Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 838 (2006) 107–112
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

HPLC determination of a novel aroylhydrazone iron chelator (*o*-108) in rabbit plasma and its application to a pilot pharmacokinetic study

Petra Kovaříková [a,*], Jiří Klimeš [a], Martin Štěrba [b], Olga Popelová [b], Vladimír Geršl [b], Přemysl Poňka [c]

[a] *Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic*
[b] *Department of Pharmacology, Faculty of Medicine in Hradec Králové, Šimkova 870, 500 05 Hradec Králové, Czech Republic*
[c] *Lady Davis Institute for Medical Research, McGill University, Montreal, PQ, Canada H3A 2T5*

Received 13 February 2006; accepted 14 April 2006
Available online 24 May 2006

Abstract

A high performance liquid chromatographic method for the determination of a biocompatible iron chelator, pyridoxal 2-chlorobenzoyl hydrazone (*o*-108), in rabbit plasma was developed and validated. The separation was achieved on a C18 column with the mobile phase composed of a mixture of 0.01 M phosphate buffer (pH 6) with the addition of EDTA (2 mM), methanol and acetonitrile (42:24:14; v/v/v). The method was validated with respect to selectivity, linearity (0.8–150 μg/mL), intra- and inter-day variability and stability. This method was successfully applied to the analysis of the samples obtained from a pilot pharmacokinetic experiment, in which the chelator was administered intravenously to rabbits.

Keywords: Pyridoxal 2-chlorobenzoyl hydrazone; *o*-108; Iron chelator; HPLC; Pharmacokinetics

1. Introduction

Iron chelation therapy represents the only effective principle of how to reduce iron burden in patients suffering from chronic iron overload [1]. Furthermore, recent information strongly suggests that free iron plays an important role in a number of human pathologies [2]. Thus, the principle of selective iron chelation represents a unique approach for a novel drug development. To date, iron chelators have been reported to demonstrate antioxidative, antiproliferative, antimicrobial, cardioprotective and neuroprotective effects [3–9].

Pyridoxal 2-chlorobenzoyl hydrazone (Fig. 1) is a selective iron chelator belonging to the group of pyridoxal isonicotinoyl hydrazone (PIH) analogues. *Ortho*-108 is a yellowish, crystalline substance which can be readily prepared by Schiff-base condensation from pyridoxal and 2-chlorobenzoic hydrazide [10]. Since this chelator is more liphophilic than its lead compound (PIH), it can reach the intracellular targets more effectively. Furthermore, *o*-108 is more effective in the mobilization of iron from reticulocytes and K506 cells [11,12]. *Ortho*-halogenated analogues of PIH were shown to be less toxic than the *meta* and *para* substituted ones [12]. A recent study has shown good tolerability and safety of *o*-108 after weekly repeated administration to rabbits [13]. Oral and intraperitoneal administration of *o*-108 has induced significantly dose-dependent cumulative excretion of radiolabelled iron in a model of the iron-overloaded rat [14].

Although *o*-108 seems to have promising pharmacodynamic properties, there is no precise and selective analytical method suitable for the determination of *o*-108 or another pyridoxal benzoyl hydrazone derivative in biological material. From that point, it is clear why bioavailability, half-life of elimination and other basic pharmacokinetic parameters of these compounds remain undetermined. While nearly hundreds of aroylhydrazones have been synthesized and successfully tested for biological activity, there is only our recent analytical contribution which reports about the development of a HPLC technique suitable for the determination of a structurally related agent—salicylaldehyde isonicotinoyl hydrazone (SIH) in plasma [15]. However, chromatographic conditions for the analysis of purity and stability of

* Corresponding author. Tel.: +420 495 067 111; fax: +420 49 55 124 23.
E-mail address: Petra.Kovarikova@faf.cuni.cz (P. Kovaříková).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.04.020

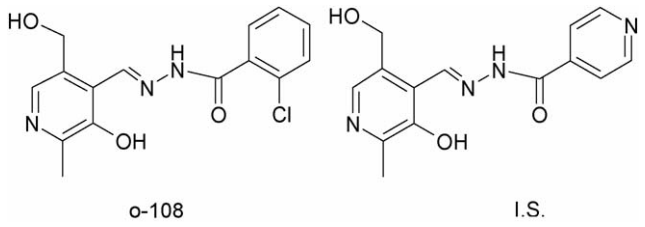

Fig. 1. The chemical structures of *o*-108 and the internal standard (PIH).

o-108 have already been developed in our previously reported paper [16].

