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Interference by metabolites and the corresponding troubleshooting during the LC–MS/MS bioanalysis of G004, a bromine-containing hypoglycemic agent

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ABSTRACT

The quantitative determination of drugs in bio-samples may be interfered by the drug-related metabolites during the high-throughput LC–MS/MS analysis. When quantifying bromine or chlorine containing compounds, the $^{81}Br/^{37}Cl$ isotopic forms of their mono-hydroxylated metabolites after in-source dehydration could produce ions which are isobaric with the precursor ions of the parent compounds at the $^{79}Br/^{35}Cl$ isotopic form. In this report, we described the identification of an interfering hydroxylated metabolite of G004, a novel bromine-containing hypoglycemic agent, during LC–MS/MS analysis of plasma samples. Several different MRM transitions were tested and evaluated to minimize the metabolite influence on the quantification of G004. Furthermore, the standard addition method using incurred samples was used to evaluate the matrix effect caused by the interfering metabolite. The lower limit of quantitation of the established method was 0.2 ng/ml, which was 10 times lower than the existing one. The method was successfully applied to investigate the single-dosing pharmacokinetic profile of G004 in beagle dogs. The above results indicated that when quantifying chlorine or bromine containing compounds, evaluation of the interference from mono-hydroxylation or dehydrogenation metabolites should be undertaken, and if such metabolites existed, their impact on quantification of the parent compounds could be eliminated by the proper selection of the MRM transitions.

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1. Introduction

In high-throughput LC–MS/MS analysis of biological samples, the co-eluting metabolite interference could be observed in some cases [1–3]. The prevalent use of short chromatographic run times may increase the risk of suffering from the metabolite interference during bioanalysis of pharmacokinetic samples [3–5]. Particularly during the determination of bromine or chlorine containing compounds, such quantitative interference may exist when their metabolites could generate the ions called [M-2] species [6]. The *m*/*z* values of these [M-2] species are 2 Da less than the precursor ions of the parent compounds. In this way, the $^{81}Br/^{37}Cl$ forms of such metabolites could interfere with the accurate quantification of the parent compounds at the $^{79}Br/^{35}Cl$ forms. Because the [M-2] species of such metabolites at the $^{81}Br/^{37}Cl$ forms are isobaric with the precursor ions of the parent compounds at the $^{79}Br/^{35}Cl$ isotopic forms. For example, the mono-hydroxylated metabolites at the $^{81}Br/^{37}Cl$ isotopic forms followed by in-source dehydration could produce ions which possess the *m*/*z* values of 2 Da less than the protonated molecular ions ($[M+H]^+$) or the deprotonated molecular ions ($[M-H]^-$) of the parent compounds at the $^{81}Br/^{37}Cl$ forms. Such metabolites could interfere with the quantification of the parent compounds when the $[M+H]^+$ or $[M-H]^-$ of the parent compounds at the $^{79}Br/^{35}Cl$ forms are chosen as the precursor ions.

G004 (1-(4-(2-(4-bromobenzenesulfonamino)ethyl)phenylsulfonyl)-3-(trans-4-methylcyclohexyl)urea, see Fig. 1A) is a novel agent of sulfonylurea derivative, which contains a bromine atom [7,8]. The pharmacological study favors G004 as a promising novel hypoglycemic agent. It aims at reducing vascular complications as well as controlling glucose excursion for type II diabetes [9–14].

The reported methods for determination of G004 include HPLC-UV [15] and HPLC–MS methods [16,17]. Those methods have the problems of either longer run times or lower sensitivity. In this report, we established a more rapid and sensitive LC–MS/MS method for the quantification of G004 in plasma samples. Since there is a bromine atom in the structure, interference from the metabolites was specifically checked. The mono-hydroxylation metabolite at the ^{81}Br isotopic form followed by in-source dehydration could produce the [M-2] species which are identical to the precursor ions of the parent compound at the ^{79}Br isotopic form. For this reason, the mono-hydroxylated metabolite could interfere with the accurate quantification of G004. The approaches

