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Journal of Chromatography B, 751 (2001) 107–115

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

High-performance liquid chromatographic assays for a second-generation novel oral iron chelator (APCP363) and their application to pharmacokinetic studies in rats

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Received 4 May 2000; received in revised form 10 May 2000; accepted 10 August 2000

Abstract

Sensitive and specific HPLC assays for APCP363 in biological matrices (rat plasma, urine and feces) were developed. The recovery of APCP363 ranged from 81.2 to 99.9% in plasma, from 82.1 to 92.8% in urine, and from 65 to 68% in feces. Standard deviations were below 10% for all analyses. The limits of quantitation were 0.1, 10 and 30 μg/ml in plasma, urine and feces, respectively. The HPLC assays, which are the first reports for APCP363 analysis in biological matrices, have been successfully applied to preliminary pharmacokinetic studies in rats. The stool assay is the first non-radiolabeled method for hydroxypyridinones in feces.

Keywords: Pharmacokinetics; Iron chelator; APCP363

1. Introduction

Patients with severe anemia, such as β-thalassemia major, require regular blood transfusions [1]. Blood transfusions not only improve the anemia but also suppress secondary features related to excessive erythopoiesis. However, regular blood transfusions lead to severe conditions of iron overload in such patients due to their inability to excrete iron produced from the breakdown of hemoglobin [2]. Complications associated with the toxicity of iron after blood transfusion can, to a large extent, be remedied by the use of specific metal scavenging or chelating agents to trap and allow excretion of excess and potentially toxic forms of iron from the body.

Deferoxamine (DFO) and deferiprone (L1) are iron chelators available for the treatment of iron-overload conditions. DFO can only be given parenterally, and requires an infusion regimen, either subcutaneously or intravenously, over 8–12 h for 5–7 days/week [3]. L1, a bidentate hydroxypyridinone compound, is the first generation of oral iron chelators. Clinical trials of L1 showed that it could promote urinary iron excretion comparable to DFO [4]. Analyses of L1 in plasma and urine of rats and man have been reported [5–8]; however, no method, aside from radioactivity assays, has been reported for fecal L1 [9,10].

APCP363 (Fig. 1), also a hydroxypyridinone, is a novel second-generation oral iron chelator. The

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00457-6

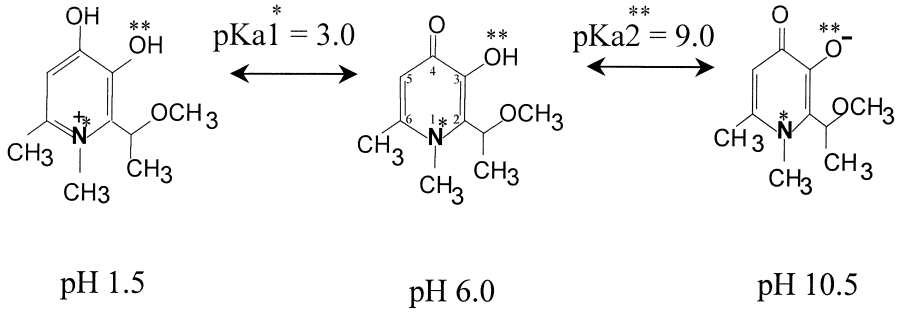

Fig. 1. Chemical structures of APCP363 at different pH values.

stability constant and p*M* of APCP363 are $10^{35.5}$ and 19.89, respectively. In a ferritin iron-loaded rat model, the efficacy of iron removal by APCP363 was 5–10-times that for L1 and DFO [11]. This report describes sensitive and specific methods for determination of APCP363 in plasma, urine, and feces so as to determine its fate in animals and man.

