Development of a HPLC-MS/MS method for the pharmacokinetic studies of Pirfenidone in pig plasma

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1. INTRODUCTION

Pirfenidone is a drug active against idiopathic pulmonary fibrosis, but recently its use was evaluated also in the myocardial infarction to reduce the heart damage[1-2]. In fact, It has been demonstrated that, in canine model, congestive heart failure (CHF) produces significant atrial fibrosis and the substrate for sustained atrial fibrillation (AF). This atrial remodeling is a potential therapeutic target. It was also showed that Pirfenidone is a possible candidate against this pathological situation.

2. AIM OF THE WORK

The aim of this study was the development and validation of a method for the quantitative determination of Pirfenidone in pig plasma for preliminary pharmacokinetic studies in order to support the activity against heart attack. Due to low concentrations of Pirfenidone in plasma samples and the complexity of the matrices a HPLC-QqQ with ESI source was chosen. The pharmacokinetic studies involved both *in vivo* and *in vitro* experiments to evaluate the different distribution of Pirfenidone for 3 different kind of administrations (oral or Os, intramuscular or IM, intravenous or IV) and the chemical stability.

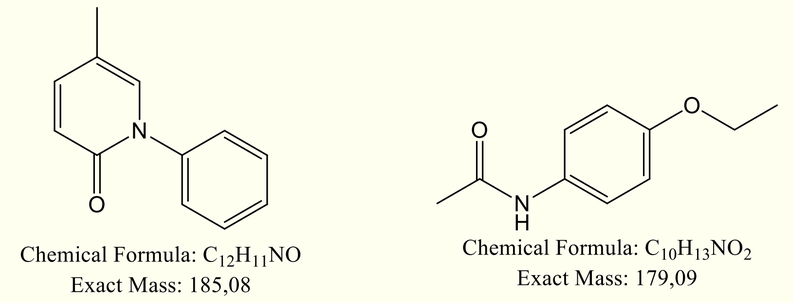


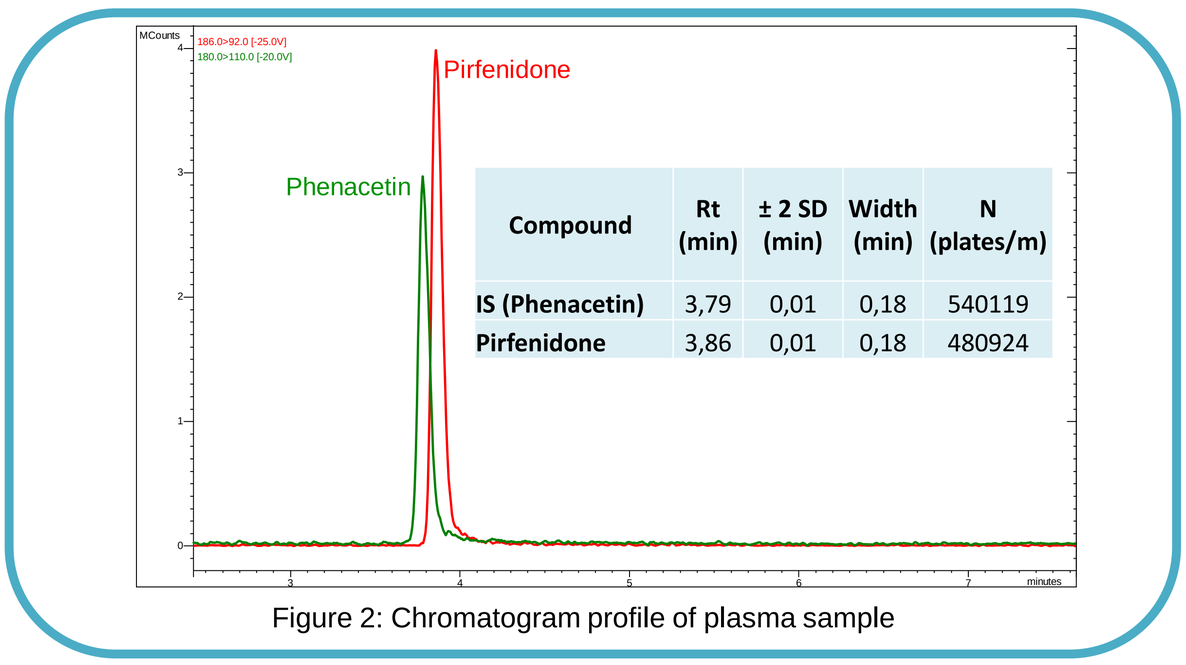
Figure 1: Chemical structures of Pirfenidone e Phenacetin

