexed for a few seconds and then kept on the autosampler rack refrigerated at 4°C pending injection.

2.4. HPLC apparatus and chromatography

The HPLC system consisted of a Model 305 pump from Gilson (Villiers-le-Bel, France), an autosampler ASPEC from Gilson, a Model UV-975 Jasco detector from Merck (Nogent sur Marne, France) monitoring at a wavelength of 295 nm. A chromatography workstation, Model X-Chrom from LabSystems (Issy-Les-Moulineaux, France), was used to perform data acquisition. Chromatographic separations were performed at room temperature on an Alltima C_{18} column 150 mm×4.6 mm, 5 μm particle size, supplied by Alltech (Templemare, France). The analytical column was protected with a Lichrospher RP18 precolumn, 5-μm particle size, supplied by Merck.

The mobile phase, 0.05 *M* di-sodium hydrogen phosphate and 0.01 *M* tetrabutylammonium hydrogen sulfate–acetonitrile–methanol (41:9:50, v/v/v), was delivered at a flow-rate of 1.3 ml/min.

2.5. Clinical study

Blood was taken from a thalassemic patient following single oral administration of a 10-mg/kg dose of ICL670. It was collected into lithium-heparinized tubes, cooled on ice immediately after collection and centrifuged for 10 min at ~2000 rpm at 3–5°C. The plasma was frozen within 15–20 min after collection and kept below −18°C pending analysis.

3. Results and discussion

3.1. Chromatographic behavior

In the millimolar to micromolar concentration range at pH 7.4, Fe–$[ICL670]_2$ is the predominant iron-containing ICL670 species. With decreasing pH, the incomplete complex Fe–ICL670 is formed and both complexes are dissociated at acidic pH. The chromatography was consequently performed at a pH ~7, so that the iron complex Fe–$[ICL670]_2$ was stabilized. Both ICL670 and the complex are negatively charged at this pH. Addition of an ion-pair reagent to the mobile phase, tetrabutylammonium hydrogen sulfate, permitted to obtain a correct separation of the two compounds within a single run. Dilution of the supernatant with water prior to injection improved peak shape by decreasing the eluting strength of the injected solvent and prevented peak splitting for the complex. Peaks with no tailing were obtained on an endcapped Alltima C_{18} column, the interactions between the residual silanols and the triazole group of ICL670 being minimized.

The iron complex of ICL670 is expected to be less prone to dissociation than that of deferiprone during analysis since the pM-value of ICL670 is higher. Nevertheless, as reported for deferiprone [8], dissociation of Fe–$[ICL670]_2$ was found to occur during chromatography when acetonitrile was used as the organic modifier in the mobile phase. As shown in Fig. 2A, the base-line between the peaks of ICL670 and Fe–$[ICL670]_2$ did not drop to its normal level. The absorbance zone between the two peaks might have been due to the presence of ICL670 in the effluent as a result of continuous conversion of the complex to ICL670 during the displacement of