2. Experimental

2.1. Materials

Unless stated otherwise, reagents were analytical grade. APCP363 was provided by Dr. R.C. Hider (King's College, London, UK). Caffeine and 8-chlorotheophylline (internal standards) were obtained from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA), concentrated phosphoric acid, anhydrous dibasic potassium phosphate, NaOH solution (10 *M*), HPLC-grade acetonitrile (ACN) and MeOH, were purchased from Fisher Scientific (Toronto, Canada). Water was purified in the laboratory, via a Milli-Q system (Millipore, Milford, MA, USA). Human blank plasma was obtained from the blood bank of the Hospital for Sick Children (Toronto, Canada). Sprague–Dawley rats (body weight around 0.3 kg) were purchased from Charles River Labs. (Montreal, Canada).

2.2. Preparation of buffer solutions

The pH 1.5 or 2.5 TFA, and pH 10.5 NaOH aqueous buffer solutions were prepared by adjusting the pH of water with TFA and NaOH, respectively. The pH 6.0 buffer solution was prepared by adjusting the pH of 50 m*M* dibasic potassium phosphate solution with concentrated phosphoric acid.

2.3. Preparation of stock solutions, working solutions, and working standards

2.3.1. Plasma analysis

Separate aqueous stock solutions of 1.0 mg/ml APCP363 and 5.0 μg/ml caffeine (internal standard) were prepared. The APCP363 working solutions, with concentrations from 0.1 to 100 μg/ml, were prepared by diluting the stock solution with water. Human plasma spiked with the APCP363 stock solution generated working standards with concentrations from 0.1 to 100 μg/ml.

2.3.2. Urine analysis

Separate aqueous stock solutions of 50.0 mg/ml APCP363 and 50.0 μg/ml 8-chlorotheophylline (internal standard) were prepared. The working solutions with concentrations from 10 to 1000 μg/ml were prepared by diluting the stock solutions with water. Rat blank urine samples were collected, as described in Section 2.6.4, and pooled. The pooled rat urine spiked with the APCP363 stock solution generated the working standards with concentrations from 10 to 1000 μg/ml.

2.3.3. Stool analysis

Separate aqueous stock solutions of 10 mg/ml caffeine (internal standard) and 50 mg/ml APCP363 were prepared. The working solutions with APCP363 concentrations at 30, 240 and 1200 μg/ml were prepared by diluting the stock with water. Blank rat stool samples were collected, as described in Section 2.6.4, ground and pooled. The pooled rat

blank feces spiked with the APCP363 stock solution generated the working standards with concentrations at 30, 240 and 1200 μg/g feces.

2.4. Ultrafiltration

All ultrafiltration was conducted by using Amicon Centrifree (molecular mass cut-off 30 000) at 500 *g* for 20 min.

2.5. Equipment

The Hamilton PRP-1 Peek column (5 μm, 150×4.6 mm) was purchased from Mandel Scientific (Guelph, Canada). The Zorbax Rx-C18 column (5 μm, 150×4.6 mm) was obtained from MAC-MOD Analytical (Chadds Ford, PA, USA). The Amicon Centrifree was obtained from Millipore (Bedford, MA, USA). The Polytron homogenizer and Eppendorf centrifuge were obtained from Brinkmann (Canada) Instruments (Rexdale, Canada). The shaker was purchased from New Brunswick Scientific (Edison, NJ, USA). The Hewlett-Packard 1050 Series high-performance liquid chromatography (HPLC) system (Palo Alto, CA, USA) consisted of a HP 1050 Series pump, a HP 1050 Series autosampler, and a HP 1050 Series UV–visible detector. The HPLC system was controlled by a personal computer employing the HP ChemStation software (Rev. A.06.03).

2.6. Procedures

2.6.1. Optimization of HPLC conditions

In optimizing the chromatographic conditions, the pH of the mobile phase, most suitable column, UV detection wavelength, and organic modifier were explored. Unless otherwise specified, the following standard conditions were used: (a) 1 μl of aqueous APCP363 (100 μg/ml) injected; (b) a Hamilton PRP-1 Peek column (5 μm, 150×4.6 mm); (c) mobile phase composed of 20% ACN and 80% pH 1.5 aqueous TFA at 1.0 ml/min; (d) UV detection at 282 nm; (e) ambient temperature.

