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Short communication

Simultaneous determination of ciclesonide and its active metabolite desisobutyryl-ciclesonide in human plasma by LC–APCI-MS/MS: Application to pharmacokinetic study in healthy Chinese volunteers

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A B S T R A C T

A sensitive and highly selective liquid chromatography tandem mass spectrometric (LC/MS/MS) method was developed and validated for the determination of ciclesonide (CIC) and its active metabolite, desisobutyryl-ciclesonide (des-CIC), in human plasma. Plasma samples were extracted using methyl tert-butyl ether with mifepristone as an internal standard (IS). Separation was carried out on a C_{18} column using a mixture of 0.1% formic acid solution and methanol as the mobile phase with linear gradient elution. The detection was operated with positive atmospheric pressure chemical ionization (APCI) by selective multiple reaction monitoring (SRM). The chief benefit of the present method was the high sensitivity, with the lower limit of quantification (LLOQ) as low as 10 pg/mL and the linearity ranging from 10 to 10,000 pg/mL for both CIC and des-CIC. The method was fully validated and successfully applied to determine CIC and des-CIC simultaneously in human plasma and proved to be suitable for phase I clinical pharmacokinetic study of inhaled ciclesonide in healthy Chinese volunteers.

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1. Introduction

Inhaled corticosteroids (ICSs) have been the most effective long-term control drugs available for the treatment of persistent asthma. The pharmacologic characteristics desirable for an ideal ICS include low oral bioavailability, high lung deposition, potent activity in the lungs, potent glucocorticoid receptor binding affinity, prolonged pulmonary retention time, high plasma protein binding, a short physiologic half-life and rapid clearance from the circulation [1]. Ciclesonide (CIC), the recently developed inhaled corticosteroid, possesses several of these properties [2]; clinical data suggest that it could safely and significantly improve lung function with high tolerance in patients with mild-to-moderate persistent asthma [3–5] because of its low systemic activity and minimal incidence of oropharyngeal adverse events [2].

Ciclesonide has been administered as a pro-drug in an aerosol solution, and it is converted to its pharmacologically active metabolite, desisobutyryl-ciclesonide (des-CIC, Fig. 1), in the lungs, the target organ, by endogenous esterase [6]. The dose of CIC is at the level of several hundred micrograms, and the plasma concentrations of CIC and des-CIC in the terminal elimination phase after inhalative administration have been in the low pg/mL levels in human plasma. To elucidate the pharmacokinetics and pharmacodynamics of CIC and des-CIC, a highly sensitive and selective method is required for their simultaneous determination. To date, several LC–MS methods for the determination of CIC and/or its metabolite, des-CIC, were briefly mentioned in CIC pharmacokinetic studies [7–9], but the details of these methods were not described. Mascher et al. [10] developed an LC–MS/MS method using atmospheric pressure photo ionization (APPI). However, the application of an APPI ionization source has not been as widely used as atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI). Furthermore, it employs an internal standard that is not commercially available, which makes it difficult to reproduce.

The goal of this study was to develop a sensitive, specific and reproducible LC–MS/MS method using APCI to simultaneously estimate CIC and des-CIC in human plasma. The method was fully validated and successfully applied for a pharmacokinetic study of inhaled ciclesonide in healthy Chinese volunteers.

2. Experiment

2.1. Reagents and materials

Ciclesonide aerosol, the reference substance of ciclesonide (purity: >99% HPLC), desisobutyryl-ciclesonide (purity: >99% HPLC)

