

Original Research Paper

Novel LC-MS/MS Method for CJ-023423 (Grapiprant) Determination in Rabbit Plasma

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Abstract: Grapiprant is a new anti-inflammatory drug that preferentially targets the EP4 receptor of prostaglandin E2 limiting the wide range of adverse effects caused by the classical non steroidal anti-inflammatory drugs. The aim of this study was to develop and validate a new, simple, sensitive and rapid Liquid Chromatography tandem Mass Spectrometry method (LC-MS/MS) in order to quantify this novel drug in plasma. The method involved a simple liquid extraction followed by a gradient elution with formic acid 0.2% in water and acetonitrile in reverse phase chromatography. The method was validated according to international guidelines determining selectivity, linearity, sensitivity, recovery, matrix effect and precision. Linearity was obtained over a range of 5-1000 ng mL⁻¹. The obtained Limit of Quantitation (LOQ) and of Determination (LOD) were of 5 and 1.5 ng mL⁻¹ respectively. Extraction recovery was >73% for all the tested concentrations. Matrix effect, expressed as ion suppression, was ≤9%. The intraday and inter-day precision results showed good RSD values. All the validation parameters were satisfactory making this new method an interesting tool for scientists to further investigations on pharmacokinetics parameters. This validated method was applied to assess the pharmacokinetic of grapiprant in one rabbit administered with 0.5 mg kg⁻¹.

Keywords: Grapiprant, LC-MS/MS, Plasma, Pharmacokinetics

Introduction

Grapiprant (CJ-023,423) is the pioneer drug of the newly recognized class of drug named priprant (WHO, 2013). Its discovery plays an important role in the treatment of osteoarthritis and inflammatory pain (Giorgi, 2014). Commonly these pathologies are handled with Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) and selective Cyclooxygenase (COX)-2 inhibitors. Although these commonly used drugs are actually efficient in the reduction of pain and inflammation, their use is correlated with well-known side effects. Indeed NSAIDs act by inhibiting the two COX isoforms (COX-1 and COX-2 isoforms). The COX-1 inhibition is shown to induce adverse gastrointestinal effects such as gastric erosion, ulceration and hemorrhage (Khan and McLean, 2012). The COX-2 inhibition has been recently found to correlate with kidney toxicity (Kim and Giorgi, 2013).

For these reasons the discovery of a new drug, able to block only the prostaglandin receptor without impacting the other prostanoid produced by the COX enzymes, constitutes an important breakthrough (Rausch-Derra *et al.*, 2015). Currently, grapiprant is under development in humans and approved in dogs (USA) against the inflammation related to osteoarthritis (Giorgi, 2014). Grapiprant acts through a selective antagonism of the PGE2 receptor EP4 that has been identified as a major receptor in the mediation of pain associated with rheumatoid arthritis, osteoarthritis and inflammatory states (Chen *et al.*, 2010; Clark *et al.*, 2008; Rausch-Derra *et al.*, 2016a). Given its novelty, very few data are reported in literature about this new drug especially regarding its pharmacokinetic profile (Rausch-Derra and Rhodes, 2014; 2016; Rausch-Derra *et al.*, 2016b; Łebkowska-Wieruszewska *et al.*, 2017a,b; De Vito *et al.*, 2017). To date, only one study is available in

literature regarding the quantification of grapiprant in canine plasma using a validated method through liquid chromatography associated with a spectrofluorometric detector (De Vito *et al.*, 2016). Actually, LC-MS/MS techniques are largely used for the quantification of xenobiotics in biological matrices given their high sensitivity, selectivity and total analysis time reduction compared with other analytical methods. The aim of this study was to develop and validate a new LC-MS/MS method able to quantify grapiprant in order to explore its pharmacokinetic profile in rabbits.

Materials and Methods

Chemicals and Reagents

Grapiprant analytical standard (>99.0% purity) was obtained from Cayman Chemical (Ann Arbor, MI, USA). The Internal Standard (IS) 1,1-cyclohexanediacetic acid monoamide was purchased from Sigma Aldrich, (Milano, Italy). All reagents and solvents used were of the highest commercial quality and were obtained from Sigma-Aldrich (Milan, Italy). Deionized and distilled water was filtered through a Milli-Q water system (Millipore, Billerica, MA, USA). Stocks standard solutions of grapiprant 1 mg mL^{-1} were prepared in methanol and used to prepare working solutions. Calibration samples were prepared by spiking working solutions into blank rabbit plasma to yield concentrations of 5.0, 10, 50, 100, 200, 400, 800 and 1000 ng mL^{-1} . Rabbit plasma samples were supplied by the blood bank of Veterinary Hospital at the University of Pisa.