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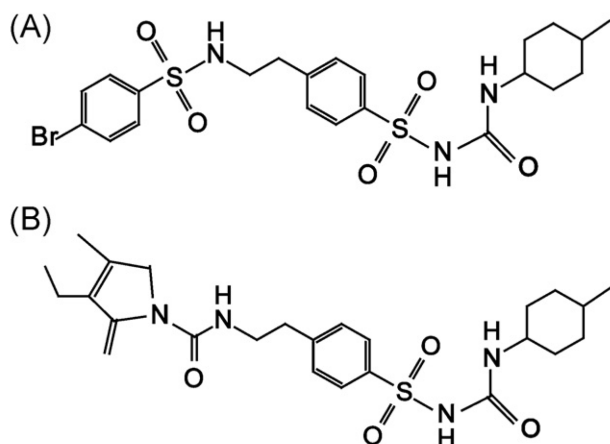

Fig. 1. Chemical structures of G004 (A) and glimepiride (B).

to overcoming this interference were also demonstrated. Chromatographic resolution was given up for the cost of longer run times and lower throughput. Several MRM transitions were evaluated to overcome the isotope-related metabolite interference in this report, which would enable more reliable parent compound quantification without the need for chromatographic separation of the parent drugs from their metabolites [6,21,22]. Furthermore, a standard addition method for the metabolite-related matrix effect evaluation was established by using the incurred plasma samples, which can be applied to the LC–MS/MS method validation for other drugs facing the problem of the metabolite interference. Finally, the established method, with an LLOQ of 0.2 ng/ml, offered increased sensitivity and shorter analysis run time. It was validated and successfully applied to the evaluation of pharmacokinetic profiles of G004 in beagle dogs after oral administration.

2. Experimental

2.1. Chemicals and reagents

The reference standard of G004 was kindly supplied by Center of Drug Discovery, China Pharmaceutical University (Nanjing, China). The internal standard (IS), glimepiride, was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile was obtained from Merck KGaA (Darm-stadt, Germany). Ammonium acetate and methyl tert-butyl ether was analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (SCRC, Shanghai, China). Acetic acid and hydrochloric acid were analytical grade purity and purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

2.2. Chromatographic and mass spectrometric conditions

2.2.1. Chromatographic conditions

The liquid chromatography was performed on an Agilent 1200 Series liquid chromatography (Agilent Technologies, Palo Alto, CA, USA), which included an Agilent 1200 binary pump (model G1312B), vacuum degasser (model G1322A), Agilent 1200 autosampler (model G1367C), temperature controlled column compartment (model G1330B). The chromatographic separation was archived on a Hedera ODS-2 column, 5 μm, 150 mm × 2.1 mm i.d. (Hanbon Science and Technology) protected by a security guard C18 column, 4 mm × 2.0 mm i.d., 5 μm (Phenomenex, Torrance, CA, USA). The mobile phase was consisted of acetonitrile–5 mM ammonium acetate buffer solution containing 0.05% acetic acid (68:32, v/v) and eluted at a flow rate of 0.35 ml/min. Injection volume was 5 μl. The analytical column and the autosampler tray were maintained at 38 °C and 10 °C, respectively.

2.2.2. Mass spectrometric conditions

The LC system was coupled with an Agilent 6410B triple quadrupole mass spectrometer (USA) equipped with an electrospray source (model G1956B). The electrospray ionization source was set with a drying gas (N_2) flow of 12 l/min, nebulizer pressure of 40 psig, drying gas temperature of 350 °C, capillary voltage of 4.0 kV and the negative ion mode. The fragmentor voltage for G004 and the IS was 170 V and 150 V, respectively. The fragmentation transitions for the multiple reaction monitoring (MRM) were m/z 558.1 → 419.0 for G004 with a collision energy of 18 eV, and m/z 489.3 → 367.2 for glimepiride with a collision energy of 16 eV, as shown in Fig. S1 of Supplementary Information. Data were collected and analyzed by the Masshunter Qualitative Analysis Software B.03.01 Build 346 (Applied Agilent Technologies). The MS data acquisition started at 2.6 min after sample injection, and the stream selection valve was set to waste until data acquisition started.