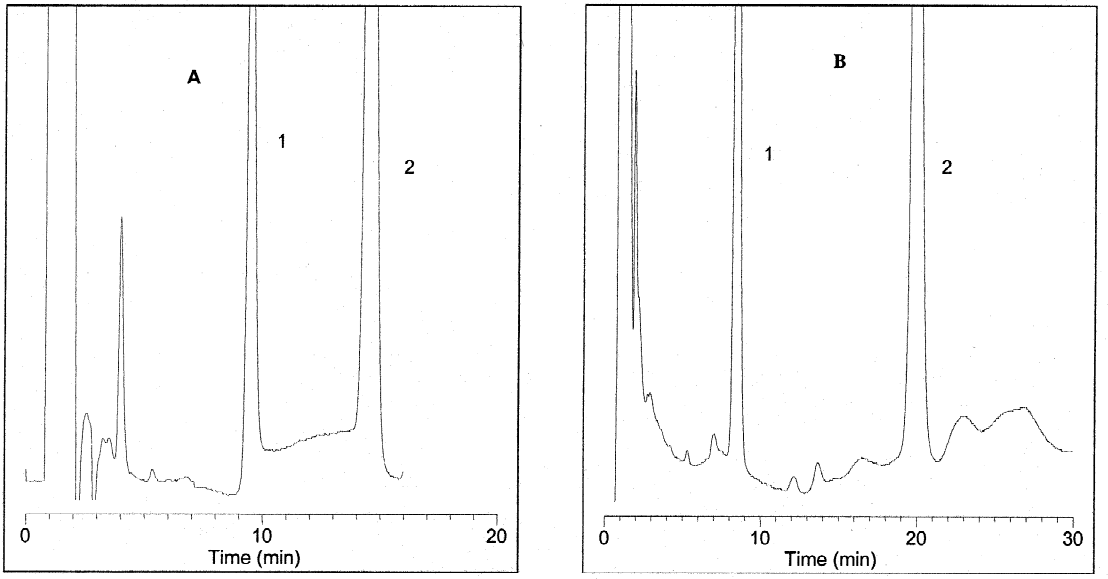

Fig. 2. Chromatograms from normal human plasma samples spiked with ICL670 and Fe–$[ICL670]_2$ in a ratio of 1/1. Peak 1: Fe–$[ICL670]_2$; peak 2: ICL670. (A) Mobile phase: 0.05 *M* Na_2HPO_4 and 0.01 *M* tetrabutylammonium hydrogen sulfate–acetonitrile (63:37, v/v); nominal concentrations: 6 μg/ml. (B) Mobile phase: 0.05 *M* Na_2HPO_4 and 0.01 *M* tetrabutylammonium hydrogen sulfate–acetonitrile–methanol (42:10:48, v/v/v); nominal concentrations: 10 μg/ml.

the elution band of the complex along the column. Such anomalous chromatographic behavior was minimized when acetonitrile was partly replaced with methanol as shown in Fig. 2B. Then, only small amounts of free ICL670 (less than 10% of Fe–$[ICL670]_2$ peak height) were found upon injection of Fe–$[ICL670]_2$ at a low concentration of 2 μg/ml (Fig. 3A), indicating that the dissociation of the complex during chromatography was low. Acetonitrile was not completely removed from the mobile phase because the difference in retention times of ICL670 and Fe–$[ICL670]_2$ drastically increased when only methanol or ethanol were used.

ICL670 is expected to remove less labile iron from the chromatographic system and the analytical column than desferrioxamine due to its lower iron-affinity binding, but more than deferiprone. An endcapped column was used for the assay of ICL670 in order to decrease the amount of iron bound to the residual silanols in the column. No further special equipment was used. Only small amounts of Fe–$[ICL670]_2$ (~5% of ICL670 peak height) were found upon injection of ICL670 at a low concentration of 2 μg/ml as shown in Fig. 3B. This indicates that the formation of Fe–$[ICL670]_2$ during chromatography was not significant. This was also confirmed at a high ICL670 concentration level (Section 3.5.2).

3.2. Protein precipitation

Plasma proteins were precipitated with either acetonitrile or methanol. With acetonitrile, the results obtained for ICL670 were not reproducible and low recovery (below 50%) was obtained for Fe–$[ICL670]_2$. With methanol, reproducible results were obtained for the two compounds. The recovery was ~100% for ICL670 despite its high protein binding (>99.5%), and ~75% for Fe–$[ICL670]_2$. Hence, methanol was selected as the precipitating agent.

Acetonitrile is more effective in precipitating proteins than methanol [9] and is consequently expected to more extensively direct a compound bound to plasma proteins into the precipitate. Therefore, the differences in the recovery of Fe–$[ICL670]_2$