Sample Extraction

Simple liquid-liquid extraction was developed in order to extract grapiprant from rabbit plasma. Four mL of acetonitrile were added to $500 \mu\text{L}$ of plasma. After vortexing for 30 sec, the mixture was transferred to an ultrasonic bath for 10 min and then centrifuged at 20000 g for 10 min at 25°C . The supernatant was collected into a glass vial and evaporated to dryness under nitrogen. The dry residue was reconstituted in $500 \mu\text{L}$ of a mixture containing 100 ng mL^{-1} of IS in H_2O and CH_3CN 80:20.

LC-MS/MS Analysis

LC-MS/MS analyses were performed on a Varian 310-MS triple quadrupole mass spectrometer (Varian, Palo Alto, CA, USA). Grapiprant and 1,1-cyclohexanediacetic acid monoamide were detected in positive ionization (ESI+) and in multiple reaction monitoring mode (MRM). For the optimization of detection conditions, direct infusions of standard solutions ($500 \mu\text{g mL}^{-1}$) were made using a T connection with mobile phase at a flow of $400 \mu\text{L min}^{-1}$. The ESI

source conditions were: Capillary voltage, 50 V; drying gas temperature, 200°C ; nebulizer gas pressure, 50 psi (both nebulizer and drying gas were high-purity nitrogen); electron multiplier voltage, 1350 V. For operation in MS/MS mode, the collision gas used was argon with a pressure of 2 mTorr in the collision cell. The collision energies were optimized for a maximum detection of each product ion. For grapiprant and IS, three and two transitions were used respectively; the most abundant was used for the quantitation and the others were used to confirm the analyte identity. Samples were injected into a $5 \mu\text{L}$ loop. Chromatographic separation was achieved with a ProStarTM 300 HPLC system (Varian, Palo Alto, CA, USA) on a LUNA C_{18} column ($5 \mu\text{m}$, $100 \times 2.1 \text{ mm ID}$, Phenomenex, Bologna, Italy) fitted with a Phenomenex C_{18} security guard cartridge ($4 \times 2.0 \text{ mm ID}$). A linear gradient, using a flow rate of 0.2 mL min^{-1} , with 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B) was performed as follows: 1 min at 20% B; in 0.06 min solvent B was increased from 20 to 80% and remained constant at 80% B till 3.30 min. Then in 30 sec solvent B was decreased from 80 to 20% and it remained constant for 4 min to restore initial mobile phase conditions for the next injection. The total runtime was 8 min.

Method Validation

Validation was based on the Food and Drug Administration guidelines for Bioanalytical Method Validation (US DHHS, 2013). The determined parameters were selectivity, linearity, Limit of Determination (LOD), Limit of Quantitation (LOQ), accuracy, precision, matrix effect and analyte stability. The selectivity of the method is the ability to differentiate and quantify the analyte also in presence of other components in the sample: It was determined analysing six blank plasma samples. Linearity was assessed by the construction of a calibration curve obtained fortifying blank plasma with known amounts of grapiprant ranging from 5 ng mL^{-1} to $1 \mu\text{g mL}^{-1}$ and each concentration was analysed in triplicate. Calibration curve was derived from the peak area ratios (grapiprant/IS) using $1/x^2$ weighted linear least-squares regression of the area ratio versus the concentration of the corresponding standard.

The Limit of Detection (LOD) and the Limit of Quantitation (LOQ) were calculated using the signal-to-noise criteria of 3 and 10, respectively.

Accuracy was determined as % recovery. To assess this parameter, blank samples were spiked to final concentrations of 50, 400 and 800 ng mL^{-1} ($n = 5$). Recovery was calculated by comparing the analytical results for extracted samples at the three concentrations mentioned above with unextracted standards that represent 100% of recovery.

The precision of the method was determined using the concentrations of 50, 400 and 800 ng mL⁻¹ and expressed as the percent Relative Standard Deviation (RSD). The sample Standard Deviation (SD) was calculated for five replicates for each level for the intra-day precision and over five consecutive days for the inter-day precision.

