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Determination of casopitant and its three major metabolites in dog and rat plasma by positive ion liquid chromatography/tandem mass spectrometry

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ABSTRACT

A sensitive, selective and quantitative method for the simultaneous determination of casopitant, a potent and selective antagonist of the human Neurokinin 1 (NK-1) receptor, and its three major metabolites M12, M13 and M31 was developed and validated in dog and rat plasma. Acetonitrile containing stable labeled internal standards for the four analytes was used to precipitate proteins in plasma. Chromatographic separation was obtained using a reversed phase column with multiple reaction monitoring turboionspray positive ion detection. The lower and upper limits of quantification for casopitant and its metabolites were 15 and 15,000 ng/mL, using a 50 μL of dog or rat plasma aliquot, respectively. The inter-day precision (relative standard deviation) and accuracy (relative error) in dog plasma, derived from the analysis of validation samples at 5 concentrations, ranged from 4.1% to 10.0% and −10.8% to 8.7%, respectively, for casopitant and its 3 major metabolites. The intra-day precision (relative standard deviation) and accuracy (relative error) in rat plasma, derived from the analysis of validation samples at 5 concentrations, ranged from 3.9% to 6.6% and −9.6% to 8.3%, respectively, for casopitant and its three metabolites. All analytes were found to be stable in analytical solutions for at least 43 days at 4 °C, in dog and rat plasma at room temperature for at least 24 h, at the storage temperature of −20 °C for at least 6 months, and following the action of three freeze–thaw cycles from −20 °C to room temperature. All analytes were also found to be stable in processed extracts at 4 °C for at least 72 h. This assay proved to be accurate, precise, fast and was used to support long-term toxicology studies in dog and rat.

1. Introduction

Casopitant, also known as GW679769 (Fig. 1), is a piperidine derivative that has been shown to be a potent and selective antagonist of the human Neurokinin subtype-1(NK-1) receptor, the primary receptor of substance P (SP), both in vitro and in vivo with good brain penetration properties [1]. NK-1 receptors are widely distributed in the peripheral and central nervous system including areas thought to be involved in the regulation of affective behavior and neurochemical response to stress [2–4]. NK-1 receptors are also found in non-neural tissues such as endothelial and inflammatory cells as well as gastrointestinal, respiratory, and genitourinary tissues. Blocking NK-1 neurotransmitter receptors has been demonstrated to be effective for the treatment of major depressive disorder, one or more anxiety disorders [5,6] and to prevent chemotherapy-induced and post-operative nausea and vomiting [7,8]. Based on this mode of action it has been evaluated for the prevention of chemotherapy-induced and post-operative nausea and vomiting [9,10]. In addition, casopitant has been investigated in a number of chronic dosing indications where the NK-1 receptor is believed to play a role, such as anxiety, depression, insomnia, and over-active bladder.

The nonclinical safety package of casopitant included investigations appropriate for both acute and chronic indications [11]. Following a single oral administration in rats and dogs, casopitant has been shown to be extensively metabolized, widely distributed with quite long retention time in tissues and slow rate of elimination mainly in dog [12]. Of the many metabolites which were found circulating in humans [13], three were considered as major: M12 (coded as GSK631832), M13 (coded as GSK525060) and M31 (coded as GSK517142). Their structures are shown in Fig. 1.

In order to provide safety cover data in the clinical phase, casopitant and its major metabolites M12, M13 and M31 had to be quantified in long-term toxicity studies. For this purpose, a precise, accurate and high-throughput method for the simultaneous quantification of these four analytes had to be developed and validated. The target was to develop a multi-analyte assay with a similar

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; HPLC, high performance liquid chromatography; MRM, multiple reaction monitoring; DMF, dimethylformamide; SIL, stable isotope labeled; IS, internal standard; QC, quality control; LIMS, laboratory information management system; MF, matrix factor; NMF, normalized matrix factor.

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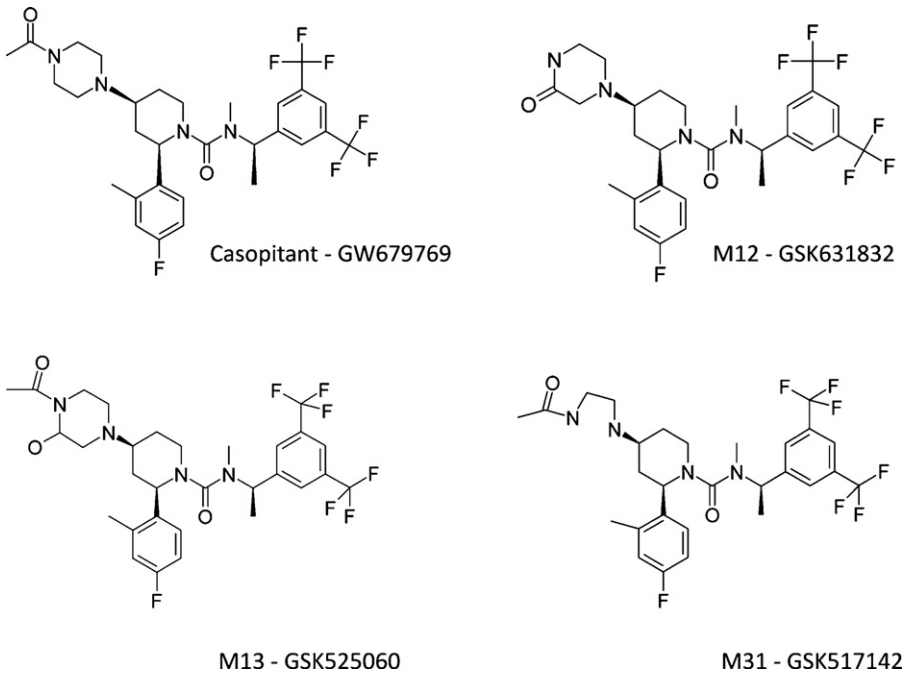