2.6.1.1. Effect of pH of the mobile phase. Aliquots of the APCP363 working solution (100 μg/ml) were analyzed by HPLC using: (1) 80% pH 1.5 TFA aqueous solution+20% ACN; (2) 10–80% pH 6.0 phosphate buffer+the complementary percentage of ACN; (3) 80% pH 10.5 sodium hydroxide solution+20% ACN.

2.6.1.2. Effect of column. Aliquots of the APCP363 working solution (100 μg/ml) were analyzed by HPLC using different columns: (1) Hamilton PRP-1 polymer column, and a mobile phase containing 80% pH 1.5 aqueous TFA and 20% ACN; (2) Zorbax Rx-C18 column, and a mobile phase containing 5–80% pH 2.5 aqueous TFA and the complementary percentage of ACN.

2.6.1.3. Effect of UV detection wavelength. A 100-μl volume of 0.1, 10 and 30 μg/ml APCP363 of spiked plasma, urine and feces, respectively, was ultrafiltered using the Amicon Centrifree (molecular mass cut-off 30 000) at 500 *g*, followed by analyses of filtrates by HPLC (50 μl) at 213 and 282 nm. These wavelengths represent the λ_{max} in the absorbance spectrum.

2.6.1.4. Effect of organic modifier. The effect of two organic modifiers, i.e., ACN, MeOH, on selectivity and resolution was investigated for plasma, urine and stool samples. The mobile phase was composed of varied proportions of pH 1.5 TFA aqueous solution and ACN or MeOH. Biological matrices tested were: blank plasma, urine and stool; 4-h plasma, 24-h urine and stool after drug administration. All were collected as described in Section 2.6.4. Samples of plasma, urine and stool homogenates were ultrafiltered, followed by analyses of filtrates by HPLC (50 μl) using the aforementioned different mobile phases.

2.6.2. Recovery of APCP363 from different sample matrices

Unless otherwise specified, the HPLC standard conditions were used.

2.6.2.1. Spiked human plasma. An aliquot of each plasma working standard was ultrafiltered, and 50 μl of the filtrate was analyzed by HPLC. For comparison, 50 μl of each working solution was directly

injected. Five replicate analyses of each working standard and working solution were accessed. In these studies, the mobile phase was composed of 10% ACN and 90% pH 1.5 aqueous TFA.

2.6.2.2. Spiked rat urine. An aliquot of each working standard was prepared, and 100 μl of the filtrate was analyzed by HPLC. For comparison, 100 μl of each working solution was directly injected. Two replicate analyses of each working standard and working solution were conducted. In these studies, the mobile phase was composed of pH 1.5 TFA and ACN; a gradient system with increasing ACN was employed: 2%/0 min; 21%/18 min; 80%/18.5–21 min.

2.6.2.3. Spiked rat feces. A 1-g amount of each working standard (or, spiked feces) was homogenized using 10 ml of different solvents, i.e., water, pH 1.5 water, MeOH and pH 1.5 MeOH, respectively, employing the Polytron homogenizer for 5 min. The homogenates were treated via various ways, i.e., shaking, water bath (45°C), and sonication, respectively, for various length of time, and then the homogenates were centrifuged at 2000 *g* for 30 min. The supernatant was ultrafiltered, followed by HPLC analyses of the filtrates (100 μl). For comparison, 100 μl of each working solution was directly injected. Three replicate analyses were made for working standards and working solutions at each concentration level (μg/g feces). In these studies, the mobile phase was composed of 60% pH 1.5 TFA and 40% MeOH.

Rat stool samples collected (total to 24 h) after oral administration of APCP363 were treated and analyzed as described above.

2.6.3. Calibration curve of APCP363

Mobile phases employed, respectively, for spiked human plasma, rat urine and feces were the same as described above for each biological matrix.