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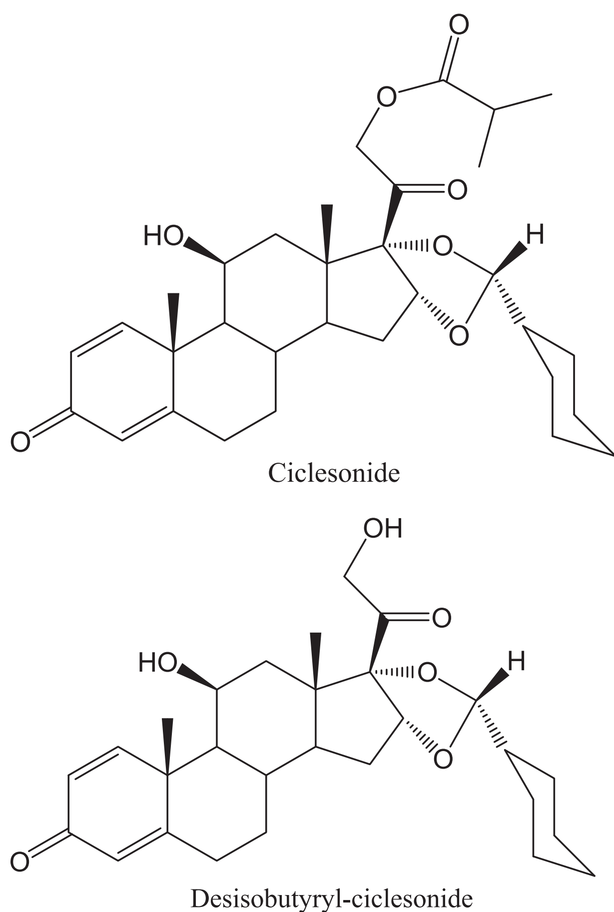

Fig. 1. Chemical structures for ciclesonide and desisobutyryl-ciclesonide.

and IS mifepristone (purity: >99% HPLC) were supplied by Xianju Pharmaceutical Co., Ltd. (Zhejiang, PR China). Methanol of HPLC/Spectro grade was obtained from Tedia Company Inc. (Fairfield, OH, USA). Other chemicals were all of analytical grade and purchased from Nanjing Chemical Reagent Factory (Nanjing, PR China). Pefabloc SC, a serine protease inhibitor, was obtained from Roche Diagnostics. Deionized water was purified through a PL5242 Purelab Classic UV (PALL Co., Ltd., USA) before use. Blank plasma was supplied by the Red Cross Society of China, Nanjing Branch.

2.2. Instrumentation

A Thermo-Finnigan TSQ Quantum Ultra AM tandem mass spectrometer equipped with atmospheric pressure chemical ionization (APCI) source (San Jose, CA, USA), a Finnigan surveyor LC pump and an autosampler were used for the LC–MS/MS analysis. Data acquisition was performed with Xcalibur 1.4 software (Thermo-Finnigan, San Jose, CA, USA). Peak integration and calibration were carried out using LC Quan software (Thermo-Finnigan).

2.3. LC/MS/MS conditions

A Lichrospher ODS-2 column (5 μm, 150 mm × 4.6 mm i.d.) was used for all of the chromatographic separations. The mobile phase ‘A’ was 0.1% formic acid, and the mobile phase ‘B’ was methanol. The linear gradient elution program was performed as follows: 0–1 min (80%B) → 1.5–5 min (97%B) → 5.1–7 min (80%B), which was pumped at a flow-rate of 1.0 mL/min. To assure the reproducibility of the retention time, the column temperature was maintained at 35 °C.

Samples were ionized by positive-ion atmospheric pressure chemical ionization mode ($APCI^+$) and monitored in multiple selective reaction monitoring (SRM) mode. The MS ionization and acquisition conditions were tuned and optimized by using infusion injection of each analyte solution in the mobile phase of 1 mg/L. The discharge current was set at 12 μA. Nitrogen was used as the sheath and auxiliary gas at pressures of 241 and 34 kPa, respectively. The capillary temperature was selected as 200 °C. Collision-induced dissociation (CID) studies were performed, and argon was used as the collision gas with a collision gas pressure of 0.17 Pa and an energy of 35 eV. Based on the full-scan MS and MS/MS spectra of the drug, the most abundant fragment ion was selected and the mass spectrometer was set to monitor the transitions m/z 541.1 → 147.1 for CIC, m/z 471.1 → 147.1 for des-CIC and m/z 430.1 → 134.1 for the IS.

2.4. Pharmacokinetic study design and sample collection

An open-label, randomized study was performed at the phase I Clinical Research Institute of the First Affiliated Hospital of Nanjing Medical University (Nanjing, PR China), and the protocol was approved by the Independent Ethics Committee (IEC) of that hospital. Ten healthy Chinese volunteers (5 females and 5 males) with a mean age of 22.8 ± 1.3 years and a body weight of 55.4 ± 6.9 kg were enrolled in the study. All volunteers provided their signed, informed consent to participate in the study according to the principles of the Declaration of Helsinki and Good Clinical Practice (GCP). Participants were required to abstain from alcohol and caffeine-containing products or green tea before and during treatment periods. The use of any prescription or nonprescription medications was not permitted during the study unless deemed necessary by the clinician for treatment.