Fig. 1. Chemical structures of casopitant (coded as GW679769) and its metabolites M12 (coded as GSK631832), M13 (coded as GSK525060) and M31 (coded as GSK517142).

productivity, selectivity, precision and accuracy of the previous validated bioanalytical methods which allowed quantification of casopitant, only.

2. Experimental

2.1. Chemicals and materials

Casopitant and its metabolites GSK631832, GSK525060 and GSK517142 were obtained from Chemical Development at GlaxoSmithKline (Tonbridge, UK); stable labeled internal standards (SIL) [$^2H_3{}^{13}C$]-GW679769, [$^2H_3{}^{13}C$]-GSK631832, [$^2H_3{}^{13}C$]-GSK525060 and [$^2H_3{}^{13}C$]-GSK517142 (Fig. 2) were obtained from Isotope Chemistry at GlaxoSmithKline (Stevenage, UK). Dog and rat plasma were obtained from Laboratory Animal Sciences (GSK Verona) and were derived by ethical approved procedures. HPLC grade acetonitrile was obtained from J.T. Baker (Deventer, The Netherlands). Analytical grade ammonium acetate was obtained from Sigma–Aldrich (Steinhein, Germany) and water was from a Millipore Milli-Q system (Billerica, MA).

2.2. Equipment

A Heraeus MultiFUGE (Milan, Italy) centrifuge with a rotor capacity for four 96-well plates and a Mettler AT261 balance (Hightown, NJ, USA) were used. A Tecan Genesis150RSP liquid handler (Zurich, CH) was used for plasma transfer. The HPLC system consisted of an Agilent 1100 G1312A binary pump equipped with an Agilent 1100 G1322A degasser (Waldbronn, Germany). The autosampler was a CTC Analytics HTS PAL (Zwingen, CH). The chromatographic system consisted of a Thermo Hypersil Gold column 3.0 mm × 50 mm, 5 μm (Milan, Italy). Mass spectrometric detection was performed on an Applied Biosystems/MDS Sciex API4000 triple quadrupole (Concord, Ontario, Canada) operating in positive turboionspray mode controlled by Analyst software (version 1.1).

2.3. LC–MS/MS conditions

An isocratic HPLC method was employed for separation. The mobile phase consisted of aqueous 5 mM ammonium acetate/acetonitrile (35:65, v/v). The flow rate was set at 0.7 mL/min. The autosampler was programmed to inject 2.5 μL sample aliquots every 1.5 min.

The API4000 triple quadrupole turboionspray source of the mass spectrometer was operated in positive ion mode, with the curtain gas (nitrogen), ion source 1 and ion source 2 gasses (purified air) set at 25, 50 and 45 psi, respectively. The IonSpray voltage was set at 3500 V, the source temperature was maintained at 600 °C and the source parameters were optimized for casopitant, M12, M13, M31 and their internal standards (IS) in multiple reaction monitoring (MRM) mode. In MRM mode, casopitant was monitored at the transition *m/z* 617–167 and its internal standard [$^2H_3{}^{13}C$]-GW679769 was monitored at the transition *m/z* 621–171. For metabolites of casopitant, the MRM transitions monitored were *m/z* 589–453 for M12 and *m/z* 593–493 for its IS [$^2H_3{}^{13}C$]-GSK631832, *m/z* 633–479 for M13 and *m/z* 637–483 for its IS [$^2H_3{}^{13}C$]-GSK525060, *m/z* 591–184 for M31 and *m/z* 595–184 for its IS [$^2H_3{}^{13}C$]-GSK517142. The product ions were generated with collision energy of 28, 35, 25 and 45 eV for casopitant, M12, M13 and M31, respectively. The collision gas thickness was set at the instrument value of 6. The Declustering Potential (DP), Entrance Potential (EP) and Collision Cell Exit Potential (CXP) were set for all compounds at 65, 10 and 10 V, respectively. A dwell time of 100 and 50 ms was used for the transitions of analytes and internal standards, respectively. The pause time was 5 ms.