2.3. Preparation of standards and quality control (QC) samples

The stock solution of G004 was prepared at a concentration of 1 mg/ml in methanol. Dilutions prepared in methanol at concentrations of 10 ng/ml, 30 ng/ml, 100 ng/ml, 300 ng/ml, 1.0 μg/ml and 10 μg/ml were used as the working solutions to prepare the calibration standards. Appropriate amount of the working solutions were added into 10 ml test tubes. After evaporated to dryness under a stream of nitrogen, the residues were dissolved in 0.4 ml blank plasma to construct the calibration standards of G004. The calibration standards were prepared at the concentration levels of 0.2, 1, 3, 10, 30, 100, 200, 400 and 800 ng/ml. The quality control (QC) samples were designed at three G004 concentrations of 0.5 ng/ml (low QC), 20 ng/ml (medium QC) and 700 ng/ml (high QC) in the plasma. The QC samples were prepared independently using the working solutions different from those for preparing the calibration standards.

The stock solution (1 mg/ml) of the IS was prepared in methanol and was further diluted with methanol to 2 μg/ml and used for all analyses. All the solutions were stored at −20 °C and were brought to room temperature before use.

2.4. Sample preparation

Aliquot of 0.4 ml plasma sample was added with 15 μl IS solution (2 μg/ml) and 50 μl of 0.1 M hydrochloric acid solution. After a thorough vortex mixing for 30 s, mixtures were extracted with 4 ml of methyl tert-butyl ether, vortex-mixed for 5 min, and centrifuged at 4000 rpm for 10 min. The organic layer was transferred and evaporated under a stream of nitrogen gas in the water-bath of 37 °C until completely dry. The dried residue obtained was dissolved in 100 μl of mobile phase, vortex-mixed for 5 min, centrifuged at 15,600 × g for 10 min, and 5 μl of the supernatant liquid was injected into the LC–MS/MS system.

2.5. Method validation

A full validation was preformed on the LC–MS/MS method described above based on the Guidance for Industry, Bioanalytical Method validation issued by the US Food and Drug Administration. Method validation experiments included specificity, matrix effect (*ME*), extraction recovery, linearity, LLOQ, precision, accuracy and stability.

The selectivity of the method was evaluated by comparing the chromatograms of the blank plasma samples from six different batches with the corresponding spiked plasma samples. Each blank

plasma sample was tested using the proposed preparation procedure and the LC–ESI-MS conditions to ensure no interference of the analytes from the plasma.

The *ME* of the method was evaluated by comparing the peak areas of the analytes resolved in blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution) (A) with those resolved in mobile phase (B). The *ME* was calculated by using the formula: *ME* (%) = A/B × 100. Three different concentration levels of G004 at 0.5, 20 and 700 ng/ml and the IS of 2 μg/ml were evaluated by analyzing five replicates at each level. The blank plasmas used in this study were from five different batches of dog blank plasma.

Each respective extraction recovery of G004 and the IS was determined at three different concentration levels of QC samples by comparing peak areas of analytes extracted from plasma samples with peak areas obtained from the pure standard without the procedure of extraction. Five replicates of extracted samples and three replicates of unextracted samples were run at each concentration level.

Calibration standard samples were prepared by adding different volume of working standards at different concentrations. The solutions were dried under nitrogen gas flow and then, aliquot of 0.4 ml plasma was added. The final concentrations from 0.2 to 800 ng/ml for G004 in plasma were prepared. Samples were quantified using the ratio of the peak area of G004 to that of the IS. The peak area ratios were plotted against nominal concentration of G004, and the standard curves were calculated using linear regression analysis with $1/x^2$ weighting. The lower limit of quantification (LLOQ) was the lowest concentration on the calibration curve that can be measured with acceptable accuracy and precision. The LLOQ was established using five samples independent of standards. The precision and accuracy at LLOQ should fall within the range of 80–120%.

Accuracy and precision were assessed by the determination of QCs at three concentration levels in five replicates in three validation batches. The precision was expressed by relative standard deviation (RSD) and the accuracy by relative error (RE). The intra- and inter-batch precisions are required to be below 15%, and the accuracy to be within ±15%.

The stability of each analyte in blank dog plasma was assessed by analyzing three concentration levels of QC samples at different conditions, including kept at room temperature for 5 h (short-term stability), stored at −80 °C for 16 weeks (long-term stability) and after three freeze–thaw cycles. The autosampler stability was conducted by reanalyzing the extracted QC samples kept under autosampler conditions (10 °C) for 7 h.