Matrix effect in ion suppression or enhancement was determined using the post extraction method: Blank extracted plasma was fortified with 50, 400 and 800 ng mL⁻¹ of the analyte and obtained areas were compared with those obtained by the standards solutions at the same concentrations using the following equation:

$$\text{Matrix effect (\%)} = \frac{RP - RS}{RS} \times 100$$

where, *RP* is the “mean peak area of reconstituted extract in plasma” and *RS* is the “mean peak area of the reference solution”.

The freeze-thaw stability of the analyte was determined in plasma at two concentrations (50 and 400 ng mL⁻¹) in triplicates. Bench-top stability in plasma up to 4 h, freeze-thaw stability from -20°C to ambient temperature in plasma (up to three cycles) and the stability of the analyte in stock solution at both 4°C (for 37 days) and room temperature (for 17 h), were evaluated.

Animals

Blood samples were obtained from one healthy male New Zealand Rabbit administered with an intravenous injection of grapiprant (0.5 mg kg⁻¹) in the arterial of the right ear. The drug was prior dissolved in ethanol (10 mg mL⁻¹), then diluted with sterile water for injection (9:1 v:v) and immediately injected (injection rate 5 mL min⁻¹). Blood (2 to 3 mL) was collected via catheter, previously inserted in the left arterial of the ear, at assigned times (0, 15, 30, 45 min and 1, 2, 4, 6, 8, 10 and 24 h) and immediately placed into collection tubes containing lithium heparin. The samples were centrifuged at 1,006 g within 30 min of collection and the harvested plasma was frozen immediately and stored at -20°C. Samples were analysed within 1 week from the collection. Immediately prior to the analysis, the samples were thawed at room temperature. Standard animal care and handling were performed according to the Directive 2010/63/UE.

Pharmacokinetic Analysis

The pharmacokinetic calculations were carried out using WinNonlin v 5.3.1 (Pharsight Corp, Sunnyvale, CA, USA). The area under the concentration *versus* time curve (AUC_{0-∞}) was calculated using the linear trapezoidal rule. Changes in plasma concentrations of grapiprant were evaluated using the standard non compartmental analysis and the relative pharmacokinetic parameters were determined using standard non compartmental equations (Gibaldi, 1982).

Results

Optimization of the Extraction Method

Sample clean-up is a critical step especially when complex matrices such as plasma are analyzed. Two liquid-liquid extractions were compared in the first phase of this work using CHCl₃ and CH₃CN in order to identify the solvent that best fitted our purpose. CH₃CN gave the best result with a mean recovery of 82.2% against a mean recovery of 66.4% obtained with CHCl₃.

LC-MS/MS Analysis and Method Validation

Preliminary experiments were carried out by analysing six blanks plasma to evaluate the interference of the matrix (selectivity) and no interfering endogenous substances at the retention times of the analyte and IS were detected. Figure 1A shows the chromatogram of a plasma sample spiked with 50 ng mL⁻¹ of grapiprant and 100 ng mL⁻¹ of IS. Quantitative analysis was carried out in ESI positive and in MRM by selecting three precursor/product ion transitions for grapiprant and two for the internal standard. The selection of the MRM transitions was made on the basis of the best signal-to-noise ratios obtained. The transitions used for the quantitation were 495.5 *m/z* → 295.5 *m/z* (CE = -21eV) and 200.4 *m/z* → 182.1 *m/z* (CE = -9eV) for grapiprant and IS respectively. With the chromatographic conditions used, grapiprant and IS had a retention time of 2.6 and 2.7 minutes respectively. The linearity of the chromatographic response in the investigated range of concentrations (5-1000 ng mL⁻¹) was evaluated with the construction of a calibration curve in rabbit plasma fortified with standard solutions of grapiprant and the correlation coefficient (*r*) was 0.99. The obtained LOD and LOQ were 1.5 and 5 ng mL⁻¹ respectively, suggesting a good sensitivity of the method. Recoveries, assessed at 50, 400 and 800 ng mL⁻¹, ranged from 73.3 to 97.1%; matrix effect, expressed as ion suppression, was ≤9%. The intraday and inter-day precision results showed good RSD values, all within the acceptable limits (Table 1).

No significant loss or deterioration of the analyte was observed after 4 h at room temperature and after three freeze-thaw cycles from -20°C to room temperature. The analyte stability was also verified up to 37 days at 4°C and for 17 h at room temperature (data not shown). Obtained results indicate the absence of stability-related problems during a pharmacokinetic analysis of grapiprant.

