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Simultaneous quantification of 22*R* and 22*S* epimers of budesonide in human plasma by ultra-high-performance liquid chromatography–tandem mass spectrometry: Application in a stereoselective pharmacokinetic study

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ABSTRACT

Budesonide (BUD) is used as a mixture of 22*R* and 22*S* epimers for the topical treatment of asthma, rhinitis, and inflammatory bowel disease. To study stereoselectivity in the pharmacokinetics of each epimer, we developed a stereoselective and sensitive ultra-high-performance liquid chromatography–tandem mass spectrometry method for the quantitative determination of 22*R* and 22*S* epimers of BUD in human plasma. The epimers of BUD were extracted from plasma using *n*-hexane/dichloromethane/isopropanol (2:1:0.1, v/v/v) under alkaline conditions. Baseline separation was obtained within 7 min on an Acquity UPLC BEH C_{18} (50 mm × 2.1 mm, 1.7 μm) column using an isocratic mobile phase consisting of acetonitrile/5 mM ammonium acetate/acetic acid (29:71:0.142, v/v/v) at a flow rate of 0.7 mL/min. Mass spectrometric detection was performed in a multiple reaction monitoring mode using the *m*/*z* 489 → 357 transition for BUD epimers and the *m*/*z* 497 → 357 transition for the internal standard d_8-BUD epimers. Calibration curves were linear over the concentration ranges of 5.0–500 and 5.0–3000 pg/mL for 22*R*-BUD and 22*S*-BUD, respectively. The lower limit of quantification was 5.0 pg/mL for both epimers. The method was successfully applied in a pharmacokinetic study of BUD controlled-release capsules in humans. Consistent differences in the pharmacokinetics of the 22*R* and 22*S* epimers were observed, the $AUC_{(0-\infty)}$ of 22*S*-BUD was approximately six times higher than that of 22*R*-BUD, and the 22*S*-/22*R*-BUD ratio of total body clearance was 0.17.

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1. Introduction

Budesonide [BUD; 22(*R*,*S*)-16α,17α-butylidenedioxy-11β,21-dihydroxypregna-1,4-diene-3,20-dione] (Fig. 1) is a non-halogenated glucocorticoid with high local anti-inflammatory activity. It is commonly used by inhalation to treat asthma and intranasally for allergic rhinitis [1] or by oral administration to treat inflammatory bowel disease [2]. The drug is a mixture of two epimers with 22*R* and 22*S* configurations at an approximately 1:1 ratio. Although they have similar qualitative pharmacological effects, 22*R*-BUD is more potent than 22*S*-BUD by two- to threefold [3].

The pharmacokinetics of the epimers has been investigated in six healthy male subjects after intravenous administration of 500 μg of ^{3}H-BUD [4]. The concentration of 22*S*-BUD was higher than that of 22*R*-BUD at all times, and the distribution volume and plasma clearance of 22*R*-BUD were almost twice as large as those of 22*S*-BUD. Pedersen et al. [5] also reported the pharmacokinetics of the epimers in six children with asthma. After single-dose intravenous and inhaled administrations, differences in the distribution volume and plasma clearance between the two epimers were determined to be in accordance with the results obtained for adults. In contrast to such reports of epimer-selective disposition, Minto et al. [6] did not detect consistent differences in the pharmacokinetics of the two epimers after 5 days of repeat-dose inhalation. All these studies indicated that 22*R*-BUD possessed higher tissue affinity and was more readily metabolized than 22*S*-BUD. The 6β-hydroxylation to 6β-hydroxybudesonide pathway and acetal splitting to 16α-hydroxyprednisolone pathway are the main metabolic pathways of BUD [7]. Research has shown that the former pathway proceeded with both epimers and that the latter was stereoselective for 22*R*-BUD [8]. To the best of our knowledge, no study on stereoselective pharmacokinetics of BUD epimers after oral administration has been reported.

The pharmacokinetics of BUD is characterized by extensive first-pass elimination, low oral bioavailability, a large volume of

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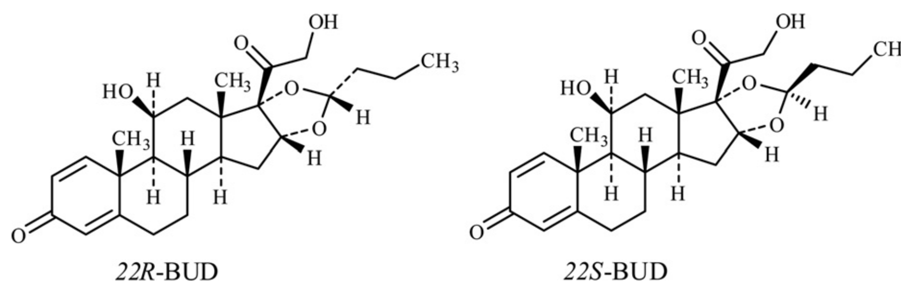

Fig. 1. Structures of BUD epimers.

distribution, and high systemic clearance, all of which lead to low plasma concentrations [3,9]. Thus, a robust, sensitive, and selective method is required for the determination of BUD in plasma. The determination of total BUD levels in biological fluids for pharmacokinetic studies has been widely investigated [10–17]. The method described by Qu et al. [14] showed a lower limit of quantification (LLOQ) of 5.0 pg/mL using capillary LC–tandem mass spectrometry (MS/MS) with d_7-triamcinolone acetonide as internal standard (IS). Kronkvist et al. [11] reported an automated bioanalytical method for the determination of BUD in plasma samples by LC–MS/MS using d_8-BUD as IS. The LLOQ under this method was 6.45 ng/mL. Some of these methods applied solid-phase extraction, whereas the others used liquid–liquid extraction with ether [13] or ethyl acetate [15].