3. DEVELOPMENT OF THE METHOD

For this study, a HPLC Varian Prostar coupled with a triple quadrupole Varian 1200L was employed. The development of the HPLC-MS/MS method started with an Energy Resolved Mass Spectrometry (ERMS) experiment on the Pirfenidone for study its fragmentation in order to select the best MRM transitions. Phenacetin, was chosen as internal standard due to its structural and molecular weight similarities. The chromatographic parameters employed to analyze the samples, were finely tuned to minimize the run time, maintaining the sensitivity and the reliability requested for the study. The best performances were obtained by using a Phenomenex Luna PFP 30x2 mm, 3 μm of particle size column with a gradient elution of 10 mM formic acid and 5 mM ammonium formate solution (Solvent A), 5 mM formic acid and 10 mM ammonium formate in methanol solution (Solvent B). The elution gradient was started at 90 % solvent A, then decreased to 5 % in 4.0 min, kept for 4.0 min, returned to initial conditions in 0.1 min and maintained for 3.9 min for reconditioning, to a total run time of 12 min. Finally, the mobile phase flow, sample injection and column temperature were kept at 0.25 mL/min, 5 μL and 20° C respectively. Each sample was prepared by simply protein precipitation and dilution of supernatant, in order to increase productivity.

Compound	Precursor ion (m/z)	Quantifier ion (m/z) [CE (v)]	Qualifier ion (m/z) [CE (v)]
IS (Phenacetin)	180	110 [20]	
Pirfenidone	186	92 [25]	158 [20]

Table 1: MRM experiments



Compound	Rt (min)	± 2 SD (min)	Width (min)	N (plates/m)
IS (Phenacetin)	3,79	0,01	0,18	540119
Pirfenidone	3,86	0,01	0,18	480924

Figure 2: Chromatogram profile of plasma sample

4. VALIDATION OF THE METHOD

Considering that the pharmacokinetics studies have been made in plasma samples it was deemed necessary to calculate a matrix effect. For this purpose it was decided to follow the guidelines of Matuszewski et al[3] using Pig plasma.

Accuracy and Precision experiments were also performed to validate the proposed HPLC-MS/MS method. For Accuracy it was decided to add 3 different known concentrations (low, medium and high) in pig plasma and has been calculated by expressing it as a percentage. Every concentration was prepared in 6 duplicate so the RSD% obtained could be considered the Precision of the method.

5. IN VIVO AND IN VITRO STUDIES

The pharmacokinetic studies involved three different Pirfenidone administrations to Pigs (IV, IM and Os). Then plasma samples in different times (0, 30, 60, 90, 120, 180 and 240 minutes) have been collected.

Moreover, in order to establish the chemical stability of Pirfenidone in studied samples, a series of experiments in PBS buffer and pig plasma were carried out incubating Pirfenidone in the two matrices for 4 times (0, 30, 60 and 120 minutes). The samples have been prepared as described in chapter 3 (*development of the method*).