In Vivo Application

The proposed LC-MS-MS method was applied to a pharmacokinetic study in a rabbit after a single 0.5 mg kg⁻¹ intravenous injection. Plasma was collected at established times and the developed analysis confirmed the presence of grapiprant in time related amounts (Fig. 2). The concentrations of grapiprant in plasma ranged from

1106.66 ng mL⁻¹ (after 15 min from the injection) to 5.8 ng mL⁻¹ (after 6 h from the injection). The analyte was quantified up to 6 h from the injection. The described method allowed monitoring of the concentration *versus*

time curve of the drug and the calculation of the basic pharmacokinetic parameters (Table 2). A chromatogram of the plasma sample collected after 15 min is reported in Fig. 1B.

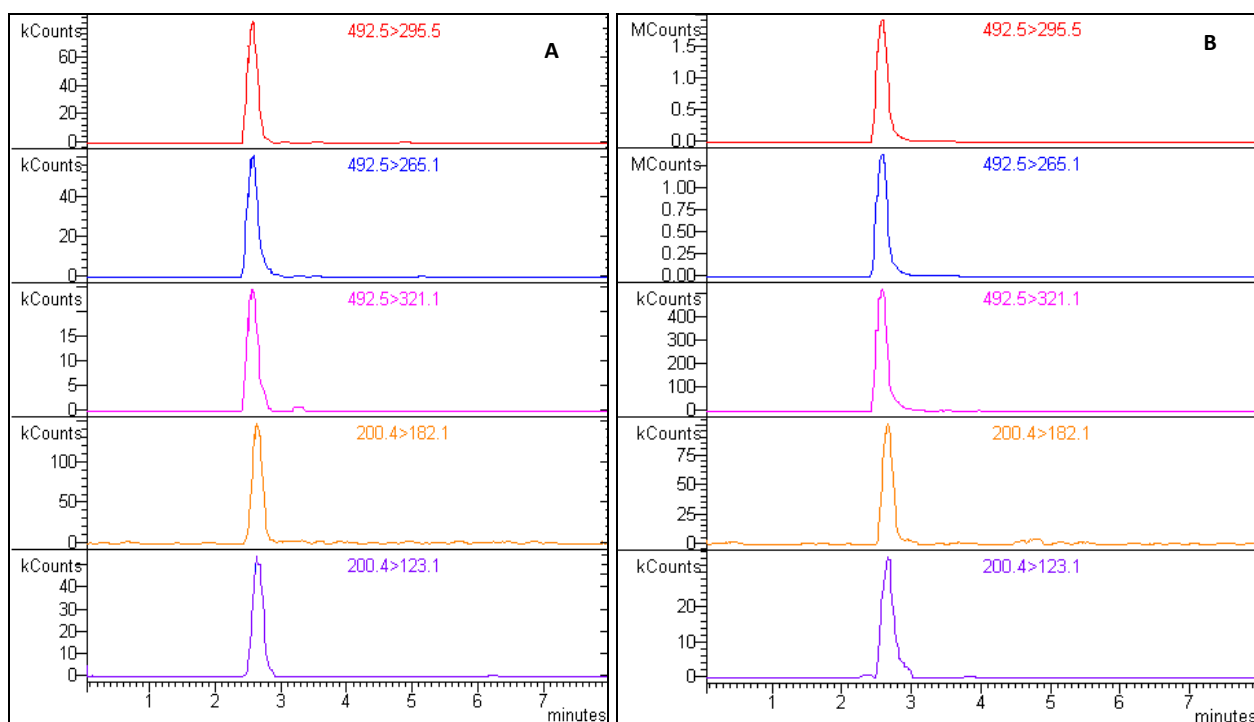


Fig. 1: Chromatogram of a rabbit plasma sample spiked with 50 ng mL⁻¹ of grapiprant and 100 ng mL⁻¹ of IS (A); Chromatogram of a rabbit plasma sample collected after 15 min from the grapiprant (0.5 mg Kg⁻¹) intravenous injection (B)