2.6.3.1. Spiked human plasma. A 100-μl volume of each working standard and 10 μl of the internal standard stock solution were mixed thoroughly. An aliquot of the mixture was ultrafiltered, and 50 μl of the filtrate was analyzed by HPLC.

2.6.3.2. Spiked rat urine. The working standards were diluted 10 times using distilled water, then 200 μl of each diluted working standard and 20 μl of the internal standard stock solution were mixed thoroughly. An aliquot of the mixture was ultrafiltered, and 100 μl of the filtrate was analyzed by HPLC. Two replicate analyses were conducted for each working standard.

2.6.3.3. Spiked rat feces. A 1-g amount of each working standard was added with 50 μl of the internal standard stock solution, and homogenized employing 10 ml of pH 1.5 water using the Polytron homogenizer for 5 min. The homogenate was shaken for 0.5 h at 400 rpm using a shaker from New Brunswick Scientific, and then centrifuged at 2000 *g* for 30 min. The supernatant was ultrafiltered, followed by HPLC analysis of the filtrate (100 μl).

2.6.4. Pharmacokinetics of APCP363 in rats

Four days after jugular vein pre-catheterization, Sprague–Dawley rats were given an intravenous (i.v.) bolus of aqueous APCP363 (25 mg/kg; $n=2$). Two additional rats were given a single dose by oral gavage of 250 mg/kg APCP363 dissolved in water. Serial blood samples were collected over 48 h, while urine and feces were collected for 3 days. Heparinized blood was centrifuged to obtain plasma. A 100-μl volume of the plasma fraction and 10 μl of the internal standard stock solution were mixed thoroughly. An aliquot of the mixture was ultrafiltered, and 50 μl of the filtrate was analyzed by HPLC.

Urine samples were diluted 10-fold using distilled water. Then 200 μl of each diluted samples and 20 μl of the internal standard stock solution were mixed thoroughly. An aliquot of the mixture was ultrafiltered, and 50 μl of the filtrate was analyzed by HPLC.

A 1-g amount of a stool sample was added with 50 μl of the internal standard stock solution, and homogenized using 10 ml of pH 1.5 water employing the Polytron homogenizer for 5 min. The homogenate was shaken at 400 rpm for half an hour,

and then centrifuged at 2000 *g* for 30 min. The supernatant was ultrafiltered, followed by HPLC analysis of the filtrate (50 μl).

3. Results and discussion

3.1. Optimization of HPLC conditions

3.1.1. Effect of the mobile phase pH

Attempts to use mobile phases of pH 6.0 or pH 10.5 failed, either because of peak tailing or absent peaks. Only the pH 1.5 mobile phase yielded a sharp and symmetrical chromatographic APCP363 peak.

Based on the two pK_a values of APCP363 (pK_1 about 3.0 and pK_2 about 9.0), APCP363 becomes neutral around pH 6.0, positively charged at pH 1.5, and negatively charged at pH 10.5, as shown in Fig. 1. Strong interaction between the aromatic ring of the neutral drug and the stationary phase of the polymer column [poly(styrene–divinylbenzene)] around pH 6.0 might result in unacceptably long retention times. At pH 10.5, APCP363 contains a lone pair of electrons at positions 3 and 4 which are available for chelation; chelation between APCP363 and metal ions in the HPLC system might conceivably yield a small and severely tailing peak. At pH 1.5, because oxygens at both the 3 and 4 positions of APCP363 are protonated, chelation is avoided; a sharp and symmetrical peak with reasonable retention time is observed at this pH.

We conclude that successful APCP363 chromatography is critically determined by the pH of the HPLC mobile phase; pH 1.5 is optimal.

3.1.2. Column selection

Attempts to use a Zorbax Rx-C18 column to achieve a desirable elution peak of APCP363 failed. Zorbax Rx-C18 (mobile phase of pH 3.0) yielded small, severely tailing peaks, in contrast to sharp and symmetrical peaks obtained with a polystyrene column (mobile phase of pH 1.5).

