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Development and validation of a LC–MS/MS method for the quantification of the regioisomers of dihydroxybutylether in human plasma

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ABSTRACT

Dihydroxybutylether (DHBE), a strong choleretic drug, is a mixture of three regioisomers: 4-(3-hydroxybutoxy)-2-butanol (I), 3-(4-hydroxy-2-butoxy)-1-butanol (II) and 3-(3-hydroxylbutoxy)-1-butanol (III). A liquid chromatography–tandem mass spectrometry method was developed and validated for the quantification of dihydroxybutylether (DHBE) regioisomers in human plasma. After plasma samples were deproteinized with 10% perchloric acid, the post-treatment samples were analyzed on a Capcell Pak C_{18} MGII column interfaced with a triple quadrupole tandem mass spectrometer in positive electrospray ionization mode. Methanol and water was used as the mobile phase with a gradient elution at a flow rate of 1 mL/min. Acetaminophen was used as an internal standard (IS). Multiple selected reaction monitoring was performed using the transitions *m/z* 163 → 55 and *m/z* 152 → 110 to quantify DHBE regioisomers and IS, respectively. Five DHBE isomers (a, b, c, d and e) were separated under the present chromatographic condition. The assay was linear over the concentration range of 5.0–200 ng/mL for DHBE isomers a, b and c, and 10.0–400 ng/mL for DHBE isomers d and e. The intra- and inter-day precision was within 13.6% in terms of relative standard deviation (RSD%) and the accuracy within 7.3% in terms of relative error. This simple and sensitive and easily reproducible LC–MS/MS method was successfully applied to the pharmacokinetic study of DHBE regioisomers in healthy male Chinese volunteers after an oral dose of 1.0 g DHBE.

1. Introduction

Dihydroxybutylether (DHBE), a strong choleretic drug, is used for the treatment of gallstone and hepatic disorders due to its choleretic activity and hepatoprotective action [1–3]. DHBE is obtained as a mixture of three regioisomers, including 4-(3-hydroxybutoxy)-2-butanol (I), 3-(4-hydroxy-2-butoxy)-1-butanol (II) and 3-(3-hydroxylbutoxy)-1-butanol (III) (Fig. 1) [4]. There are two chiral centers in each regioisomer. Thus, regioisomers I and II possess a pair of enantiomers plus a meso form, and regioisomer III possesses two pairs of enantiomers.

Although DHBE has been used in clinic since 1970s, the pharmacokinetics of DHBE has not been well studied because of lack of effective analysis methods for the quantification of DHBE in bio-matrices. To date, only Porta and Fregnan investigated the absorption, distribution and excretion of DHBE in rats but by using radio labeled assay with ^{14}C [5]. Recently, Staccioli et al. developed a GC–EI method to identify the regioisomers of DHBE based on the volatility of DHBE, however it was not applicable for the quantification of DHBE in bio-matrices [4].

To the best of our knowledge, no analytical method has been reported for the determination of DHBE regioisomers using liquid chromatography tandem mass spectrometry (LC–MS/MS). Hence, the primary aim of this study was to develop and validate a sensitive, specific and highly reproducible LC–MS/MS method for the quantification of DHBE regioisomers in human plasma. Furthermore, this validated method was applied to the pharmacokinetic study of DHBE.

2. Experimental

2.1. Chemicals and reagents

DHBE regioisomers I, II and III were supplied by Sirentang Pharm Co. Ltd. (Guizhou, China). Acetaminophen, the internal standard (IS), was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). DHBE capsules were purchased from Laphal Laboratories (Cedex, France). Heparinized drug-free plasma was provided by Liaoning Provincial Blood Center (Liaoning, China). HPLC-grade methanol was obtained from Yuwang industrial Co. Ltd. (Shandong, China). Distilled water,