2.6. Application of the method to a pharmacokinetic study

Six beagle dogs (weight, 10 ± 1 kg, 3/sex) were purchased from Experimental Animal Center of Shanghai Xingang and were kept in an environmentally controlled room (temperature: 22 ± 1 °C, humidity: 50 ± 20%, 12 h dark–light cycle) for at least 1 week before the experiment. The dogs were fasted overnight before drug administration. All animal experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals of Southeast University (Nanjing, China).

The pharmacokinetics of G004 in beagle dogs was studied after oral administration of G004 (0.9 mg/kg). Blood samples (2 ml) were collected immediately before and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16 and 24 h after dose. Blood samples were collected directly into the heparinized tubes and centrifuged at 5500 rpm for 10 min to isolate the plasma. The plasma samples were stored at −80 °C until analysis.

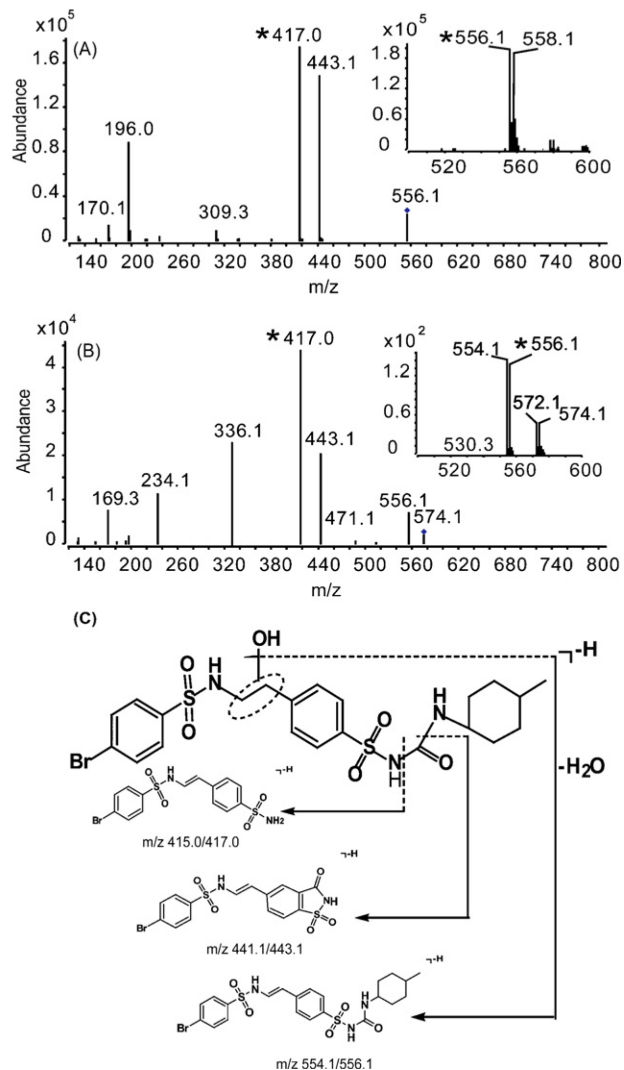

Fig. 2. The product ion scanning spectrum of the precursor ion at *m/z* 556.1 of G004 (A). Inset, the Q1 full-scan spectrum of G004. The product ion scanning spectrum of the precursor ion at *m/z* 574.1 of metabolite M1 (B). Inset, the Q1 full-scan spectrum of metabolite M1. The proposed fragmentation pathway of M1 (C).

