HHS Public Access

Author manuscript

Anal Methods. Author manuscript; available in PMC 2016 November 07.

Published in final edited form as:

Anal Methods. 2016 May 7; 8(17): 3550–3563. doi:10.1039/C6AY00202A.

Quantitative determination of betamethasone sodium phosphate and betamethasone dipropionate in human plasma by UPLC-MS/MS and a bioequivalence study

Man-Yun Chen[a,b], **Yong-Jun Tang**[a,b], **Yi-Cheng Wang**[a,b], **Chong-Zhi Wang**[c], **Chun-Su Yuan**[c], **Yao Chen**[a,b], **Zhi-Rong Tan**[a,b,**], **Wei-Hua Huang**[a,b,c,*], and **Hong-Hao Zhou**[a,b]

[a]Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha 410008, China

[b]Institute of Clinical Pharmacology, Hunan Key Laboratory of Pharmacogenetics, Central South University, Changsha 410078, China

[c]Tang Center for Herbal Medicine Research, The Pritzker School of Medicine, University of Chicago; 5841 South Maryland Avenue, MC 4028, Chicago, IL 60637, USA

Abstract

The compound medicine of betamethasone sodium phosphate (BSP) and betamethasone dipropionate (BDP) is widely used for diverse glucocorticoid-sensitive acute and chronic diseases such as asthma, rheumatoid arthritis and systemic lupus erythematosus. It will be useful and beneficial to validate sensitive method for the determination of BSP, BDP and their metabolites for their pharmacokinetic study. Hereby, an ultra-high pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) has been validated for the determination of BSP, BDP and their metabolites betamethasone (BOH), betamethasone 17-monodipropionate (B17P) and betamethasone 21-monodipropionate (B21P) in human plasma. Liquid-liquid extraction with ether and n-hexane (v/v, 4:1) was used for sample preparation of BDP, BOH, B17P and B21P with beclomethasone dipropionate as internal standard (IS), while solid phase extraction was adopted for sample preparation of BSP using prednisolone as IS. The chromatographic separation was performed on a Hypurity C_{18} column (150 mm×2.1 mm, 5 μm) for BOH, BDP, B21P and B17P, and a Luna C_{18} (2) column (150 mm×2.0 mm, 5 μm) for BSP. Electrospray ionization interfaced with positive multiple reaction monitoring (MRM) scan mode was used for mass spectrometric detection. The standard calibration curves were linear within the range of 2.525×10^{-9}–403.9×10^{-9} mol·dm^{-3} for BSP, 0.125×10^{-9}–55.81×10^{-9} mol·dm^{-3} for BDP, 0.278×10^{-9}–74.95×10^{-9} mol·dm^{-3} for BOH, 0.098×10^{-9}–4.688×10^{-9} mol·dm^{-3} for B17P and 0.226×10^{-9}–5.411×10^{-9} mol·dm^{-3} for B21P, respectively. The validated method was successfully applied to a

*Corresponding author. Tel.: +86 731-8480 5380, fax: +86 731-8235 4476; endeavor34852@aliyun.com (W.-H. Huang).
**Corresponding author. Tel.: +86 731-8480 5380, fax: +86 731-8235 4476; tanzr@163.com (Z.-R. Tan).

Author Contributions

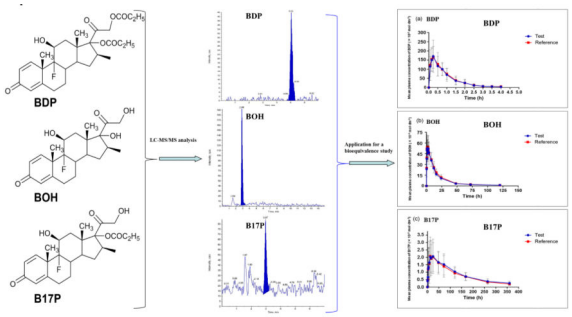
R.-Z. T., W.-H. H. and H.-H. Z. designed this study. M.-Y. C., Y.-C. W. and Y.-J. T. performed the experiments. W.-H. H., R.-Z. T., Y. C., C.-Z. W. and C.-S. Y. analyzed the data and described the figures. M.-Y. C. and W.-H. H. wrote the manuscript. All authors have read and approved the final manuscript.

Competing interests

All the authors declare that they have no conflict of interests.

bioequivalence study in 23 healthy subjects after they were injected with this compound medicine BSP and BDP.

Graphic abstract



Keywords

betamethasone; betamethasone sodium phosphate; betamethasone dipropionate; UPLC-MS; human plasma

1. Introduction

Both Betamethasone sodium phosphate (BSP) and betamethasone dipropionate (BDP) are synthesized glucocorticoid, which were able to reduce the production of inflammatory mediator.[1] The compound betamethasone intramuscular injection consists of BSP and BDP, which were usually used for diverse glucocorticoid-sensitive acute and chronic diseases such as asthma, rheumatoid arthritis, systemic lupus erythematosus.[2, 3] BSP and BDP could be hydrolyzed by phosphatase as fast-release phosphate prodrug and esterase enzymes as sustained-release dipropionate prodrug into active pharmaceutical ingredients betamethasone (BOH), betamethasone 17-monodipropionate (B17P) and betamethasone 21-monodipropionate (B21P), respectively.[4, 5] Because the activating enzymes such as phosphate and esterase were highly efficient and ubiquitous in human blood, BSP that was highly ionized and hydrophilic could be rapidly absorbed into blood from administration place and then be metabolized quickly into active betamethasone (BOH) without rate-limiting step.[6, 7] However, BDP was so highly lipophilic that it was slowly to release into the fluids of the intercellular space from muscle fibers, which resulted in the lower plasma concentration, longer half-life and slower metabolism process than BOH, B17P and B21P after intramuscular injection.[6] Therefore, it was useful and important to determine BSP, BDP and their metabolites in human plasma by a sensitive method.

As synthesized glucocorticoid, the adverse effects are inevitable ranging from skin fragility to full-blown iatrogenic Cushing syndrome such as obesity, hypertension, osteoporosis and other diseases.[8, 9] Prolonged use of BSP and BDP may lead to suppression of the hypothalamic-pituitary-adrenal axis due to the impact on endogenous cortisol concentration as well as a circadian pattern.[8, 10] Moreover, as a kind of antenatal steroid therapy to prevent respiratory distress syndrome in premature neonates, it had been reported that maternal

betamethasone administration could cause the risk of fetal growth restriction and preterm birth.[11, 12] Hereby, developing and validating a sensitive analytical method for the quantification of BSP and BDP as well as their metabolites will be useful and valuable for their pharmacokinetic studies.

In the past three decades, there were several analysis methods validated to quantify BDP, BOH, B17P and B21P in human plasma, and the reported methods compared with this developed method will be discussed below. As mentioned above, BSP was rapidly and completely converted to BOH *in vivo* so that BSP was rarely determined for the quantification in human plasma *in vitro* and *in vivo*.[13-15] In most studies, the pharmacokinetic processes of BSP and BDP were reflected by their main metabolites BOH and B17P due to the low blood concentration and poor receptor-binding affinity at the glucocorticoid receptor of B21P compared with B17P.[16-18] In this study, the analytical method was accordingly validated to quantify BSP and BDP as well as their metabolites BOH, B17P and B21P in human plasma for a bioequivalence study.

As on-going development as the analytical instruments, ultra-performance liquid chromatography (UPLC) system had evolved from high performance liquid chromatography (HPLC), which usually had packing material with smaller particle size less than 2 μm. Since UPLC has greatly improved the column efficiency and resolution, it could shorten the analysis time.[19] Especially equipped with mass spectrometer (MS), the sensitivity of UPLC-MS/MS was 3 to 5 times higher than HPLC-MS/MS.[20] Therefore, in this study, a rapid, simple and sensitive UPLC-MS/MS had been validated for the determination of BSP, BDP and their metabolites BOH, B17P and B21P in human plasma. Then, the validated method was successfully applied to their pharmacokinetic studies in 23 healthy subjects after they were injected with this compound medicine BSP and BDP.

2. Experimental

2.1. Chemicals and reagents

Standards of betamethasone sodium phosphate (BSP, purity: 99.4%, batch no. 20120501), betamethasone dipropionate (BDP, purity: 99.7%, batch no. 120222), betamethasone (BOH, purity: 99.5%, batch no. 20101001), betamethasone 17-monopropionate (B17P, purity: 98.1%, batch no. 20100601) and betamethasone 21-monopropionate (B21P, purity: 99.3%, batch no. 20100602) were provided by Chongqing Huapont Pharm. Co., Ltd (Chongqing, China). The internal standard prednisolone (IS-1, purity: 99.6%, batch no. 100153-199603) and beclomethasone dipropionate (IS-2, purity: 99.9%, batch no. 100119-200603) were obtained from National Institute for Food and Drug Control (Beijing, China). Sodium cacodylate (purity: 99.5%, batch no.C1308011) was purchased from Aladdin Industrial Corporation (Shanghai, China). Potassium fluoride (purity: 99.2%, batch no. 20120331) was bought from Chengdu Kelong Chemical Co. Ltd (Chengdu, China). The structures of all the analytes and ISs were shown in Fig.1.The test drug (batch No.: 2012004) was supplied by the Chongqing Huapont Pharm. Co., Ltd, and the reference drug (batch No.: 2BBKA18A01) was purchased from Schering Plough pharmaceutical Co. Ltd (Shanghai, China).

