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High performance liquid chromatography analysis of a 4-anilinoquinazoline derivative (PD153035), a specific inhibitor of the epidermal growth factor receptor tyrosine kinase, in rat plasma

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Abstract

The quinazoline derivative, 4-*N*-(3′-bromo-phenyl)amino-6,7-dimethoxyquinazoline (PD153035), has recently been identified as a potential drug for the treatment of proliferative disease. Here, we report a sensitive high performance liquid chromatography (HPLC)-based quantitative detection method for measurement of PD153035 levels in rat plasma. Sample pretreatment involved a two-step extraction with chloroform. The analytes were separated on a column packed with OmniSpher C18 material and eluted with acetonitrile–0.1 M ammonium acetate, pH 7.2 (70:30, v/v). The column effluent was monitored by UV detection at 330 nm. A linear response was achieved over the concentration range 0.50–100.00 μM using multilevel calibration with an internal standard. The analytical method inter- and intra-run accuracy and precision were better than ±15%. The lower limit of quantification was 0.50 μM. The method has been applied to study the preclinical pharmacokinetics of this compound in rats.

Keywords: 4-Anilinoquinazoline derivatives; Liquid–liquid extraction; High performance liquid chromatography

1. Introduction

The 4-(phenylamino)pyrimidine is a pharmacophore of distinct ATP-competitive protein kinase inhibitors [1]. We have recently synthesised a series of quinazoline derivatives with pharmacological potential [2–4]. It is noteworthy to mention that, similar compounds have been shown to possess highly potent and selective inhibitory activity against tyrosine-kinase receptors, which might explain most of their pharmacological properties [1,5–9]. One such compound shown in Fig. 1, PD153035, shares with 4-anilinoquinazoline derived compounds the required elements for highly potent enzyme inhibition. These include electron-donating substituents at the 6- and 7-positions, a free NH− at the 4-position, free CH groups at the 2-, 5- and 8-positions of the quinazoline core, and a lipophilic substituent at the aniline *meta*-position [8,9].

Although compounds of the PD153035 family have been largely studied [1,5–9] in animals and humans due to their pharmacodynamics and pharmacological effects, detailed analytical methods assessing attained levels in biological fluids are still lacking. Therefore, we have developed a UV detection HPLC-based method for the quantitative detection of PD153035 in rat plasma samples.

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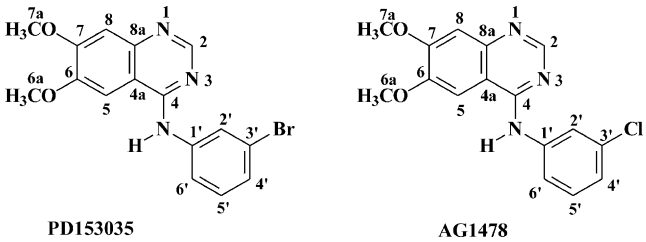

Fig. 1. Chemical structures of PD153035 and its internal standard AG1478.

2. Experimental

2.1. Chemicals and reagents

Compound PD153035 [4-*N*-(3′-bromo-phenyl)amino-6,7-dimethoxyquinazoline hydrochloride] and its internal standard AG1478 [4-*N*-(3′-chloro-phenyl)amino-6,7-dimethoxyquinazoline hydrochloride] were synthesised, as previously described [2–4], and their chemical structures are shown in Fig. 1. Both compounds were >99% purity as determined by elemental analysis, HPLC, mass spectrometry and ^{1}H and ^{13}C NMR [3]. All solvents and reagents used in this study were HPLC grade. Water was purified and deionised by the Milli-Q-UF system (Millipore, Milford, Mass, USA) and used throughout in all aqueous solutions. Acetonitrile was obtained from Tedia (São Paulo, SP, Brazil). Chloroform, dimethylsulfoxide (DMSO) and ammonium acetate were purchased from VETEC (São Paulo, SP, Brazil). The mobile phase used in the HPLC system was vacuum-filtered through a 0.45 μm filter (Millipore, Milford, Mass, USA). Using a 0.01 M ammonium hydroxide solution, the 0.1 M ammonium acetate buffer's pH was adjusted to a 7.2 value.