The aim of this study was to develop and validate a chromatographic method for the determination of *o*-108 in rabbit plasma and to employ this method in a pilot pharmacokinetic experiment.

2. Experimental

2.1. Materials

Pyridoxal 2-chlorobenzoyl hydrazone (*o*-108) and PIH were prepared according to the procedures described in our previous study [14]. The structure and purity of these compounds were confirmed by FTIR (a Nicolet Impact 400 spectrophotometer, Thermo, Madison, USA), ^{1}H and ^{13}C NMR (a Varian Mercury-Vx BB 300 instrument, Palo Alto, USA, operating at 300 MHz for ^{1}H, 75 MHz for ^{13}C) and HPLC methods [14]. Methanol, acetonitrile, phosphate buffer ($NaH_2PO_4{\cdot}2H_2O$), EDTA, and phosphoric acid were purchased from Lachema (Brno, Czech Republic). Water was purified employing reverse osmosis. Solid phase extraction (SPE) tubes—Discovery® DSC-C8 1 mL/100 mg and a vacuum SPE manifold were obtained from Sigma Aldrich (Munich, Germany). Drug-free rabbit plasma was obtained either from the ZOO Servis—(Dvur Kralove, Czech Republic) or from the Faculty of Medicine in Hradec Králové (Hradec Králové, Czech Republic).

2.2. Chromatographic system and conditions

The chromatographic system LC 20A (Shimadzu, Duisburg, Germany) consisted of a DGU-20A3 degasser, LC-20 AD pumps, a SIL-20 AC autosampler, a CTO-20AC column oven, a SPD-20AC UV–vis detector, and a CBM-20AC communication module was used in this study. The separation was achieved on a analytical chromatographic column 250 mm × 4.6 mm (LiChrospher 100, RP-18, 5 μm) protected with a guard column (Purospher RP-18, 5 μm) purchased from Merck (Darmstadt, Germany). The column oven was set at 25 °C. The mixture of a phosphate buffer (0.01 M NaH_2PO_4 with addition of appropriate amount of EDTA to obtain a concentration of 2 mM, pH 6.0—adjusted with the use of phosphoric acid), methanol and acetonitrile in a ratio 42:24:14 (v/v/v) was employed as a mobile phase. The flow rate 1.0 mL/min was used for the analyses. The detection was performed at 288 nm, the injection volume was 100 μL and the samples in the autosampler were kept at 15 °C. The chromatographic data were processed employing the LC-solution software, version 2.21 SP1 (Schimadzu, Duisburg, Germany).

2.3. Preparation of the stock and spiking solutions

The stock solutions of *o*-108 and I.S. (9 and 4 mg/mL, respectively) were prepared by dissolving an appropriate amount of the substance in methanol. The spiking solutions of *o*-108 (9.0–0.048 mg/mL) and I.S. (2.5 mg/mL) were obtained by appropriate dilution of the stock solution with methanol. These solutions were used to prepare the calibration and quality control (QC) samples. The stock and spiking solutions were stored at 4 °C for 3 days.

2.4. Calibration and QC samples preparation

The calibration samples were prepared by addition of 10 μL of appropriate spiking solutions to 600 μL of drug-free plasma to get nine different samples. Each sample contained *o*-108 in a defined concentration (150–0.8 μg/mL) and I.S. in a concentration of 40 μg/mL. Before SPE extraction, 10 μL of 10 mM EDTA were added into each sample. The same procedure was used to obtain QC samples. These samples were used for the validation procedure and stability testing proposes.