Methanol (HPLC grade, batch no. I638607219) was purchased from Merck Company (Darmstadt, Germany). Deionized water was purified by a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). The centrifuge (Type: Biofuge prime R) was produced by Heraeus company (Osterode, Germany). Formic acid (HPLC grade, batch no. HX090818), ammonium formate (HPLC grade, batch no. SX68594) and ammonium acetate (HPLC grade, batch no. SC12R2504) were supplied by CNW technologies GmbH (Düsseldorf, Germany). Ether (analytical grade, batch no. 20080311) and hexane (analytical grade, batch no. T20090225) were provided by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Ammonia (analytical grade, batch no. 20090315) was purchased from Kaixin Chemical Reagent Co.,Ltd (Hunan, China). Blank human plasma was supplied by Changsha Blood Center (Changsha, China).

2.2. Liquid chromatographic conditions

The Waters Acquity UPLC system (Acquity, Waters, USA) consisted of a vacuum degasser, a binary pump, an autosampler and a column compartment, which was used for chromatographic separation of samples. An API4000 mass spectrometer produced by AB Sciex (Concord, Ontario, Canada) was coupled with the UPLC system for the analytes detection. The Analyte version 1.4.2 software (Concord, Ontario, Canada) was used for collecting and analyzing data. The Hypurity C_{18} column (150 mm × 2.1 mm, 5 μm) supplied by Thermo Fisher Scientific Inc. (Waltham, MA, USA) was installed for the separation of BOH, BDP, B21P and B17P with the maintenance temperature at 40 °C, while the Luna C_{18} (2) column (150 mm × 2.0 mm, 5μm) purchased from Phenomenex Inc. (Torrence, California, USA) was used for the isolation of BSP with the maintenance temperature at 40 °C. The C_{18} columns of Security Guard Cartridges (4.0 mm × 3.0 mm, 5 μm, Part no.: AJ0-4378) purchased from Phenomenex Inc. (Torrence, California, USA) was assembled as the guard columns. The mobile phase used for all the analytes and the run-time were shown in Table 1, the flow rate of which was set at 0.3 mL/min. The temperature of the auto-sampler was maintained at 15 °C. 10 μL of the samples was injected into the UPLC-MS/MS system.

2.3. Mass spectrometric conditions

The effluent from the UPLC system was directed into the ESI probe. The mass spectrometer was operated under the positive mode, the operating parameters of which were optimized with multiple reactions monitoring (MRM) detection to achieve maximal sensitivity by syringe infusion of working solutions containing each compound by peristaltic pump. Herein, the appropriate mass spectrometric signal responses of the precursor and product ions were calculated for identification and quantification of those compounds. In this study, the protonated precursor ions of BSP, BDP, BOH and B21P were chosen, while the precursor ion $[M+Na]^+$ was used for B17P due to its stronger response. The different and specific MS/MS parameters optimized for the developed method were presented in Table 2.

2.4. Standard and quality control sample preparation

All stock solutions were prepared with methanol at the concentrations as following, 2.997×10^{-3} mol·dm^{-3} for BOH, 2.344×10^{-3} mol·dm^{-3} for B17P, 2.705×10^{-3} mol·dm^{-3} for B21P, 2.232×10^{-3} mol·dm^{-3} for BDP, 2.109×10^{-3} mol·dm^{-3} for BSP, 2.871×10^{-3}

mol·dm^{-3} for beclomethasone dipropionate (IS-1) and 2.750 × 10^{-3} mol·dm^{-3} for prednisolone (IS-2), which were all stored at 4 °C until use.

All working solutions were generated by diluting the stock solutions of all compounds step by step with the binary mixtures of methanol and 10mM ammonium formate (1:1, v/v). The upper limits of all analytes concentrations in the plasma samples used for standard calibration curves were prepared by spiking the drug-free human plasma with appropriate final working solutions. And, the quality control (QC) working solutions were prepared with the same procedures (Table 3). In order to obtain the final concentration of calibration curves, 50 μL working solution of each compound was spiked with 450 μL human blank plasma to make the desired concentration. Because BSP is labile in human blank plasma, the resulting plasma solutions of BSP were mixed with human blank plasma, 2M sodium cacodylate and potassium fluoride (50%) (40:1:1, v/v/v) in advance (Table 3). The Beclomethasone dipropionate (IS-1) stock solution was further diluted with the binary mixtures of methanol and 10 mM ammonium formate (1:1, v/v) as well to obtain the working IS solution at concentration of 287.1 × 10^{-9} mol·dm^{-3} used for BOH and 28.71 × 10^{-9} mol·dm^{-3} used for B17P, B21P, and BDP. The prednisolone (IS) working solution was prepared at the concentration of 550.0 × 10^{-9} mol·dm^{-3} used for BSP.

2.5. Sample preparation

500 μL of the collected human plasma was added into a disposable Eppendorf tube, which was followed by an addition of 50 μL of IS working solution, and subsequently vortexed for 30s on a vortex mixer (IKA VIBR AX, Germany). Continuously, one step of liquid-liquid extraction (LLE) was employed to extract BDP, BOH, B17P, B21P and IS from the human plasma, while solid phase extraction (SPE) was adopted for BSP sample preparation. For LLE, 2 mL of Ether/n-hexane (4: 1, v/v) as extractant was added into each tube with vortex-mixing for 10 min. Then, the well-mixed samples were centrifuged at 4000 rpm for 10 min, and 1.5 mL of supernatant organic layer was transferred into another Eppendorf tube and dried by a slow stream of nitrogen in water bath at 40 °C. Following, the residue was re-dissolved with 150 μL of methanol/10 mM ammonium formate (1:1, v/v) for BDP, BOH, B21P and methanol/water (7:3, v/v) for B17P by vortexing approximate 3 min, which was re-centrifuged at 13000 rpm for another 5min before analysis. At last, 10 μL of supernatant was injected into UPLC system.

To the sample preparation of BSP, 500 μL of plasma sample mixed with 50 μL of IS working solution (550.0 × 10^{-9} mol·dm^{-3}) was loaded into an activated OASIS HLB cartridge. After eluted by 1 mL of water/formic acid (50:1, v/v) and 0.5 mL of water, the HLB cartridge was subsequently eluted by 1 mL of water/formic acid (50:1, v/v), 0.5 mL of water and 1 mL methanol/ammonia (20:1, v/v) under centrifuging at 1500 rpm for 1 min, respectively. 0.6 mL of methanol/ammonia eluent was transferred into another Eppendorf tube and dried by nitrogen under water bath at 40 °C. The residue was re-dissolved with100 μL of methanol/20 mM ammonium acetate (1:1, v/v). After vortexed for approximately 3 min and centrifuged at 13000 rpm for 5 min, 10 μL of supernatant was injected into the UPLC system.

2.6. Method validation

The analytical method was validated according to FDA guidelines,[21] which were pertinent to selectivity, sensitivity, linearity, precision, matrix effect, extraction recovery and stability reported on our previous study.[22] The peak area ratios generated from the analytes of QC samples to IS were interpolated into the calibration curves plotted on the same day to directly calculate the concentrations of all the analytes. The QC samples were used in the procedure of evaluating the precision, accuracy and stability in three consecutive days.

2.6.1. Selectivity—The selectivity was evaluated by comparing the chromatographic profiles of six blank plasma samples from six different healthy subjects with those of corresponding plasma samples spiked with the analytes and IS, and those of plasma samples from drug subjects who had been injected with the compound medicine BSP and BDP.

2.6.2. Linearity and lower limit of quantification—The calibration standards of all the analytes at seven concentrations were determined in three independent runs to validate the linearity. To avoid the interferences in human plasma, blank plasma samples were also analyzed. The peak area ratios for these compounds and IS were calculated to construct the calibration curves by plotting the peak area ratios of these compounds to IS versus the concentrations (x) of these analytes with weighted least squares linear regression (1/x). The lower limit of quantification (LLOQ) was defined as the lowest concentration, which usually was the lowest concentration of the calibration curve with an acceptable precision (relative standard deviation, RSD) and accuracy (relative error, RE). The values of LLOQs were at least the signal-to-noise ratios (S/N) of about 10. Five replicates were analyzed with an acceptable precision (RSD, within 20%) and accuracy (RE, 80–120%) in this study.

2.6.3. Precision and accuracy—In order to evaluate intra-day precision and accuracy, six parallel and repeated QC samples on high, middle, low concentration levels of these analytes were determined to obtain the calculated concentration using a calibration curve that was prepared and analyzed on the same batch and day. The inter-day precision and accuracy was demonstrated by evaluating QC samples in three consecutive days. Six parallel and repeated QC samples on high, middle, low concentration levels of these analytes were determined on each day. The accuracy would be calculated by using mean concentration results of QC samples at each level versus the nominal concentration, which must be within 85%-115% (80%-120% for LLOQ).

2.6.4. Extraction recovery and matrix effect—The extraction recoveries of these analytes were analyzed and operated for six times by using the QC samples at different concentration levels. Recoveries were calculated by comparing the analyte/IS peak area ratio (A1) determined from extracted plasma samples with those (A2) from these QC sample solutions at the same concentrations. The matrix effects were calculated by comparing the peak areas of samples spiked post-extraction (A2) with those of the working solutions containing equivalent amounts of these compounds (A3). The ratio values (A2/A3) ×100% were regarded to be the matrix effects. The extraction recovery and matrix effect of ISs were also calculated and operated with the same procedure.