2.2. Chromatography

The HPLC system consisted of a Shimadzu series SCL-10Avp (Kyoto, Japan) equipped with a binary pump (LC-10AD), an autosampler (SIL-10ADvp), a degasser and an UV–vis Model SPD-10A spectrophotometric detector model, which was set at 330 nm. Chromatographic separations of the compounds (PD153035 and AG1478) were achieved at ambient temperature, using a reversed-phase OmniSpher C18 column (250 mm × 4.6 mm i.d.; 5 μm particle size; purchased from Varian, CA (USA) with a ChromoSep guard column SS (10 mm × 3 mm). The mobile phase consisted of a mixture of acetonitrile–0.1 M ammonium acetate buffer, pH 7.2 (70:30, v/v) and was daily prepared and filtered. The column was equilibrated and eluted under isocratic conditions, using a flow-rate of 1.0 mL/min at ambient temperature. Preliminary experiments indicated that, within a range of 190–400 nm, the peak maximum wavelength for PD153035 and AG1478 internal standard occurred at 330 nm. The chromatographic run time for each analysis was 8 min.

System control, data acquisition and processing were performed with a PC-Pentium IV Processor personal computer, operated with Microsoft Windows NT version 4.0 and Shimadzu CLASS-VP version 6.12 SP2 chromatography software with the system suitability option installed. Calibration graphics were calculated by linear regression analysis of the peak area ratio of PD153035 and the internal standard AG1478 versus the PD153035 concentration of the nominal standard, using Microsoft Excel 1997/Origin version 5.0. The zero concentration sample (blank) was used to verify the purity of the reagents and the lack of other potentially interfering (endogenous) substances, but was not considered for the regression analysis of standards. The goodness-of-fit of various calibration models were evaluated by visual inspection, the correlation coefficient and by intra- and inter-run accuracy and precision values.

2.3. Preparation of stock solutions, calibration standards and quality control samples

Stock solutions of PD153035 and AG1478 internal standard were prepared independently in duplicates by dissolving 4.00 mg of PD153035 and 3.52 mg of AG1478 in 1 mL of dimethylsulfoxide (DMSO), to obtain a final concentration of 10 mM. The solutions were stored in glass flasks at 4 °C for up to four weeks. The difference in PD153035 and AG1478 internal standard concentration in each of the duplicate stock solutions, estimated from the average peak area following repeat analysis of a dilution of the stock, was determined to be within 5%.

A solution of acetonitrile–0.1 M ammonium acetate buffer, pH 7.2 (50:50, v/v) was used to dilute stock solutions of PD153035 to a 500.00 μM working concentration. Moreover, stock solutions of internal standard AG1478 were diluted in chloroform to a 50.00 μM concentration. Serial dilution (100.00, 50.00, 25.00, 10.00, 2.00, and 0.50 μM) from 500 μM PD153035 solution was obtained with drug-free rat heparinized plasma. These calibration standards were prepared freshly in triplicate to each analytical run. A sample of blank rat plasma (without PD153035 and AG1478) was also prepared. Pools of quality control standards of PD153035 in rats plasma were prepared similarly at concentrations of 75.00, 7.50, 0.75 and 0.50 μM, and stored at −80 °C. The samples were prepared from a stock solution that was different from that used to generate standard curve samples. These quality control samples were used to investigate intra- and inter-run variations.

2.4. Sample preparation

Calibration standard, quality control and unknown plasma samples were processed by a two-step liquid–liquid extraction with chloroform. For determination of PD153035 levels in plasma samples, a 100 μL volume of each sample was transferred to a clean glass tube (100 mm × 14 mm), followed by addition of 30 μL of the internal standard at 50 μM (to yield a final concentration of 1.1 μM).