3. Results and discussion

3.1. Method development and optimization

3.1.1. Detection of the metabolite interference in incurred samples

The base peaks in the negative mass spectra of G004 obtained were the deprotonated molecular ions $[M-H]^-$ at *m/z* 556.1/558.1. The two peaks of the isotopes (^{79}Br and ^{81}Br) were in nearly equivalent abundance in the mass spectra. While in natural isotopic distribution of bromine atom, the distribution of ^{79}Br (100.0%) is a little higher than ^{81}Br (97.3%). The product ion spectrum of the deprotonated molecular ion $[M-H]^-$ at *m/z* 556.1 showed fragment ions at *m/z* 443.1, 417.0, 196.0 and 170.1, as shown in Fig. 2A. Due to the product ion at *m/z* 417.0 is the base peak in the product ion spectrum, the MRM transition of *m/z* 556.1 → 417.0 was chosen for G004 in the LC–MS/MS analysis at first. Fig. 3 shows the MRM chromatograms, monitored under the MRM transition of *m/z* 556.1 → 417.0, of a blank plasma sample (A), a medium QC plasma sample spiked with G004 at 20 ng/ml (B), and an incurred dog plasma sample (C), in which the retention time of G004 is about 3.9 min. Fig. 3A and B shows no interfering peak to G004. However,

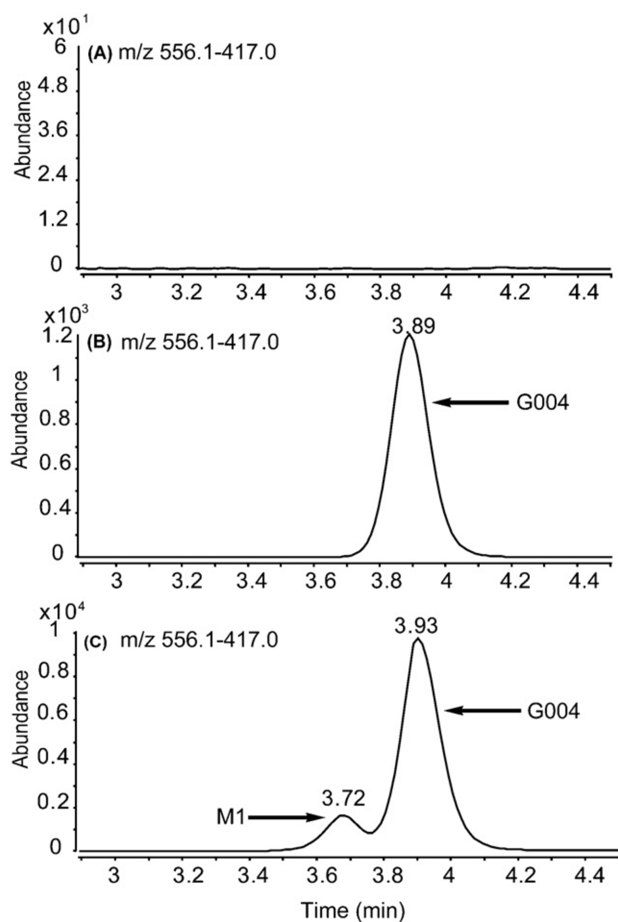

Fig. 3. MRM chromatograms monitored under the transition of m/z 556.1 → 417.0 for blank sample (A), plasma spiked with G004 at 20 ng/ml (B), and a dog plasma sample collected at 3 h after single dose administration of G004 (C).

an interfering peak with the retention time of 3.7 min was noted in the MRM chromatogram of the incurred samples, see Fig. 3C. It suggested that the unexpected peak might be derived from a putative metabolite (M1) rather than an artifact from the sample preparation process.

Additional evidence for the metabolite interference quantification came from inspection of full scan and product ion spectra of G004 and the putative metabolite (M1). The full scan spectra of metabolite M1 in the concentrated incurred plasma samples showed that the deprotonated molecule ions $[M-H]^-$ of M1 at m/z 572.1/574.1. At the same time, the in-source fragment ion $[M-H-H_2O]^-$ of M1 at m/z 554.1/556.1 (see Fig. 2B) could be seen in the mass spectra as well, indicating that M1 might be a hydroxylated metabolite retaining the bromine atom of the parent compound G004. The product ions of M1 deprotonated molecular ion (m/z 574.1) at m/z 556.1, 443.0, 417.0 (the base peak), 336.1 and 234.1 further suggested that the potential hydroxylation might possibly take place on the ethyl chain in G004 (see Fig. 2B). Moreover, the fragmentation patterns of M1 are quite similar with those of the hydroxylation metabolites from the other sulphonylureas [23–27], and the proposed fragmentation pathway of M1 was shown in Fig. 2C. Of note, product ion spectra derived from the predominated fragment ion of the M1 (m/z 574.1) showed prominent M1 product ion at m/z 417.0, which was identical to the major product ion in the MRM transition that was used for quantifying G004. It was apparent that the MRM transition used to quantify G004 in the study plasma samples (m/z 556.1 → 417.0) was susceptible to quantitative interference as a consequence of its ability to detect the isotopic ^{81}Br forms of hydroxylation metabolite in these samples.