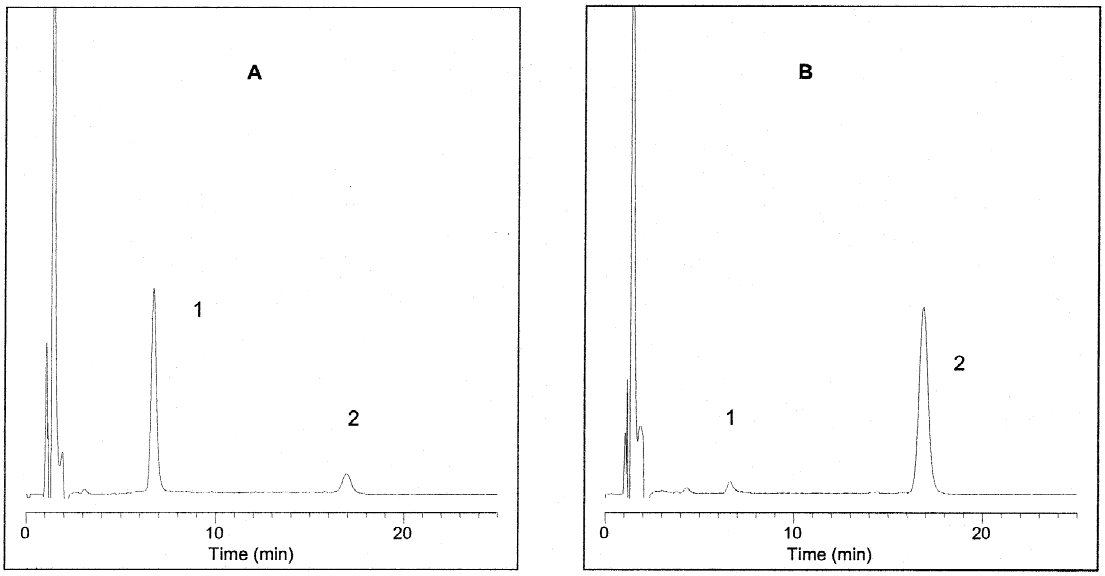

Fig. 3. Direct injection of a solution of (A) Fe–$[ICL670]_2$ (2 μg/ml), (B) ICL670 (2 μg/ml). Peak 1: Fe–$[ICL670]_2$; peak 2: ICL670. Mobile phase: 0.05 *M* Na_2HPO_4 and 0.01 *M* tetrabutylammonium hydrogen sulfate–acetonitrile–methanol (42:10:48, v/v/v).

as a function of the solvent used might have been due to co-precipitation of the complex, but also to some dissociation of the compound in the presence of acetonitrile as observed during chromatography when using high content of acetonitrile in the mobile phase (Section 3.1).

3.3. Internal standard

An attempt was made to use an analog of ICL670 as internal standard. The analog used presented one atom of fluorine on each phenol group instead of one atom of hydrogen and had coordinating nitrogen and oxygen atoms as ICL670. It was consequently able to bind iron. Following the addition of this ICL670 analog and Fe–$[ICL670]_2$ to plasma, the peak area obtained for Fe–$[ICL670]_2$ was lower than expected, and the reproducibility of the results was poor. It was assumed that the analog used as internal standard removed the iron from Fe–$[ICL670]_2$ to itself form a complex. Hence, no internal standard was used for quantitation in order not to displace the equilibrium between ICL670 and Fe–$[ICL670]_2$.

3.4. Determination in spiked normal human plasma

A more pronounced dissociation of Fe–$[ICL670]_2$ is expected when it is alone in a medium than in the presence of ICL670 since the two compounds are in equilibrium. Therefore, ICL670 and Fe–$[ICL670]_2$ were spiked either alone or together in normal human plasma, and the samples were analysed according to the procedure described in Section 2. When each compound was spiked alone in plasma, its peak area was similar to that obtained in the presence of the other compound, except with the complex at the lowest concentration level, 0.5 μg/ml (Table 1). A difference of 18% was then observed. As after direct injection of the complex (Section 3.1), low amounts of free ICL670 were found for the plasma sample spiked with Fe–$[ICL670]_2$ (Fig. 4). This indicates that a slight dissociation of the complex may occur during analysis when it is the major compound in plasma. On the other hand, no relevant dissociation may occur in presence of ICL670.