2.5. Sample preparation and solid-phase extraction procedure

The SPE tubes (C8) were conditioned using 1 mL of methanol, followed by 1 mL of phosphate buffer (pH 7). The plasma samples (calibration, QC and *in vivo* study samples) were added into the SPE tube and passed through using vacuum with suction of 5 mmHg. The column was further washed using 1 mL of phosphate buffer (0.01 M NaH_2PO_4 with addition appropriate amount of EDTA to get a concentration of 2 mM; pH 6.0). Finally, the analytes were eluted using 0.7 mL of methanol. Before HPLC analysis, the samples were diluted using 0.7 mL of phosphate buffer (0.01 M NaH_2PO_4 with 2 mM EDTA, pH 6.0).

2.6. Method validation

2.6.1. Selectivity

Selectivity of the method was assessed by analyses of blank plasma samples obtained from eight rabbits.

2.6.2. Linearity

The linearity of the method was evaluated using calibration samples of nine different concentrations of *o*-108 (150, 105, 83, 40, 33, 17, 4, 1 and 0.8 μg/mL) in the presence of 40 μg/mL of internal standard. The calibration curves were constructed by plotting the peak area ratios (*o*-108/I.S.) to the corresponding concentrations of *o*-108. The accuracy of the calibration curves was tested by a comparison of the back-calculated concentrations and the nominal concentrations for all calibration levels.

2.6.3. Precision, accuracy and the lower limit of quantification (LLOQ)

The QC samples of five different concentrations (150, 83, 29, 1.2 and 0.8 μg/mL) were prepared and analysed according to the procedures described in Sections 2.4 and 2.5. The precision (R.S.D.) and accuracy (percentage of recovery) of these analyses were calculated to obtained intra-day variation. The inter-day variation was calculated from the results of the analyses of the QC samples performed at 3 different days. LLOQ was determined as the concentration which can be evaluated with the precision and accuracy of at least 20% and 80–120%, respectively. Furthermore, the analyte response at this level should be at least five-times higher than the response of the blank sample [17].

2.6.4. Recovery of the extraction procedure

The recovery of the extraction procedure was evaluated using the QC samples at three different concentrations of *o*-108 (150, 40 and 0.8 μg/mL) and one concentration of I.S. (40 μg/mL). The recovery was calculated by comparing the peak areas of extracted samples and peak areas of unextracted standards.

2.6.5. Stability

The stability of the stock and spiking solutions stored at 4 °C for 3 days was tested. The stability of the samples kept in the autosampler (15 °C) for 24 h was assessed as well. The stability of the chelator in plasma (both freeze–thaw stability and the long-term stability) was tested using the QC samples ($n = 30$) of three different concentrations of *o*-108 (150, 40 and 1 μg/mL). These QC samples were prepared (Section 2.4) and frozen immediately (−80 °C). After 1 h, a half of these samples (five per each concentration) were thawed at room temperature and treated immediately. The rest of the samples were stored at −80 °C for 30 days. After this time period, the samples were thawed at room temperature and analysed instantly. The stability of the chelator in plasma was expressed as the difference between the concentrations of *o*-108 determined in the QC samples analysed immediately after preparation and the QC samples analysed after the stability procedure. The conditions used in the stability study have reflected the situations encountered during the real samples handling, storage, and preparation.

2.7. Pharmacokinetic experiment

The Chinchilla male rabbits ($n = 5$) weighing 3.5–4.5 kg were used in this pilot pharmacokinetic study. The animals were housed under a 12-h light cycle, constant temperature and humidity. The animals had free access to water and a standard laboratory pellet diet. The animals were fasted overnight before entering the pharmacokinetic study. The experiments were performed in accordance with the "Guide for the care and use of laboratory animals" (1996) and were approved by The Ethical Committee of the Medical Faculty in Hradec Králové.