The extraction procedure involved the addition of a total chloroform volume of 6 mL (2 × 3 mL) in increments of 1 mL. The mixtures were vortexed for 1 min following each 1 mL addition of chloroform to the plasma samples. After adjusting the chloroform volume to 3 mL, the aqueous layer was frozen in a −80 °C ethanol-dry-ice bath and the organic phase was transferred into a clean glass tube. The aqueous layer was allowed to thaw. Afterwards, the sample extraction second-step was carried out following the above first step extraction procedure. Thus, the combined organic extracts (6 mL) were evaporated to dryness in a water bath (60–70 °C). The resulting residues were reconstituted in 200 μL of acetonitrile followed by the addition of 200 μL of 0.1 M ammonium acetate buffer, pH 7.2. Samples were vortex-mixed for 1 min and transferred to plastic tubes for centrifugation (12,000 rpm, 6 min). Aliquots of 40 μL of the reconstituted extracts were injected into the HPLC system.

2.5. Validation procedures

Rat plasma samples with known and unknown amounts of PD153035 were analyzed by the analytical assays described above. Method validation was performed according to previously reported procedures [10–15]. Validation chromatographic runs were carried out on five days and included a calibration curve processed in triplicate, and quality control samples at four different concentrations of PD153035 (0.50, 0.75, 7.50 and 75.00 μM), in six replicates.

Calibration graphics in the range of 0.50–100.00 μM of PD153035, in rat plasma, were plotted based on the peak-area ratios of PD153035 to internal standard AG1478 (PD153035/AG1478) (axis *x*) against the respective nominal concentrations (axis *y*). All calibration curves were required to have a correlation value of at least 0.998. The intra- and inter-run precision and accuracy of the assay were determined by percentage of coefficient of variance (C.V.) and the percentage deviation (DEV%) values, respectively, based on reported guidelines [14,15]. Precision (C.V.) and accuracy (DEV%) were calculated by following Formulas (1) and (2):

$$\text{C.V.}(\%) = \left[\frac{\text{S.D.}}{\text{Average calculated concentration}}\right] \times 100 \quad (1)$$

$$\text{DEV}(\%) = 1 - \left[\left(\frac{\text{Average calculated concentration}}{\text{Nominal concentration}}\right)\right] \times 100 \quad (2)$$

where S.D. stands for standard deviation.

Intra-run precision and accuracy measurements were performed on the same day on rat plasma samples, containing 0.50, 0.75, 7.50 and 75.00 μM concentrations of analyte ($n = 6$). Inter-run precision and accuracy of the analytical method were determined simultaneously from the results of calibration curve and quality control samples run on five days. Each set of quality control samples, containing the concentrations of PD153035 was evaluated from recently obtained calibration curves.

The assay lower limit of quantification (LLOQ) was determined to be 0.50 μM for PD153035. The LLOQ was determined by following two criteria: (i) the signal-to-noise ratio larger than 5 and (ii) the values for accuracy and precision were less than 20% [14,15].

The percentage recoveries for PD153035 and the AG1478 internal standard extractions were established by comparing the averaged chromatographic peak areas of the sample from rat plasma, against the results of compounds in acetonitrile–0.1 M ammonium acetate buffer (pH 7.2; 50:50, v/v). Recoveries, after plasma extraction, were measured at PD153035 concentrations of 2.00, 10.00 and 50.00 μM, containing 30 μL of AG1478 (1.10 μM) ($n = 6$). The extraction recovery (ER) was calculated according to formula: $\%\text{ER} = \{\text{peak area } [\text{PD153035}]_{\text{extracted}}/\text{peak area } [\text{PD153035}]_{\text{unextracted}}\} \times 100$. Blank rat plasma samples ($n = 6$) were used to investigate the potential interference of endogenous components.