3.1.2. Troubleshooting of the metabolite interference

Chromatographic resolution of parent compounds from their metabolites usually results in extended chromatographic run times and lower sensitivity of the analyte [2,18–22]. We therefore explored MS/MS-based approaches to solve *the above mentioned metabolite interference* problem. For comparison of the product ion profiles of G004 and M1, alternative MRM transitions were evaluated. The abundant product ion at m/z 196.0 of the G004 was not present in the product ion spectra of metabolite M1. The structure of this ion does not contain the bromine atom, so the impact of M1 at the ^{81}Br form on quantification of G004 could be eliminated. As a result, the transition at 556.1 → 196.0 was selected and tested. As shown in Fig. S2 of Supplementary Information, the interfering peak disappeared, however, an approximately more than 70% loss of sensitivity was suffered at the same time. Such a sensitivity loss might not be acceptable, according to the sensitivity requirements for this assay. Another alternative MRM transition consist of the deprotonated molecular ion of G004 at the ^{81}Br isotopic form (m/z 558.1) as the precursor ion coupled with its most abundant product ion (m/z 419.0). This transition not only provided ample selectivity but also retained the same sensitivity as the original MRM transition of m/z 556.1 → 417.0, for the two naturally occurring isotopes of bromine (^{79}Br and ^{81}Br) are in nearly equivalent abundance. As a result, the transition m/z 558.1 → 419.0 (Fig. S2 of Supplementary Information) was finally chosen for the LC–MS/MS analysis. The LC–MS/MS parameters were optimized to maximize the response for the analyte and IS. Chromatography conditions were also evaluated. Regarding the acidic properties of G004, mobile phase with the use of different concentrations of acetic acid was compared to test their effects on MS peak intensity [28,29], and peak efficiencies were improved when acetonitrile instead of methanol was used. Finally, an elution system (acetonitrile–0.05% acetic acid containing 5 mM ammonium acetate) as previously described was chosen.

3.1.3. Evaluation of metabolite-related matrix effect using incurred biological samples

The matrix effect caused by the co-eluting metabolites has been largely ignored, because the QC samples used for the routine validation of an assay are prepared in the same matrix as the calibration samples. Thus, they will not reveal metabolite-related matrix effects observed in the incurred samples [22,30,31]. During the early-stage of preclinical study, the lack of metabolite reference makes it difficult to obtain analyte-free with metabolite containing samples of the authentic biological matrix. However, the incurred samples obtained from animals dosed with the drug could be easily collected. So the standard addition method using the incurred samples could be used to evaluate the metabolite-related *ME*. The incurred plasma samples used in this study were from six dogs after administration of G004 (3 mg/kg). The higher concentration of M1 could be reached in the incurred plasma samples of six dogs under this condition. The incurred plasma samples obtained from each treated dog were pooled together respectively to give six different batches of the incurred dog plasma samples.

The metabolite-related matrix effects (*MR-ME*) of G004 was examined by comparing the peak areas of the G004 among three different sets of the samples. In set 1, the incurred plasma of each dog was prepared and assayed as the procedures described in Section 2.4, and the obtained peak areas of G004 was defined as A. In set 2, G004 was resolved in the corresponding incurred sample's reconstituted solution (the final solution of incurred plasma after extraction and reconstitution), the obtained peak area of G004 was defined as B. In set 3, G004 was resolved in the mobile phase, and the obtained peak area of G004 was defined as C. The

Table 1
Precision and accuracy data for the analysis of G004 in dog plasma (five replicates per run).

	Concentration levels (ng/ml)		RSD (%)		RE (%)
	Added	Measured	Intra-day	Inter-day	Accuracy
Low QC	0.5855	0.6005	4.3	5.1	0.03
Middle QC	23.42	23.87	6.8	4	0.02
High QC	819.7	856.7	2.6	4.5	0.05

RSD: relative standard deviation; RE: relative error.