The rabbits were anaesthetized with pentobarbital (Nembutal, 30 mg/kg, i.v.). The left carotid artery was prepared and a PE-cannula was introduced through the artery to the aorta to allow prompt sampling of blood. Due to the limited solubility *o*-108, the solution for intravenous administration was designed using saline and pharmaceutically acceptable co-solvents PEG 300 and ethanol in a ratio of 5:4:1 (v/v/v). The chelator *o*-108 was administered at a dose of 10 mg/kg by a slow (3 min) intravenous injection through a cannula (Neoflon) inserted into the marginal ear vein. Blood sampling (1.5 mL) was performed 5 min before drug administration (blank), during drug administration (1st, 2nd and 3rd min), and subsequently in the selected intervals (through a range of 4–210 min). The volume loss was compensated with sterile saline. The blood was harvested into heparinized tubes (Vacuette) and centrifuged as soon as possible ($2000 \times g$). Plasma was collected and frozen immediately (−80 °C). The non-compartmental pharmacokinetic analysis was performed using Kinetica software, version 4 (Thermo Electron Corporation, Waltham, USA).

3. Results and discussion

3.1. Method development

The chromatographic separation previously developed for the determination of purity of *o*-108 was modified to be suitable for analyses of this chelator in plasma. Due to the similarity in the chemical structure and anticipated closeness in the extraction recovery, PIH was chosen as the internal standard. The composition of the mobile phase was changed in order to reach a sufficient separation of *o*-108 and I.S. in an acceptable run-time and without interferences with possible metabolites of the chelator (pyridoxal and 2-chlorobenzoyl hydrazone).

Different SPE conditions were tested to reach an acceptable and reproducible recovery of both *o*-108 and I.S. At the beginning of the experiment, the effectiveness of the isolation procedure on different SPE columns (C18, C8 and phenyl) was investigated. Since the highest recovery was reached on a C8 column, this sorbent was chosen for further sample clean up procedure optimization. The addition of a small amount of EDTA into the phosphate buffer (used to wash the SPE column) improved the recovery of both *o*-108 and I.S. Two different organic solvents (methanol, acetonitrile or their mixture) were tested to remove the analytes from the SPE columns. In comparison with acetonitrile, methanol gave higher recoveries of both compounds.

3.2. Method validation

3.2.1. Selectivity

The chromatogram of the blank plasma sample is shown in Fig. 2. No interferences were detected at the retention times of either *o*-108 or I.S.

3.2.2. Linearity

The relationship between the peak area ratios (*o*-108/I.S.) and appropriate concentrations was linear over the tested range (0.8–150 μg/mL). The typical equation of the calibration curve ($n = 3$) was: $y = 0.0362x + 0.007$. The correlation coefficient (r^2)