2.6. Application of the method

Male Wistar rats ($n = 5$) were dosed orally by gavages with 88 mg/kg body weight of PD153035 suspended in 10% (w/v) arabic gum aqueous solution as vehicle, to demonstrate the application of the assay. The control groups ($n = 5$) received vehicle only. Blood samples (∼250 μL) were collected from a catheter previously (24 h) implanted into the femoral artery under ketamine-diazepan (80:20, v/v, i.p.) anesthesia. Samples were collected in plastic tubes, containing heparin at control, 0.25, 0.5, 1, 2, 3, 4, 5 and 6 h. Afterwards, plasma samples were separated by centrifugation (12,000 rpm, 6 min), the aliquots were transferred to clean flasks and stored at −80 °C, until analysis, using the above method. Rats were from the university's Central Animal House. The rats were housed with food and water provided ad libitum. All procedures and the care of the rats were in accordance with institutional guidelines for animal use in research.

3. Results and discussion

3.1. Chromatography

Fast and reproducible separation of the compounds was achieved using a silica reversed-phase column, eluted isocratically with acetonitrile–0.1 M ammonium acetate buffer, pH 7.2 (70:30, v/v), at ambient temperature. Isolation of PD153035 and the AG1478 internal standard from plasma was initially performed using the one-step chloroform solvent extraction procedure previously reported for other 4-anilinoquinazoline derivatives [10–12]. However, due to the lower extraction efficiency obtained in the current target compounds, the two-step extraction method described in this report was developed. In addition to an increase in extraction

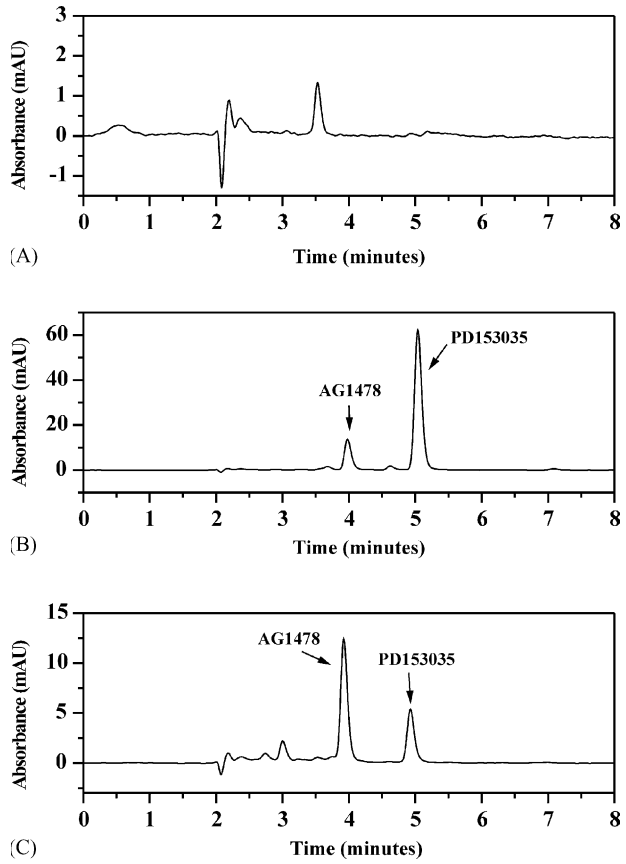

Fig. 2. Representative HPLC chromatograms of plasma extracts: (A) an extract of a blank rat plasma sample, (B) an extract of a plasma sample spiked with PD153035 at a concentration of 50 μM, (C) and an extract of a plasma sample taken 2 h after a single oral dose of 88 mg/kg of PD153035. First peak corresponds to AG1478 (t_R = 3.97 min) and the second to PD153035 (t_R = 5.01 min).

efficiency, a second advantage of the two-step approach was the partial elimination of endogenous matrix interferences.