Single dose and multiple doses (7 days administration, once daily) were designed with 400 μg CIC dosage (four puffs of 100 μg each, ex-valve). The subjects were cared for by the physicians and nurses. Venous blood samples, each about 3 mL, were collected at 0 (pre-dose), 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 480 and 720 min post-dose into heparinized tubes. To prevent the ex vivo or in vitro hydrolysis of CIC, 0.3 mg Pefabloc SC (an esterase inhibitor) in 50 μL of water was also added into the heparinized tubes before sample collection [11]. Plasma samples were separated, decanted, frozen and stored at −20 °C for analysis within two weeks.

2.5. Preparation of standard solutions

Standard stock solutions of CIC and des-CIC were prepared by dissolving an appropriate amount of each chemical reference substance in a 100-mL volumetric flask with methanol to achieve a concentration of 100 μg/mL. Stock solutions for the IS were also prepared in methanol and diluted to 0.8 ng/mL. All of the solutions were then stored at 4 °C and were brought to room temperature before use.

2.6. Preparation of calibration and QC samples

The mixture standard solutions containing both CIC and des-CIC were prepared using serial dilutions of the stock solutions, and the serial concentrations were obtained as follows: 0.10, 0.20, 0.40, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, and 100.0 ng/mL. Calibration standard solutions were prepared by spiking 100 μL standard stock solutions with 1.0 mL blank human plasma to achieve the concentrations of 10, 20, 40, 100, 200, 400, 1000, 2000, 4000 and 10,000 pg/mL for both CIC and des-CIC in plasma, respectively. Quality control (QC) samples in plasma were prepared in a similar way with a low, middle and high concentration, 40, 400, and 4000 pg/mL, for both CIC and des-CIC.