Table 2
Summary of stability of G004 in dog plasma under various storage conditions ($n = 3$).

Conditions	Concentration (ng/ml)		RSD (%)	RE (%)
	Added	Measured		
Room temperature, 5 h	0.5855	0.6133	1.5	4.7
	23.42	24.67	3.3	5.3
	819.7	861.965	6.6	5.2
Autosampler for 7 h (10 °C)	0.5855	0.5667	1.1	−3.2
	23.42	23.85	3.9	1.8
	819.7	839.2	7.1	2.4
Three freeze/thaw cycles	0.5855	0.6109	4.8	4.3
	23.42	24.27	3.6	3.6
	819.7	879.1	7.2	7.2
−80 °C, 16 weeks	0.5855	0.6323	1.1	8.0
	23.42	22.77	1.5	−2.8
	819.7	884.6	11.1	7.9

RSD: relative standard deviation; RE: relative error.

metabolite-related matrix effects were calculated using the formula: *MR-ME* (%) = (B − A)/C × 100. The *MR-ME* for G004 was evaluated at the added plasma concentration level of 100 ng/ml, and the average *MR-ME* value calculated using the data from the above mentioned six different batches of the incurred dog plasma was (101.0 ± 6.7)%, indicating that the co-eluting metabolite did not influence the ionization of G004.

3.2. Method validation

The specificity of this method was confirmed. Fig. 4 shows that chromatograms of blank plasma (A), blank plasma spiked with G004 at 0.2 ng/ml (LLOQ) and the IS at 2 μg/ml (B) and plasma sample of a dog obtained at 1 h after oral administration of G004 (C). Under the described chromatographic conditions, G004 and IS were well separated, the retention times were 3.8 and 3.3 min for G004 and the IS, respectively, and no interfering endogenous peaks around their retention times were observed.

The *ME* of five different batches of dog plasma were (105.8 ± 7.2)%, (98.7 ± 4.2)% and (101.3 ± 1.3)% for G004, and the *ME* value for the IS (2 μg/ml in plasma) was (102.3 ± 4.3)%. No significant matrix effect for G004 and the IS were observed. These results indicated that no co-eluting substance influenced the ionization of the analytes. The extraction recovery of G004, estimated at three concentration levels of 0.5, 20 and 700 ng/ml were (91.6 ± 7.5)%, (84.0 ± 7.0)% and (89.5 ± 3.3)% ($n = 5$), respectively. The extraction recovery of the IS at the concentration level of 2 μg/ml was (85.2 ± 7.0)% ($n = 5$).

The calibration curve was constructed by plotting the peak area ratios (y) of G004 to the IS *versus* the concentrations (x) of G004 using weighted least squares linear regression (the weighting factor was $1/x^2$). The typical calibration curve for G004 was $y = 0.001534 + 0.005980x$ ($n = 5$), and $r \geq 0.997$ over the concentration range of 0.2–800 ng/ml. The LLOQ of the method was 0.2 ng/ml of G004 in dog plasma, the precision was 7.9% ($n = 5$), and the accuracy was 3.6% ($n = 5$).

The accuracy and precision data from QC samples demonstrate the suitability of the method. The precision was calculated by using one-way ANOVA. In this assay, the intra-run precision was 5.1% or less, and the inter-run precision was 6.8% or less for each QC level of G004, see Table 1. The results above demonstrate that the values are within the acceptable range and the method is accurate and precise.

The stability results of G004 in dog plasma are summarized in Table 2. G004 in dog plasma were found to be stable after being placed at room temperature for 5 h, stored at −80 °C for at least 16