Although several combinations of mobile phase solvents have been reported for quinazolines derivatives [10–13], indicated increases in the organic modifier content of the mobile phase reported by Chen et al. [10] was shown to yield increases in both the selectivity and efficiency of the separation. Detection was obtained by UV detector monitoring of PD153035 and AG1478 at 330 nm.

Fig. 2 shows representative chromatograms obtained from an extract of a blank mouse plasma sample (A), an extract of a plasma sample spiked with PD153035 at 50 μM (B) and an extract of a plasma sample taken 2 h after a single oral dose of 88 mg/kg of PD153035 (C). The chromatograms depicted in this figure were obtained according to the described conditions. The selectivity for the PD153035 and AG1478 is indicated by the sharp and symmetrical resolution of the peaks, as well as the lack of significant interfering peaks from the biological matrix. Further indications of improvements in the chromatographic efficiency were indicated by a reduction in the chromatographic run time from 8 min to respective retention times (t_R) of 3.97 and 5.01 min for AG1478 and PD153035.

Table 1
Extraction recovery (average ± S.D.) of PD153035 and AG1478 internal standard over the working range of the assay (n = 6)

Compound	Added concentration (μM)	%Recovery (±S.D.)	Average (%)
PD153035	2.00	89.07 (±8.67)	
	10.00	93.92 (±6.54)	92.26
	50.00	93.78 (±2.77)	
AG1478	1.10	88.28 (±6.48)	88.28

3.2. Recovery

The recoveries of PD153035 and AG1478 internal standard, from spiked plasma samples, were calculated by comparing the peak area of extracted samples at concentrations of 2.00, 10.00 and 50.00 μM with those obtained from the analysis of corresponding unextracted samples directly injected into the column (n = 6). The obtained results are summarised in Table 1.

High recovery percentages were obtained for both compounds (average recovery for PD153035 equals to 92.26% and that for AG1478 equals to 88.28%). Furthermore, they also presented similar behavior towards the extraction method, allowing AG1478 to be used as an internal standard for PD153035 analysis.

3.3. Assay linearity, accuracy, precision and sensitivity

The statistics for the five calibration curves, obtained during inter-run validation for plasma sample assay are shown in Table 2. The standard calibration curves for known amounts of PD153035, ranging from 0.50 to 100.00 μM, against the ratio of peak areas for PD153035 and AG1478, were linear (R > 0.999) and could be described by the linear regression equation: $y = 11.7213x + 0.09088$ (n = 6, S.D. = 0.14105, R = 0.99999), in which y is the PD153035 concentration in μM and x is the chromatogram peak area ratio (PD153035/AG1478).

The 4-anilinoquinazoline analogue AG1478 (Fig. 1) was chosen as internal standard, since it is eluted before the PD153035, has similar extraction characteristics and shows excellent stability in the auto-sampler over the assay duration. Furthermore, it is generally believed that the use of internal standard in HPLC analyses would improve the accuracy and precision of the assay. Consistent with this notion, when we initially used the current assay methodology in the absence of any internal standard, substantial variability in the results was observed. This variability was most likely related to the additional steps in the sample preparation, which required separation of organic and aqueous phases, besides a transfer of the organic layer to a different tube. In addition, partial evaporation of the sample solution for injection into the HPLC equipment might contribute to this variability. There-

Table 2
Inter-run variation – accuracy, precision and linearity of standard curve samples from five separate assays[a]