ICL670 and Fe–$[ICL670]_2$ concentrations were

Table 1
Assessment of an eventual shift of the equilibrium between ICL670 and Fe–$[ICL670]_2$ spiked in normal human plasma

Nominal conc. (μg/ml)	Peak area of ICL670		Peak area of Fe–$[ICL670]_2$	
	ICL670 spiked alone	Both compounds spiked	Fe–$[ICL670]_2$ spiked alone	Both compounds spiked
0.5	8.45	9.03	6.83	8.34
2.5	50.1	52.7	35.0	36.6

ICL670 and Fe–$[ICL670]_2$ were spiked in normal human plasma either alone or together in a concentration ratio of 1/1.

not modified upon incubation in spiked normal human plasma at ambient temperature for 24 h (Table 2). Iron is mainly bound to transferrin in plasma of normal individuals, one-third of the protein only being saturated with iron. Therefore, ICL670 does not remove the iron specifically bound to transferrin in vitro.

3.5. Determination in spiked iron-overloaded plasma

In patients with iron overload, plasma transferrin becomes completely saturated, and non-specific iron appears in the plasma [10]. This non-transferrin bound iron (NTBI), i.e. iron that circulates in plasma unbound to transferrin, is potentially toxic because it is capable of taking part in free-radical-mediated reactions that result in irreversible tissue damage [11]. The nature of NTBI is not well known. It is assumed to represent a heterogenous group of low-molecular-weight iron containing species, mainly iron–citrate complexes, high-molecular-weight forms of NTBI being also likely to exist [12,13].

Desferrioxamine has been shown to remove NTBI in iron-overloaded patients undergoing intravenous desferrioxamine infusion [14], and to slowly bind NTBI in vitro [13]. Deferiprone has been shown to remove iron from saturated transferrin following its administration to iron-overloaded patients, and in vitro following incubation at 37°C in iron-overloaded patient plasma [15]. These findings suggest that ICL670 may be prone to bind NTBI and saturated transferrin in actual plasma samples, either before or during sample preparation. Therefore, the feasibility of ICL670 conversion to Fe–$[ICL670]_2$ during analysis and the rate and extent of iron transfer from plasma onto ICL670 at physiological conditions, i.e. at 37°C, was investigated in iron-overloaded plasma. The plasma was obtained from patients with β-thalassemia who stopped Desferal® treatment at least 3 days before blood sampling. The determinations were performed versus calibration standards prepared in normal human plasma.

3.5.1. Temperature influence during sample preparation

Drug-free plasma samples from patients with β-thalassemia were spiked with ICL670 and Fe–$[ICL670]_2$ in a concentration ratio of 1/1 and the complex formation in these samples was assessed. When the samples were prepared at ambient temperature, up to 50% of the introduced amount of ICL670 was converted to Fe–$[ICL670]_2$. When the samples were cooled during sample preparation and prepared rapidly, the conversion of ICL670 to the complex was not significant. This shows that, at ambient temperature, ICL670 decays during sample preparation, and that the rate of conversion of ICL670 is slowed down by cooling the sample. Therefore, it is important to cool the plasma samples during sample preparation and to prepare them as rapidly as possible.

3.5.2. Incubation at ambient temperature

After 15 min of incubation at ambient temperature, the accuracy (found/nominal concentration in %) found for ICL670 and Fe–$[ICL670]_2$ was within 80–120% (Fig. 5). Afterwards, the lower the concentration, the more important the conversion of ICL670 to the complex. Therefore, ICL670 is stable in normal human plasma (Section 3.4), but slowly decays into Fe–$[ICL670]_2$ in iron-overloaded plasma. As a consequence, for pharmacokinetic studies, the blood samples taken from patients given ICL670 must be cooled immediately after collection and the plasma prepared and frozen as rapidly as possible.