Only a few studies have been conducted to determine the 22*R* and 22*S* epimers of BUD in human plasma by LC–MS/MS. Li et al. [18,19] developed a complex method: BUD was first isolated from human plasma using C_{18} solid-phase extraction, acetylated with acetic anhydride and triethylamine, and finally detected by LC–MS/MS. In 1996, Li et al. reported an LOQ of 0.25 ng/mL for each epimer [18] and in 2001, reported an LOQ of 0.05 ng/mL for BUD [19]. Streel et al. [20] simplified the sample preparation process, with the samples detected directly without derivatization. The LLOQ of both epimers was approximately 50.0 pg/mL. No baseline separation was achieved, and a very long (>10 min) chromatography run was observed in this method.

The present study developed a highly sensitive and selective ultra-high-performance liquid chromatography (UHPLC) method to determine BUD epimers in human plasma. The assay was then successfully applied in a stereoselective pharmacokinetic study of BUD controlled-release capsules after oral administration in healthy Chinese volunteers.

2. Experimental

2.1. Chemicals and reagents

BUD (chemical purity, 100%) was provided by Shanghai Sine Promod Pharmaceutical Co. Ltd. (Shanghai, China), 22*R*-BUD (chemical purity, 99.5%) and 22*S*-BUD (chemical purity, 100%) were provided by Shandong New Age Pharmaceutical Co. Ltd. (Shandong, China). d_8-BUD (chemical purity, 99%; isotopic purity, 98%) was purchased from TLC Pharmachem., Inc. (Toronto, Canada). Methanol and acetonitrile of HPLC grade were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium acetate and acetic acid of HPLC grade were purchased from Tedia (Fairfield, OH, USA). Na_2CO_3, *n*-hexane, dichloromethane, and isopropanol of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Deionized water (18.2 mΩ, TOC $\leq$ 50 ppb) was purified using a Millipore Milli-Q Gradient Water Purification System (Molsheim, France).

2.2. Instrumentation

The UHPLC system consisted of an LC-30AD pump and an SIL-30AC autosampler (Shimadzu, Kyoto, Japan). Mass spectrometric detection was conducted on an AB Sciex Qtrap 5500 System (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray ionization interface. The data acquisition software used was Analyst^{TM} Version 1.5.2 (Applied Biosystems).

2.3. LC–MS/MS conditions

The analytes were separated on an Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters, Milford, MA, USA) and maintained at 40 °C. The mobile phase consisted of acetonitrile/5 mM ammonium acetate/acetic acid (29:71:0.142, v/v/v) and was delivered at a flow rate of 0.7 mL/min. The autosampler was conditioned at 4 °C, and the injection volume was 20 μL.

The mass spectrometer was operated in negative ion mode using an electrospray ionization (ESI) source for the analytes. Quantification was performed using multiple reaction monitoring (MRM) of the m/z 489 → 357 transition for BUD epimers and the m/z 497 → 357 transition for IS d_8-BUD epimers, with a scan time of 150 ms per transition. The optimal MS parameters were set as follows: curtain gas, 35 psi; nebulizer gas (GS1), 50 psi; turbo gas (GS2), 50 psi; ion spray voltage, −3800 V; source temperature, 550 °C. The optimized collision energy and declustering potential for all analytes and ISs were −21 and −40 eV, respectively.

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

Stock solutions of 22*R*- and 22*S*-BUD were prepared in methanol at 1.0 mg/mL for both solutions. The solutions were then serially diluted with 50% aqueous methanol to obtain 22*R*-/22*S*-BUD working solutions of 50.0/50.0, 120/120, 300/300, 625/900, 1250/2500, 2500/5000, 5000/15,000, and 0/30,000 pg/mL. IS d_8-BUD stock solution was also prepared in methanol and then diluted to obtain the final concentration of 20.0 ng/mL. Calibration curves for 22*R*-/22*S*-BUD were prepared by spiking blank plasma with working solution to obtain a ten times dilution giving the final concentrations of 5.0/5.0, 12.0/12.0, 30.0/30.0, 62.5/90.0, 125/250, 250/500, 500/1500, and 0/3000 pg/mL. QC samples were independently prepared in blank plasma at four concentrations (LLOQ, low, medium, and high at 5.0/5.0, 10.0/10.0, 75.0/300, and 400/2400 pg/mL for 22*R*-/22*S*-BUD, respectively). All solutions were stored at 4 °C and brought to room temperature before use. QC samples were stored at −20 °C.