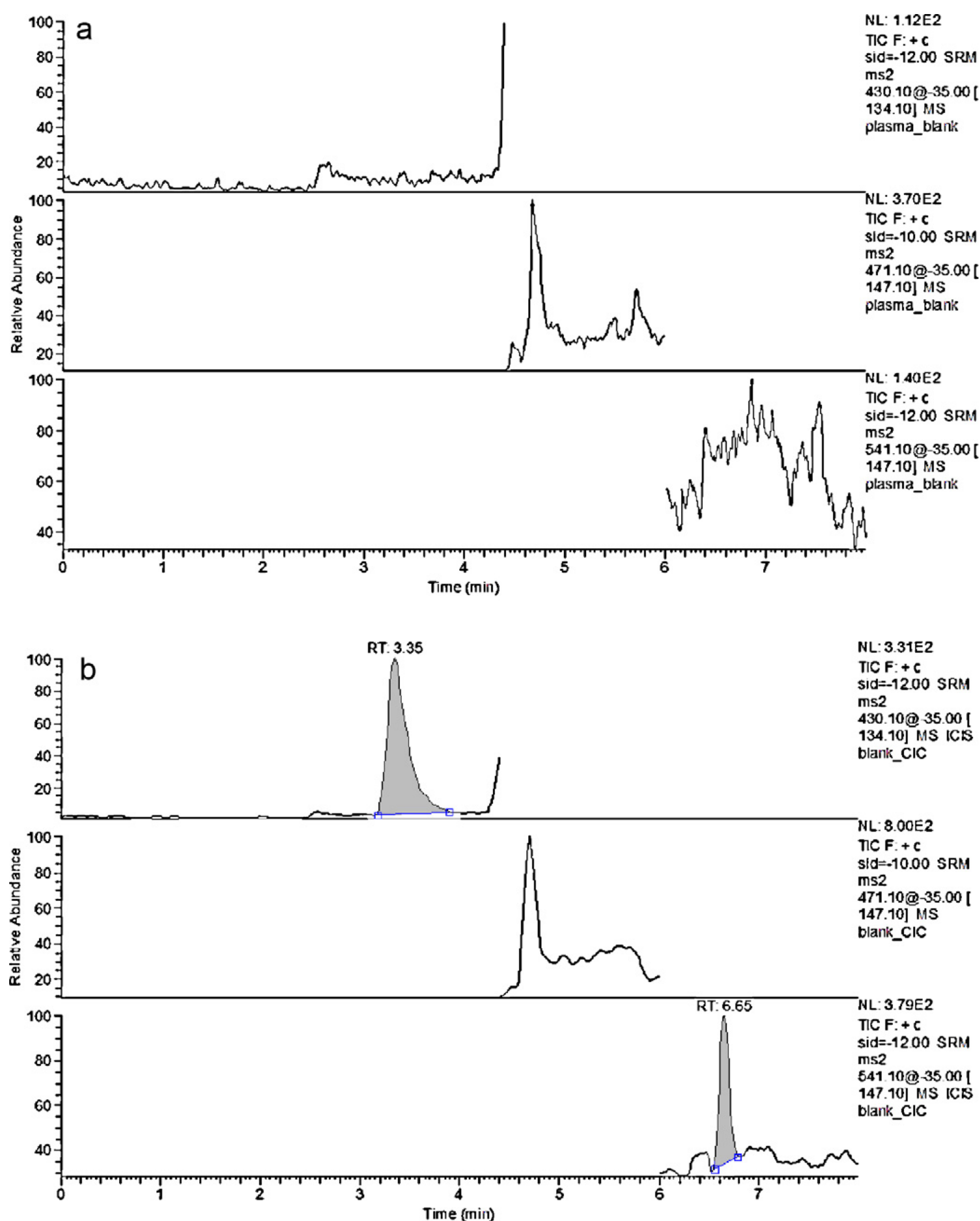

Fig. 2. Chromatograms by selective reaction monitoring (SRM): (A) blank plasma (CIC, des-CIC and IS free); (B) blank plasma spiked with CIC (10 pg/mL, LLOQ, t_R = 6.65 min) and IS (80 pg/mL, t_R = 3.35 min); (C) blank plasma spiked with des-CIC (10 pg/mL, LLOQ, t_R = 5.06 min) and IS (80 pg/mL, t_R = 3.36 min); (D) plasma sample of a subject 15 min after inhalation of ciclesonide aerosol at a 400 μg dosage.

2.7. Sample preparation

An aliquot of 1 mL of plasma in a 10-mL glass centrifugation tube was spiked with 100 μL of methanol (when preparing calibration and QC samples, standard solution was added instead of methanol) and 100 μL of IS solution (0.8 ng/mL). After vortex mixing for 30 s, 5 mL methyl tert-butyl ether was added and vortexed for 4 min. An aliquot of 4 mL of the supernatant was taken after centrifuging at 1550 × *g* for 5 min and evaporated to dryness under a stream of nitrogen at 40 °C. The residual was reconstituted in 0.15 mL of a mixture of 0.1% formic acid and methanol (20:80, v/v) and centrifuged at 16,000 × *g* for 5 min; an aliquot of 80 μL was then injected into the chromatographic system. For optimal stability, the auto-sampler temperature was set at 4 °C.

3. Results and discussion

3.1. MS and chromatographic conditions optimization

An HPLC–MS/MS with APPI method for the simultaneous determination of CIC and des-CIC has been reported [10]. In our study, the APCI was employed, and a similar LLOQ of 10 pg/mL was obtained. The most sensitive mass transitions were *m*/*z* 541.1–147.1 for CIC, *m*/*z* 471.1–147.1 for des-CIC and *m*/*z* 430.1–134.1 for IS. These are different from the literature [10], in which the *m*/*z* 599.4–339.2 for CIC and *m*/*z* 529.4–357.2 for des-CIC were chosen with the acetic acid adducts. This phenomenon was perhaps caused by the different ionization mechanism. Especially, the APCI discharge needle was kept clean for effective ionization and sensitive detection of the analytes during the entire operation. Furthermore, the gradient elu-