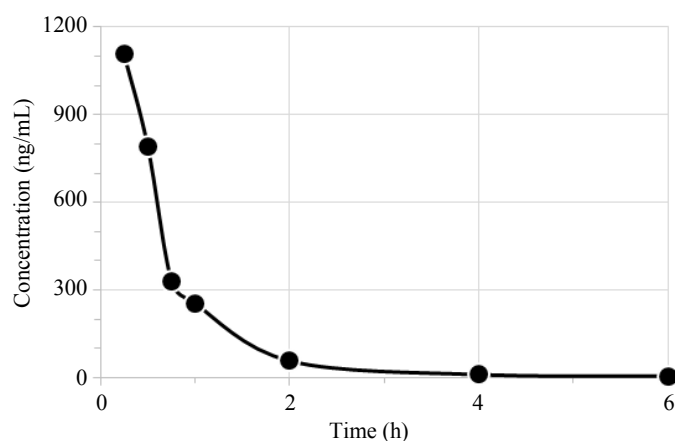


Fig. 2: Plasma concentrations of grapiprant after a single intravenous administration (0.5 mg/kg BW) in one rabbit

Table 1: Recovery, matrix effect and precision

Matrix	Grapiprant (ng/mL)	Recovery (%) (n = 10)	Matrix effect (%) (n = 10)	Intraday RSD (%) (n = 5)	Interday RSD (%) (n = 5)
Plasma	50	97.1	-9.0	5.9	10.1
	400	76.3	-8.7	3.5	12.1
	800	73.3	-8.5	4.8	7.5

RSD: Relative Standard Deviation

Table 2: Main pharmacokinetics parameters of grapiprant following single Intravenous (IV) 0.5 mg kg⁻¹ administration in one healthy rabbit

Parameters	Grapiprant
R ²	0,936
λz (1/h)	0,7937
t _{1/2} λz (h)	0,8734
T _{max} (h)	0,25
C ₀ (μg/mL)	1545,82
Vd (mL/kg)	613,025
Cl (mL/h/kg)	486,531
AUC _{0-last} (h μg/mL)	1022,95
AUC _{0-∞} (h μg/mL)	1030,26
AUMC _{0-∞} (h ² μg/mL)	734,332
MRT (h)	0,6963
Vss (mL/kg)	338,795

R² = correlation coefficient; λz = terminal phase rate constant; t_{1/2}λz = terminal half-life; T_{max} = time of peak; C₀ = peak plasma concentration at time zero; Vd = volume of distribution; Cl = total body clearance; AUC_{0-last} = area under the plasma concentration-time curve; AUC_{0-∞} = area under the plasma concentration-time curve extrapolated to infinity; AUMC_{0-∞} = area under the first moment curve from zero to infinity; MRT = mean resident time; Vss = Volume of distribution at the steady state

Discussion

This is the first report to describe a validated method for the quantification of grapiprant in plasma matrix through LC-MS/MS. In recent years, the LC-MS/MS technique is considered the most suitable one for the quantitation of drugs in biological matrices because of its high sensitivity and selectivity. In addition, LC-MS/MS showed the advantage of unambiguous analyte identification without further time consuming and error-prone confirmation steps with excellent sensitivity and specificity. Compared with the HPLC-FL method reported in literature by De Vito *et al.* (2016), an improvement in the LOQ and in the total analysis time is evident. More importantly, the sensitivity of the method reported here was well below the minimum effective concentration of grapiprant reported for dogs (114-164 ng mL⁻¹) (Nagahisa and Okumura, 2017) and suggested that the method would be suitable for further *in vivo* studies of grapiprant in plasma.

Conclusion

The analytical method described in this study provides selective and accurate analysis of grapiprant without the need for expensive clean up steps, solvent consuming flows or time consuming procedures. Moreover, only small amounts of plasma are required, which is important for pharmacokinetic studies of grapiprant in smaller animals (e.g., cats). The low LOQ shows that the method could be useful for drug

measurement even when administered in sub-clinical doses. These features make the described method suitable for pharmacokinetic investigations including drug-drug interaction and potential future applications such as guidance for dose adjustment.

Authors Contributions

Elena Baralla: Carried out the experiment, contributed to sample preparation took the lead in writing the manuscript.

Virginia De Vito: Carried out the experiment, contributed to sample preparation.

Maria Vittoria Varoni: Aided in interpreting the results and worked on the manuscript.

Mario Giorgi: Conceived of the presented idea, designed and directed the project.

Maria Piera Demontis: Worked out almost all of the technical details and performed the numerical calculations of the suggested experiment.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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