2.5. Sample preparation

A 50-μL aliquot of IS solution (20.0 ng/mL d_8-BUD, final plasma concentration = 2.0 ng/mL), 50 μL of 50% aqueous methanol, and

200 μL of 2 M Na_2CO_3 aqueous solution were added to 500 μL of plasma sample. The sample was vortex-mixed and extracted with 3 mL of *n*-hexane/dichloromethane/isopropanol (2:1:0.1, v/v/v) by shaking for 5 min. The organic and aqueous phases were separated by centrifugation at 3500 × *g* for 5 min. The upper organic phase was transferred to another tube and evaporated to dryness at 40 °C under a stream of nitrogen in a TurboVap evaporator (Zymark, Hopkinton, MA, USA). The residue was reconstituted in 150 μL of the mobile phase, and an aliquot of 20 μL was injected into the UHPLC–MS/MS system for analysis.

2.6. Method validation

The method was validated for selectivity, linearity, precision and accuracy, matrix effect (ME), recovery, stability and carry-over according to EMA guidelines [21].

Selectivity was evaluated by analysing six sources of human blank plasma and 12 spiked plasma samples at the LLOQ level to test interference at the retention times of the analytes and the IS. The peak areas of the endogenous compounds co-eluted with the analytes should be less than 20% of the peak area of the LLOQ standard and less than 5% of the peak area of the IS.

Linearity was assessed by plotting the peak area ratios of the analyte to the IS against the concentrations of analyte in human plasma using a linearly weighed ($1/x^2$) least-squares regression method in duplicate on three consecutive validation days. A correlation coefficient (r^2) greater than 0.99 was required to determine linearity. The deviations of the calculated concentrations should be within ±15% of the nominal concentrations, except for the LLOQ, for which a deviation of ±20% was permitted.

Precision and accuracy were determined by assessing six replicates of QC samples at three levels (10.0/10.0, 75.0/300, and 400/2400 pg/mL for 22*R*-/22*S*-BUD) on three consecutive days. Precision was expressed as relative standard deviation, whereas accuracy was reported as relative error. The intra- and inter-day precision values were required to be below 15% and the accuracy to be within ±15%.

LLOQ was established by analysing six blank plasma samples spiked with 5.0 pg/mL of both epimers with acceptable precision and accuracy (less than 20% for each criterion).

Blank plasma from six lots was extracted and then spiked with analytes and IS to evaluate the ME of each epimer and IS. The corresponding peak area ratios of the analytes to IS in spiked plasma post-extraction samples (A) were then compared with those of the water-substituted samples (B) at equivalent concentrations. The ratio (A/B × 100%) is defined as the IS-normalized matrix factor (MF). The variability in MFs should be less than 15%.

The recovery of each epimer was determined by comparing the peak area ratios of the analytes to IS in the regularly pre-treated QC samples at three concentration levels (six samples each) with those of spiked post-extraction samples. Similarly, the recovery of ISs (d_8-22*R*-BUD and d_8-22*S*-BUD) was determined at 1.0 ng/mL.

The stabilities of each epimer in human plasma were evaluated by analysing triplicates of plasma samples at two concentration levels (10.0/10.0 and 400/2400 pg/mL of 22*R*-/22*S*-BUD), which were exposed to the following conditions: (1) short-term stability at room temperature for 6 h, (2) long-term stability at −20 °C for 30 days, (3) autosampler stability at 4 °C for 24 h, and (4) freeze–thaw stability after three freeze–thaw cycles at −20 °C. Analytes were considered stable when the accuracy bias was within ±15% of the initial concentration.

In addition, the long-term stabilities of the stock solutions of both epimers were analysed after 12 days at 4 °C. Solutions were deemed stable if the peak area difference between the stored solution and a freshly prepared solution was ≤10%. The stability of the isotopic IS was determined in a working solution (20 ng/mL) at 4 °C for 12 days. The solution was considered stable when the peak area difference of both epimers between the blank plasma sample with an IS solution (stored at 4 °C for 12 days) and the plasma samples (at the LLOQ level) was ≤20%.

Carry-over was assessed by injecting blank samples after calibration standard at the upper limit of quantification (ULOQ). If it is unavoidable, specific measures should be considered, tested during the validation and applied during the analysis of the study samples.

2.7. Application of method in a clinical pharmacokinetic study

The validated method was used to investigate the plasma profiles of 22*R*- and 22*S*-BUD following single-dose and repeat-dose oral administrations of 6 and 9 mg of BUD controlled-release capsules (Shanghai Sine Promod Pharmaceutical Co. Ltd.) once daily in 20 healthy Chinese volunteers (10 males and 10 females; mean age, 26.5 years; weight, 60.3 kg; height, 1.65 m; body mass index range, 19–24 kg/m^2). The study was of an open, randomized, parallel group, single-centre design. The 20 healthy volunteers were divided into two groups (each group consisted of 5 males and 5 females). Volunteers of the two groups received oral administration of BUD controlled-release capsules at doses of 6 and 9 mg, respectively. The pharmacokinetic study was approved by the Ethics Committee of the Shanghai Changhai Hospital. All volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples (4 mL) were collected into sodium heparin-containing tubes at 0 (pre-dose), 1, 1.33, 1.67, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 24, 36, and 48 h after administration of the first dose. There was 7 days washout period between single-dose and repeat-dose administration. After 6 days of repeat-dose oral administration, blood samples were collected at the same time points as for the single-dose oral administration. The blood samples were centrifuged at 2000 × *g* for 10 min, and the plasma samples were stored at −20 °C prior to analysis.