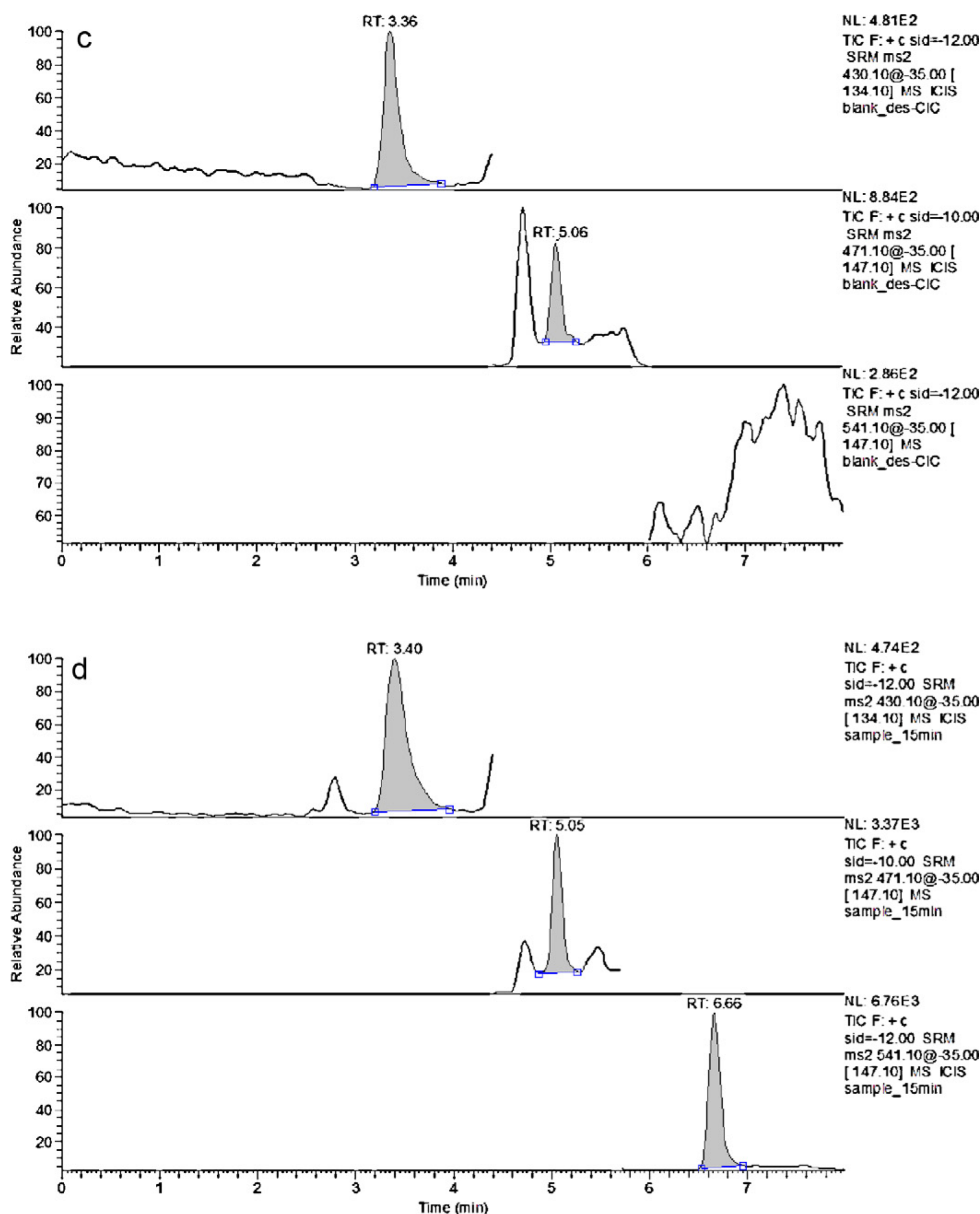

Fig. 2. (*Continued*).

tion program was employed to obtain an acceptable run time with accurate and precise quantitation.

3.2. Preparation of plasma samples

Both liquid–liquid extraction [10] and solid-phase extraction [8,9] were exploited for sample preparation. Mascher et al. [10] used diisopropylether as the extraction solvent; however, DMSO had to be added before evaporation to prevent low recovery. Furthermore, it would take longer to evaporate the solvent to dryness with diisopropylether because of its higher boiling point. In our study, methyl tert-butyl ether was chosen instead of diisopropylether for its merits in terms of both recovery and specificity. In the final process step, a mixture of 0.1% formic acid and methanol (20:80, v/v) was used for the reconstitution of the residue, which could avoid the effect of solvents and give good peak shapes. Owing to the possibility of hydrolysis of CIC [11], the esterase inhibitor, Pefabloc SC, was also added in whole blood to improve the stability of CIC. The amount of the inhibitor used was 0.1 mg/mL blood.