2.4. Preparation of standards and quality control samples

Stock solutions for both casopitant and its metabolites were prepared in DMF, while working solutions were prepared in acetonitrile/water (50:50, v/v). Stock solutions of casopitant, M12, M13 and M31 (separate weighing for calibration standards and QC's) were prepared at a concentration of 5 mg/mL. Stock solutions of their stable labeled internal standards were prepared in DMF at a concentration of 1 mg/mL, which were combined and diluted to a unique working SIL solution at 200 ng/mL in acetonitrile. Stock solutions of casopitant and its metabolites were combined and further diluted to obtain working solutions containing all analytes at the concentration of 750, 225, 75, 22.5, 7.5, 2.25 and 0.75 μg/mL.

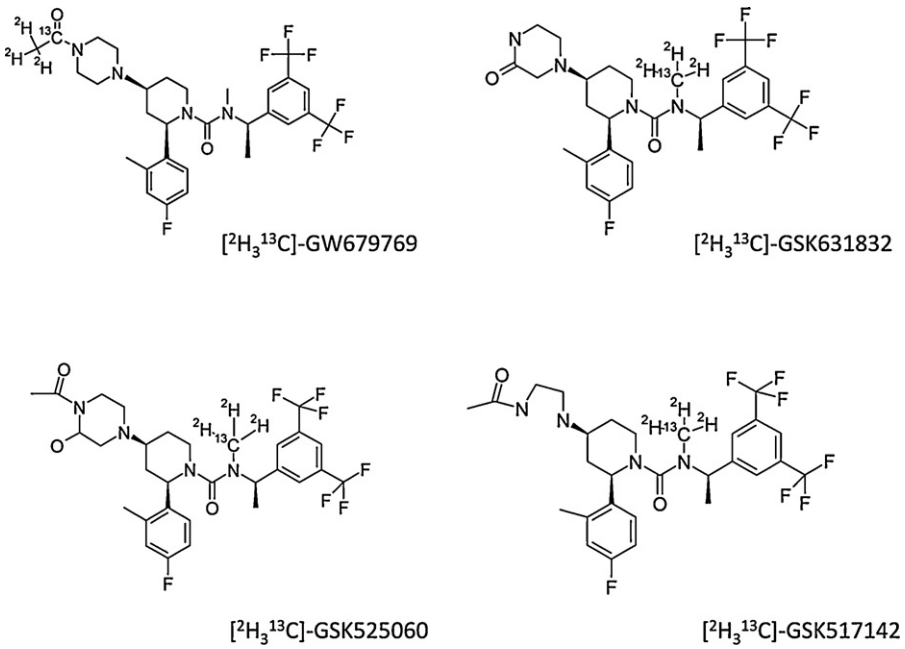

Fig. 2. Chemical structures of stable labeled internal standards (SIL) [$^2H_3{}^{13}C$]-GW679769, [$^2H_3{}^{13}C$]-GSK631832, [$^2H_3{}^{13}C$]-GSK525060 and [$^2H_3{}^{13}C$]-GSK517142.

Dog or rat plasma (490 μL) was spiked with each working solution (10 μL) to provide calibration standards at the concentration of 15, 45, 150, 450, 1500, 4500 and 15,000 ng/mL. For QC samples, stock solutions of casopitant and its metabolites were combined and further diluted to obtain five working solutions containing all analytes at the concentration of 750, 600, 22.5, 3 and 0.75 μg/mL. Dog or rat plasma (490 μL) was spiked with these working solutions (10 μL) to provide quality controls at the concentration of 15, 60, 450, 12,000, 15,000 ng/mL for each analyte. All stock and working solutions were stored at 4 °C. QC samples were divided into 0.5 mL aliquots and frozen at −20 °C or extracted immediately. In the first validation run for both species, freshly prepared QC samples were analyzed against freshly prepared calibration standards in plasma from the corresponding species. For the subsequent validation runs in dog, frozen replicate aliquots of the QC samples were thawed at ambient temperature and analyzed against a freshly prepared standard curve in dog plasma.

2.5. Sample preparation

Dog or rat plasma samples were pipetted into 96-well plates by using a Tecan Genesis150RSP. Plasma (50 μL) was extracted by protein precipitation with acetonitrile (150 μL, containing the internal standards at the concentration of 200 ng/mL for each analyte) and vortex mixed for 2 min. Ultra pure water (100 μL) was then added to each well, extracts were briefly vortex mixed for 10 s and then centrifuged at 3000 × *g* for 10 min. 96-well plates were then placed in the autosampler kept at 4 °C. A typical injection volume of 2.5 μL (full loop injection mode) was used.

2.6. Assay validation procedures

All validation experiments were performed according to departmental working practices, AAPS [14], and FDA [15] regulatory guidelines and the following parameters were assessed for casopitant and its metabolites M12, M13 and M31: selectivity, sensitivity and linearity, precision and accuracy, extraction recovery and matrix effect. The stability of all analytes was determined in analytical solutions, in dog and rat plasma at room temperature, at the storage temperature of −20 °C, under the action of freeze–thaw cycles from −20 °C to room temperature and in processed extracts stored in the autosampler at 4 °C.

Validation samples were prepared and analyzed to evaluate the intra-day and inter-day accuracy and precision of the analytical method in dog plasma. The assay was then validated in rat plasma by performing an abbreviated (1 run) validation.