The pharmacokinetic parameters of 22*R*- and 22*S*-BUD were calculated by non-compartmental analysis using WinNonlin 5.3 (Pharsight, St. Louis, MO, USA). The maximum plasma concentrations (C_{max}), the minimum plasma concentrations (C_{min}) for repeat-dose study, and their times (T_{max}/T_{min}) were obtained directly from the experimental data. The elimination rate constant (k_e) was calculated using log-linear regression of the terminal portion of each curve. The elimination half-life ($t_{1/2}$) was calculated as $0.693/k_e$. The area under the curve [$AUC_{(0-t)}$, from 0 to the last measurable plasma concentration (C_t)] was calculated using the linear trapezoidal method and was extrapolated to infinity [$AUC_{(0-\infty)}$] using the following formula: $AUC_{(0-\infty)} = AUC_{(0-t)} + C_t/k_e$. For repeated dosing, AUC_{ss} was calculated to 24 h after dosing.

2.8. Incurred sample reanalysis (ISR)

An incurred sample reanalysis (ISR) was performed to evaluate the reproducibility and accuracy of the determination results. For the ISR study, the samples obtained from several subjects close to the expected maximal concentration (C_{max}) and in the elimination phase. The difference between the two values obtained should be within 20% of the mean for at least 67% of the repeats [21].

3. Results and discussion

3.1. Mass spectrometric conditions

BUD is a non-steroidal compound with low polarity. It exhibits MS responses in both atmosphere pressure chemical ionization (APCI) and ESI. During the early stage of method development, use of an ESI source and that of an APCI source was investigated. The results showed that ESI could offer much higher signal intensity

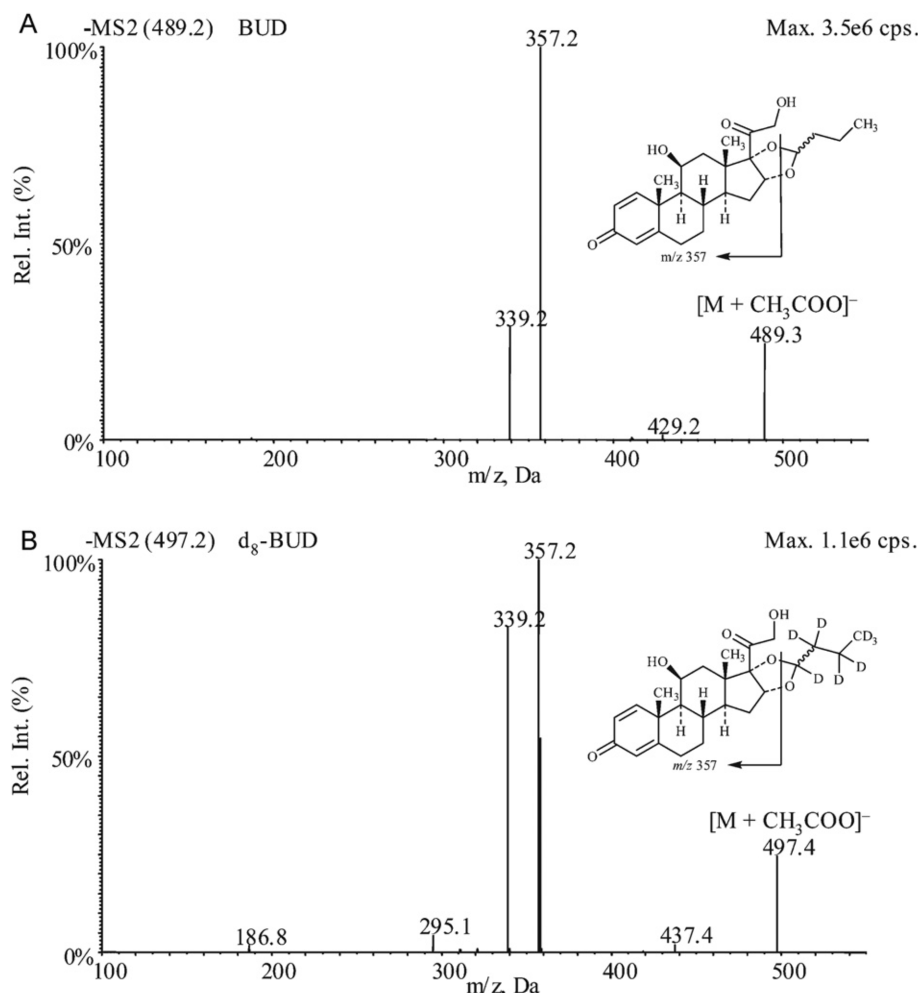

Fig. 2. Product ion spectra of $[M+CH_3COO]^-$ of BUD (A), d_8-BUD (B), and their proposed fragmentation patterns.

for the analytes than APCI. Consequently, ESI was chosen as the ionization source in the present experiment. BUD has also been analysed using both positive and negative ion modes. In the positive ion mode, the protonated molecule $[M+H]^+$ of BUD (*m*/*z* 431) was fragmented to the intense product ion *m*/*z* 413 (M−18) and the minor product ion *m*/*z* 323 (M−108). The major problem with this mode is that BUD forms intensive $[M+Na]^+$ and $[M+K]^+$ ions, thereby reducing the sensitivity of the assay. This was solved using the negative ion mode and assaying the fragments originating from the acetate adducts. Use of acetate adducts as the parent ion of BUD has been reported previously [11,12,15,20].

