Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 796 (2003) 105–112
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Improved high-performance liquid chromatographic method for the pharmacokinetic studies of a novel iron chelator, CP502, in rats

Jasmina Novakovic, Angelo Tesoro, Michael Spino, Jake Thiessen*

Leslie Dan Faculty of Pharmacy, University of Toronto, 19 Russell Street, Toronto, Ont., Canada M5S 2S2

Received 21 February 2003; received in revised form 11 August 2003; accepted 11 August 2003

Abstract

An improved reverse-phase high-performance liquid chromatographic method (RP-HPLC) for the determination of a novel iron chelator CP502 (1,6-dimethyl-3-hydroxy-4-(1H)-pyridinone-2-carboxy-(*N*-methyl)-amide hydrochloride) in rat plasma, urine and feces was developed and validated. The separation was performed on a polymeric column using a mobile phase composed of 1 mM ethylenediaminetetra-acetic acid disodium salt (EDTA), acetonitrile, methanol and methylene chloride. Separation of CP502 from plasma, urine or feces endogenous compounds was achieved by gradient elution. Retention times of CP502 and its major metabolite (glucuronide) were about 13 and 4 min, respectively. The method was validated in terms of limit of detection (LOD), limit of quantification (LOQ), selectivity (endogenous from plasma, urine or feces), linearity, extraction recovery, robustness (column selection, mobile phase composition, detection mode, internal standard (IS) selection, analyte stability), day-to-day reproducibility and system suitability (repeatability, peak symmetry and resolution). The method is applicable to bioavailability and pharmacokinetic studies of CP502 in rats.

Keywords: Pharmacokinetics; CP502; Iron chelator

1. Introduction

The removal of excess body iron would ideally occur via an orally administered chelator that is functionally non-toxic, efficient and bio-specifically removes iron from selected organs, tissues or cells. While the ideal chelator has not yet been found, 3-hydroxypyridin-4-one (HPO) compounds have shown great promise as orally active iron chelating agents [1], with deferiprone being the first clinically approved molecule. These bidentate chelators bind to iron in a 3:1 ratio. The introduction of an amido substituent in the 2-position leads to an enhancement of pFe^{3+} values. Among these compounds, CP502 (1,6-dimethyl-3-hydroxy-4-(1H)-pyridinone-2-carboxy-(*N*-methyl)-amide hydrochloride: molecular weight 232.67, Fig. 1) exhibited the highest in vitro iron chelation activity ($pFe^{3+} = 21.7$) [2]. Critical properties, when identifying a new candidate molecule, are bioavailability, metabolic profile

* Corresponding author. Tel.: +1-416-978-6483; fax: +1-416-978-8511.
E-mail address: jj.thiessen@utoronto.ca (J. Thiessen).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.08.013

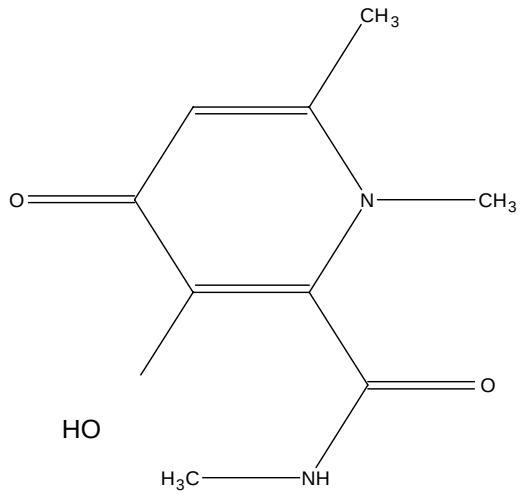

Fig. 1. CP502 chemical structure.

and disposition. To test these traits for CP502, validated bioanalytical methods were needed in different biological matrices (e.g. plasma, urine and feces).