2.6.5. Stability—The stability of all the analytes in human plasma was performed by analyzing triple human plasma samples at different concentrations of QC samples for the sample storage and processing procedures. The freeze-thaw stability was conducted on QC plasma samples at different concentration levels, which were frozen at −40 °C for 24 h and thawed at room temperature (25 °C). The freeze-thaw cycles were operated twice, and then the samples were determined accordingly. The short-term stability was evaluated after the exposure of the selected QC samples for 6h at room temperature during the period that exceeded the routine time of sample preparation and the ready-to-inject samples. The long-term stability was assayed by processing QC samples at different concentration levels stored at low temperature (−40 °C) for a period of 40 days. The post-preparative stability was performed by analyzing QC samples stored under auto-sampler condition (4 °C) for 12 h. The stock solution stability at room temperature was evaluated by determination of concentration of all analytes and IS stock solutions that were deposited at room temperature for 6 hours.

2.7. Application to pharmacokinetic study

According to the guidelines, there was no gender factors considered for the pharmacokinetic study, twenty-four healthy male volunteers (Age: 22 ± 4.5 years; Height: 172 ± 15.5 cm; Weight: 75.5 ± 15.5 kg.) were enrolled in accordance with the clinical protocols in the study. All the healthy subjects signed the informed consent form prior to assessing medical history, physical examination, electrocardiogram and standard laboratory test results of blood cell, urinalysis and biochemical profile. There was one drop-out in this clinical study.

The method was applied to evaluate the pharmacokinetics of the compound medicine in the healthy subjects. Twenty-four healthy male volunteers received a single intramuscular injection dose of Compound Betamethasone Injection (BSP (2 mg) and BDP (5 mg)) in an open label, two-periods, single-dose, two-way randomized crossover design with a washout period of forty-three days. Forearm venous blood samples were collected from each subject at pre-dose(0 h), 5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 8 h, 12 h, 16 h, 24 h, 48 h, 72 h, 120 h, 168 h, 264 h and 360 h post-dose. 6 mL blood samples were transferred to heparinized tubes which contained 90 μL 2M sodium cacodylate for 0-4 h. And 5 mL blood samples were transferred to heparinized tubes which contained 75 μL 2M sodium cacodylate for 360 h. All of blood samples were mixed immediately and placed on the ice. After centrifuged at 4000 rpm for 10 min (4 °C), about 2-3 mL plasma fractions were separated into Eppendorf tubes which contained 50% (w/v) sodium fluoride to stabilize BSP. Then, the collected plasma samples were stored at −40 °C prior to analysis. The pharmacokinetic parameters were calculated with non-compartment model, using the “Drug and statistics for windows” software (DAS), version 3.2.2. All of the pharmacokinetic parameters were calculated by using 90% geometric confidence interval of the ratios (*T/R*) from the ANOVA with least-squares means of the ln-transformed parameter.

The established method was applied to analyze the plasma concentrations of the analytes, and then their pharmacokinetic parameters were calculated respectively. The maximum plasma concentration (C_{max}) and their time of occurrence (T_{max}) were directly recorded on the concentration-time curve. The elimination rate constant (k_e) was calculated by the log-

linear regression of concentrations observed during the terminal phase of elimination, and the elimination half-life ($T_{1/2}$) was then calculated as $0.693/k_e$. The area under the plasma concentration–time curve (AUC_{0-t}) from the start of the infusion to the time of the last determined concentration was calculated using the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$.

3. Results and discussion

3.1. Method development and optimization

3.1.1. Sample preparation—The sample preparation was critical and imperative for any bio-analysis method. A good sample preparation procedure could benefit valid chromatographic separation, reliable signal response, higher sensitivity and selectivity. Solid phase extraction (SPE), liquid–liquid extraction (LLE) and protein precipitation (PP) are conventional and typical sample preparation approaches to avoid interferences from plasma samples. However, PP was limited by low-selectivity with the reason that the supernatant contained un-precipitated plasma components,[23] which maybe influence the mass spectrometry signal response. Moreover, PP was harmful to lengthen the lifetime of column for a large number of samples. Therefore, LLE was finally processed for the plasma sample preparation of BDP, BOH, B17P and B21P due to the good lipophilic property of these analytes and the drawbacks of PP.

Compared with previously reported extractant such as methyl tert-butyl ether (MTBE),[24] diethyl ether-hexamethylene,[4] acetone–chloroform (5:1, v/v),[9] ether-cyclohexane(4:1, v/v)[6] and methylene chloride,[8] ether/n-hexane (4:1, v/v) was chosen as an ideal solvent to extract the BDP, BOH, B17P and B21P with high extraction efficiency and minimal matrix effects. But a non-negligible drawback of LLE was that hydrophilic compounds could not be extracted by water-immiscible solvent. Thus, SPE was more appropriate as the pre-treatment approach of the plasma samples containing BSP, which was a kind of highly ionized sodium phosphate. In order to prevent that BSP was hydrolyzed *in vitro* by phosphatase and plasma esterase during the blood collection and plasma reparation or storage, 200 μL 2M sodium cacodylate and 200 μL potassium fluoride (50%) were added into the blank plasma samples as stabilizer.[25]

3.1.2. Optimization of the chromatographic condition—Comparing with the reported columns including C_{18} RP column[26, 27] and C_8 column,[28] a Hypurity C_{18} column (150 mm × 2.1 mm, 5 μm) was used for the separation of BDP, BOH, B17P and B21P, while a Luna C_{18} (2) column (150 mm×2.0 mm, 5μm) that could tolerate a broad pH value range (1.5-10) was installed for the analysis of BSP. The mobile phase was a major factor to affect the retention time, resolution and peak shape of analytes and IS. It was reported that acetonitrile preferred to produce abundant solvent adduct ions $[M+H+CH_3CN]^+$ which could be observed on a triple-quadrupole mass spectrometry,[24] so methanol was optimized as the organic solvent with appropriate mass response. The pH value of mobile phase was another critical factor that not only influenced the retention behaviors on column but also affected the ionization efficiency of the interested compounds. Ammonium formate and

ammonium acetate were used as the pH modifier to keep the acidity of mobile phase, which would easily keep the analytes positively charged.

The chromatographic conditions were optimized according to both chromatographic resolution and shorter retention time. Finally, a mobile phase consisting of methanol and water (70:30, v/v) was used for isocratic elution and quantification of BDP and B17P in human plasma as well as methanol and water (63:37, v/v) for BOH. Besides, a mobile phase consisting of methanol and water with 20 mM ammonium acetate (70:30, v/v) was optimized for BSP as well as methanol and water with 10 mM ammonium acetate (70:30, v/v) for B21P. Other chromatographic conditions were presented in Table 1.

3.1.3. Optimization of the LC-MS/MS conditions—Different ionization sources such as electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI) and atmospheric-pressure photoionization (APPI) possessed different ionization processing for different targeting compounds. ESI, APCI and APPI were usually used for polar, moderately polar and non-polar compounds, respectively.[29] According to the structures of BSP, BDP, BOH, B17P and B21P (Fig. 1), ESI was selected for its higher ionization efficiency. The positive mode was used for the mass detection. In the full-scan spectra of precursor and product ions, the multiple reaction monitoring (MRM) scan mode was used for the quantification of the analytes with the transitions at *m/z* 473.4→435.4 for BSP, *m/z* 505.5→411.3 for BDP, *m/z* 393.2→373.3 for BOH, *m/z* 471.5→397.2 for B17P, *m/z* 449.4→411.2 for B21P, *m/z* 521.6→503.4 for IS-1 and *m/z* 361.5→343.2 for IS-2 (Fig. 2).

In the ionized molecules, different neutral small molecules were the fragments from the analytes and IS, respectively, which provided different product ion fragments monitored for quantitative analysis. The chemical fragmentation schemes were described in Fig. 2. The collision energy was optimized to satisfy the sensitivity in the MRM mode, and the optimal values were provided at different voltage in Table 2.

3.2. Method validation

3.2.1. Selectivity—The developed method showed a good selectivity by comparing the typical MRM chromatograms of all the compounds. Fig.3 (A-H) provided the typical MRM chromatograms of the blank plasma, the blank plasmas spiked with different analytes at their LLOQ concentrations. The typical MRM chromatograms of plasma samples from a healthy subject at different time after injection of BSP and BDP were shown in Fig.4 (A-D). As indicated in the chromatograms, there was no endogenous substance to interfere the analysis of all the compounds, which proved the chromatographic conditions suitable and specific to the target analytes. The retention time in the chromatograms was approximately 4.88, 5.04, 2.86, 2.99 and 3.01 min for BSP, BDP, BOH, B17P and B21P in human plasma, respectively.