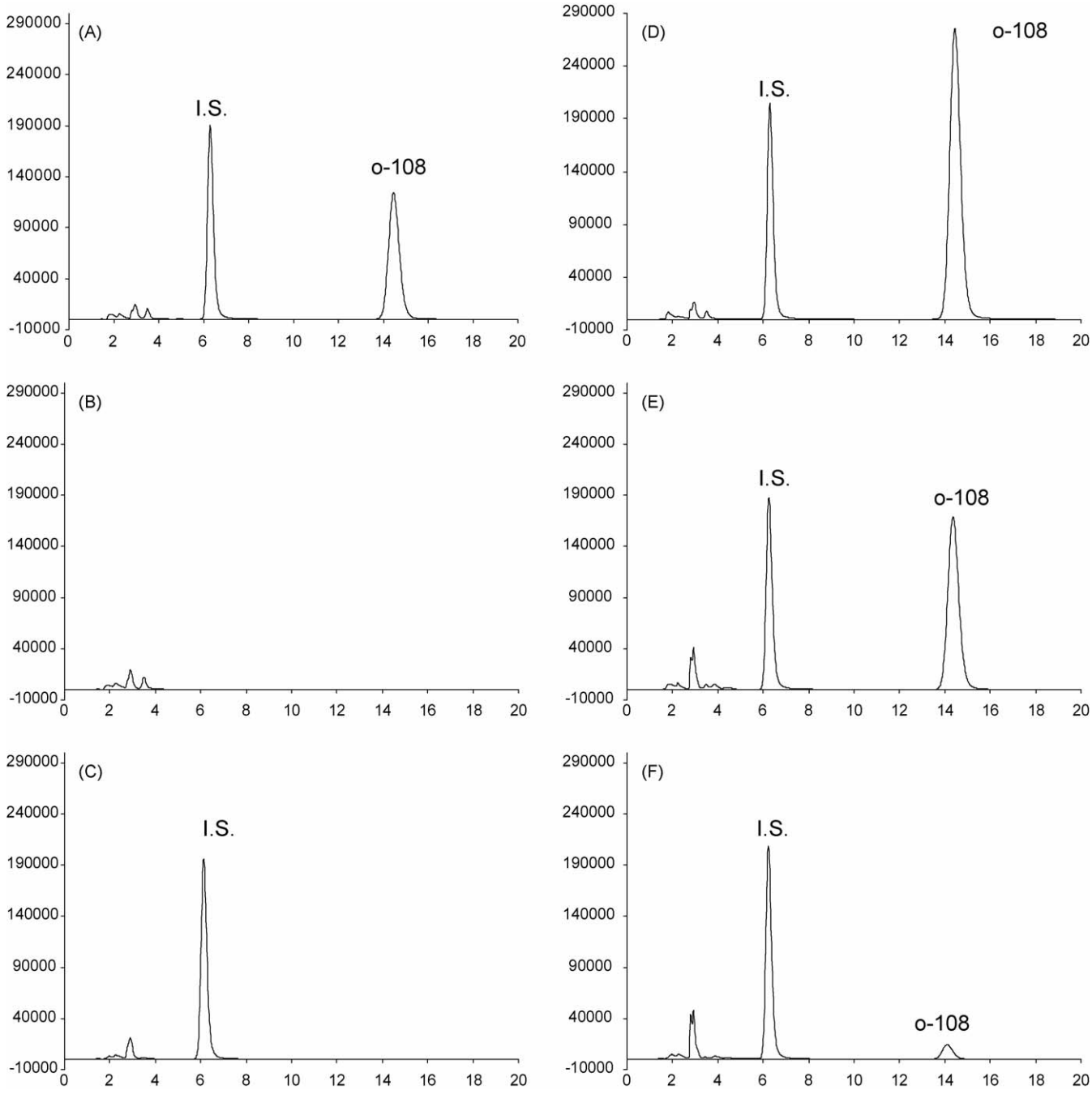

Fig. 2. The chromatograms of extracted plasma samples: (A) spiked samples (40 μg/mL both *o*-108 and I.S); (B) blank sample; (C) spiked sample (40 μg/mL of I.S.); (D) sample taken in the 3th min of the pharmacokinetic experiment (c_{max} = 101 μg/mL); (E) sample taken in the 5th min of the pharmacokinetic experiment (45 μg/mL); (F) sample taken in the 60th min of the *in vivo* experiment (3 μg/mL).

of all calibration curves was ≥0.999. The back-calculated concentrations for all concentration levels are shown in the Table 1. The representative chromatogram of the sample spiked with *o*-108 and I.S. is shown in Fig. 2.

3.2.3. Precision, accuracy and the lower limit of quantification, extraction recovery

The intra- and inter-day precision and accuracy through the concentration range of the QC samples are shown in

Table 1
Summary of the back-calculated concentrations of the calibration curves for *o*-108

Theoretical concentration (μg/mL)	150.0	105.0	83.3	40.0	33.3	17.0	4.17	1.0	0.8
Back-calculated concentration (μg/mL)	146.40	107.56	82.22	39.64	32.78	16.58	3.96	0.90	0.72
Back-calculated concentration (%)	97.60	97.62	98.70	99.10	98.45	97.51	94.90	90.13	90.45

Table 2
The intra-day precision and accuracy of the assay

Concentration added (μg/mL)	Concentration found (μg/mL) ± S.D.	Precision (R.S.D.%)	Accuracy (%)	*n*
Intra-day				
150.0	152.05 ± 1.90	1.25	101.37	5
83.3	80.63 ± 1.51	1.87	96.79	5
29.0	28.30 ± 0.17	0.60	98.59	5
1.2	1.275 ± 0.56	4.39	106.31	5
0.8064	0.8924 ± 0.0174	1.95	110.67	5