Previously reported reverse-phase high-performance liquid chromatographic method (RP-HPLC) methods for analysis of highly hydrophilic HPOs in biological materials were unsatisfactory due to their poor resolution from interfering endogenous substances [3]. Therefore, Skinner et al. suggested RP-HPLC combined with a baseline subtraction method [4]. Hider and others proposed an ion-pair HPLC method for CP502 determination in bile and urine using 1-heptanesulfonic acid as an ion-pair reagent, with the mobile phase adjusted to pH 2.0 [5]. We describe herein a new selective RP-HPLC method for CP502 determination in plasma, urine and feces using a polymeric column. The applicability of our improved method is also illustrated in preliminary PK studies of CP502 in rats.

2. Experimental

2.1. Materials and reagents

CP502 and CP363 (3-hydroxy-2-(1-methoxyethyl)-1,6-dimethylpyridin-4(1H)-one hydrochloride) were provided by Dr. R.C. Hider (King's College, London, UK). Isocaffeine (IC) and β-hydroxyethyltheophylline (HETh) (internal standards) deferoxamine mesylate (DFO), MOPS (3-[*N*-morpholino]propanesulfonic acid), and EDTA were purchased from Sigma (St. Louis, MO, USA). Methylene chloride (CH_2Cl_2), acetonitrile (ACN) and methanol (MeOH), all of HPLC grade purity, were obtained from Fisher Scientific (Toronto, Canada). Water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Rat plasma was obtained from the Division of Comparative Medicine, University of Toronto (Toronto, Canada). Male Sprague–Dawley rats (body weight about 250 g) were purchased from Charles River Laboratories (Montreal, Canada). The animal experiments were approved by the University of Toronto Animal Care Committee and the experimental procedures conformed to the requirements of the Canadian Council on Animal Care.

2.2. Stock solutions

Stock solutions of CP502 (1.0 mg/ml (A); 0.1 mg/ml (B); and 0.01 mg/ml (C)), HETh (0.1 and 0.2 mg/ml) and CP363 (0.2 mg/ml) were prepared in purified water. The IC stock solution (0.1 mg/ml) was prepared in a mixture of methanol and water (1:9, v/v).

2.3. Calibration solutions for CP502 determination in biological matrices

2.3.1. Plasma and urine

Calibration solutions were prepared by combining 0.5 ml of internal standard (IS) stock solution (HETh), and an appropriate volume of CP502 stock solutions (A, B or C), and diluting the resulting solution with water, urine (diluted 1:50 with water), or plasma to a final volume of 5.0 ml. CP502 concentrations in water, urine (diluted 1:50), and plasma were from 0.1 to 90 μg/ml, while the IS concentration was 10 μg/ml. Plasma samples were processed by ultrafiltration using Amicon Centrifree® (Millipore, Milford, MA, USA; 30,000 molecular mass cut-off) at 3000 g for 20 min. A 100 μl aliquot was chromatographed.

2.3.2. Feces

Frozen blank feces (control rats) sample was lyophilized overnight, weighed and finely pulverized.

Dried feces (0.5 g) was mixed with 10 ml of an extraction medium (60 mM MOPS containing 5 mM DFO, pH adjusted to 7.0 with 0.1 M sodium hydroxide). The extraction was facilitated with a Polytron homogenizer (Brinkmann, Germany), followed by shaking overnight. The sample was centrifuged 20 min at 4000 rpm (IECCentra-8R, International Equipment Company, USA) and the supernatant was thereafter ultracentrifuged 45 min at 40,000 rpm (140,000 g; Beckman model L5-75 ultracentrifuge, Rotor Ti 75). Clear supernatant was diluted with water (1:20) and spiked with CP502 stock solutions to obtain CP502 concentrations from 0.25 to 50 μg/ml. A 5 μl aliquot of internal standards solution (HETh and CP363, 0.2 mg/ml each in water) was added to 200 μl aliquot of spiked solution, mixed well and 100 μl was chromatographed.