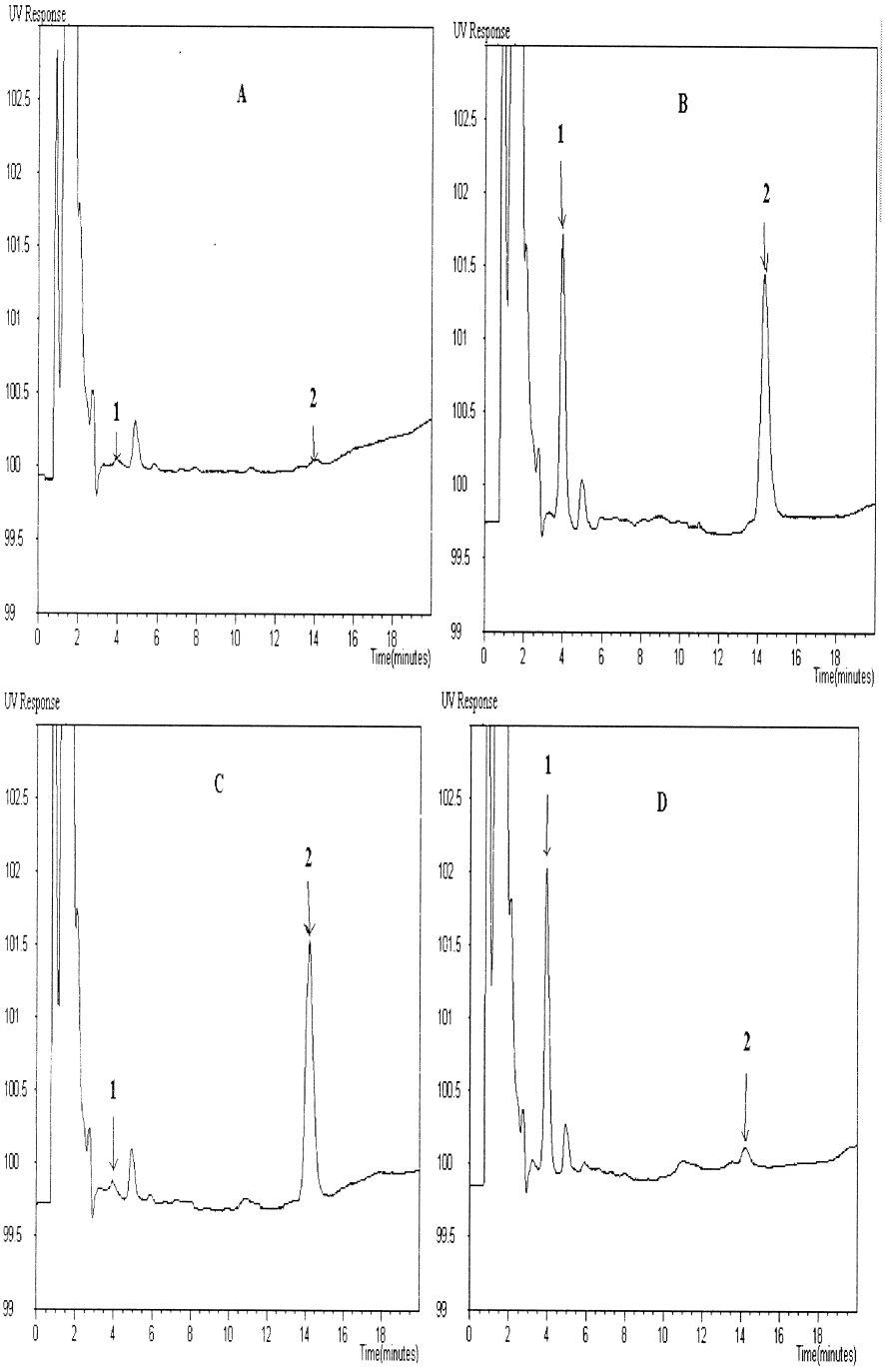

Fig. 4. Chromatograms from normal human plasma samples spiked with ICL670 and/or Fe–$[ICL670]_2$. Mobile phase: 0.05 *M* Na_2HPO_4 and 0.01 *M* tetrabutylammonium hydrogen sulfate–acetonitrile–methanol (41:9:50, v/v/v). Peak 1: Fe–$[ICL670]_2$; peak 2: ICL670. (A) Drug-free plasma, (B) plasma spiked with 2.5 μg/ml ICL670 and Fe–$[ICL670]_2$, (C) plasma spiked with 2.5 μg/ml ICL670, (D) plasma spiked with 2.5 μg/ml Fe–$[ICL670]_2$.