3.2.2. LLOQ and linearity—The calibration curves were constructed daily, which showed good linearity in the test range of all these analytes and the mean regression equations were calculated from five different calibration curves for each analyte in this study (Table 4). The peak-area ratio of the analyte to IS versus the nominal concentration exhibited a good linear relationship within the calibration range, respectively. The ideal IS for LC-MS quantitation

studies are deuterated versions of their target compounds, because only limited kinetic isotope effects these compounds represent the most accurate mimic of the target compound, its properties, retention, and response during ionization and detection inside a mass spectrometer. Generally, there are two types of ISs, which are structural analog and stable isotope labeled ISs. Whenever possible, stable isotope labeled ISs should be used because they are most effective. Therefore, the deuterated compounds will be the best internal standard used for the quantification of the analytes. In this study, the desirable performances of stable isotope labeled ISs are not available for us and too expensive to synthesize so that the structural analogs were used as ISs. The selected internal standard that has the similar chemical structure with the analytes and would not affect the analysis of the target compounds was employed for the quantitative determination.[30, 31] The precision (RSD%) and accuracy of each calibration curve was around 1.32-14.7% and 92.4-109.4% for BSP, 0.25-20.4% and 88.0-106.1% for BDP, 0.46-12.0% and 97.7-102.8% for BOH, 0.04-13.8% and 92.9-111.4% for B17P as well as 0.96-7.49% and 87.1-109.4% for B21P, respectively. The correlation coefficients of all the calibration curves were all >0.99, which satisfied the linear regression requirement of biological samples. Moreover, the appropriate linearity showed higher responses between the concentrations and peak areas of analytes and ISs within the test ranges. The test ranges of all the objectives in the calibration curves were provided in Table 4.

The precision and accuracy of LLOQs of five investigated compounds were 14.3% and 94.7% for BSP, 11.6% and 88.1% for BDP, 9.10% and 111.3% for BOH, 18.4% and 97.7% for B17P, 17.0% and 81.2% for B21P, which were all within ±20% and 80-120%, respectively. As the LLOQs shown in Table 4, the method was sensitive for quantification of trace BSP, BDP and their metabolites BOH, B17P and B21P in human plasma.

3.2.3. Intra- and inter-day precision and accuracy—The intra- and inter-day precision (RSDs) and accuracy (RE) for all those analytes were presented in Table 5. In this validation, the inter- and intra-day precision variations were within 4.41-17.1% and 2.86-15.0%, while the inter- and intra-day accuracy variations were within 88.2-108.7% and 86.1-114.0%, respectively. The overall data was effective with the requirements that precision must be ≤15% (≤20% for around LLOQ) and accuracy will be based on the mean concentration, which must be within ±15% (±20% for around LLOQ) of the nominal concentration. The results suggested that the developed method was accurate for quantifying investigated compounds.

3.2.4. Extraction recovery and matrix effect—In the process of sample preparation, the LLE and SPE had been chosen to extract and clean up the biological samples. Because the analytical signals may be suppressed or enhanced for the presence of co-eluting substances of matrix to alert the ionization of droplets,[32] which affected the sensitivity, accuracy and precision of the method, the extraction recovery and matrix effect were significant and essential to validate the developed methods. The extraction recovery and matrix effects were exhibited in the Table 6. All the extraction recoveries values of all those compounds were within 64.5–113.8 %. All results showed good efficiency of extraction and no endogenous co-elutes interfering the ionization of all those investigated compounds.

3.2.5. Stability—Table 7 summarized the freeze–thaw stability, short-term stability, long-term stability and auto-sampler stability data of all those analytes. The results indicated that all samples were not labile without any obvious degradation during the routine analysis of biological samples. Table 7 presented the stock solution stability data of all those analytes and ISs. The results indicated that all the working samples were reliable under the routine operating conditions.

3.3. Clinical applications

The validated method was successfully applied to the pharmacokinetic studies of the compound medicine BDP and BSP. A randomized, two periods and single-dose protocol was adopted. In the clinical trial study, twenty-four healthy male subjects were recruited, but one subject dropped out during the studies due to suffering from fainting at the administration of injection. The representative chromatograms of the plasma samples were plotted in Fig. 4, which was collected at different time from a subject after injecting administration with BSP and BDP sustain-release drug.

As a sustained release parent drug, the plasma concentration of BSP in human body was so low that could not be detected in the healthy subjects' plasma samples. Hereby, the pharmacokinetic parameters of BSP were unable to be calculated for estimating the bioequivalence of the two pharmaceutical preparations. Instead, the pharmacokinetic data of BOH and B17P, the main active metabolite of BSP, could be calculated to provide sufficient information to compare the bioequivalence of the test and reference formulations. In this study, as an inactive metabolite of BDP, the plasma concentration of B21P was slightly above the LLOQ (0.226×10^{-9} mol·dm^{-3}) of B21P. Moreover, the trace plasma concentration of B21P could only be detected at a few blood points. Therefore, B21P was also not fixed to evaluate the bioequivalence of these two injections.

The pharmacokinetic data of BDP, BOH and B17P were applied to evaluate the bioequivalence of two intramuscular compound betamethasone injections. Additionally, it has been reported that BDP had a shorter elimination half-life time (0.43 h) than BOH and B17P.[5] Moreover, the plasma concentration of BDP could not be determined in 4 h after the administration of this compound medicine. Thus, the plasma samples collected from 0 h to 4 h after the healthy volunteers injected this drug were adopted to calculate the pharmacokinetic parameters of BDP. Due to the reported elimination half-life time of BOH (9.6 h) and B17P (80.8 h),[4] the collected plasma samples from 0 h to 120 h and 0 h to 360 h were analyzed to calculate the pharmacokinetic parameters of BOH and B17P, respectively.

The pharmacokinetic parameters of BDP, BOH and B17P with intramuscular injection of the test and reference formulations were displayed in Table 8. It could be noted that a few extreme variance in human pharmacokinetic data reached beyond +/− 50% for some measurements. Actually, the pharmacokinetic data were calculated for the selected target compounds from their measured human plasma concentrations. The value variance beyond +/− 50% for some measurements appeared due to possibly some extensive metabolizers and some poor metabolizers of the compound medicine, betamethasone in the healthy volunteers. Additionally, the different gene types of the healthy subjects, which is responsible for the metabolism of this drug, might result in the significant difference of the

pharmacokinetic data. Therefore, we will further investigate the pharmacogenomics and pharmacogenetics of this drug in Chinese people. The mean plasma concentration-time profiles of BDP, BOH and B17P were depicted in Fig. 5. Using logarithmic conversion and variance analysis of $AUC_{0\rightarrow4}$ (BDP), $AUC_{0\rightarrow120}$ (BOH), $AUC_{0\rightarrow360}$ (B17P), $AUC_{0\rightarrow\infty}$ and C_{max}, the results showed that the pharmacokinetic parameters showed no significant differences ($p > 0.05$) between the different compound medicine BSP and BDP formulations. Two one-sided *t*-test of $AUC_{0\rightarrow4}$ (BDP), $AUC_{0\rightarrow120}$ (BOH), $AUC_{0\rightarrow360}$ (B17P), $AUC_{0\rightarrow\infty}$ and C_{max} indicated that T_{high} and T_{low} were greater than unilateral $T_{0.05}$. The $AUC_{0\rightarrow4}$ (BDP), $AUC_{0\rightarrow120}$ (BOH), $AUC_{0\rightarrow360}$ (B17P), $AUC_{0\rightarrow\infty}$ and C_{max} of test formulation did not exceed the range of parameters with defined statistical 90% confidence intervals within the 80–125%. The point estimate values of $AUC_{0\rightarrow4}$ (BDP), $AUC_{0\rightarrow120}$ (BOH), $AUC_{0\rightarrow360}$ (B17P), $AUC_{0\rightarrow\infty}$ and C_{max} were calculated. The number of subjects with an extrapolated part of $AUC_{infinity}$ of 20% was 0. The confidence intervals for *t*-test of log $AUC_{0\rightarrow4}$ (BDP), log $AUC_{0\rightarrow120}$ (BOH), log $AUC_{0\rightarrow360}$ (B17P), log $AUC_{0\rightarrow\infty}$ and log C_{max} were evidenced to be equivalent with statistical 90% confidence intervals within the 80–125% for test/reference ratios of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} according to the US Food and Drug Administration.

3.4. Comparison with reported methods

The major advantage of above-mentioned method had good sensitivity of all those analytes (LLOQs: 2.525×10^{-9} mol·dm^{-3} for BSP, 0.125×10^{-9} mol·dm^{-3} for BDP, 0.278×10^{-9} mol·dm^{-3} for BOH, 0.098×10^{-9} mol·dm^{-3} for B17P and 0.226×10^{-9} mol·dm^{-3} for B21P), which were lower than the methods previously reported on their LLOQs: 3.875×10^{-9} mol·dm^{-3} for BSP,[13] 0.6-1.6 $\times 10^{-9}$ mol·dm^{-3} for BDP,[8] 0.112×10^{-9} mol·dm^{-3} for B17P and 0.816×10^{-9} mol·dm^{-3} for BOH,[4] especially without any LLOQ for B21P reported. In the past decades, most of the validated HPLC-MS/MS were used for the quantification of BDP, BOH and B17P in both drug formulations and biological samples,[4-6, 8, 9, 28] but few methods were developed for the determination of BSP and B21P in human plasma. Although BSP was not detected in the collected human plasmas from the healthy subjects due to its rapid degradation in human body, the validated method might be useful for the determination of the sustained-release preparations containing BSP developed in the future. Besides, GC-MS was also used for the analysis of BDP, BOH and B17P, but the sample preparation procedure was labor-consuming and adding steps.[29, 33, 34] In this study, the UPLC system shortened the retention time of all these analytes, which were all less than 6 min. The shorter run time was prerequisite condition to allow high sample throughput for therapeutic drug monitoring such as the compound betamethasone intramuscular injection. Thus, the developed methods were useful and meaningful for the determination of BSP, BDP, BOH, B17P and B21P in human plasma.

4. Conclusions

A sensitive and reliable UPLC-MS/MS had been validated to quantify BSP, BDP and their metabolites BOH, B17P and B21P in human plasma. The developed method was compliantly validated according to FDA guidelines. The validated method showed a good sensitivity for the quantification of the mentioned compounds in different individuals'

human plasma samples. This method has successfully been applied to analyzing samples in bioequivalence studies of different compound medicine BSP and BDP formulations, which were demonstrated to be equal.