2.4. Chromatography

A Hewlett-Packard model series 1050 HPLC system with an autosampler and diode-array detector, and a PLRP-S stainless steel column (Polymer Laboratories Ltd., Shropshire, UK; 150 mm × 4.6 mm i.d., 5 μm) were used in the present study. Gradient-based chromatography was performed using a quaternary mobile phase (Table 1). The total elution time was 30 min at a mobile phase flow rate of 0.8 ml/min. UV detection wavelengths were 280 and 430 nm. All the measurements were performed at ambient temperature.

2.5. Recovery of CP502

2.5.1. Plasma and urine

A 0.5 ml aliquot containing CP502 and IS in water (0.5 mg/ml for each) was diluted with urine (diluted 1:50 with water) or blank plasma to 5.0 ml to obtain plasma and urine samples containing 50 μg/ml of both compounds. By serial dilution of the spiked samples with plasma and urine, respectively, a series of samples containing 0.5, 1.0, 5.0, 10.0 and 20.0 μg/ml CP502 and IS were obtained. The sample preparation and analysis were performed as described under Sections 2.3.1 and 2.4, respectively.

Table 1
Gradient chromatography conditions

Time	EDTA (1 mM) (%)	CH_2Cl_2 (%)	ACN (%)	MeOH (%)
0	98.4	0.3	0.3	1
12	91.4	0.3	0.3	8
17	78.7	0.3	6	15
25	78.7	0.3	6	15
26	98.4	0.3	0.3	1

2.5.2. Feces

To obtain well-resolved and symmetric chromatographic CP502 peaks it was necessary to remove interfering iron from the feces. Therefore DFO was added in the extraction medium. The sample preparation and analysis were performed as described under Sections 2.3.2 and 2.4, respectively. Recovery, tested for CP502 concentrations of 0.5, 1.5 and 4.0 μg/ml, was 95.98% (R.S.D. 8.8%).

2.6. Pharmacokinetic study of CP502 in rats

Four days after jugular vein pre-catheterization, six Sprague–Dawley rats were placed individually in metabolic cages. Isotonic CP502 solution (0.1538 M = 35.78 mg/ml) was prepared in distilled water (Millipore) and diluted with 0.9% sodium chloride to the final CP502 concentration of 25 mg/ml. Three rats were given an intravenous (i.v.) dose of isotonic aqueous solution of CP502 (150 mg/kg). Three additional rats were given a single oral dose of the same CP502 solution (150 mg/kg) by gavage. Venous blood samples were withdrawn from the cannulated jugular vein before the dosing (blank samples) and at defined time points up to 28 h post-dosing. Urine and feces samples were collected before the dosing (blank samples) and up to 72 h post-dosing in 24 h intervals.

Heparinized blood was centrifuged to obtain plasma. A 200 μl aliquot of plasma and 20 μl of HETh stock solution were mixed thoroughly and ultrafiltration was performed as described under Section 2.3. A 100 μl aliquot was chromatographed.

Urine samples were diluted 1:50 with distilled water. A 200 μl aliquot of diluted urine and 20 μl of HETh stock solution were mixed thoroughly and 100 μl was chromatographed. Feces samples were prepared as described under Section 2.3.2.

3. Results and discussion

3.1. Method selectivity and robustness

It was previously reported that chromatography of HPOs on a C18 column resulted in broad, asymmetrical and tailing peaks [3,4]. Polymeric columns, offer a number of advantages, including pH stability from 1 to 13, excellent sample recoveries due to lack of acidic silanol groups, compatibility with the mobile phases containing 0–100% organic solvent, and greater surface area than alkyl bonded silicas, were found to be more suitable for HPOs chromatography [6]. These advantages were essential in separating CP502, as a highly polar compound, from endogenous materials. To test the robustness in terms of the polymeric column selection, columns from two manufactures were tested, namely, Hamilton PRP-1 (Hamilton, USA) and PLRP-S column (Polymer Laboratories, UK). The columns (150; 4.6 mm i.d.; 5 μm) were comparable in terms of selectivity (CP502 separation from endogenous material). PLRP-S column was superior in terms of theoretical plates ($N = 1200$) compared to the Hamilton PRP-1 column ($N = 900$).