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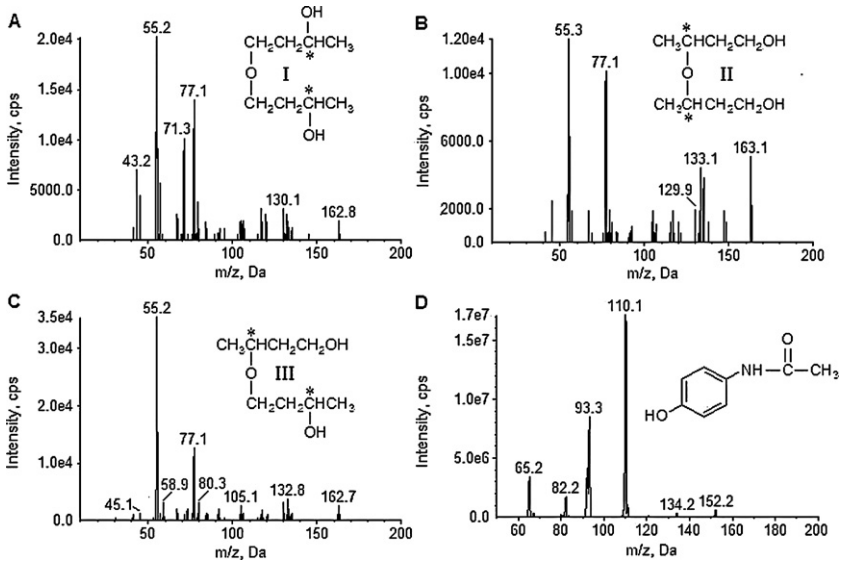

Fig. 1. Full scan MS/MS spectra of $[M+H]^+$ of DHBE regioisomers I (A), II (B), III (C) and acetaminophen (IS, D).

prepared from demineralized water, was used throughout the study.

2.2. Preparation of stock and standard solutions

Stock solutions of DHBE regioisomers I, II and III were prepared by dissolving the accurately weighed substances in methanol separately, resulting in a concentration of 5.0 mg/mL for each regioisomer. The stock solutions of DHBE regioisomers were mixed proportionally and diluted with water to prepare working solutions at different concentrations. The concentrations of regioisomers I, II and III in the working solutions were in the range of 0.20–8.0, 0.05–2.0 and 0.10–4.0 μg/mL, respectively. The working solutions were used to prepare calibration curve and quality control (QC) samples. Acetaminophen (IS) stock solution of 1.0 mg/mL was prepared in methanol and successively diluted with water to prepare a working solution at 800 ng/mL. All stock solutions were kept at −20 °C until analysis and they were found to be stable for at least 40 days (data not shown). All working solutions were prepared immediately before use.

2.3. Preparation of calibration curve and quality control samples

Calibration samples were prepared by spiking 10 μL of working solutions to 100 μL of drug-free human plasma on the day of analysis. The final concentrations of the calibrators were 20.0, 50.0, 100, 200, 400 and 800 ng/mL for regioisomer I, 5.0, 12.5, 25.0, 50, 100 and 200 ng/mL for regioisomer II, and 10.0, 25.0, 50.0, 100, 200 and 400 ng/mL for regioisomer III. Quality control samples (QCs) were prepared using separate stock solutions of DHBE regioisomers to obtain low, middle and high plasma concentrations: 40.0, 160 and 640 ng/mL for regioisomer I, 10.0, 40.0 and 160 ng/mL for regioisomer II, and 20.0, 80.0 and 320 ng/mL for regioisomer III. Additional validation QCs were prepared to test the lower limit of quantification (LLOQ): 20.0, 5.0 and 10.0 ng/mL for regioisomers I, II and III, respectively.

2.4. Plasma sample preparation

A 10 μL aliquot of the IS solution (800 ng/mL of acetaminophen in water) and 10 μL of water were added to 100 μL of plasma sample. The mixture was vortex mixed for 10 s. A 50 μL aliquot of 10% perchloric acid ($HClO_4$) was added and vortex mixed for another 60 s. Then, the sample was centrifuged at 12,000 rpm for 5 min and 100 μL supernatant was quantitatively transferred to a clear centrifuge tube. A 10 μL aliquot of the supernatant was injected for LC–MS/MS analysis.