As a result, we chose ESI(−) for analysis. In the Q1 full scan mode, the base peaks for BUD and IS were acetate adducts $[M+CH_3COO]^-$. Fig. 2 presents the product ion spectra of ions from the BUD and IS, as well as their proposed fragmentation patterns. The most abundant and stable fragment ion at *m*/*z* 357 was selected in the MRM transitions for both BUD and IS.

3.2. Chromatographic conditions

Chromatographic conditions were optimized to obtain high sensitivity, good peak shape, and short retention time. The log *P* values of 22*R*- and 22*S*-BUD were the same at 3.05 [calculated using Advanced Chemistry Development (ACD/Labs) software, ADME Suite, Version 5.0, ACD/Labs Inc., Toronto, Ontario, Canada]. The most difficult challenge was achieving good resolution (R_s, which was calculated as $R_s = (2(t_{R2} - t_{R1}))/(W_1 + W_2)$, where t_R is the retention time and *W* is the peak width). A number of HPLC columns were used in our experiment to separate BUD epimers, including Waters Atlantis C_{18}, Agilent XDB C_{18}, and Hypersil GOLD C_{18}. Poor resolution and long retention time (>15 min) were achieved for 22*R*- and 22*S*-BUD on these columns, although various percentages of acetonitrile, methanol, and acid/base modifier were tested. We then evaluated the UHPLC system for better separation and sensitivity. Three types of UHPLC columns were tested, namely, Acquity UPLC BEH C_{18}, Acquity UPLC HSS T_3, and Phenomenex Kinetex C_{18}. The results showed that the Acquity UPLC BEH C_{18} column performed the best.

22*R*- and 22*S*-BUD have the same mass spectra. Therefore, clear baseline separation is necessary to reduce the interference from each other. Various combinations of methanol/acetonitrile along with buffers (ammonium acetate/acetic acid/formic acid) having different ionic strengths (1–10 mM) in the pH range of 3.4–6.4 and volume ratios were tested to find the best eluting solvent system. With the use of methanol as the organic mobile phase, the analytes remained strong. With methanol replaced by acetonitrile, the resolution and peak shape improved and the resolution of the epimers significantly increased from 1.00 to 1.73. Moreover, the lower viscosity of acetonitrile contributed to lower column pressure and higher efficiency. The use of ammonium acetate as a buffer in the mobile phase improved the peak symmetry and reproducibility of retention time, and the use of acetic acid to adjust the aqueous mobile phase to pH 3.6 improved the signal and the resolution of BUD epimers. Based on these findings,

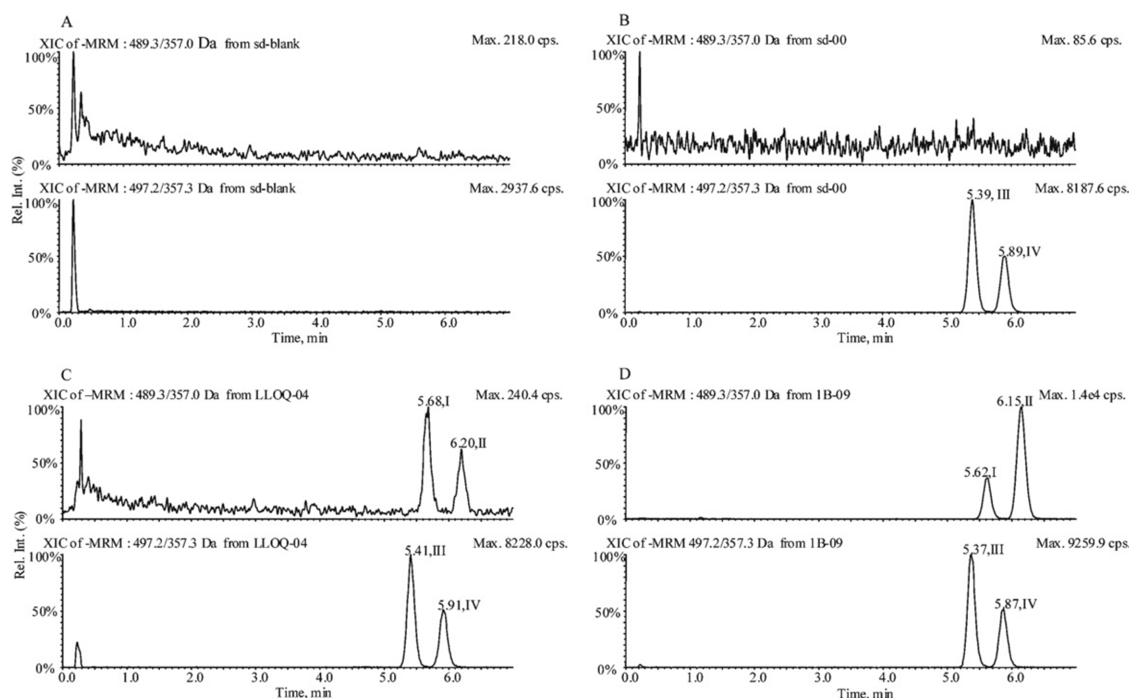

Fig. 3. Representative MRM chromatograms for 22*R*-BUD (I), 22*S*-BUD (II), d_8-22*R*-BUD (IS; III), and d_8-22*S*-BUD (IS; IV) in human plasma. (A) Blank plasma sample. (B) Blank plasma sample spiked with d_8-BUD (2.0 ng/mL). (C) Blank plasma sample spiked with 22*R*-BUD (5.0 pg/ml), 22*S*-BUD (5.0 pg/ml), and d_8-BUD (2.0 ng/mL). (D) Plasma sample 5 h after oral administration of 6 mg of BUD to a subject on the first day.