The mobile phase composition is critical for the method selectivity. Metal contamination in the chromatographic system, resulting in chelate formation during chromatography, must be minimized. Therefore EDTA (1 mM) was added to the mobile phase in our analysis. The chromatography remained robust over varying EDTA concentrations in the mobile phase (0.5–2 mM) and to laboratory temperature variations ($25 \pm 3\,^{\circ}C$). To optimize separation of CP502 from endogenous plasma compounds a gradient strategy was used as described in Table 1. Chromatograms of blank plasma, and spiked plasma (containing 1 μg/ml of CP502 and 10 μg/ml of HETh (IS)) are presented in Fig. 2A and B. The same chromatographic conditions were suitable for CP502 analysis in urine. Chromatograms of blank urine, and spiked urine (containing 5 μg/ml of CP502 and 10 μg/ml of HETh (IS)) are given in Fig. 3A and B. Chromatograms of blank feces and feces spiked with 0.25 μg/ml of CP502 are given in Fig. 4A and B. Added DFO, to remove fecal iron, did not interfere with HPOs.

HETh was chosen as IS for the following reasons: it is not an endogenous compound does not possess iron-chelation properties, and the DAD spectra are

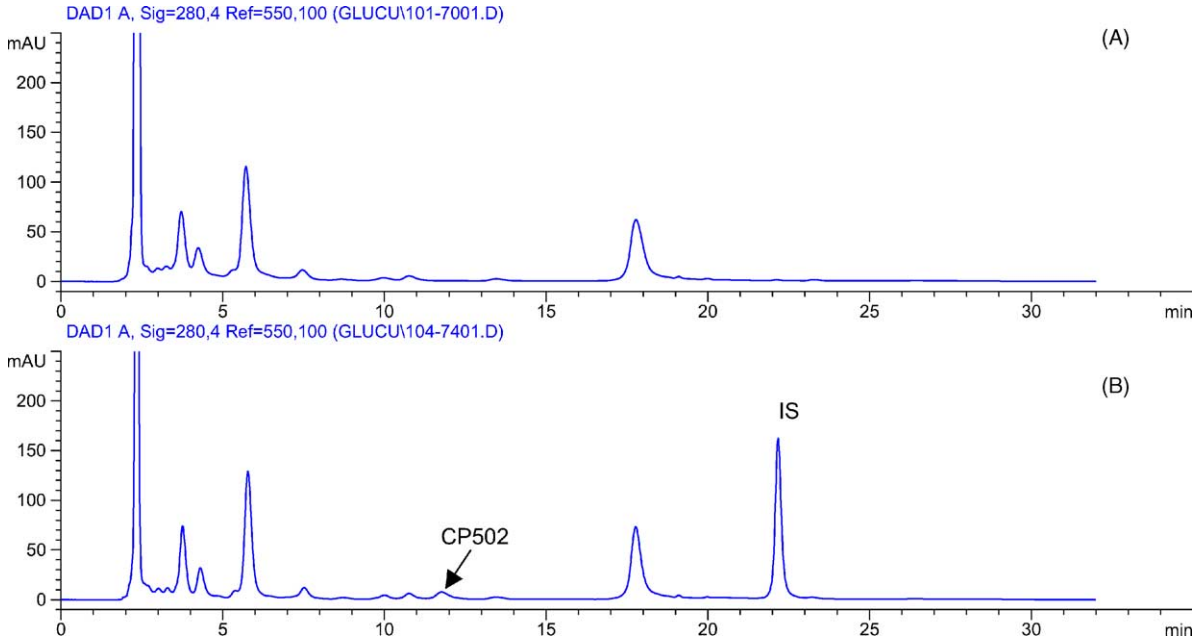

Fig. 2. HPLC chromatograms of blank plasma (A); and plasma spiked with 1 μg/ml of CP502 and 10 μg/ml of IS (B).