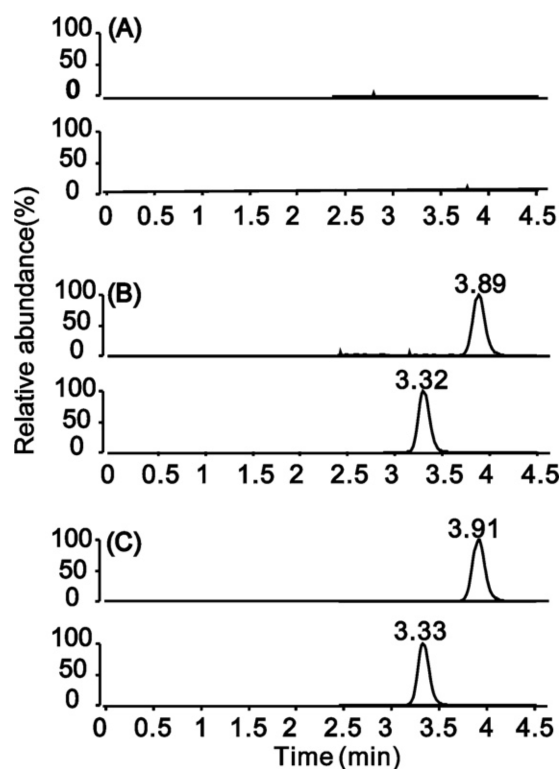

Fig. 4. Representative MRM chromatograms of drug-free plasma (A), plasma spiked with 0.2 ng/ml (LLOQ) G004 and the IS (B), and a plasma sample of a beagle dog withdrawn at 3 h after oral administration of 0.9 mg/kg (C).

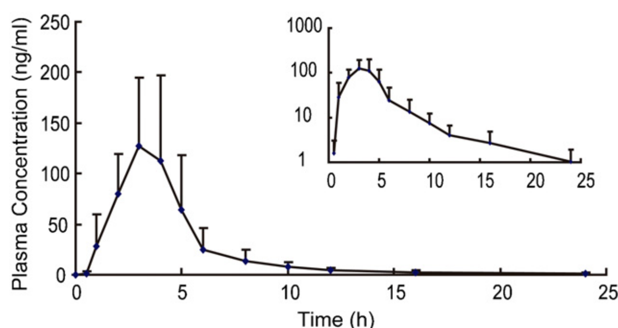

Fig. 5. The plasma concentration *versus* time profiles of G004 of beagle dogs after administrations of 0.9 mg/kg G004 ($n = 6$). Inset, semilogarithmic scale.

Table 3
Mean pharmacokinetic parameters of G004 following the single dose of 0.9 mg/kg to beagle dogs ($n = 6$, mean ± SD).

Dose (mg/kg)	0.9
T_{max} (h)	2.8 ± 0.8
C_{max} (ng/ml)	185.3 ± 68.5
$t_{1/2}$ (h)	4.2 ± 0.4
MRT (h)	4.87 ± 0.9
CL/*F* (l/h/kg)	1.4 ± 0.6
Vd/*F* (l/kg)	11.5 ± 4.3
AUC_{0-24} (ng h/ml)	537.3 ± 194.9
$AUC_{0-\infty}$ (ng h/ml)	521.7 ± 194.5

weeks and through three freeze–thaw cycles. Furthermore, samples after treatment were stable at 10 °C in autosampler for a period of 7 h, which indicated that a large number of samples could be determined in each analytical run.

3.3. Applicability to pharmacokinetic studies

To our knowledge, there was no publication on the pharmacokinetic study of G004 in beagle dogs. Mean plasma concentration *versus* time profile following the administration are presented in Fig. 5. The pharmacokinetic parameters are summarized in Table 3.

4. Conclusion

This study reported a method which is sensitive enough to determine the G004 plasma concentrations in the PK studies of G004. It offered advantages of wide linear concentration range, good sensitivity and high selectivity. It was successfully applied to characterize the pharmacokinetics of G004 in beagle dogs.

More generally, this study stressed that it is prudent to determine whether mono-hydroxylation or dehydration metabolites may be present in the study samples, when quantifying chlorine- or bromine-containing molecules in biological systems *in vivo* or *in vitro*, full scan spectrums would warrant careful evaluation by directly identifying the remnants of hydroxylation metabolites that are undergoing in-source decomposition or any metabolite precursor ions which contained [M-2] species. Furthermore, the use of presumptive MRM transitions that consist of [M-2] species coupled to various parent-compound-derived product ions would be valued either. If such metabolites are indeed shown to be present, chromatography-based or mass-based approach and the standard addition method using incurred sample can be used.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.04.027.

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