the mobile phase was finally optimized as acetonitrile/5 mmol/L ammonium acetate solution with 0.2% acetic acid using an isocratic elution. Along with increasing column temperature, the viscosity of acetonitrile, column pressure, and resolution of the two epimers decreased. In view of the resolution, column pressure, and peak shapes of the analytes and IS, the optimal column temperature was maintained at 40 °C. With increasing ratio of the aqueous mobile phase, the resolution increased but the retention time of the analytes extended. The flow rate was increased to 0.7 mL/min to reduce the retention time. The analytical run time under optimized chromatographic conditions of each sample was 7 min, and the retention times of 22*R*-BUD, 22*S*-BUD, d_8-22*R*-BUD, and d_8-22*S*-BUD were 5.6, 6.2, 5.4, and 5.9 min, respectively.

3.3. Sample preparation

The chromatographic column packed with small particles for UHPLC was more easily blocked than the common one under the HPLC system, indicating that sample extraction is very important. Liquid–liquid extraction was chosen as the method for sample preparation because this technique could produce not only purified but also concentrated samples. Extraction with ethyl acetate, ethyl ether, or *n*-hexane/dichloromethane/isopropanol (2:1:0.1, v/v/v) after pH modification of plasma with 2 mol/L sodium carbonate (500 μL of plasma and 200 μL of buffer) was performed. The results showed that the mixture solvent of *n*-hexane/dichloromethane/isopropanol (2:1:0.1, v/v/v) was the most suitable extraction solvent, with extraction recovery rates of the analytes at approximately 100%, and that interferences were not detected at the retention times of the analytes and IS.

3.4. Method validation

3.4.1. Assay selectivity

Selectivity of the method was assessed by comparing the chromatograms of blank human plasma from six sources with the corresponding spiked plasma at the LLOQ concentration. Fig. 3 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with IS (d_8-BUD 2 ng/mL), a blank plasma sample spiked with 22*R*- and 22*S*-BUD at the LLOQ and IS, and a plasma sample obtained 5 h after oral administration of 6 mg of BUD to a volunteer. No significant endogenous interference co-eluting with analytes and IS was observed in the blank human plasma. Quantitative analysis of the reference standards revealed that the MS response of 22*R*-BUD was approximately two times higher than that of 22*S*-BUD.

3.4.2. Linearity of calibration curve and LLOQ

Linear regression curves were obtained over the concentration ranges of 5.0–500 and 5.0–3000 pg/mL for 22*R*-BUD and 22*S*-BUD, respectively. The following typical equations of the calibration curve were used:

$$\text{22}R\text{-BUD}: \quad y = 0.00360x + 0.00282 \quad (r = 0.9986)$$
$$\text{22}S\text{-BUD}: \quad y = 0.00374x + 0.00629 \quad (r = 0.9982)$$

where *y* is the peak area ratio of analytes to IS and *x* is the concentration of analytes.

The LLOQ of both 22*R*- and 22*S*-BUD was 5.0 pg/mL. The precision at the LLOQ of the two epimers was between 9.3% and 13.3%, and their accuracy was between −4.6% and 0.8%. With the present LLOQ, the plasma concentrations of 22*R*- and 22*S*-BUD could be determined for up to 36 h after oral administration of 6 and 9 mg of BUD controlled-release capsules to human volunteers, which were sensitive enough to allow for the investigation of the pharmacokinetic behaviour of 22*R*- and 22*S*-BUD.

3.4.3. Precision and accuracy

Intra- and inter-day precision and accuracy values for the QC samples are summarized in Table 1. In this assay, the intra- and inter-day precision values for 22*R*-BUD were less than 11.1%, whereas the accuracy values ranged from −9.6% to 1.4%. For

Table 1
Precision and accuracy data for analysis of 22*R*- and 22*S*-BUD in human plasma (3 days with six replicates per day).

Analyte	Nominal conc. (pg/mL)	Mean ± SD (pg/mL)	Interday RSD (%)	Intraday RSD (%)	Relative error (%)
22*R*-BUD	5.0	4.77 ± 0.52	11.0	10.8	−4.6
	10.0	9.04 ± 0.61	6.0	11.1	−9.6
	75.0	75.0 ± 2.8	1.7	9.7	0.0
	400	406 ± 14	1.8	8.6	1.4
22*S*-BUD	5.0	5.04 ± 0.65	13.3	9.3	0.8
	10.0	9.78 ± 1.04	10.7	10.3	−2.2
	300	286 ± 11	2.3	9.9	−4.8
	2400	2292 ± 66	1.2	7.8	−4.5

22*S*-BUD, intra- and inter-day precision values were less than 10.7% and accuracy was between −4.8% and −2.2%.

3.4.4. ME and recovery

The MEs from six lots of blank plasma were in the range of 99.7–104% for all compounds. Inter-subject variability of the IS-normalized MFs, as measured by their relative standard deviation, was lower than 12.9%. Thus, ion suppression or enhancement from the plasma matrix was negligible under the current conditions.