C_{18} columns contain silanol groups which may form hydrogen bonds with the positively charged APCP363, thereby causing severe tailing of the eluted peak. On the other hand, a Hamilton PRP-1 polymer column yields a desirable APCP363 peak.

3.1.3. Effect of UV detection wavelength

The UV spectrum of APCP363 at pH 1.5 showed two major λ_{max} values, i.e., 213 and 282 nm, with the molar extinction coefficient at 213 nm being 2–3-times that at 282 nm. However, simultaneous HPLC analyses of samples of plasma, urine, and feces containing 0.05 mg/ml APCP363 showed that the signal-to-noise ratio of the APCP363 peaks detected at 282 nm was much higher than that at 213 nm.

Furthermore, plasma, urine and feces, contained various endogenous materials which absorbed strongly at 213 nm, but not 282 nm. Thus, detection at 213 nm exposed interfering peaks, while 282 nm not only avoided interference from endogenous materials but also improved sensitivity.

3.2. Effect of organic modifier and selectivity of HPLC separation

The optimal chromatography produced by the organic modifiers, i.e., ACN and MeOH, was not identical for the three biological matrices. Although ACN and MeOH yielded comparable baseline separation for APCP363 and endogenous materials for both human and rat plasma, as well as rat urine samples, ACN was preferred over MeOH, because it proved to be less viscous and produced lower pump head pressure than MeOH. Figs. 2 and 3 show chromatograms of blank rat plasma and urine samples, and representative rat plasma and urine samples (100 μl), respectively, employing ACN as the organic modifier.

For rat stool samples, co-elution of the drug and endogenous materials was always a problem when ACN was used as the organic modifier, even if the capacity factor was adjusted though varying the proportion of ACN. In contrast, baseline separation for APCP363 and endogenous materials was achieved when MeOH was used as the organic modifier, as shown in Fig. 4.

3.3. Recoveries of APCP363

Table 1 shows the mean recoveries of APCP363 at the five concentrations tested in human plasma. Recovery of APCP363 over the quoted concentration

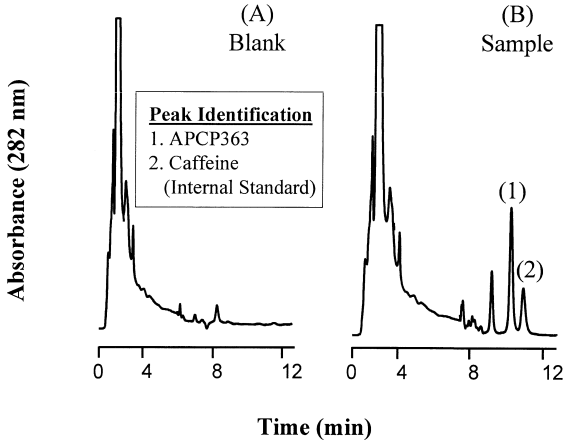

Fig. 2. Chromatograms of (A) plasma blank, (B) a 50-μl filtrate of the rat plasma sample obtained 4 h after i.v. 25 mg/kg APCP363 administration.

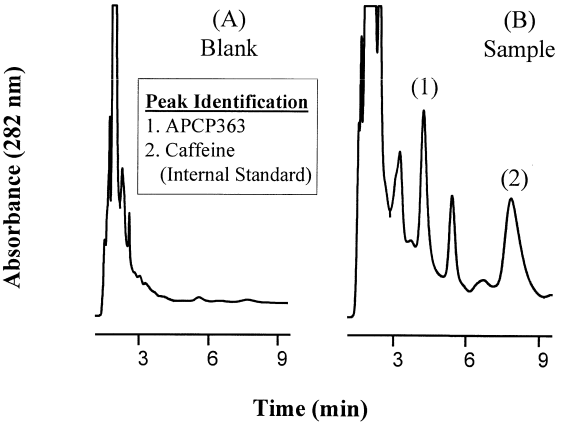

Fig. 4. Chromatograms of (A) rat stool blank, (B) a 50-μl of the homogenate filtrate of the bulk rat stool obtained 0 to 24 h after 250 mg/kg oral APCP363 administration.

range spanned from 81.2 to 99.9%. Precision (relative standard deviation, RSD) varied from 4.2 to 9.4%.