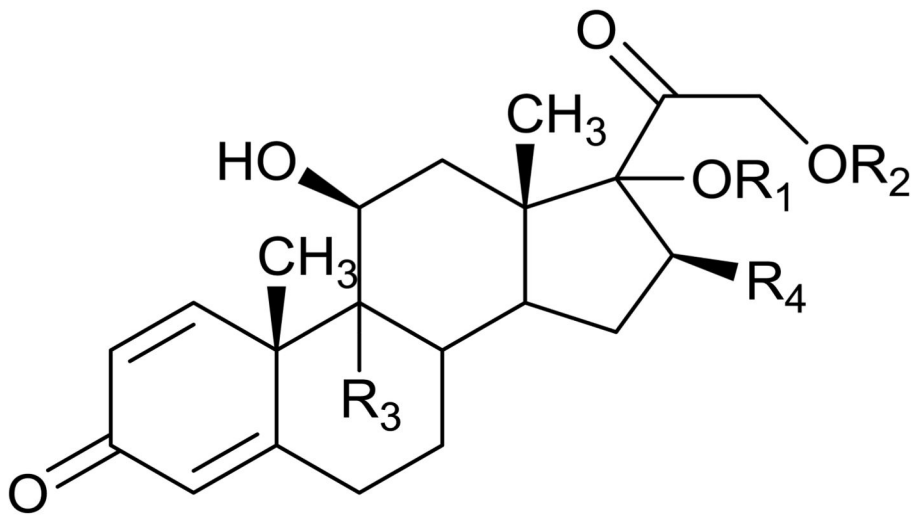
Acknowledgements

This work was supported by the National Natural Scientific Foundation of China (no. 81001471 to Z.-R. Tan, 31400306 to W.-H. Huang), Hunan Provincial Natural Science Foundation of China (No.2015JJ3156 to W.-H. Huang), China Postdoctoral Science Foundation (No. 2015M570692) and the fundamental research funds for the central universities of Central South University (no. 1681-7608040003 to W.-H. Huang).

References

1. Zhang YK, Yang H, Zhang JY, Song LJ, Fan YC. Int J Clin Pract. 2014; 68:633. [PubMed: 24472084]
2. Dauden E, Bewley A, Lambert J, Girolomoni G, Cambazard F, Reich K. J Eur Acad Dermatol Venereol. 2014; 28:22. [PubMed: 24684740]
3. David Czock FK, Rasche FM, Aussler UH. Clin Pharmacokinet. 2005; 44:61. [PubMed: 15634032]
4. He C, Fan H, Tan J, Zou J, Zhu Y, Yang K, Hu Q. Arzneimittel-Forschung. 2010; 61:417. [PubMed: 21899210]
5. Salem II, Najib NM. Clin Ther. 2012; 34:214. [PubMed: 22154197]
6. Zou JJ, Dai L, Ding L, Xiao DW, Bin ZY, Fan HW, Liu L, Wang GJ. J Chromatogr B Analyt Technol Biomed Life Sci. 2008; 873:159.
7. Hirano K, Ichihashi T, Yamada H. J Pharm Sci. 1982; 71:495. [PubMed: 7097491]
8. Taylor RL. Clin Chem. 2004; 50:2345. [PubMed: 15486026]
9. Shamsipur M, Jouybari TA, Barati A, Mahmoudi M, Amin N, Pashabadi A. Anal Methods-Uk. 2014; 6:4542.
10. Petersen M, Nation R, McBride W, Ashley J, Moore R. Eur J Clin Pharmacol. 1983; 25:643. [PubMed: 6662164]
11. Liggins G, Howie R. Pediatrics. 1972; 50:515. [PubMed: 4561295]
12. Moss TJM, Doherty DA, Nitsos I, Harding R, Newnham JP. Am J Obstet Gynecol. 2003; 189:1751. [PubMed: 14710109]
13. Salem II, Alkhatib M, Najib N. J Pharm Biomed Anal. 2011; 56:983. [PubMed: 21855245]
14. Ahmed S, Atia NN. J Pharm Biomed Anal. 2013; 74:250. [PubMed: 23245258]
15. Helmy SA, El-Bedaiwy HM. Ther Drug Monit. 2014; 36:100. [PubMed: 24270045]
16. Samtani MN, Lohle M, Grant A, Nathanielsz PW, Jusko WJ. Drug Metab Dispos. 2005; 33:1124. [PubMed: 15860658]
17. Fialho SL, Siqueira RC, Jorge R, Silva-Cunha A. J Drug Deliv Sci Tec. 2007; 17:93.
18. Salem II, Najib NM. Clin Ther. 2012; 34:214. [PubMed: 22154197]
19. Gumustas M, Kurbanoglu S, Uslu B, Ozkan SA. Chromatographia. 2013; 76:1365.
20. Plumb R, Castro-Perez J, Granger J, Beattie I, Joncour K, Wright A. Rapid Commun Mass Spectrom. 2004; 18:2331. [PubMed: 15384155]
21. Industry, GF. Guidance for Industry, Bioanalytical Method Validation. May. 2001 http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory/Information/Guidances/UCM070107.pdf
22. Huang WH, Hu K, Shao L, Chen Y, Zhang W, Zhou HH, Tan ZR. Anal Methods-Uk. 2014; 6:8258.
23. Novakova L, Vlckova H. Anal Chim Acta. 2009; 656:8. [PubMed: 19932811]
24. Guan F, Uboh C, Soma L, Hess A, Luo Y, Tsang DS. J Mass Spectrom. 2003; 38:823. [PubMed: 12938103]
25. Petersen MC, Nation RL, Ashley JJ. J Chromatogr B Analyt Technol Biomed Life Sci. 1980; 183:131.

26. Matabosch X, Pozo OJ, Monfort N, Pérez-Mañá C, Farré M, Segura J, Ventura R. Drug Test Anal. 2015; 7:663. [PubMed: 25545348]
27. Wang L, Yang YY, Chung TS, Chen XQ. J Pharm Biomed Anal. 2002; 28:629. [PubMed: 12008142]
28. Qu TT, Zhang R, Wang BJ, Liu XY, Yuan GY, Guo RC. Yao xue xue bao. 2008; 43:402. [PubMed: 18664204]
29. Schiewek R, Lorenz M, Giese R, Brockmann K, Benter T, Gäb S, Schmitz OJ. Anal Bioanal Chem. 2008; 392:87. [PubMed: 18661124]
30. Taylor PJ. Clin Biochem. 2005; 38:328. [PubMed: 15766734]
31. Tan, A.; Awaiye, K. Use of internal standards in LC-MS bioanalysis. In: Li, WK.; Zhang, J.; Tse, FLS., editors. Handbook of LC-MS Bioanalysis: Best Practices, Experimental Protocols, and Regulations. New Jersey: 2013. p. 217-229.
32. Bronsema KJ, Bischoff R, van de Merbel NC. J Chromatogr B Analyt Technol Biomed Life Sci. 2012; 893-894:1–14.
33. Ados, S. Pereira; Oliveira, LS.; Mendes, GD.; Gabbai, JJ.; De Nucci, G. J Chromatogr B Analyt Technol Biomed Life Sci. 2005; 828:27.
34. Courtheyn D, Vercammen J, Logghe M, Seghers H, De Wasch K, De Brabander H. Analyst. 1998; 123:2409. [PubMed: 10435270]



Compound	Molecular Formula	R_1	R_2	R_3	R_4
BSP	$C_{22}H_{28}FNa_2O_8P$	H	PO_3Na_2	F	CH_3
BDP	$C_{28}H_{37}FO_7$	COC_2H_5	COC_2H_5	F	CH_3
BOH	$C_{22}H_{29}FO_5$	H	H	F	CH_3
B17P	$C_{25}H_{33}FO_6$	COC_2H_5	H	F	CH_3
B21P	$C_{25}H_{33}FO_6$	H	COC_2H_5	F	CH_3
IS-1	$C_{28}H_{37}ClO_7$	COC_2H_5	COC_2H_5	Cl	CH_3
IS-2	$C_{21}H_{28}O_5$	H	H	H	H

Fig.1. Chemical structures of all the analytes and internal standards (IS)