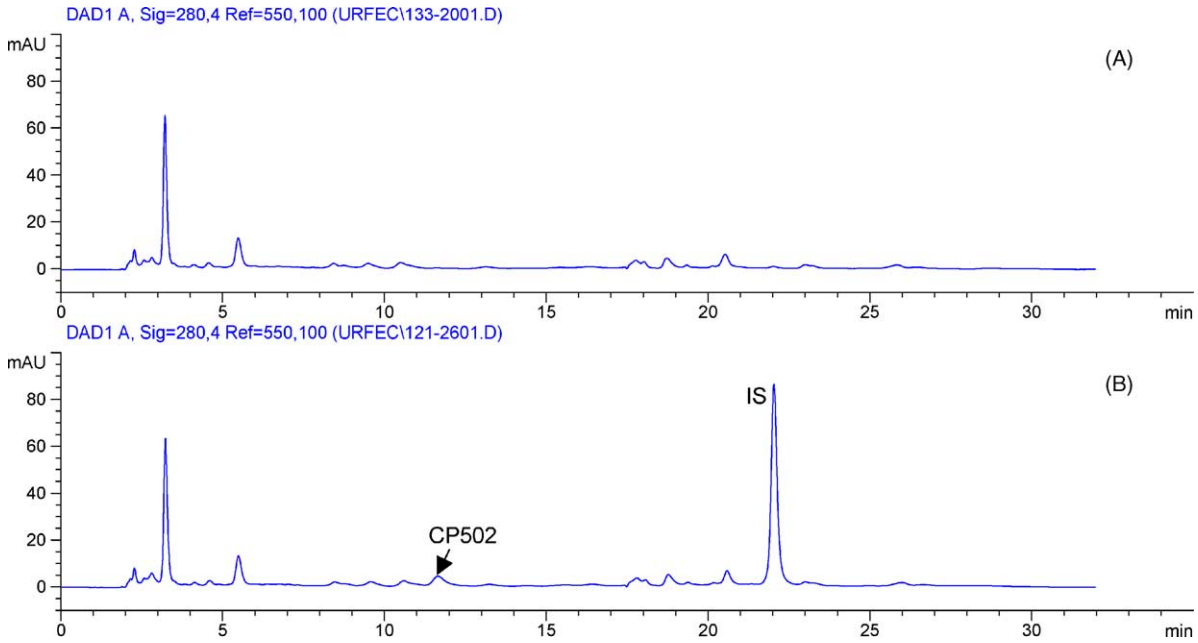

Fig. 3. HPLC chromatograms of blank urine (A); and urine spiked with CP502 (0.5 μg/ml) and HETh (10 μg/ml) (B).