2.7. Data analysis

HPLC–MS/MS data were acquired and processed (integrated) using the proprietary software application Analyst™ (version 1.1 for acquisition and version 1.4.1 for processing, Applied Biosystems/MDS Sciex, Canada). Calibration plots of analyte/internal standard peak area ratio versus casopitant, M12, M13 and M31 concentrations were constructed and a weighted $1/x^2$ linear regression applied to the data. Concentrations of casopitant, M12, M13 and M31 in validation samples were determined from the appropriate calibration line, and used to calculate the bias and precision of the method with an in-house LIMS (SMS2000, version 1.5, GlaxoSmithKline).

3. Results and discussion

3.1. LC–MS/MS conditions

LC–MS/MS was used as the most powerful analytical tool in preclinical pharmacokinetics for its selectivity, sensitivity and linearity. The objective of this study was to develop and validate a simple, accurate and high-throughput assay method for the quantitative analysis of casopitant and its three major metabolites in plasma samples from toxicokinetic studies. Protein precipitation, a simple, generic extraction technique was utilized for the pretreatment of both dog and rat plasma samples. Chromatographic conditions, in particular the composition and nature of the mobile phase, were optimized through several trials to achieve the best peak shape, retention and sensitivity as well as a short run time for casopitant and its metabolites M12, M13 and M31. The use of ammonium acetate in the mobile phase gave the best ion response with low background noise. It was found that without adjusting the pH, the mixture of aqueous 5 mM ammonium acetate/acetonitrile

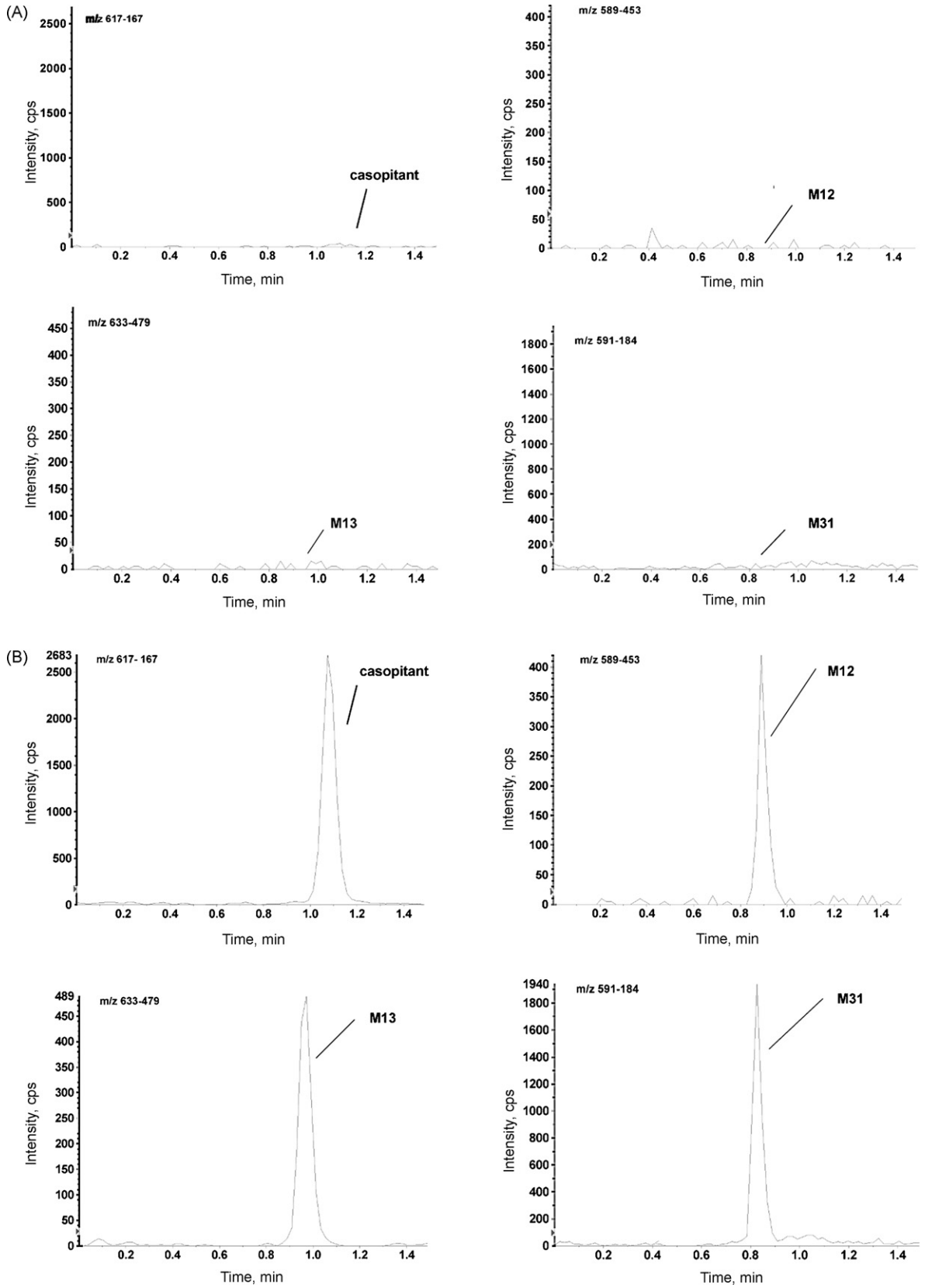

Fig. 3. Representative chromatograms of casopitant and its metabolites M12, M13 and M31: (A) blank dog or rat plasma and (B) spiked dog or rat plasma containing 15 ng/mL of each analyte.