Table 1 also shows the mean recoveries of APCP363 at the five concentrations tested in rat urine. Recovery of APCP363 over the quoted concentration range spanned from 82.1 to 92.8%. Precision (RSD) varied from 4.8 to 9.4%.

Rat stool presented a challenge because APCP363 was either physically adsorbed on feces, or chemically bonded to fecal endogenous substances. As shown in Table 2, there was little difference in the recovery of APCP363 from spiked feces or rat 24-h stool samples, whether water or MeOH was used to achieve a so-called extraction. In contrast, pH seemed to play an important role in the extraction of the drug. Using pH 1.5 water or acidified MeOH (pH 1.5) yielded much higher recoveries than non-acidified solvents. However, there appeared to be no difference between pH 1.5 water and acidified MeOH (pH 1.5).

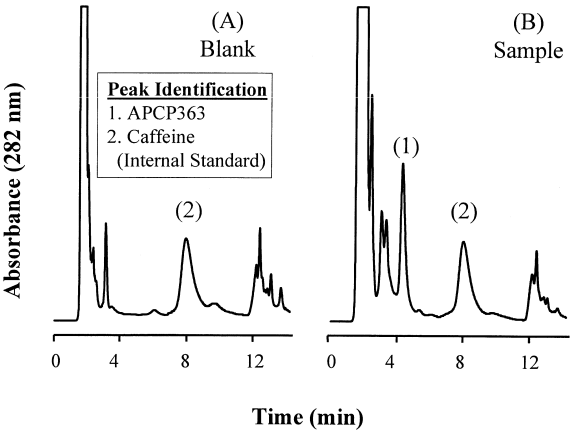

Fig. 3. Chromatograms of (A) urine blank containing internal standard caffeine, (B) 50 μl filtrate of the diluted bulk urine sample obtained 0 to 24 h after 250 mg/kg oral APCP363 administration.

We speculate that at the normal pH of rat feces (pH 6.0), the uncharged drug seems to attach to the stool matrix more tightly and yields a lower recovery. Furthermore, non-polar substances in feces, such as steroid and fatty acids, might contribute to the strong affinity of the neutral drug to the stool matrix and lower recovery at neutral pH. At pH 1.5, the drug becomes positively charged and more readily dissolves in the extraction solvent.

Our recovery studies also concluded that different extraction procedures, i.e., shaking, water bath at 45°C, and sonication, showed no difference in recovery of APCP363, as also shown in Table 2. Shaking longer than 30 min did not further enhance the extraction efficiency.

In summary, the optimized extraction conditions are the following: pH 1.5 water will be used as the extraction solvent, and the homogenized stool will be shaken for half an hour at room temperature.

Table 1 also shows the mean recoveries of

Table 1
Mean recoveries of APCP363 from human plasma ($n=5$), rat urine ($n=2$), and rat feces ($n=3$)[a]

Plasma			Urine			Feces		
Concentration (μg/ml)	Recovery (%, mean±SD)	RSD (%)	Concentration (μg/ml)	Recovery (%, mean±SD)	RSD (%)	Concentration (μg/ml)	Recovery (%, mean±SD)	RSD (%)
0.25	87.2±5.4	5.4	10	82.1±7.7	9.4	3	64.5±3.4	5.3
0.5	99.9±4.2	4.2	50	86.5±7.7	8.9	24	63.7±2.2	3.5
1.0	81.2±6.6	8.2	100	87.8±7.6	8.7	120	68.1±1.7	2.5
5.0	84.2±6.8	8.1	500	92.8±6.7	7.2			
10.0	85.9±8.1	9.4	1000	90.3±4.3	4.8			

[a] The table shows recoveries (%), mean and standard deviations (mean±SDs) and relative standard deviation (RSDs, %).