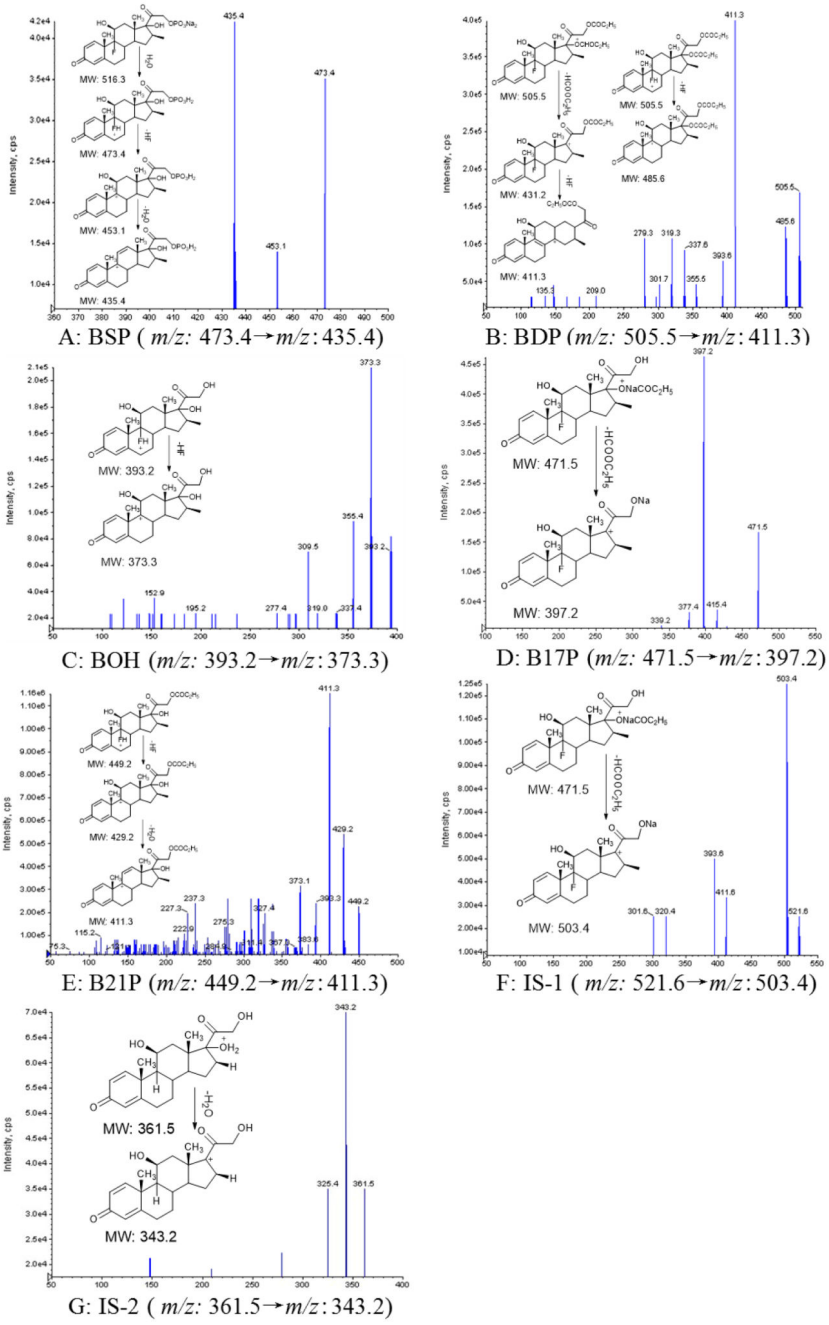


Fig.2. Positive ion mass scan spectra and fragmentation pathways of BSP (A), BDP (B), BOH (C), B17P (D), B21P (E), IS-1 (F) and IS-2 (G)

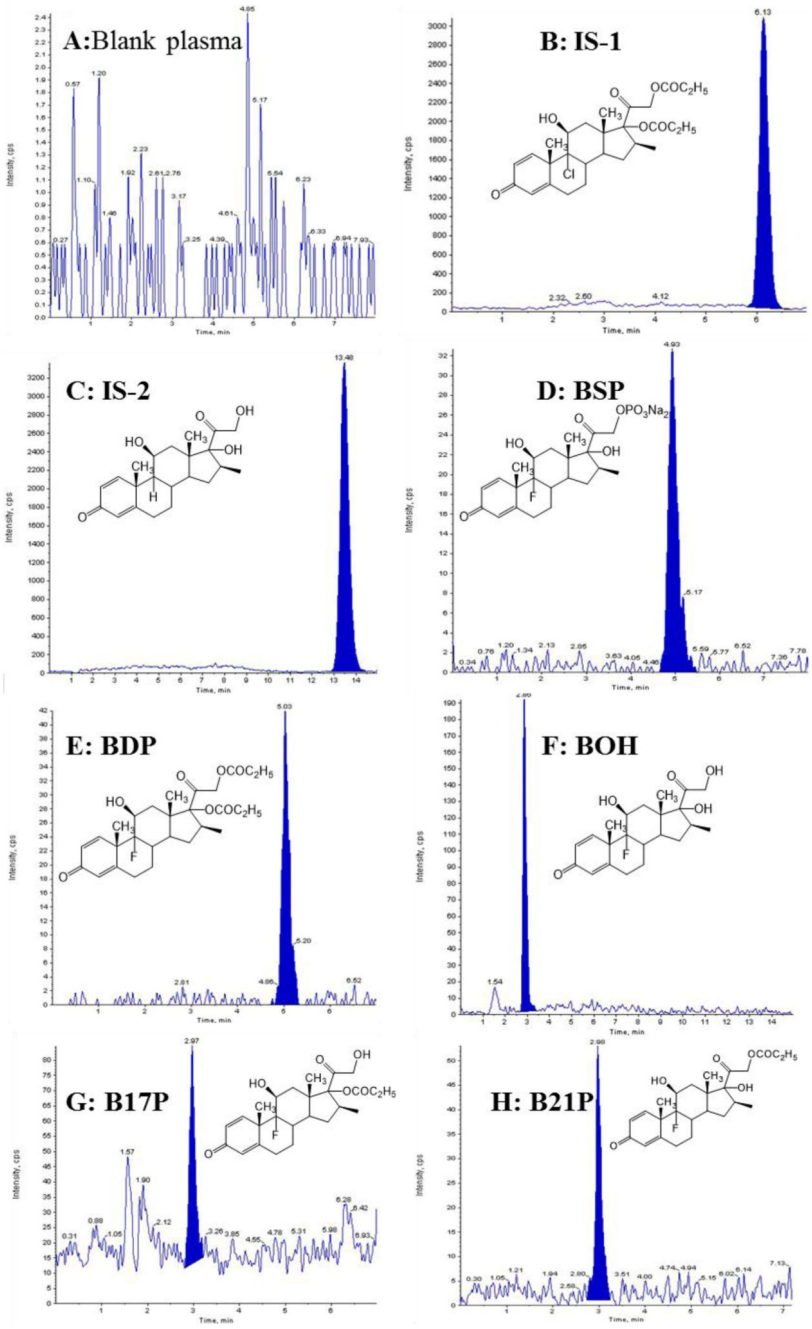


Fig.3.

The typical MRM chromatograms of a blank plasma (A), blank plasma spiked with IS-1 (28.71×10^{-9} mol·dm^{-3}**) (B) and IS-2 (**550.0×10^{-9} mol·dm^{-3}**) (C), LLOQ of BSP (D**, 2.525×10^{-9} mol·dm^{-3}**), BDP (E**, 0.125×10^{-9} mol·dm^{-3}**), BOH (F**, 0.278×10^{-9} mol·dm^{-3}**), B17P (G**, 0.098×10^{-9} mol·dm^{-3}**) and B21P (H**, 0.226×10^{-9} mol·dm^{-3}**).**

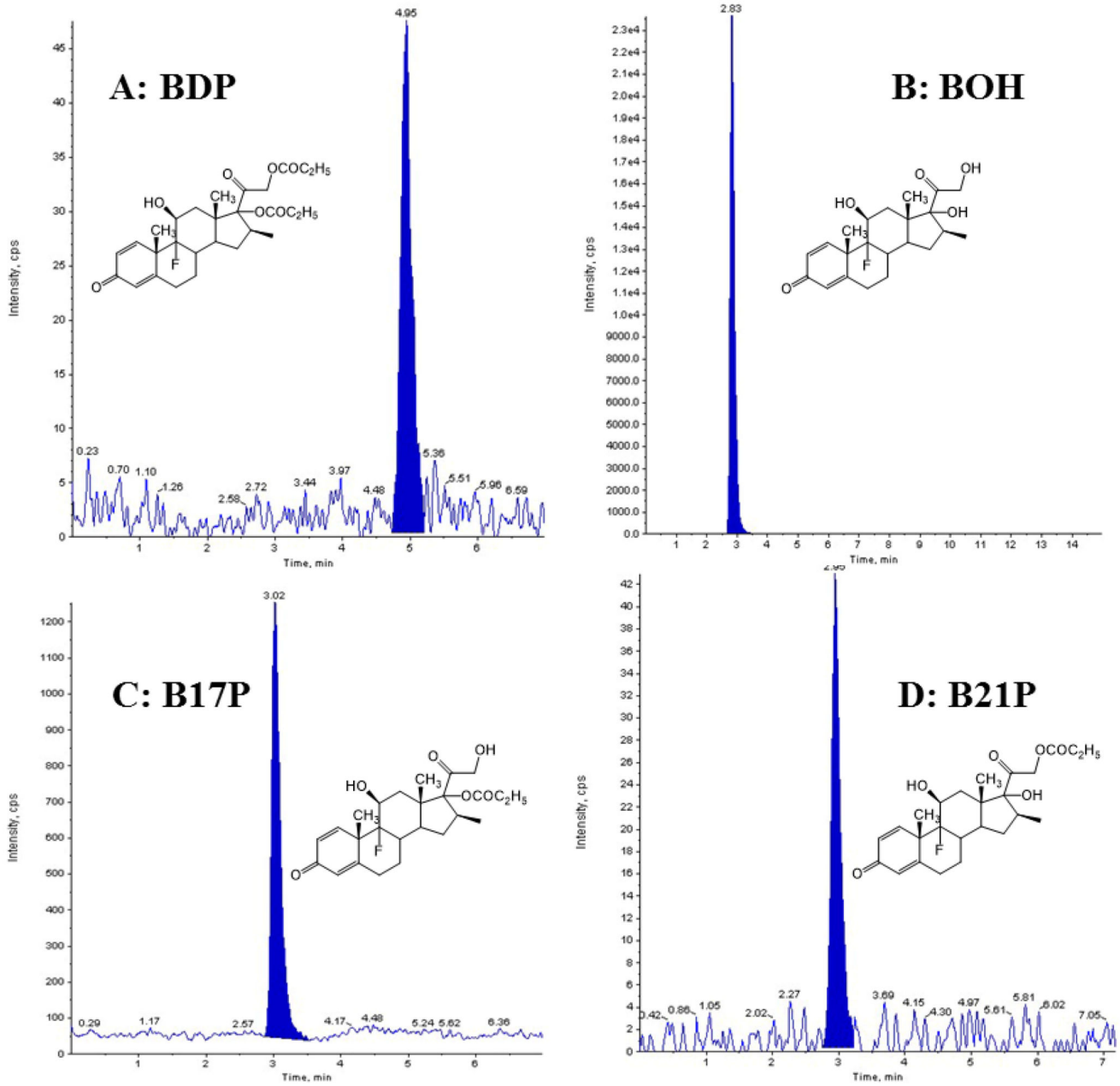


Fig.4. The MRM chromatograms of human plasma samples from a healthy subject after injected with the compound medicine BSP and BDP, which were collected in 4 h (BOH (B); B17P (C)) and 12 h (BDP (A); B21P (D)), respectively