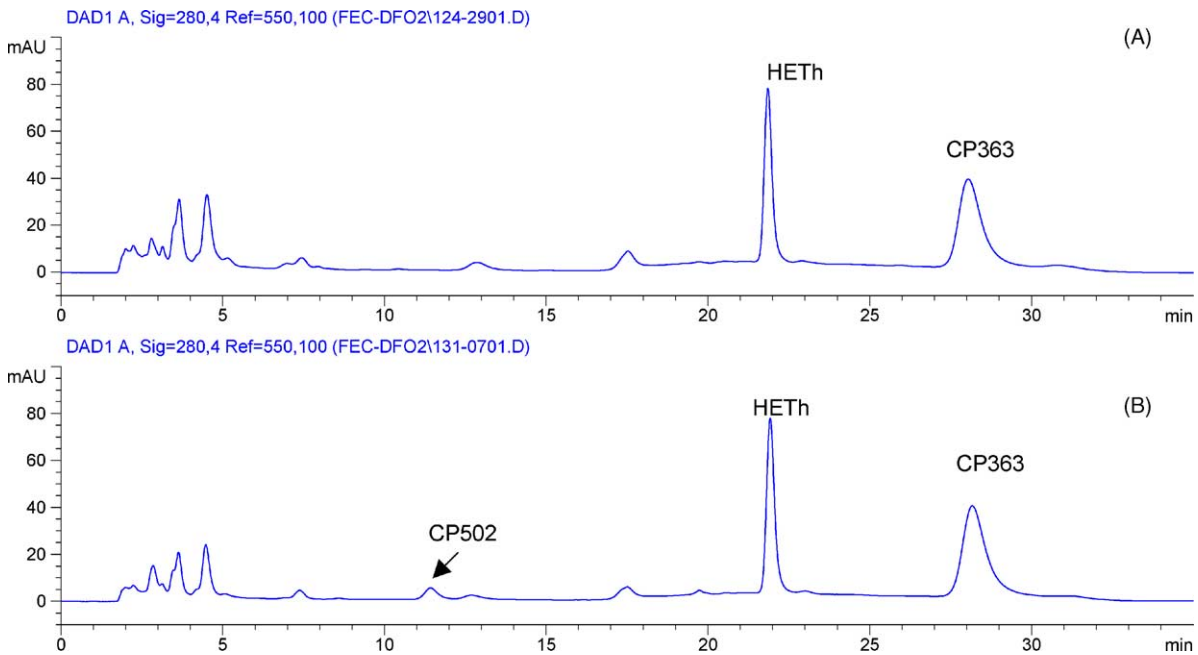

Fig. 4. HPLC chromatograms of blank feces (A); and feces spiked with HETh and CP363 (internal standards) and CP502 (0.25 μg/ml) (B).

similar to CP502. Detection was performed at 280 nm and the chromatographic quantitation remained robust to small changes of the wavelength (±2 nm). Due to complexity of feces (many endogenous diet-based components) and its extraction procedure, there was a risk that HETh peak might overlap with "unknown" eluting compounds. Therefore CP363, which exhibits much longer chromatographic retention, was chosen as a second internal standard for feces analysis.

3.2. Extraction recoveries of CP502 and HETh

Recoveries of both CP502 and HETh were tested separately in spiked plasma and urine samples (diluted 1:50) at six different concentrations from 0.5 to 50 μg/ml. The recoveries of CP502 in plasma calculated through peak area ratio of the compound in plasma and in water were from 73.19 to 97.53% (mean 85.33%, R.S.D. 9.19%), while the estimated mean recovery value via slope ratio of the calibration plots of the compound in plasma and water, was 84.92%. The recoveries of HETh in plasma calculated through peak area ratio of the compound in plasma and water ranged from 74.54 to 99.56% (mean 87.78%, R.S.D. 9.43%) while recovery estimated via the slope ratio of the calibration plots of the compound in plasma and water was 90.29%.

The recoveries of CP502 in urine calculated through peak area ratio of the compound in urine and in water were from 73.19 to 95.53% (mean 84.65%, R.S.D. 9.24%), while recovery calculated via slope ratio of the calibration plots of the compound in urine and water was 95.57%. The recoveries of HETh in urine calculated through peak area ratio of the compound in urine and water were from 74.54 to 105.94% (mean 94.72%, R.S.D. 12.25%) while recovery calculated via slope ratio of the calibration plots of the compound in urine and water was 94.62%.

The recovery of CP502 in feces at concentrations of 0.5, 1.5 and 4.0 μg/ml was 95.98% (R.D.S. 8.8%).

3.3. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

3.3.1. Plasma and urine

A linear dependence of CP502 and IS peak area ratio on CP502 concentration was tested in water, spiked plasma and spiked urine samples (diluted 1:50) from 0.1 to 80 μg/ml. The parameters of linear regression ($y = mx + b$), calculated by least squares methods, for water, plasma and urine samples are shown in Table 2. An on-column injection of 0.01 μg was determined as the LOQ based on a signal-to-noise ratio (S/N) > 10 and R.S.D. of noise <20%. LOD in plasma and urine (non-diluted) based on S/N > 3 was 0.1 and 5.0 μg/ml.