Table 1
Intra- and inter-assay performance data for casopitant in dog plasma samples ($n = 6$ for each concentration level in each individual run).

Nominal concentration (ng/mL)	15	60	450	12,000	15,000
Run 1					
Mean concentration (ng/mL)	13.4	61.5	457.5	11411.0	14363.4
S.D.	0.4	1.8	8.3	181.4	285.8
Precision (%CV)	2.8	2.9	1.8	1.6	2.0
Accuracy (% Bias)	−10.8	2.5	1.7	−4.9	−4.2
Run 2					
Mean concentration (ng/mL)	13.6	61.3	466.3	11758.8	14551.2
S.D.	0.4	2.2	3.4	327.9	317.9
Precision (%CV)	2.9	3.7	0.7	2.8	2.2
Accuracy (% Bias)	−9.1	2.1	3.6	−2.0	−3.0
Run 3					
Mean concentration (ng/mL)	14.7	59.0	461.7	12296.7	15275.0
S.D.	0.8	1.6	13.9	434.1	370.3
Precision (%CV)	5.3	2.7	3.0	2.8	2.4
Accuracy (% Bias)	−2.1	−1.7	2.6	2.5	1.8
Overall (inter-run) statistics					
Mean concentration (ng/mL)	13.9	60.6	461.8	11822.2	14729.9
S.D.	0.8	2.1	9.7	465.3	507.5
Between-run precision (%)	4.7	1.9	Negligible	3.6	3.1

35:65 (v/v) could achieve this purpose and was finally adopted as the mobile phase. The MS optimization was performed by direct infusion of the casopitant, M12, M13 and M31 into the TurboIonSpray source of the mass spectrometer. During optimization, the ion source of the mass spectrometer was operated at the same conditions (nebulizer and auxiliary gas flow rate, LC flow rate, temperature and IonSpray voltage) used for the analysis of samples. Particular attention had to be paid for the selection of the MS/MS transitions to be monitored. In fact, the three metabolites M12, M13 and M31 have a chemical structure which is quite similar to the casopitant one, with parent ions generating common daughter ions in the MS/MS collision cell of the mass spectrometer. Hence, specific transitions were selected for the four analytes and their internal standards, in order to avoid clashes which could cause cross-talk effects and potential over-estimate of some of the analytes. The collision gas thickness was optimized to yield a maximum sensitivity for all analytes. Collision energies were optimized for each compound in order to generate the most specific daughter ions to be monitored and maximize the MS/MS sensitivity. The choice of the selective MRM transitions made the complete chromatographic separation of the four analytes not necessary and this helped to provide a simple and very fast LC–MS/MS method for their quantification.

3.2. Assay validation

3.2.1. Assay selectivity

The selectivity of the method was established by the analysis of samples of control dog and rat plasma from 6 individual animals. The selectivity of the method was also assessed by the inclusion of blank and double blank samples prepared from pooled control dog and rat plasma in validation assays. HPLC–MS/MS chromatograms of the blanks and validation samples were visually examined and compared for chromatographic integrity and potential interferences.

Using a 3:1 (v/v) acetonitrile to plasma protein precipitation ratio, the separation power of the chromatographic column, and the selectivity of tandem mass spectrometry minimized potential interferences. Representative chromatograms of blank dog and rat plasma samples showing the lack of interfering peaks are represented in Fig. 3.

3.2.2. Linearity

Calibration standards in duplicate ranging from 15 to 15,000 ng/mL in dog plasma were analyzed in three separate runs for casopitant and its metabolites M12, M13 and M31. In rat plasma, calibration standards in duplicate were analyzed in a single run. Ratios of peak areas of each analyte versus its internal standard were calculated for each point and standard curves were constructed by least square linear regression analysis using a weighting factor of $1/x^2$, in which x is the concentration in ng/mL. Linear responses in the analyte/internal standard peak area ratios were observed over the range 15–15,000 ng/mL for all analytes in dog and rat plasma. In dog plasma, the correlation coefficients obtained using $1/x^2$ weighted linear regression were better than 0.9991 for casopitant, 0.9967 for M12, 0.9978 for M13, and 0.9991 for M31. In rat plasma, the correlation coefficients were 0.9984 for casopitant, 0.9964 for M12, 0.9980 for M13, and 0.9990 for M31.