3.3. Method validation

Representative chromatograms of blank human plasma, the LLOQs for CIC and des-CIC with IS in plasma and volunteer plasma samples are shown in Fig. 2. The typical retention times for CIC, des-CIC and IS were 6.6 ± 0.1 min, 5.0 ± 0.1 min and 3.3 ± 0.1 min, respectively. The total run time was about 8 min. Blank human plasmas from 6 different individuals were allowed to run longer, for up to 20 min, and no late-eluting interfering peak was observed.

Linearity was assessed by 10-level calibration curves in human plasma in duplicate on three consecutive days. The calibration curve was fitted to a $1/x^2$ weighted linear regression (where x was the concentration of the analyte). The calibration curve was linear over the concentration range of 10–10,000 pg/mL for each analyte

Table 1
Intra- and inter-batch precisions and recoveries for the determination of CIC and des-CIC in human plasma by LC–MS/MS.

Quality control	Nominal concentration (pg/mL)	Intra-batch assay (n = 5)		Inter-batch assay (bath = 3, n = 5)		Recovery (n = 5) (mean ± SD, %)
		Mean (pg/mL)	Precision (CV, %)	Mean (pg/mL)	Precision (CV, %)	
CIC						
QC-low	40	38.6	10.5	40.6	10.7	99.6 ± 5.8
QC-medium	400	412.6	4.9	401.3	8.1	96.0 ± 9.9
QC-high	4000	4222	0.7	3968.7	8.8	88.3 ± 4.4
des-CIC						
QC-low	40	39.4	11.7	41.2	8.5	91.8 ± 2.6
QC-medium	400	394.6	2.4	394.8	7.2	89.5 ± 9.0
QC-high	4000	3974	8.0	3926	9.6	90.7 ± 7.1

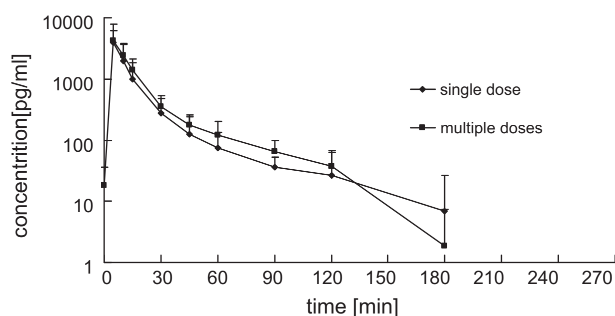

Fig. 3. Mean plasma concentration–time curve of CIC at a single dose and multiple doses of 400 μg CIC with inhaled administration (n = 10).

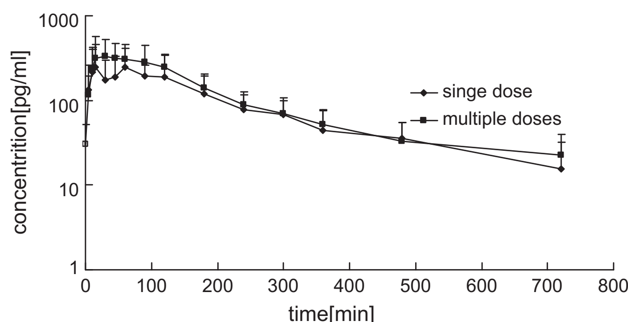

Fig. 4. Mean plasma concentration–time curve of des-CIC at a single dose and multiple doses of 400 μg CIC with inhaled administration (n = 10).

(CIC and des-CIC). The mean linear regression equation of the calibration curve for CIC was $y = 299.38(\pm 43.38)x - 7.12(\pm 4.42)$ and for des-CIC was $y = 218.60(\pm 28.91)x - 2.57(\pm 3.40)$, where y was the concentration of the analyte and x was the peak area ratio of the analyte to IS. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.997 for CIC and 0.996 for des-CIC. The % CV at each level varied from 2.5 to 10.5 for CIC and from 2.7 to 11.6 for des-CIC. The LLOQ was found to be 10 pg/mL for both CIC and des-CIC in human plasma, which could meet the requirement in human pharmacokinetic study.