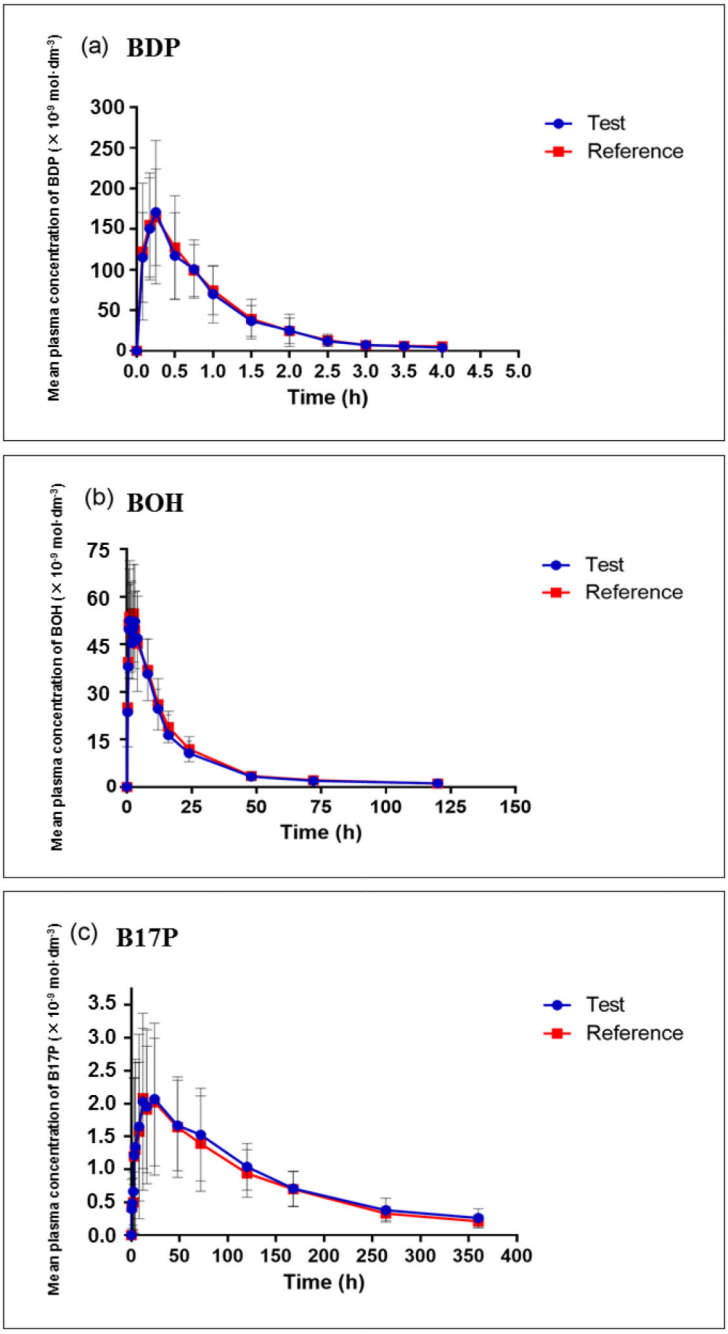


Fig.5. Mean plasma concentration-time profiles of BDP, BOH and B17P in human plasma after injecting with the compound medicine BSP and BDP of reference or test drug, each point and bar represented the mean ± SD (n = 23)

Table 1

Liquid chromatographic conditions for all the analytes

Analyte	Mobile phase			Run-time (min)	R.T.[a] (min)	Chromatographic Column
	A	B	A/B(v/v)			
BSP	CH_3OH	H_2O (20 mM CH_3COONH_4)	70:30	8	4.95	The Luna C_{18} (2) column (150 mm × 2.0 mm, 5 μm)
BDP	CH_3OH	H_2O	70:30	7	5.05	The Hypurity C_{18} column (150 mm × 2.1 mm, 5 μm)
BOH	CH_3OH	H_2O	63:37	15	2.82	The Hypurity C_{18} column (150 mm × 2.1 mm, 5 μm)
B17P	CH_3OH	H_2O	70:30	7	2.96	The Hypurity C_{18} column (150 mm × 2.1 mm, 5 μm)
B21P	CH_3OH	H_2O (10 mM $HCOONH_4$)	70:30	7	2.98	The Hypurity C_{18} column (150 mm × 2.1 mm, 5 μm)

[a] R.T. = Retention Time (mean values)

Table 2

The optimized mass spectrometric parameters of all the compounds[a]

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (V)	DT (ms)	CAG (psi)	CUR (psi)	GS1 (psi)	GS2 (psi)	SV (V)	TEM (°C)	DP (V)	EP (V)
BSP	473.4	435.4	17	300	6	10	35	35	4500	350	60	10
BDP	505.5	411.3	16	200	6	10	30	30	4500	350	105	10
BOH	393.2	373.3	15	300	6	10	35	35	4500	450	80	10
B17P	471.5	397.2	25	300	6	20	30	30	5000	550	45	10
B21P	449.2	411.3	15	300	6	20	30	30	5000	450	50	10
IS-1	521.6	503.4	14	200	6	10	35	30	4500	400	93	10
IS-2	361.5	343.2	16	300	6	10	35	35	4500	360	50	10

[a]CE: collision energy; DT: dwell time; CAG: collision gas; CUR: curtain gas; GS1: the nebulizing gas of nitrogen; GS2: turbo spray gas; SV: ion spray voltage; DP: declustering potential; EP: entrance potential.

Table 3

The solution concentrations of samples for standard calibration curves and quality controls (× 10^{-9} mol·dm^{-3})[a]

NO.	BSP		BDP		BOH		B17P		B21P	
	WS	SCCS	WS	SCCS	WS	SCCS	WS	SCCS	WS	SCCS
S8	4039	403.9	558.1	55.81	749.5	74.95	46.88	4.688	53.99	5.411
S7	2019	201.9	279.1	27.91	374.7	37.47	33.48	3.348	38.55	3.855
S6	1010	504.8	139.5	13.95	187.4	18.74	23.44	2.344	26.99	2.699
S5	404.1	40.41	46.51	4.651	93.68	9.368	11.72	1.172	19.28	1.928
S4	202.0	20.20	23.25	2.235	46.84	4.684	7.813	0.781	13.50	1.350
S3	101.0	10.10	7.751	0.751	23.42	2.342	3.906	0.391	8.998	0.900
S2	50.50	5.051	2.584	0.258	11.71	1.171	1.953	0.195	4.499	0.450
S1	25.25	2.525	1.292	0.129	5.855	0.586	0.978	0.098	2.249	0.226
HQCS	3231	323.1	446.4	44.64	599.5	59.95	37.50	3.750	43.18	4.318
MQCS	403.9	40.39	46.51	4.651	99.92	9.992	11.72	1.172	19.28	1.929
LQCS	50.48	5.048	2.480	0.248	5.551	0.556	1.953	0.195	4.499	0.450

[a]WS: working solution; SCCS: standard calibration curve solution; HQCS: high quality control solution; MQCS: middle quality control solution; LQCS: low quality control solution.