APCP363 at the three concentrations tested under the aforementioned optimized conditions. Recovery of APCP363 over the quoted concentration range spanned from 63.7 to 68.1%. Precision (RSD) varied from 2.5 to 5.3%.

3.4. Calibration curve of APCP363

The calibration curve of the working standard for APCP363 was constructed from the least-squares linear regression of peak-height ratios of the drug to internal standard versus concentrations of the compound. The linear range of APCP363 analysis spanned from 0.1 to 100 μg/ml [$n=5$: $y=0.5391$ (±0.0670)x, $r^2=0.9933$ (±0.0031)] in human plasma, 10 to 1000 μg/ml [$n=3$: $y=0.0109$ (±0.0027)$x+0.0176$ (±0.0071), $r^2=0.9925$ (±0.0043)] in urine, and 29.5 to 1178 μg/ml [$n=2$: $y=0.01135$ (±0.0014)$x+0.0262$ (±0.0015), $r^2=0.9984$ (±0.0022)] in feces.

The calibration curve constructed from spiked human plasma was used for the analysis of rat plasma samples. The human blank plasma showed a chromatogram similar to the rat blank plasma, and the HPLC method yielded baseline separation for APCP363, the internal standard (caffeine), and endogenous materials from both spiked human and rat plasma.

3.5. Limit of quantitation

Based on a signal-to-noise ratio ($S/N=10$) (RSD<20%), the limit of quantitations (LOQs) of APCP363 were 0.1 μg/ml in plasma, 10 μg/ml in urine and 29.5 μg/ml in feces. The precision and accuracy of the present methods at the limit of quantification are within the recommended value of ±20% for a valid analytical method used in bioavailability and pharmacokinetic studies.

3.6. Pharmacokinetics of APCP363 in the rat

Fig. 5 shows the plasma APCP363 concentration–time profiles in the four rats. Since APCP363

Table 2
Effect of various work-up conditions on the recovery of APCP363 from spiked feces ($n=3$) and a rat fecal sample ($n=2$)

Condition	Solvent 1[a], pH 1.5 water	Solvent 2[a], pH 1.5 MeOH	Solvent 3[a], MeOH	Shaking[b]	Water bath[b], 45°C	Sonication[b]
Spiked feces[c]	68.1±1.7	65.4±1.5	37.5±2.2	68.1±1.7	69.2±1.4	62.9±1.9
RSD (%)	2.5	2.3	5.9	2.5	2.0	3.0
Fecal sample[d]	4.4±0.3	4.9±0.4	2.8±0.1	4.4±0.3	4.2±0.2	4.1±0.3
RSD (%)	6.8	7.2	5.1	6.8	4.7	8.2

[a] The fecal homogenates were treated via shaking for half an hour.
[b] pH 1.5 water was used as the extracting solvent.
[c] Recoveries of spiked feces (120 μg/ml), mean and standard deviations (mean±SDs) and relative standard deviations (RSDs, %).
[d] The amount of APCP363 (mg) recovered from a rat fecal sample, mean and standard deviations (mean±SDs) and relative standard deviations (RSDs, %).