Table 2
Stability in normal human plasma at ambient temperature

Nominal conc. (μg/ml)	Recovery of ICL670 (%)		Recovery of Fe–[ICL670]$_2$ (%)	
	6-h Storage	24-h Storage	6-h Storage	24-h Storage
0.25	119	97	82	116
0.5	106	110	91	88
1	99	106	97	94
10	97	100	105	105

ICL670 and Fe–[ICL670]$_2$ were spiked in normal human plasma in a concentration ratio of 1/1.

3.5.3. Incubation at 37°C

Drug-free plasma samples from six patients with β-thalassemia were spiked at high concentrations of ICL670 (5 and 40 μg/ml), and the concentration of the complex formed before and after incubation at 37°C for 1, 4 and 24 h was determined. Before incubation, the complex was not found (<0.25 μg/ml) in the plasma of four patients and was determined at levels of ~0.5 μg/ml in the plasma from patients A and B. Upon incubation, Fe–[ICL670]$_2$ formed slowly as shown in Table 3. For the spiked 40-μg/ml concentration of ICL670, the mean concentration of the complex was only 1.7 times higher at 4 h than at 1 h. Such a slow iron removal from plasma is in agreement with previous findings with desferrioxamine [13]. The transfer of iron from iron-overloaded plasma onto desferrioxamine has been shown to be a slow process.

The higher the ICL670 spiked concentration, the higher the amount of complex formed as shown in Table 3. However, for the 40-μg/ml concentration of ICL670, the mean Fe–[ICL670]$_2$ concentration at 24 h was 1.1 μg/ml (1.46 μmol/l) only. This concentration level is low compared to the plasma levels of Fe–[ICL670]$_2$ measured in patients following single oral administration of ICL670 (mean C_{max} = 4.9±3.1 μmol/l, n=6, dose: 10 mg/kg; unpublished data). It is also low compared to the NTBI levels

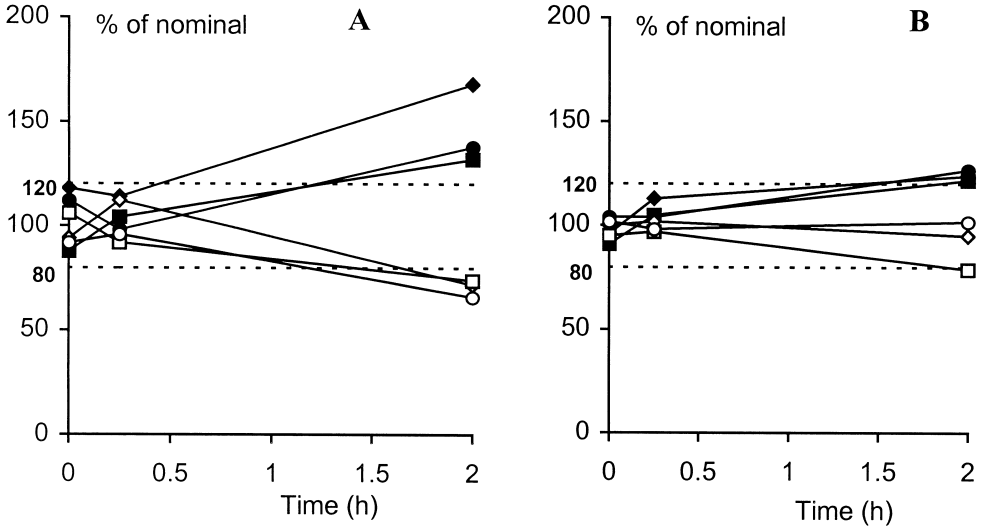

Fig. 5. Formation of the iron complex in spiked plasma samples from thalassemic patients at ambient temperature. ICL670 and Fe–[ICL670]$_2$ were spiked in plasma from three different patients at a concentration ratio of 1/1. They were determined immediately (time 0) and at 15 min and 2 h after incubation at ambient temperature. Empty symbols represent ICL670. Black symbols represent Fe–[ICL670]$_2$. Nominal concentration: (A) 0.5 μg/ml, (B) 5 μg/ml.