The intra-batch, inter-batch precision and recoveries were shown in Table 1 by analyzing QC samples at three concentration levels (40, 400 and 4000 pg/mL). For both CIC and des-CIC, the accuracies varied from 82.5% to 117.5% for low-concentration (40 pg/mL) and 87.2% to 113.3% for medium concentration (400 pg/mL) and high concentration (4000 pg/mL). The extraction recoveries of CIC and des-CIC were consistent, precise and reproducible. The recovery of IS was 97.3 ± 4.8% (n = 5).

The matrix effect of this study was evaluated by analyzing samples at three concentration levels (40, 400 and 4000 pg/mL). The mean MEs of the six different samples sources were 107.6%, 113.9% and 95.5% for CIC and 89.6%, 104.0% and 85.8% for des-CIC at the three concentration levels, respectively. In addition, the mean ME of IS was 96.3%. The results revealed no obvious ion suppression or enhancement, owing to the effective plasma sample preparation by methyl tert-butyl ether extraction clean-up.

The stock solutions were stable at room temperature for 24 h and at 4 °C for 10 days. The processed sample could be kept in the autosampler (4 °C) for at least 12 h. The stabilities of CIC and des-CIC spiked in human plasma were investigated under various storage conditions at low, medium and high QC samples. The freeze-thaw stability was determined over three freeze–thaw cycles. The short-term stability was evaluated by keeping un-extracted QC samples at 25 ± 3 °C for 5 h. The long-term stability of the analyte was determined by placing QC samples at −20 °C for 30 days. The stability test results indicated that the analytes were stable under the conditions investigated.

3.4. Application in phase I clinical pharmacokinetic study

The proposed method has been successfully used to quantify the CIC and des-CIC concentrations in the plasma of Chinese volunteers after the administration of a single and multiple 400 μg inhaled doses of CIC aerosol in a metered-dose inhaler. The main pharmacokinetic parameters of CIC and des-CIC are presented in Table 2. The mean plasma concentration–time curves of CIC and des-CIC are presented in Figs. 3 and 4, respectively.

The parameters C_{max}, AUC and $T_{1/2}$ obtained in this study were similar with those reported previously [9]. Although the pharmacokinetics of CIC have been performed by other investigators, few multiple-dose pharmacokinetic studies have been published. In our result, insignificant accumulation was found after the admin-

Table 2
Main pharmacokinetic parameters of CIC and des-CIC following single and multiple inhaled doses of 400 μg CIC (mean ± SD, n = 10).

Parameters	CIC		des-CIC	
	Single-dose	Multiple-doses	Single-dose	Multiple-doses
C_{max} (ng/mL)	4.0 ± 3.9	4.3 ± 1.7	0.31 ± 0.19	0.41 ± 0.24
T_{max} (min)	5 ± 0	6 ± 2	44 ± 35	50 ± 35
$T_{1/2}$ (min)	15 ± 15	13 ± 9	156 ± 131	224 ± 102
MRT (min)	21 ± 6	21 ± 5	265 ± 117	283 ± 104
AUC_{0-720} (min ng mL^{-1})	50.4 ± 42.2	62.2 ± 26.8	59.3 ± 34.6	72.2 ± 31.1
$AUC_{0-\infty}$ (min ng mL^{-1})	51.0 ± 43.3	62.3 ± 26.8	65.4 ± 38.3	81.4 ± 37.4

istration of multiple 400 μg inhaled doses of CIC aerosol for 7 consecutive days.

4. Conclusions

The measurement of CIC and its metabolite, des-CIC, in human plasma samples derived from pharmacokinetic studies after the inhalative administration of CIC requires a very sensitive method. This study developed and validated an LC–MS/MS method by using APCI for the simultaneous quantification of CIC and des-CIC in human plasma with the LLOQs as low as 10 pg/mL with good reproducibility and accuracy. This method was successfully applied for the clinical pharmacokinetic study of single and multiple 400 μg inhaled doses of CIC aerosol. Insignificant accumulation was found after administration for 7 consecutive days.

Acknowledgement

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