Nominal concentration (μM)	Calculated PD153035 concentration (μM) (triplicate of each concentration)					Average	S.D.[b]	DEV (%)[c]	C.V. (%)[d]
	1[e]	2[e]	3[e]	4[e]	5[e]				
0.50 (LLOQ)	0.51 ± 0.060	0.49 ± 0.010	0.47 ± 0.023	0.48 ± 0.011	0.48 ± 0.015	0.49	0.015	−2.00	3.06
2.00	2.01 ± 0.115	2.07 ± 0.049	2.02 ± 0.100	1.98 ± 0.006	2.02 ± 0.053	2.02	0.032	+1.00	1.58
10.00	10.16 ± 0.741	10.81 ± 0.115	10.51 ± 0.351	9.44 ± 0.244	9.80 ± 0.145	10.14	0.546	+1.40	5.38
25.00	24.40 ± 0.753	24.43 ± 0.360	23.86 ± 0.839	25,38 ± 3.002	24.85 ± 0.987	24.58	0.567	−1.68	2.31
50.00	49.28 ± 1.975	49.98 ± 1.334	51.66 ± 0.237	46.55 ± 1.140	48.65 ± 1.735	49.22	1.870	−1.56	3.80
100.00	98.01 ± 2.463	98.73 ± 2.985	99.46 ± 3.743	97.29 ± 4.717	100.87 ± 4.853	98.87	1.379	−1.13	1.39
R	0.99999	0.99988	0.99976	0.99972	0.99991	0.99985	0.00011	−0.015	0.01

[a] A linear curve was fitted to the data for response (PD153035/AG1478) vs. theoretical concentration as described in Section 2. The calculated concentration was derived from reading the response for each standard sample against calibration curve. Each entry (assays 1–5) corresponds to the average (±S.D.) value of triplicate analysis.
[b] S.D. = Standard deviation.
[c] Accuracy (DEV%) = the deviation of the calculated concentration from the nominal value. Calculation according to Formula (2).
[d] C.V. (coefficient of variation, precision) = calculation according to Formula (1).
[e] Assay number.

fore, an internal standard was used to improve accuracy and precision of the assay.

The limit of detection (LOD) was determined as the sample whose signal to noise ratio (S/N) was just greater than 3 and corresponded to a 0.20 μM PD153035 concentration (Fig. 3A). Likewise, the 0.50 μM sample displayed S/N equal to 10 and was established as the lower limit of quantification (LLOQ), Fig. 3B.

Validation data of the analytical method, in terms of accuracy (i.e. the percentage deviation from nominal concentration) and precision (coefficient of variance, C.V.), are shown in Tables 2 and 3.

Each assay in Table 2 was performed on different days and each calculated concentration value corresponds to the average value of triplicate analysis. In addition, Table 2 is also presenting average results of concentrations, standard deviation, accuracy and precision obtained on five days. According to [14] and [15], acceptable values for accuracy (DEV) and precision (C.V.) must be ≤15%, except for LLOQ, whose values must be no larger than 20%.

The reproducibility of the method was evaluated by analysing replicates of quality control samples of 0.50 (LLOQ), 0.75, 7.50 and 75.00 μM PD153035 concentrations. The average results are presented in Table 3. From the data for inter- and intra-run (Table 3), it can also be observed that both precision (C.V.) and accuracy (DEV) are within the acceptable limits (±15%) [14,15].

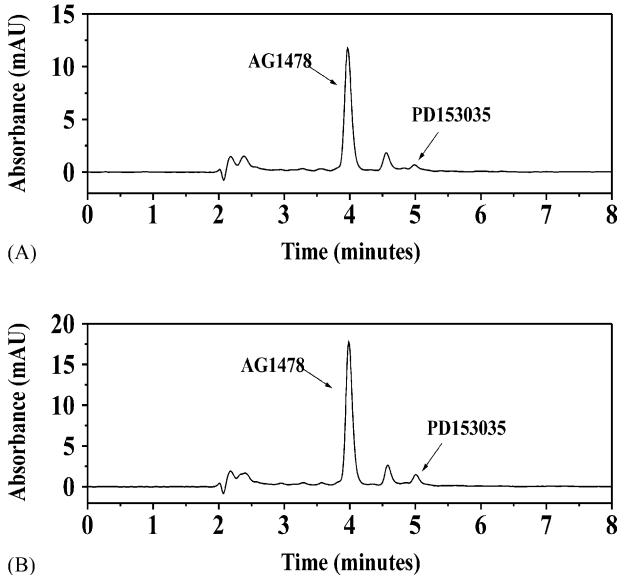

Fig. 3. Chromatograms of rat plasma samples spiked with PD153035 at 0.20 μM (A) and 0.50 μM (B). The 0.20 and 0.50 μM concentrations were determined as the LLOD and LLOQ, respectively.