3.2.3. Precision and accuracy

The precision and accuracy of the method were determined by analysis of quality control samples in dog or rat plasma as six replicates at five concentrations (15, 60, 450, 12,000 and 15,000 ng/mL). For the dog plasma assay, they were analyzed along with two sets of standard samples prepared in dog plasma on each of three days using the same instrument. Within-run precision was defined as the relative standard deviation of the six replicates and between-run precision as the relative standard deviation of the overall measured concentrations from the three days ($n = 18$). For the rat assay, within-run precision was determined, only. The accuracy was defined in terms of % Bias from nominal values. Concentrations of casopitant, M12, M13 and M31 in validation samples of dog plasma were determined from the calibration line in the same matrix on each occasion; accuracy and precision values are presented in Tables 1–4. At all validation sample concentrations examined, the bias was less than 15%, and therefore acceptable. The maximum bias observed was −10.8% for casopitant, −8.0% for M12, −10.4% for M13, and −10.8% for M31. The within- and between-run precision values were less than 15%, and are therefore acceptable. The maximum within- and between-run precision values observed were 5.3% and 4.7% for casopitant, 9.5% and 4.1% for M12, 6.8% and 6.2% for M13 and 7.4% and 10.0% for M31.

Concentrations of casopitant, M12, M13 and M31 in validation samples of rat plasma were determined from the calibration line prepared in rat plasma on one occasion, accuracy and precision values are presented in Tables 5–8. At all validation sample concentrations examined, the bias was less than 15%, and therefore acceptable. The maximum bias observed was −5.3% for caso-

Table 2
Intra- and inter-assay performance data for M12 in dog plasma samples ($n = 6$ for each concentration level in each individual run).

Nominal concentration (ng/mL)	15	60	450	12,000	15,000
Run 1					
Mean concentration (ng/mL)	14.0	60.5	448.5	11365.0	14270.3
S.D.	1.1	3.6	7.5	227.1	531.6
Precision (%CV)	7.5	6.0	1.7	2.0	3.7
Accuracy (% Bias)	−6.6	0.8	−0.3	−5.3	−4.9
Run 2					
Mean concentration (ng/mL)	13.8	59.1	466.0	11879.7	14642.9
S.D.	1.3	3.3	8.3	265.0	259.2
Precision (%CV)	9.1	5.6	1.8	2.2	1.8
Accuracy (% Bias)	−8.0	−1.5	3.5	−1.0	−2.4
Run 3					
Mean concentration (ng/mL)	14.3	58.8	442.7	12274.7	15510.5
S.D.	1.3	3.8	24.0	404.7	595.2
Precision (%CV)	9.5	6.5	5.4	3.3	3.8
Accuracy (% Bias)	−4.9	−2.1	−1.6	2.3	3.4
Overall (inter-run) statistics					
Mean concentration (ng/mL)	14.0	59.5	452.4	11839.8	14807.9
S.D.	1.2	3.4	17.6	480.5	702.1
Between-run precision (%)	Negligible	Negligible	2.3	3.7	4.1

Table 3
Intra- and inter-assay performance data for M13 in dog plasma samples ($n = 6$ for each concentration level in each individual run).

Nominal concentration (ng/mL)	15	60	450	12,000	15,000
Run 1					
Mean concentration (ng/mL)	15.3	61.7	452.1	11337.7	14805.0
S.D.	0.9	2.3	20.2	427.4	323.1
Precision (%CV)	5.6	3.7	4.5	3.8	2.2
Accuracy (% Bias)	2.2	2.8	0.5	−5.5	−1.3
Run 2					
Mean concentration (ng/mL)	13.4	60.4	462.0	12170.6	15167.8
S.D.	0.9	2.9	12.8	219.3	527.3
Precision (%CV)	6.5	4.7	2.8	1.8	3.5
Accuracy (% Bias)	−10.4	0.7	2.7	1.4	1.1
Run 3					
Mean concentration (ng/mL)	14.9	57.6	464.7	12505.1	15152.2
S.D.	1.0	3.1	16.1	271.8	405.5
Precision (%CV)	6.8	5.3	3.5	2.2	2.7
Accuracy (% Bias)	−1.0	−4.0	3.3	4.2	1.0
Overall (inter-run) statistics					
Mean concentration (ng/mL)	14.5	59.9	459.6	12004.5	15041.7
S.D.	1.2	3.1	16.6	587.1	436.5
Between-run precision (%)	6.2	2.9	Negligible	4.9	0.7

Table 4
Intra- and inter-assay performance data for M31 in dog plasma samples ($n = 6$ for each concentration level in each individual run).

Nominal concentration (ng/mL)	15	60	450	12,000	15,000
Run 1					
Mean concentration (ng/mL)	14.3	61.0	458.7	11747.4	14678.8
S.D.	1.1	2.2	9.1	195.3	265.0
Precision (%CV)	7.4	3.6	2.0	1.7	1.8
Accuracy (% Bias)	−4.7	1.6	1.9	−2.1	−2.1
Run 2					
Mean concentration (ng/mL)	13.4	60.6	460.6	11695.5	15012.0
S.D.	0.5	1.3	11.8	204.6	387.2
Precision (%CV)	3.5	2.2	2.6	1.7	2.6
Accuracy (% Bias)	−10.8	0.9	2.4	−2.5	0.1
Run 3					
Mean concentration (ng/mL)	16.3	59.1	460.7	12406.8	15180.8
S.D.	0.3	2.9	9.4	324.6	386.8
Precision (%CV)	1.9	5.0	2.0	2.6	2.5
Accuracy (% Bias)	8.7	−1.6	2.4	3.4	1.2
Overall (inter-run) statistics					
Mean concentration (ng/mL)	14.7	60.2	460.0	11949.9	14957.2
S.D.	1.4	2.3	9.6	406.8	393.5
Between-run precision (%)	10.0	0.7	Negligible	3.2	1.4

Table 5
Intra-assay performance data for casopitant in rat plasma samples ($n = 6$ for each concentration).