The mean extraction recovery rates of the two epimers and ISs (d_8-22*R*-BUD and d_8-22*S*-BUD) were between 99.1% and 112%.

3.4.5. Stability

The results of stability experiments are presented in Table 2. 22*R*- and 22*S*-BUD were stable in plasma after placement at room temperature for 6 h, undergoing three freeze–thaw cycles and storage at −20 °C for 30 days. Processed samples were stable up to 24 h at the autosampler tray. The results demonstrated good stability of 22*R*- and 22*S*-BUD throughout all steps of the determination.

The stability of stock solutions of 22*R*- and 22*S*-BUD was established at 4 °C for 12 days as part of the validation. The relative errors of both epimers were between 1.7% and 3.6%. The stability of the d_8-BUD working solution (20 ng/mL) was determined after 12 days at 4 °C. The differences between the peak areas of both epimers in the blank plasma samples with an IS solution and the plasma samples at the LLOQ level were 3.6% (22*R*-BUD) and 7.0% (22*S*-BUD), respectively.

3.4.6. Carry-over

Carryovers were not found for all analytes and ISs in extracted double blank plasma (without analyte and IS) after subsequent injection of the ULOQ (500/3000 pg/mL for 22*R*-/22*S*-BUD).

3.5. Comparison with reported methods

Only one study reporting direct analysis of 22*R*- and 22*S*-BUD using an APCI–LC–MS/MS system with hydrocortisone acetate as IS has been published in the literature [20]. The analytes were separated by Luna C_{18} (50 mm × 4.6 mm × 3.0 μm; Phenomenex) with a chromatographic run time of 12 min. Baseline separation was not observed. The LLOQ values determined by the method were 41.2 pg/mL for 22*R*-BUD and 37.6 pg/mL for 22*S*-BUD. By comparison, our method has the following advantages: first, UHPLC and a column of smaller particle size [BEH C_{18} (50 mm × 2.1 mm × 1.7 μm)] were applied for the separation and the total run time per sample was significantly reduced to 7 min. The resolution of the two epimers was 1.73, and complete baseline separation was obtained. Second, the deuterated standard would be the preferred IS in LC–MS/MS assay. In our experiment, d_8-BUD was used as IS. Under the same chromatographic conditions, d_8-BUD was also separated into two peaks of 22*R* and 22*S* for the quantification of 22*R*- and 22*S*-BUD, respectively. Third, The LLOQ of 37.6 pg/mL for 22*S*-BUD could not meet the necessary sensitivity for the pharmacokinetic study of BUD after oral administration. Our UHPLC method provided superior sensitivity with an LLOQ as low as 5.0 pg/mL for both 22*R*- and 22*S*-BUD in plasma.

Table 2
Stability of 22*R*- and 22*S*-BUD in human plasma under various storage conditions (*n* = 3).

Storage condition	Analyte	Concentration (pg/mL)		Relative error (%)
		Nominal	Mean ± SD	
−20 °C/30 days	22*R*-BUD	10.0	10.4 ± 0.8	4.0
		400	355 ± 4	−11.4
	22*S*-BUD	10.0	9.67 ± 0.93	−3.3
		2400	2268 ± 17	−5.5
−20 °C/3 freeze–thaw cycles	22*R*-BUD	10.0	9.26 ± 0.46	−7.4
		400	402 ± 4	0.4
	22*S*-BUD	10.0	10.1 ± 0.7	1.1
		2400	2233 ± 16	−6.9
Room temperature/6 h	22*R*-BUD	10.0	9.76 ± 0.55	−2.4
		400	402 ± 2	0.4
	22*S*-BUD	10.0	10.0 ± 1.4	0.4
		2400	2277 ± 34	−5.1
4 °C/24 h (extracted sample)	22*R*-BUD	10.0	9.55 ± 1.21	−4.5
		400	395 ± 6	−1.3
	22*S*-BUD	10.0	9.44 ± 0.36	−5.6
		2400	2266 ± 13	−5.6