Table 3
Concentration of Fe–$[ICL670]_2$ formed upon incubation at 37°C for 1, 4 and 24 h in blank plasma taken from 6 iron-overloaded patients and spiked with ICL670

	Concentration (μg/ml) of Fe–$[ICL670]_2$ formed during incubation													
Patient	A		B		C		D		E		F		Mean	
ICL670 (μg/ml)	5	40	5	40	5	40	5	40	5	40	5	40	5	40
1 h	0.50	0.71	0.86	1.01	0.47	0.56	–	0.45	0.31	0.52	0.36	0.75	0.50	0.67
4 h	0.76	1.11	1.15	1.41	0.83	1.22	0.53	1.03	0.44	1.01	0.57	1.00	0.71	1.13
24 h	0.85	1.27	1.46	1.81	0.59	0.81	0.50	1.08	0.54	0.79	0.51	0.89	0.74	1.11

–: Not detected (below 0.25 μg/ml).

reported in thalassemic patients, 2–18 μmol/l [12], and 2.9–4.5 μmol/l as a mean [16]. Hence, the pool of iron measured as the iron complex formed upon incubation in plasma from thalassemic patients may represent a small portion of the iron readily chelatable by ICL670 in the body following its administration.

3.6. Calibration

Calibration samples were prepared in drug-free normal human plasma since iron was found not to be removed from this matrix by free ICL670, even at ambient temperature. ICL670 and Fe–$[ICL670]_2$ were spiked together in a ratio of 1/1 since the dissociation of the complex is minimized in the presence of ICL670. The calibration samples were prepared at seven different concentrations in the range 0.25–20 μg/ml. The calibration curves, represented by the plots of the peak area of either ICL670 or Fe–$[ICL670]_2$ versus the concentration in the calibration sample, were generated using weighted $(1/x^2)$ linear regression. The curves were linear with coefficients of correlation higher than 0.997 for ICL670 and 0.999 for Fe–$[ICL670]_2$. The bias between the back-calculated and nominal concentrations did not exceed 15%.

3.7. Accuracy and precision

Replicate quality control samples were prepared in drug-free normal human plasma at five different concentrations (Table 4). They were analyzed within 1 day (intra-day reproducibility) and over 3 days

Table 4
Accuracy and precision

	Nominal conc. (μg/ml)	ICL670		Fe–$[ICL670]_2$	
		Mean accuracy (%)	Precision (C.V.) (%)	Mean accuracy (%)	Precision (C.V.) (%)
(a) Intra-day	0.25	102	7	95	20
	0.5	91	8	103	5
	2	104	6	99	9
	8	106	4	105	6
	20	109	4	104	2
(b) Inter-day	0.25	104*	9	92*	7
	0.5	91	5	102	6
	2	101	8	100	8
	8	105	7	100	4
	20	100	6	96	8

ICL670 and Fe–$[ICL670]_2$ were spiked in normal human plasma in a concentration ratio of 1/1 ($n=6$ at each concentration level, duplicate samples at each concentration level being determined on 3 analysis days for the inter-day reproducibility). *: $n=5$.

(inter-day reproducibility). The mean accuracies ranged from 91 to 109% with the C.V. ranging from 2 to 20% over the concentration range 0.25–20 μg/ml (Table 4(a)).

3.8. Stability of frozen samples

Plasma samples taken from patients given ICL670 were determined just after receipt, refrozen at −20°C, and redetermined later. They were found to be stable at −20°C for at least 1 month both for ICL670 and Fe–$[ICL670]_2$. Drug-free plasma samples from thalassemic patients spiked with ICL670 and Fe–$[ICL670]_2$ were found to be stable at −80°C for at least 3 months.

3.9. Plasma profiles in a patient given ICL670

The plasma concentrations of ICL670 and Fe–$[ICL670]_2$ were determined using the above described method from a thalassemic patient following single oral administration of a 10-mg/kg dose of ICL670. ICL670 was by far the major compound in plasma (Fig. 6). The measured concentrations of Fe–$[ICL670]_2$ were well above the level of residual concentrations (0.25–0.5 μg/ml) that could result from the conversion of ICL670 to the iron complex in vitro following sample collection (Section 3.5.2).