Nominal concentration (ng/mL)	15	60	450	12,000	15,000
Mean concentration (ng/mL)	14.5	62.4	469.1	11576.8	14203.3
S.D.	0.6	1.9	16.1	419.9	200.1
Precision (%CV)	3.9	3.0	3.4	3.6	1.4
Accuracy (% Bias)	−3.6	4.0	4.3	−3.5	−5.3

Table 6
Intra-assay performance data for M12 in rat plasma samples ($n = 6$ for each concentration).

Nominal concentration (ng/mL)	15	60	450	12,000	15,000
Mean concentration (ng/mL)	14.5	64.6	487.2	11815.2	14807.5
S.D.	1.0	2.9	19.6	451.2	267.2
Precision (%CV)	6.6	4.5	4.0	3.8	1.8
Accuracy (% Bias)	−3.4	7.6	8.3	−1.5	−1.3

Table 7
Intra-assay performance data for M13 in rat plasma samples ($n = 6$ for each concentration).

Nominal concentration (ng/mL)	15	60	450	12,000	15,000
Mean concentration (ng/mL)	14.8	57.0	469.4	12364.9	15456.1
S.D.	0.9	2.1	22.1	457.4	652.7
Precision (%CV)	6.1	3.7	4.7	3.7	4.2
Accuracy (% Bias)	−1.5	−5.0	4.3	3.0	3.0

pitant, 8.3% for M12, −5.0% for M13, and −9.6% for M31. The within-run precision values were less than 15%, and are therefore acceptable. The maximum within-run precision values observed were 3.9% for casopitant, 6.6% for M12, 6.1% for M13 and 4.9% for M31.

3.2.4. Extraction recovery

The recovery of casopitant and its metabolites M12, M13 and M31 from dog and rat plasma samples spiked at 60, 450 and 12,000 ng/mL was assessed by comparing the analyte response of the extracted samples to those of blank extracts of dog and rat plasma spiked at the same concentration after extraction. All samples were analyzed in replicates of six.

The extraction recovery from dog plasma samples ranged between 91.9% and 100.3% for casopitant, 93.4% and 98.7% for M12, 92.1% and 100.7% for M13, 86.8% and 97.9% for M31. In rat plasma samples, the observed extraction recovery ranged between 100.7% and 102.4% for casopitant, 97.3% and 102.0% for M12, 101.5% and 103.7% for M13, 96.0% and 102.1% for M31. The precision of the extraction recovery was less than 15% at all concentrations in both dog and rat plasma and was therefore acceptable. The data obtained are shown in Tables 9 and 10.

3.2.5. Matrix effect

The effects of matrix components on the HPLC–MS/MS response of casopitant and its metabolites M12, M13 and M31 in six individual lots of dog and rat plasma was assessed at 3 different concentrations (60, 450 and 12,000 ng/mL) by comparing the analyte responses of blank extracts of dog and rat plasma spiked after extraction, with the response of matrix free samples at the same concentrations. All samples were analyzed in replicates of six.

The quantitative measure of matrix effect, also termed as matrix factor (MF) [14] was determined as a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions, i.e.:

Matrix factor = (Peak response in the presence of matrix ions)/(Peak response in the absence of matrix ions).

The normalized MF was also calculated by substituting peak response with peak response ratio (analyte/IS) in the above equation for MF. The data obtained in dog and rat plasma are shown in Tables 9 and 10. An MF of 1 signifies no matrix effects. A value of MF less than 1 suggests ionization suppression while an MF greater than 1 may be due to ionization enhancement. In dog plasma, the normalized MF ranged between 0.99 and 1.04 for casopitant, 0.95 and 1.04 for M12, 0.98 and 1.06 for M13, 0.96 and 1.14 for M31. In rat plasma, the normalized MF ranged between 0.96 and 1.01 for casopitant, 0.98 and 1.02 for M12, 0.98 and 1.02 for M13, 1.00 and 1.04 for M31. The experimental data show that for all analytes the MF values are indicative of a certain degree of ion suppression or enhancement, which is generally more evident at higher concentrations. Nevertheless, all IS normalized MF values observed in different lots of blank biological matrices, in two different animal species and at three different concentrations which are representative of the dynamic range of the method clearly demonstrate that the use of stable isotope – labeled IS minimizes the influence of matrix effects most effectively since the matrix effects observed for stable isotope – labeled IS are similar to those observed for the matching analyte.

3.2.6. Stability

The stability of casopitant and its metabolites M12, M13 and M31 was examined in stock and working analytical solutions. The stability of all analytes in dog and rat plasma was examined at room

Table 8
Intra-assay performance data for M31 in rat plasma samples ($n = 6$ for each concentration).