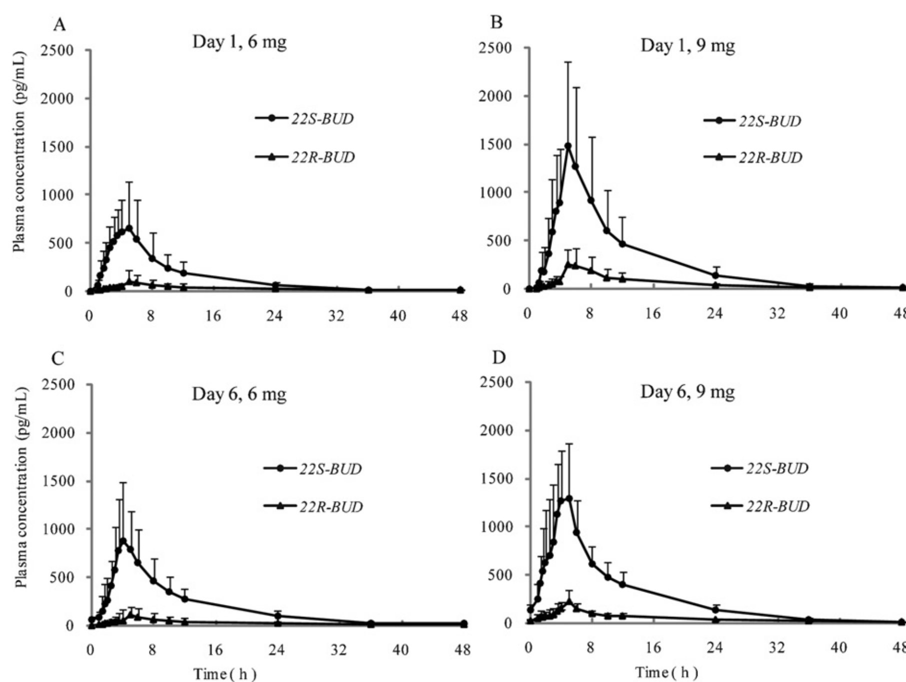

Fig. 4. Mean plasma concentration – time profiles of 22*R*- and 22*S*-BUD in two groups healthy volunteers (each group consisted of 5 males and 5 females) after the first dose (single-dose) (A and B) and after 6 days of oral administration (repeat-dose) (C and D) at 6 and 9 mg once daily.

Table 3
Pharmacokinetic parameters of 22*R*- and 22*S*-BUD obtained from two groups healthy volunteers (each group consisted of 5 males and 5 females) after the first dose (single-dose) and after 6 days of oral administration (repeat-dose) at 6 and 9 mg once daily.

Parameter	Single-dose		Repeat-dose	
	6 mg	9 mg	6 mg	9 mg
22*R*-BUD				
AUC_{ss} (ng h/mL)	NA	NA	1.54 ± 0.96	1.83 ± 0.60
$AUC_{(0-t)}$ (ng h/mL)	1.05 ± 0.95	2.43 ± 1.40	1.85 ± 1.19	1.97 ± 0.64
$AUC_{(0-\infty)}$ (ng h/mL)	1.18 ± 0.99	2.70 ± 1.42	1.97 ± 1.17	2.33 ± 0.66
$t_{1/2}$ (h)	10.1 ± 3.7	8.94 ± 3.21	9.63 ± 1.88	11.0 ± 3.9
t_{max} (h)	5.20 ± 1.03	5.65 ± 1.60	5.00 ± 0.67	4.38 ± 1.20
C_{max} (pg/mL)	110 ± 109	286 ± 181	166 ± 128	230 ± 120
C_{min} (pg/mL)	NA	NA	10.5 ± 9.4	20.1 ± 10.9
22*S*-BUD				
AUC_{ss} (ng h/mL)	NA	NA	7.69 ± 3.07	11.6 ± 3.6
$AUC_{(0-t)}$ (ng h/mL)	6.38 ± 3.50	13.2 ± 6.8	8.64 ± 3.38	12.8 ± 3.8
$AUC_{(0-\infty)}$ (ng h/mL)	6.49 ± 3.50	13.3 ± 6.8	8.81 ± 3.43	12.9 ± 3.9
$t_{1/2}$ (h)	6.19 ± 1.28	5.65 ± 0.74	9.00 ± 5.95	6.60 ± 0.68
t_{max} (h)	3.85 ± 0.91	4.60 ± 1.02	4.10 ± 1.22	4.15 ± 0.78
C_{max} (pg/mL)	733 ± 480	1560 ± 818	984 ± 588	1465 ± 616
C_{min} (pg/mL)	NA	NA	45.6 ± 26.3	112 ± 60

3.6. Clinical application

The validated UHPLC–MS/MS method was successfully applied for the assay of 22*R*- and 22*S*-BUD in healthy Chinese volunteers. Fig. 4 shows the plasma concentration vs. time profiles for 22*R*- and 22*S*-BUD. Table 3 summarizes the mean pharmacokinetic parameters after single-dose and repeat-dose oral administrations of 6 and 9 mg of BUD controlled-release capsules once daily.

The plasma concentrations of 22*S*-BUD for all time points were higher than those of 22*R*-BUD, with the 22*S*-/22*R*-BUD ratio at each time point ranging from 1.8 to 12.8. The $AUC_{(0-\infty)}$ of 22*S*-BUD was six times higher than that of 22*R*-BUD, and the 22*S*-/22*R*-BUD ratio of total body clearance (CL/F) was 0.17. Compared with previous studies [4–6], the stereoselectivity in the plasma concentration of BUD after oral administration was much more significant than that observed after injection or inhalation. 22*R*-BUD showed lower system exposure than 22*S*-BUD such that it produced lower adverse effects while offering higher local anti-inflammatory effects.

3.7. Incurred sample reanalysis

In this study, 95.8% of the ISR samples meet the acceptance criteria for both epimers. The results showed that there was no interference caused by metabolites of the drug(s), degradation products formed during sample preparation, and possible co-administered medications.

4. Conclusions

This study developed a fast, selective, and sensitive UHPLC–MS/MS method for the simultaneous determination of 22*R*- and 22*S*-BUD in human plasma. The sharp peaks and good resolution produced by UHPLC were of particular advantage. High sensitivity with an LLOQ of 5.0 pg/mL for both epimers in plasma was achieved. A short analysis time of 7 min and a relatively simple preparation procedure with one-step liquid–liquid extraction showed greater simplicity and efficiency for analysing a large number of plasma samples. The method has been successfully applied in a stereoselective pharmacokinetic study of BUD controlled-release capsules.

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