3.3.2. Feces

A linear dependence of CP502 and IS peak area ratio on CP502 concentration was tested from 0.25 to 50 μg/ml. The parameters of linear regression, calculated by least squares methods using HETh, are also presented in Table 2. An on-column injection of 0.01 μg was determined as the LOD based on signal-to-noise ratio (S/N) > 3 and R.S.D. of noise <20%. LOQ in feces based on S/N > 10 was 0.25 μg/ml (5.0 μg/ml for feces sample before dilution (1:20)).

Calibration parameters for water, plasma, urine and feces samples spiked with CP502 are presented in Table 2.

3.4. Inter-day reproducibility and sample stability

Inter-day reproducibility and sample stability were determined within a period of five consecutive days (from Monday to Friday) in the spiked plasma stored at 4 °C and aqueous solution stored at 25 °C. CP502 concentrations in the spiked plasma and aqueous solutions were 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 30, and 50 μg/ml ($n = 10$). The stability of the samples was estimated

Table 2
Summarized calibration parameters for water, plasma, urine and feces samples spiked with CP502

Parameter	Water	Plasma	Urine	Feces
Range (μg/ml)	0.1–80	0.1–80	0.1–80	0.25–50
Slope (m)	0.1259 ± 0.0021	0.1392 ± 0.0027	0.1302 ± 0.0032	0.2308 ± 0.0031
Intercept (b)	0.05295 ± 0.06995	0.07354 ± 0.08787	0.1402 ± 0.1063	−0.122 ± 0.0635
Pearson correlation (r^2)	0.9974	0.9967	0.9945	0.991

by testing the slope of the calibration regression line. The slope ratio following linear regression of working solutions in water stored at 25 °C indicated a decrease in CP502 peak area by about 5%. No change in the linear regression line slope was observed for spiked plasma stored at 4 °C. No degradation products were detected during this period under the aforementioned chromatographic conditions. To assess the sample stability, two sets of data were compared, namely, the data obtained on days 1 and 5. The statistical evaluation of the inter-day reproducibility was assessed by a one-tailed paired *t*-test [7]. The observed *t* values for the series stored at 25 and 4 °C (2.821 and 0.572, respectively) indicate sample stability at 4 °C (critical $t = 1.833$ at $P = 0.05$).

3.5. System suitability

To test the method's repeatability, a 100 μl aliquot solution of CP502 (5 μg/ml) and HETh (10 μg/ml) was injected six times during a single day. R.S.D. of CP502 peak RT and area were 0.34 and 0.89%, while R.S.D. of HETh peak RT and area were 0.02 and 0.64%. The peak symmetry measured at 5% of the peak height was 0.52 for CP502 and 0.66 for HETh. The resolution of the two peaks was >10. The minimal column theoretical plates number for CP502 was 1200.

3.6. Applicability of the method in rat study

Chromatograms of rat plasma taken 60 min after the CP502 i.v. (150 mg/kg) are presented in Fig. 5A. Chromatograms of rat urine collected 24 after the same CP502 oral dose are presented in Fig. 5B. An additional peak at about 4 min was suspected to be a metabolite of CP502. To test whether this component might be a glucuronide, the urine samples were enzymatically hydrolyzed (16 h at 37 °C) with type IX-A β-glucuronidase (1400 units in 0.1 ml). Chromatograms of the urine sample incubated overnight at 37 °C with the enzyme, are given in Fig. 5C. Furthermore, the decrease in the suspected glucuronide peak was accompanied by an increase in the CP502 peak. Similar observations were made when the above analytical procedure was applied to feces samples. Thereby, our urinary and fecal findings support a role for glucuronidation in the overall disposition of CP502.