Nominal concentration (ng/mL)	15	60	450	12,000	15,000
Mean concentration (ng/mL)	15.3	62.5	467.4	10850.6	14301.7
S.D.	0.7	1.4	7.8	249.7	387.1
Precision (%CV)	4.9	2.2	1.7	2.3	2.7
Accuracy (% Bias)	1.7	4.2	3.9	−9.6	−4.7

Table 9
Matrix factor, normalized matrix factor and extraction recovery data for casopitant and its metabolites M12, M13 and M31 in dog plasma.

Casopitant nominal concentration (ng/mL)	60	450	12,000
Matrix factor (MF)	1.012	0.9973	0.978
Normalized matrix factor (NMF)	0.962	1.011	1.013
% extraction recovery	100.3	91.9	92.3
M12 nominal concentration (ng/mL)	60	450	12,000
Matrix factor (MF)	0.955	0.907	0.908
Normalized matrix factor (NMF)	0.983	1.022	0.999
% extraction recovery	98.7	93.4	93.4
M13 nominal concentration (ng/mL)	60	450	12,000
Matrix factor (MF)	1.002	0.941	0.948
Normalized matrix factor (NMF)	0.982	0.996	1.016
% extraction recovery	100.7	93.4	92.1
M31 nominal concentration (ng/mL)	60	450	12,000
Matrix factor (MF)	1.107	1.059	0.976
Normalized matrix factor (NMF)	0.996	1.011	1.038
% extraction recovery	97.9	94.9	86.8

Table 10
Matrix factor, normalized matrix factor and extraction recovery data for casopitant and its metabolites M12, M13 and M31 in rat plasma.

Casopitant nominal concentration (ng/mL)	60	450	12,000
Matrix factor (MF)	1.583	1.227	1.025
Normalized matrix factor (NMF)	1.004	1.045	0.999
% extraction recovery	102.4	100.7	101.2
M12 nominal concentration (ng/mL)	60	450	12,000
Matrix factor (MF)	1.307	0.989	0.833
Normalized matrix factor (NMF)	1.046	1.013	0.945
% extraction recovery	102.1	97.3	100.7
M13 nominal concentration (ng/mL)	60	450	12,000
Matrix factor (MF)	1.414	1.120	0.953
Normalized matrix factor (NMF)	1.047	1.064	0.976
% extraction recovery	103.7	102.8	101.5
M31 nominal concentration (ng/mL)	60	450	12,000
Matrix factor (MF)	1.247	1.127	1.018
Normalized matrix factor (NMF)	1.136	0.982	0.956
% extraction recovery	97.5	102.1	96.0

temperature, at the storage temperature of −20 °C and after the action of three freeze–thaw cycles from −20 °C to room temperature, at the concentration of 60, 450 and 12,000 ng/mL. The analytes were considered stable in plasma when 85–115% of the initial concentration was found. The stability of stock and working solutions (kept at 4 °C) was determined by comparing peak areas ratios of stored solutions with freshly prepared solutions, after dilution to a final concentration of 0.75 μg/mL in acetonitrile/water (50:50, v/v). Stability of stock and working solutions were considered acceptable when 95–105% of the initial area ratio was found.

Analytical solutions of casopitant and its metabolites M12, M13 and M31 were found to be stable for at least 65 days when prepared in DMF (stock solutions) or acetonitrile/water (working solutions) and stored at 4 °C. In dog plasma, all analytes were found to be stable at ambient temperature for at least 24 h, at the storage temperature of −20 °C for at least 12 months, and under the action of three freeze–thaw cycles from −20 °C to room temperature. All analytes were also found to be stable in processed extracts of dog plasma, stored in the autosampler at 4 °C, for at least 72 h. In rat plasma, all analytes were found to be stable at ambient temperature for at least 4 h, at the storage temperature of −20 °C for at least 6 months, and under the action of three freeze–thaw cycles from −20 °C to room temperature. All analytes were also found to be stable in processed extracts of rat plasma, stored in the autosampler at 4 °C, for at least 48 h.

4. Conclusion

An LC–MS/MS assay for the quantitation of casopitant and its three metabolites M12, M13 and M31 in dog and rat plasma has been developed and validated. The assay incorporates a simple protein precipitation with acetonitrile, automated by means of a robotic sample processor and reversed phase fast chromatography. No significant interferences caused by endogenous components were observed in both dog and rat plasma. The use of acetonitrile as precipitant proved to be highly effective with extraction efficiencies which were quantitative for all analytes. The method proved to be unaffected by any significant matrix effect, which was reduced to a minimum upon use of the stable labeled internal standards used in the extraction technique. All analytes were found to be stable in

analytical solutions, in dog and rat plasma at the long-term storage conditions and during the analytical process. The method proved to be highly selective, accurate, precise, and fast and has demonstrated usefulness in the analysis of dog and rat plasma samples from long-term safety studies.

Disclosure statement

This study was supported by GlaxoSmithKline (GSK) Research and Development. The author was employee of GSK at the time the study was performed and was eligible for GSK stock options and has stock ownership. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties within 3 years of beginning the work submitted. No writing assistance was utilized in the production of this manuscript.

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