Table 3
Inter-day precision and accuracy of the assay

Concentration added (μg/mL)	Concentration found (μg/mL) ± S.D.	Precision (R.S.D.%)	Accuracy (%)	*n*
Inter-day				
150.0	150.56 ± 2.76	1.84	100.38	15
83.3	81.86 ± 4.38	5.36	98.28	15
29.0	28.35 ± 0.457	1.61	98.31	15
1.2	1.2618 ± 0.056	4.43	105.15	15
0.8064	0.8969 ± 0.078	8.71	113.74	15

Tables 2 and 3. The lower limit of quantification was determined to be 0.8 μg/mL. The precision and accuracy of the assay at LLQO are presented in Tables 2 and 3. The results of the validation procedure indicated a satisfying intra- and inter-day variation of the assay according to the given recommendation [17]. The recovery of the SPE procedure is shown in Table 4. Although the extraction recovery was not totally complete, it was consistent, precise and reproducible, which is in line with the FDA Guideline [16].

3.2.4. Stability

The stock and spiking solutions were stable under the stored conditions. The samples maintained in the autosampler at 15 °C were stable during the time of the study (24 h). The results of the freeze–thaw and long-term stability study are shown in Table 5. These results indicated that the stability of the chelator was within an acceptable range (±15% deviation from the nominal concentration of the analyte) [17].

3.2.5. Pharmacokinetic experiment

In a pilot pharmacokinetic experiment, the plasma concentration–time profile of the novel iron chelator *o*-108 after intravenous administration was determined. After injection of the chelator in a dose of 10 mg/kg BW, the following concentration–time profile was obtained (Fig. 3). Relatively fast distribution and elimination were observed. The maximal plasma concentrations of *o*-108 on the molar base were surprisingly higher than those previously obtained for its analogue SIH [16]. The basic pharmacokinetic parameters of *o*-108 obtained through the non-compartmental pharmacokinetic analysis are shown in Table 6. The representative chromatograms of the plasma samples taken in the different time period of the pharmacokinetic experiment are shown in the Fig. 2.

Table 4
The effectiveness of the SPE procedure for isolation of *o*-108 from rabbit plasma

Analyte	Concentration added (μg/mL)	Recovery (%) ± S.D.	R.S.D. (%)	*n*
Extraction recovery				
o-108	150.0	86.30 ± 3.18	3.68	3
	29.0	83.89 ± 1.35	1.61	3
	0.8064	83.19 ± 2.45	2.95	3
I.S.	40	78.80 ± 2.46	3.13	6

Table 5
The stability of *o*-108 in plasma after one freeze–thaw cycle and after storage at −80 °C for 30 days

Concentration added (μg/mL)	Percentage remaining	R.S.D. (%)	*n*
Stability after freeze–thaw procedure			
150.0	98.28	4.49	3
40.0	94.63	1.15	3
1.0	96.16	8.56	3
Long-term stability			
150.0	91.08	0.72	3
40.0	94.90	0.71	3
1.0	94.94	5.30	3

The results are expressed as the percentage of the concentration remaining at the end of the stability study.

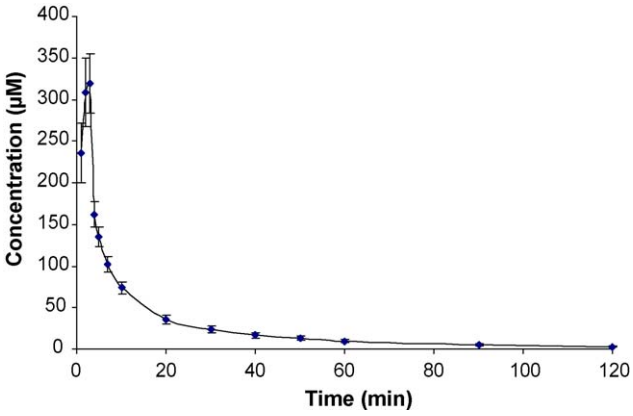

Fig. 3. The plasma concentration–time profile (mean ± S.E.M.) after i.v. administration of the chelator (*o*-108, 10 mg/kg) to rabbits ($n = 5$).