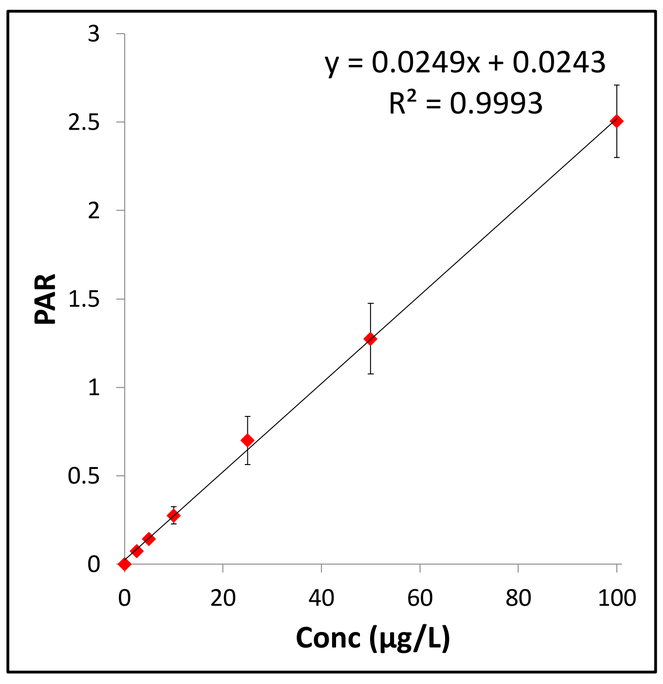


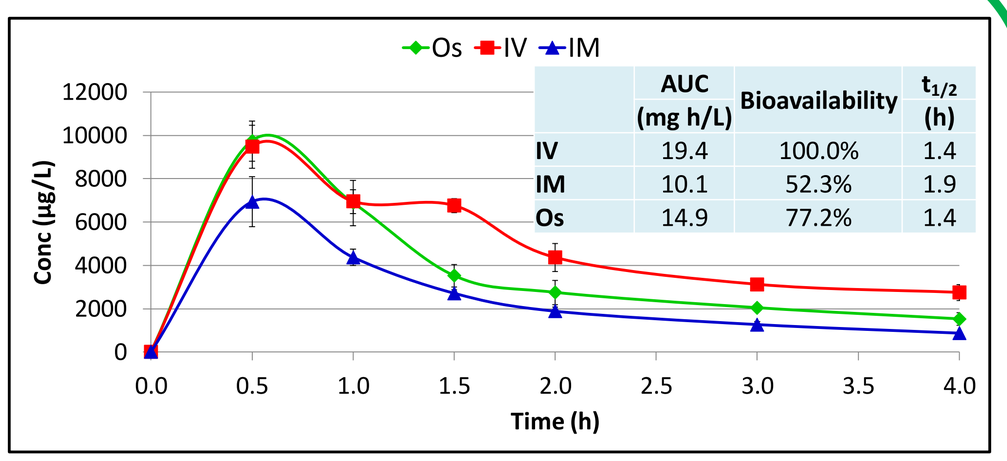
Figure 3: Calibration Curve

Y-SD LOD (µg/L)	Y-SD LOQ (µg/L)	ME (%)	RE (%)	PE (%)
1,8	5,3	97%	97%	94%

Table 2: Matrix Effect, Recovery and Process Efficency values

	Accuracy (recovery%)	Precision (RSD%)
Low Concentration	94%	4%
Medium Concentration	87%	2%
High Concentration	83%	3%

Table 3: Accuracy & Precision values



	AUC (mg h/L)	Bioavailability	$t_{1/2}$ (h)
IV	19.4	100.0%	1.4
IM	10.1	52.3%	1.9
Os	14.9	77.2%	1.4

Figure 4: Pharmacokinetics curve

DRUG PLASMA STABILITY	PBS (min)	Pig Plasma (min)
Prifenidone	>240	>240

Table 4: $t_{1/2}$ values obtained from drug plasma stabilty experiments

6. CONCLUSIONS

The proposed HPLC-MS/MS method has proven to be suitable for the determination of Pirfenidone in the pharmacokinetic studies described above (Table 2 and 3). The 3 different type of administrations showed different behavior (figure 4) and, this obtained preliminary data will be useful to support the Pirfenidone study to reduce the heart damages in the myocardial infarction. The obtained t1/2 values from the drug plasma stability experiment (table 4) showed the chemical stability of Pirfenidone so it is possible to assume that the decreasing of concentration in *in vivo* studies are due to distribution and metabolism.

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