3.4. Application of the method

We used the established HPLC method in a pilot study to determine the pharmacokinetics of PD153035 in male Wistar

Table 3
Intra- and inter-run precision and accuracy for PD153035 in quality control samples[a]

Added concentration (μM)	Measured concentration (μM)	S.D.	DEV (%)	C.V.(%)
Intra-run (n = 6)				
0.50 (LLOQ)	0.50	0.031	–	6.20
0.75	0.74	0.024	−1.33	3.24
7.50	7.39	0.239	−1.47	3.23
75.00	75.40	2.089	+0.53	2.77
Inter-run (n = 30)				
0.50 (LLOQ)	0.49	0.028	−2.00	5.71
0.75	0.77	0.037	+2.67	4.80
7.50	7.26	0.538	−3.20	7.41
75.00	74.02	3.735	−1.31	5.05

[a] The data are shown as averages, S.D. (standard deviation), accuracy (percent deviation, DEV%) and C.V. (coefficient of variation, precision). Accuracy and precision calculations were carried out by Formulas (1) and (2), respectively.

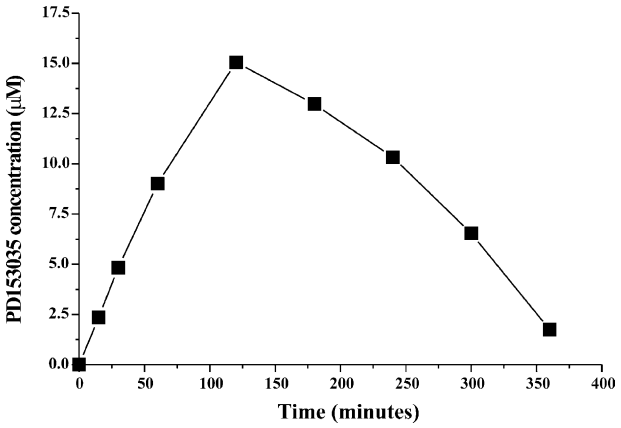

Fig. 4. Plasma concentration-time course of PD153035 after the oral administration of a single 88 mg/Kg dose of the compound to Wistar rats ($n = 5$). Data are expressed as averages.

Table 4
PD153035 concentration values in rat plasma samples and their respective times, after the oral administration of a dose of PD153035 (88 mg/kg; $n = 5$ rats)

Time (min)	Plasmatic concentration values (μM)
0	0
15	2.36 ± 0.426
30	4.84 ± 0.533
60	9.01 ± 1.079
120	15.06 ± 0.965
180	12.97 ± 1.253
240	10.98 ± 0.688
300	7.47 ± 1.062
360	1.74 ± 0.124

Data are expressed as averages (±S.D.).

rats. Graphic shown in Fig. 4 indicate the concentration of PD153035 in the rat plasma along an 8-h period of sample collections.

PD153035 maximal concentration (C_{max}) of 15.06 μM was reached at 2 h after oral administration of 88 mg/kg of compound to rats. After 6 h, PD153035 concentration approached of the limit of quantification. Table 4 shows the average values of PD153035 concentrations in sample plasma, for several periods following oral administration.

4. Conclusion

We have shown here a chromatographic method applicable to the determination of the quinazoline derivative PD153035, in plasma, at microgram levels, using a two-step liquid–liquid extraction procedure. The analytical HPLC method presented is specific, accurate, precise and selective enough to be used in plasma samples and other biological fluids. Furthermore, the availability of this assay will now allow studies describing detailed pharmacodynamics and pharmacokinetics of PD153035.

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