Table 4

The calibration curves, linear ranges and LLOQs of BSP, BDP and its metabolites BOH, B17P, B21P (× 10^{-9} mol·dm^{-3})

Analyte	Regression equation	r^2	Test range	LLOQ
BSP	$Y = (0.02519 \pm 0.00136)X + (0.01827 \pm 0.00738)$	0.9945	2.525-403.9	2.525
BDP	$Y = (1.93253 \pm 0.25396)X + (0.08710 \pm 0.07639)$	0.9982	0.125-55.81	0.125
BOH	$Y = (0.48979 \pm 0.05516)X + (0.00791 \pm 0.00765)$	0.9980	0.278-74.95	0.278
B17P	$Y = (3.98058 \pm 0.37633)X + (0.02678 \pm 0.04087)$	0.9934	0.098-4.688	0.098
B21P	$Y = (0.43080 \pm 0.03460)X + (0.00938 \pm 0.00558)$	0.9960	0.226-5.411	0.226

Table 5

Precision and accuracy for the quantification of BSP, BDP and their metabolites BOH, B17P and B21P in human plasma[a]

	Conc. Added ($\times 10^{-9}$ mol·dm^{-3})	Intra-day (n=6)			Inter-day (n=18)		
		Mean conc. measured (MCM, $\times 10^{-9}$ mol·dm^{-3}) ± SD	Accuracy (RE, %)	Precision (RSD, %)	Mean conc. measured (MCM, $\times 10^{-9}$ mol·dm^{-3}) ± SD	Accuracy (RE, %)	Precision (RSD, %)
BSP	5.048	4.578 ± 0.500	90.7	10.9	4.907 ± 0.674	97.2	13.7
	40.39	38.72 ± 5.793	95.9	14.9	41.88 ± 5.521	104	13.2
	323.1	320.0 ± 29.30	99.0	9.16	332.6 ± 28.12	103	8.45
BDP	0.248	0.260 ± 0.038	104	14.6	0.260 ± 0.044	104	17.2
	4.651	4.986 ± 0.143	107	2.86	4.736 ± 0.542	102	11.4
	44.64	42.46 ± 2.327	95.1	5.48	43.15 ± 3.746	96.7	8.68
BOH	0.556	0.584 ± 0.051	105	8.81	0.605 ± 0.071	109	12.0
	9.992	10.60 ± 0.645	106	6.09	10.77 ± 0.556	108	5.17
	59.95	54.44 ± 4.681	90.8	8.60	59.82 ± 5.888	99.8	9.84
B17P	0.195	0.223 ± 0.025	114	11.4	0.199 ± 0.031	102	16.3
	1.172	1.158 ± 0.112	98.9	9.61	1.152 ± 0.103	98.3	8.84
	3.750	3.230 ± 0.121	86.1	3.74	3.308 ± 0.183	88.2	5.54
B21P	0.450	0.468 ± 0.053	103.73	11.2	0.434 ± 0.053	96.7	12.4
	1.929	1.871 ± 0.154	97.06	8.23	1.775 ± 0.129	92.1	7.31
	4.318	4.082 ±0.143	94.52	3.47	3.976 ± 0.176	92.1	4.41

[a]Conc.: concentration; SD: standard deviation; MCM: mean concentration measured; CA: concentration added; RE% = (MCM/CA)×100; RSD%=SD/MCM×100.

Table 6

Extraction recovery and matrix effect of BSP, BDP, BOH, B17P and B21P using LLE

Analyte	Recovery (%) (n=6)				Matrix effect (%) (n=6)			
	Low conc.[a]	Middle conc.	High conc.	IS[b]	Low conc.	Middle conc.	High conc.	IS
BSP	86.2 ± 6.79	87.5 ± 3.87	83.4 ± 2.15	88.3 ± 2.55	113.9 ± 7.31	102.8 ± 1.92	104.1 ± 1.80	96.1 ± 1.68
BDP	96.1 ± 8.45	94.3 ± 7.51	93.8 ± 7.37	83.1 ± 11.7	102.4 ± 10.4	95.4 ± 3.08	90.3 ± 4.02	98.1 ± 10.3
BOH	73.8 ± 15.04	69.9 ± 5.13	64.5 ± 4.53	81.7 ± 9.93	93.4 ± 2.87	96.7 ± 3.87	93.5 ± 3.74	106.9 ± 4.27
B17P	95.4 ± 18.03	98.1 ± 6.82	89.6 ± 17.0	96.6 ± 14.7	92.3 ± 15.4	89.9 ± 9.88	85.2 ± 13.7	108.0 ± 18.0
B21P	98.9 ± 6.82	93.7 ± 9.53	91.7 ± 4.89	96.5 ± 9.81	110.4 ± 13.1	109.8 ± 8.01	110.9 ± 7.98	90.7 ± 10.9

[a] Conc.: concentration.

[b] IS: international standard (IS-1 and IS-2).

Table 7

Stability results of all the analytes in human plasma at selected QC levels (n = 5, × 10^{-9} mol·dm^{-3})[a]

Analyte	CA	Freeze–thaw (four cycles, −40 °C)		Short-term (6 h, r.t.)		Long-term (40 days, −40 °C)		The autosampler (r.t.)			
		MCM ± SD	Dev. (%)	MCM ± SD	Dev. (%)	MCM ± SD	Dev. (%)	MCM ± SD (24h)	Dev. (24h, %)	MCM ± SD (48h)	Dev. (48h, %)
	5.048	4.905 ± 0.357	−2.84	4.988 ± 0.250	−1.19	4.994 ± 0.560	−1.07	5.252 ± 0.696	4.03	5.585 ± 0.616	10.63
BSP	40.39	39.30 ± 5.785	−2.67	39.36 ± 5.767	−2.55	39.94 ± 5.576	−1.06	40.83 ± 5.209	1.08	44.38 ± 5.581	9.88
	323.1	315.1 ± 4.665	−2.50	310.9 ± 14.43	−3.78	320.0 ± 10.35	−1.02	313.6 ± 35.39	−2.94	359.2 ± 22.46	11.19
	0.248	0.276 ± 0.042	11.2	0.258 ± 0.048	4.0	0.240 ± 0.012	−3.2	0.220 ± 0.016	−11.2	0.222 ± 0.020	−10.4
BDP	4.651	4.811 ± 0.135	3.45	4.746 ± 0.155	2.04	4.734 ± 0.141	1.80	4.304 ± 0.123	−7.45	4.387 ± 0.103	−5.68
	44.64	44.94 ± 1.177	0.68	44.42 ± 0.893	−0.51	46.43 ± 1.399	4.01	41.59 ± 1.016	−6.84	43.33 ± 1.131	−2.95
	0.556	0.577 ± 0.038	3.67	0.610 ± 0.048	9.63	0.597 ± 0.064	7.34	0.584 ± 0.056	5.04	0.569 ± 0.087	2.29
BOH	9.991	10.48 ± 0.645	4.88	10.87 ± 0.633	8.82	10.57 ± 0.577	5.78	10.20 ± 0.505	−2.05	10.38 ± 0.513	3.89
	59.95	63.29 ± 2.673	5.54	64.74 ± 5.074	8.02	52.55 ± 2.816	−12.3	53.47 ± 2.906	−10.82	63.95 ± 3.102	6.68
	0.196	0.221 ± 0.335	12.5	0.214 ± 0.020	9.09	0.205 ± 0.027	4.54	0.208 ± 0.022	5.68	0.185 ± 0.025	−5.68
B17P	1.172	1.250 ± 0.112	6.62	1.196 ± 0.112	2.08	1.241 ± 0.129	5.91	1.266 ± 0.094	7.80	1.114 ± 0.129	−5.02
	3.750	3.832 ± 0.330	2.2	3.435 ± 0.353	−8.39	4.185 ± 0.616	10.5	3.295 ± 0.304	−12.14	3.574 ± 0.531	−4.7
	0.450	0.463 ± 0.024	2.97	0.463 ± 0.062	2.97	0.488 ± 0.067	8.42	0.486 ± 0.020	7.92	0.448 ± 0.038	−0.5
B21P	1.929	1.949 ± 0.147	1.08	1.873 ± 0.094	−2.87	1.891 ± 0.143	−1.98	1.993 ± 0.129	2.05	2.007 ± 0.156	−4.06
	4.318	4.211 ± 0.301	−2.48	3.944 ± 0.303	−8.64	3.924 ± 0.078	−9.13	3.960 ± 0.160	−8.3	3.924 ± 0.171	−9.13

[a]CA: concentration added; MCM: mean concentration measured; SD: standard deviation; Dev.: deviation = (MCM/CA×100) − 100.; r.t.: room temperature.

Table 8

The main pharmacokinetic parameters of BSP, BOH and B17P in Chinese male healthy subjects (mean ± SD, n = 23)

Parameter	BDP		BOH		B17P	
	Test	Reference	Test	Reference	Test	Reference
C_{max} (× 10^{-9} mol·dm^{-3})	189.7 ± 71.67	192.9 ± 76.69	52.76 ± 11.03	54.82 ± 13.23	2.250 ± 1.034	2.145 ± 1.018
T_{max} (h)	0.266 ± 0.157	0.265 ± 0.156	1.891 ± 0.956	2.033 ± 0.951	19.83 ± 12.27	17.74 ± 13.04
$T_{½}$ (h)	0.774 ± 0.385	0.660 ± 0.282	31.82 ± 16.99	31.85 ± 17.54	101.3 ± 39.43	106.6 ± 35.93
AUC_{0-t} (× 10^{-9} mol·dm^{-3}·h^{-1})	171.8 ± 58.57	176.7 ± 51.47	820.2 ± 249.4	867.1 ± 186.3	259.2 ± 89.1	243.5 ± 92.50
$AUC_{0-\infty}$ (× 10^{-9} mol·dm^{-3}·h^{-1})	176.2 ± 58.21	180.5 ± 51.15	856.6 ± 269.4	915.3 ± 206.5	305.4 ± 108.1	281.7 ± 91.50
F(%)	101.70% ± 36.10%		93.7% ± 16.3%		113.5% ± 40.6%	

$T_{½}$: half-life of elimination; T_{max}: time of maximum plasma concentration; C_{max}: maximum plasma concentration; AUC: area under the plasma concentration vs time curve; F (%): relative bioavailability between test patch and reference patch; A_{mean} ± SD: arithmetic mean ± standard deviation. The median value of T_{max} was provided in the geometric mean.