Table 6
The basic pharmacokinetic parameters of *o*-108 obtained through non-compartmental pharmacokinetic analysis

Parameter	*o*-108 (10 mg/kg, i.v.)
c_{max} (μmol/L)	236.2 ± 35.8
t_{max} (min)	2.4 ± 0.4
V_z (L/kg)	0.352 ± 0.028
Cl_{tot} (L/min kg^{-1})	0.0092 ± 0.0009
AUC (μmol/L min^{-1})	3598 ± 454.3
$t_{1/2}$ (min)	27.7 ± 2.98

c_{max}: maximal concentration determined in the study; t_{max}: time in which c_{max} was determined; V_z: apparent volume of distribution; Cl_{tot}: total clearence; AUC: area under curve of concentration–time profile; $t_{1/2}$: elimination half-life.

4. Conclusion

In this study, for the first time a simple, precise and accurate HPLC method suitable for the determination of *o*-108 in plasma was developed. Prior to HPLC analysis, the SPE procedure was used to effectively isolate the chelator from rabbit plasma. The developed analytical methodology was validated with respect to selectivity, linearity, precision, accuracy, and stability. All validation parameters were within acceptable limits and in line with the guidelines. The applicability of the method was confirmed by the analysis of the plasma sample obtained from a pharmacokinetic experiment. LLOQ of the method allowed to follow a concentration–time profile up to the120th min after i.v. administration of the chelator to rabbits. This study provides the first information about the plasma concentration–time profile and basic pharmacokinetic parameters of the novel aroylhydrazone chelator *o*-108 in rabbits.

Acknowledgement

This study was supported by the research projects MSM0021620822 and MSM0021620820.

References

[1] D.S. Kalinowski, D.R. Richardson, Pharmacol. Rev. 57 (2005) 547.
[2] T.F. Tham, R. Lejny-Toung, W. Li, Y. Wang, K. Karimian, M. Spino, Curr. Med. Chem. 10 (2003) 983.
[3] T. Simunek, C. Boer, R.A. Bouwman, R. Vlasblom, A.M.A.M. Versteilen, M. Štěrba, V. Geršl, R. Hrdina, P. Poňka, J.J. de Lange, W.J. Paulus, R.J. Musters, J. Mol. Cell. Cardiol. 39 (2005) 345.
[4] T.B. Chaston, R.N. Watts, J. Yuan, D.R. Richardson, Clin. Cancer Res. 10 (2004) 7365.
[5] E.E. Voest, G. Vreugdenhil, J.J.M. Marx, Ann. Intern. Med. 120 (1994) 490.
[6] J.L. Buss, F.M. Torti, S.V. Torti, Curr. Med. Chem. 10 (2003) 1021.
[7] S.M. Swain, P. Vici, J. Cancer Res. Clin. Oncol. 130 (2004) 1.
[8] Z.I. Cabantchik, H. Glickstein, J. Golenser, M. Loyevsky, A. Tsafack, Acta Haematol. 95 (1996) 70.
[9] D.R. Richardson, Ann. N.Y. Acad. Sci. 1012 (2004) 326.
[10] J.L. Buss, M. Hermes-Lima, P. Poňka, Adv. Exp. Med. Biol. 509 (2002) 205.
[11] J.L. Buss, E. Arduini, P. Poňka, Biochem. Pharmacol. 64 (2002) 1689.
[12] J.L. Buss, E. Arduini, K.C. Shepard, P. Poňka, Biochem. Pharmacol. 65 (2003) 349.
[13] M. Štěrba, T. Simunek, Y. Mazurova, O. Popelová, J. Kaplanova, P. Poňka, V. Geršl, Hum. Exp. Toxicol. 24 (2005) 1.
[14] G. Link, P. Poňka, A.M. Konin, W. Breuer, Z.I. Cabantchik, C. Hersko, Blood 101 (2003) 4172.
[15] P. Kovaříková, M. Mokry, J. Klimeš, K. Vavrova, J. Sep. Sci. 27 (2004) 1503.
[16] P. Kovaříková, J. Klimeš, M. Štěrba, O. Popelová, M. Mokry, V. Geršl, P. Poňka, J. Sep. Sci. 28 (2005) 1300.
[17] CDER and CVM, Bioanalytical Method Validation, Guidance for Industry, May 2001 (http://www.fda.gov/cder/guidance/index.htm).