3.10. Limit of quantitation

The lowest concentrations of ICL670 and Fe–$[ICL670]_2$ that were determined in spiked normal human plasma with an accuracy within 80–120% and a precision lower than or equal to 20% are 0.25 μg/ml (Table 4(a) and (b)). For ICL670, the limit of quantitation (LOQ) can be set to 0.25 μg/ml. For the iron complex, the LOQ should be set to 0.5 μg/ml in case some slight conversion of ICL670 to the complex occurs in actual samples at high ICL670 concentration levels following blood collection. However, ICL670 is expected to remove NTBI in vivo, so that the plasma NTBI level in patients should be low upon treatment with ICL670. As a consequence, the in vitro conversion of ICL670 to the complex following blood collection from treated patients should be marginal.

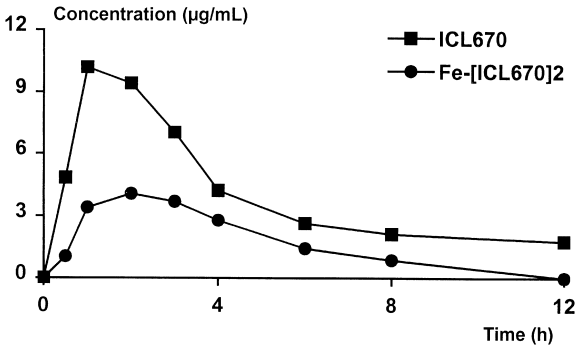

Fig. 6. Representative plasma concentration–time profiles of ICL670 and Fe–$[ICL670]_2$ in one thalassemic patient following single oral administration of a 10-mg/kg dose of ICL670.

4. Conclusion

The described HPLC–UV method permits the separate determination of ICL670 and its iron complex in plasma with adequate sensitivity. The method is simple and rapid. The dissociation of the complex during analysis was shown to be marginal. Moreover, iron removal from iron-overloaded plasma and the HPLC devices by ICL670 during analysis was not significant as shown by the low amounts of the complex formed during chromatography.

References

[1] F.N. Al-Refaie, C. Hersbko, A.V. Hoffbrand, M. Kosaryan, N.F. Olivieri, P. Tondury, B. Wonke, Br. J. Haematol. 91 (1995) 224.
[2] S. Venkataram, Y.E. Rahman, J. Chromatogr. 411 (1987) 494.
[3] S. Singh, R.C. Hider, J.B. Porter, Anal. Biochem. 187 (1990) 212.
[4] S.M. Cramer, B. Nathanael, C. Horvath, J. Chromatogr. 295 (1984) 405.
[5] H.J. Kraemer, H. Breithaupt, J. Chromatogr. B 710 (1998) 191.
[6] S. Singh, N. Mohammed, R. Ackerman, J.B. Porter, R.C. Hider, Anal. Biochem. 203 (1992) 116.
[7] J.G. Goddard, G.J. Kontoghiorghes, Clin. Chem. 36/1 (1990) 5.
[8] R.O. Epemolu, S. Singh, R.C. Hider, L.A. Damani, J. Chromatogr. 519 (1990) 171.
[9] J. Blanchard, J. Chromatogr. 226 (1981) 455.
[10] C. Hersliko, G. Graham, G.W. Bates, E.A. Rachmilewitz, Br. J. Haematol. 40 (1978) 255.
[11] C. Borgna-Pignatti, A. Castriota-Scanderbeg, Haematologica 76 (1991) 409.

[12] S. Singh, R.C. Hider, J.B. Porter, Biochem. Soc. Trans. 17 (1989) 697.
[13] M. Grootveld, J.D. Bell, B. Halliwell, O.I. Aruoma, A. Bomford, P.J. Sadler, J. Biol. Chem. 264 (8) (1989) 4417.
[14] S. Singh, R.C. Hider, J.B. Porter, Anal. Biochem. 186 (1990) 320.
[15] F.N. Al-Refaie, C.E. De Silva, B. Wonke, A.V. Hoffbrand, J. Clin. Pathol. 48 (1995) 110.
[16] J.B. Porter, R.D. Abeysinghe, L. Marshall, R.C. Hider, S. Singh, Blood 88 (2) (1996) 705.