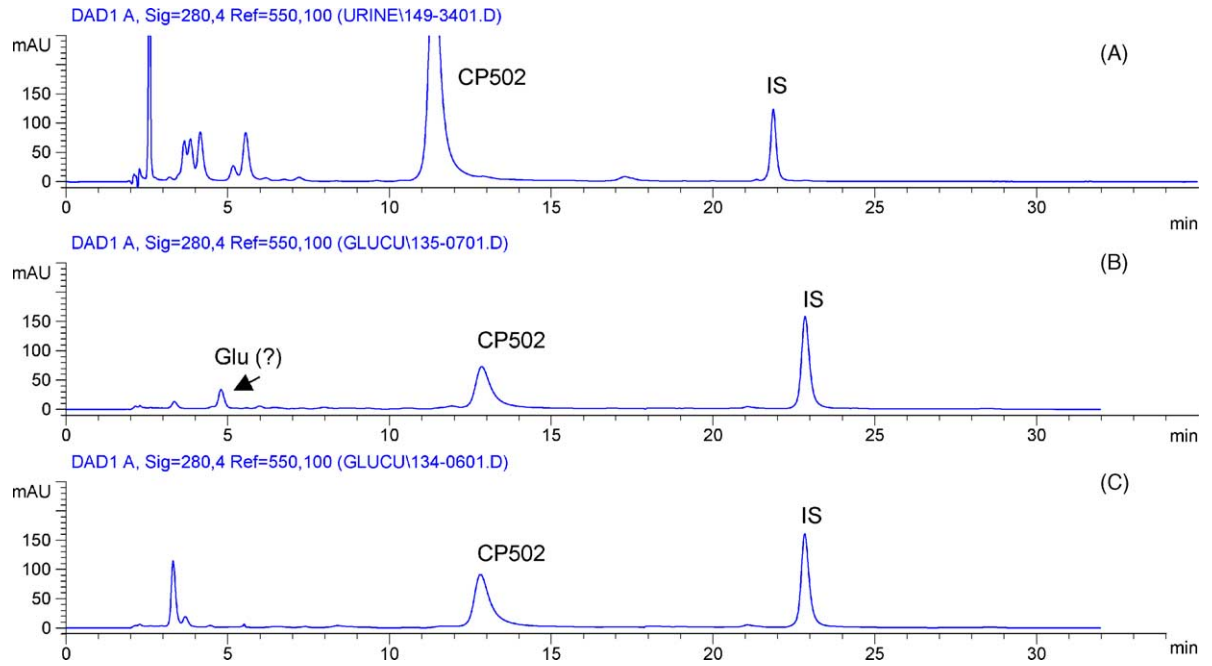

Fig. 5. Chromatograms of rat plasma collected 30 min after the CP502 i.v. dose (A); and rat urine, collected 24 h after the CP502 oral dose, before (B); and after incubation overnight with β-glucuronidase (C).

4. Conclusions

An improved, selective and sensitive RP-HPLC method for the determination of CP502 in rat plasma, urine and feces is reported herein. Our methodology, applied to urine and feces, has also uncovered CP502 glucuronidation. The analytical method is applicable to CP502 bioavailability and pharmacokinetic studies. A further communication will report in detail about these CP502 properties.

Acknowledgements

J.N. thanks the University of Toronto and Apotex Inc. for a postdoctoral fellowship.

References

[1] Z.D. Liu, R. Kayyali, R.C. Hider, J.B. Porter, A.E. Theobald, J. Med. Chem. 45 (2002) 631.
[2] Z.D. Liu, S. Piyamongkol, D.Y. Liu, H.H. Khodr, S.L. Lu, R.C. Hider, Bioorg. Med. Chem. 9 (2001) 563.
[3] R.O. Epemolu, S. Singh, R.C. Hider, L.A. Damani, J. Chromatogr. 519 (1990) 171.
[4] T.L. Skinner, K.A. Meurer, R.A. Yokel, J. Chromatogr. Sci. 34 (1996) 52.
[5] D.Y. Liu, Z.D. Liu, S.L. Lu, R.C. Hider, J. Chromatogr. B 730 (1999) 135.
[6] F. Guo, J.J. Thiessen, A. Tesoro, M. Spino, J. Chromatogr. B 751 (2001) 107.
[7] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, Ellis Horwood, Chichester, UK, 1992, p. 58.