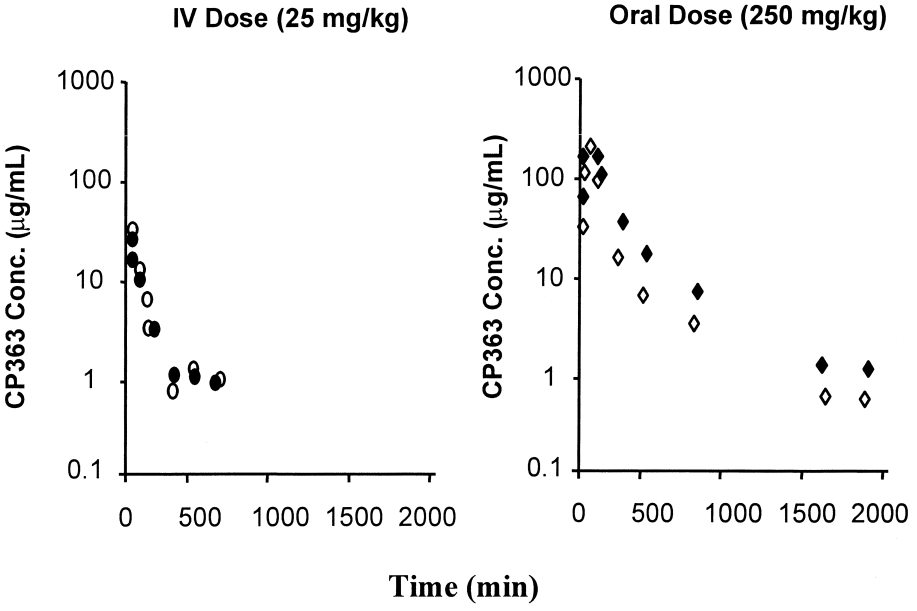

Fig. 5. Preliminary pharmacokinetic studies of APCP363 in the rat. The left panel shows two rats given 25 mg/kg i.v.; the right panel shows two rats given 250 mg/kg orally.

concentrations during the initial 2-h sampling period after oral doses were above 100 μg/ml, which is the upper limit of the linear range, a dilution factor of two was adopted for such samples.

This information constitutes the first description of APCP363 pharmacokinetics in the rat. We have observed that, in contrast to the larger oral dose, the i.v. profiles suggest a comparatively short APCP363 half-life. Such an observation may imply analytical limitations (LOQ) when comparatively small doses are administered. This, and other possible explanations, are under investigation in a detailed pharmacokinetic study.

The 48-h recovery of unchanged APCP363 in urine and feces is shown in Table 3. The majority of the unchanged drug was excreted in the first day in both urine and feces, while the excretion during the second day only accounted for one tenth of the first day. The total urinary and fecal recovery of APCP363 in 2 days accounted for about 10 and 6% of the administered dose, respectively. The fate of the remaining dose likely comprises metabolized products. This facet of the drug's disposition is under current investigation as well.

4. Conclusions

This report describes the successful development of HPLC assays that achieve specific and sensitive quantification of APCP363 in plasma, urine and feces. The pH of the mobile phase, types of column used, detection wavelength, and organic modifier

Table 3
Urinary and fecal recovery of CP363 following the 250 mg/kg oral dose

Rat	Day	Urinary recovery (%)	Fecal recovery (%)
1	1	10.86	5.32
	2	1.13	1.20
	3	0.03	NA
	Total[a]	12.02	6.52
2	1	8.79	3.97
	2	0.66	1.12
	3	0.06	NA
	Total[a]	9.51	5.09

[a] Total: total recovery (%) over 3 days.
NA: Not detected.

play important roles in chromatographic separation of APCP363. The pH of the extraction solvent was critical in recovery of APCP363 from feces. Over the aforementioned concentration ranges, the recovery of APCP363 ranged from 81.2 to 99.9% in plasma, from 82.1 to 92.8% in urine, and from 65 to 68% in feces. The RSDs were below 10% on repeated analyses for plasma ($n=5$), urine ($n=2$), and feces ($n=3$). The LOQs of APCP363 were 0.1 μg/ml ($n=5$) in plasma, 10 μg/ml ($n=4$) in urine, and about 30 μg/ml ($n=4$) in feces. These assays have been successfully applied to preliminary pharmacokinetic studies in rats.

Acknowledgements

The research has been supported by Apotex Inc. We are thankful to Professor R.C. Hider for his advice and the supply of CP363.

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