2.5. LC–MS/MS conditions

Chromatographic separation was carried out on a Capcell Pak C_{18} MGII column (150 mm × 4.6 mm, 5 μm, Shiseido, Japan). Methanol (A) and water (B) were used as mobile phase for elution. The gradient was controlled as follows: 0–5 min, 20% A, 5–10 min, 15% A, 10–12 min, 15–20% A, 12–16.5 min, 20% A. The flow rate was 1.0 mL/min. The outlet of the column was split and only 0.5 mL/min portion of the column effluent was carried into mass spectrometer.

An API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) with electrospray source (ESI) was operated in positive ion mode. The quantification was performed using multiple reaction monitoring (MRM) method with the transitions of *m/z* 163 → 55 for DHBE (regioisomers I, II and III) and *m/z* 152 → 110 for IS. The main working parameters were set as follows: ionspray voltage, 4.5 kV; ion source temperature, 600 °C; gas1, 60 psi; gas2, 60 psi; curtain gas, 20 psi. Analyte concentrations were determined using the software Analyst 1.5.

2.6. Method validation

The validation process was carried out according to Guidance for Industry – Bioanalytical Method Validation, recommended by U.S. Food and Drug Administration [6].

2.6.1. Selectivity

Selectivity of the method was evaluated by analyzing six different blank plasma samples to investigate the potential interferences at the LC retention times for the analytes and IS.

2.6.2. Calibration curve, accuracy, and precision

Linearity of the method was assessed by six-point calibration curves on 3 consecutive days. The peak area ratios of analyte/IS were plotted against the nominal analyte concentrations. Calibration curves were generated by a weighted linear least-squares regression analysis with a weighting factor of $1/x^2$ (where x is the concentration). The calibration curves had to have a correlation

coefficient (r) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at LLOQ, which was set at ±20%. The LLOQ, taken as the lowest concentration on the calibration curve that could be measured with acceptable accuracy and precision, was determined in six replicates on 3 consecutive days [6].

Precision and accuracy were evaluated by analyzing QCs at LLOQ and three other concentration levels (low, middle and high) in six replicates on 3 consecutive days. The precision was expressed by relative standard deviation (RSD%). The assay accuracy was expressed as relative error (RE), i.e. (observed concentration − nominal concentration)/(nominal concentration) × 100%. The intra- and inter-day precisions were accepted to be below 15%, and the accuracy to be within ±15% except for LLOQ at which both precision and accuracy were accepted to be within 20% [6].

2.6.3. Dilution integrity

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. Dilution integrity experiment was carried out at 4 and 16 times ULOQ concentrations in six replicates. Samples at 4 times ULOQ concentration were 5 times diluted with drug-free plasma and those at 16 times ULOQ concentration were 20 times diluted with drug-free plasma. Their concentrations were calculated by applying the dilution factors 5 and 20 against the freshly prepared calibration curve, respectively.

2.6.4. Matrix effect and recovery

The matrix effect and extraction recovery for DHBE regioisomers and IS were evaluated by assaying three groups of samples: six replicates QC samples which were prepared as described in Section 2.4 (group 1), post-extracted blank plasma samples (100 μL) from six different subjects spiked with 10 μL DHBE and 10 μL IS working solutions (group 2), and six replicates post-extracted water (100 μL) spiked with 10 μL DHBE and 10 μL IS working solutions (group 3). Samples of each group were prepared at three DHBE regioisomers levels. The matrix effect was evaluated by measuring the matrix factor, which was defined as the percentage of peak area of an analyte spiked post-extraction (group 2) to its mean peak area in the absence of plasma matrix (group 3). The acceptance criterion for the inter-subject variability of matrix effect at each concentration level was less than 15% [7]. The recovery was calculated as the percentage of the peak area of an analyte spiked prior to extraction (group 1) to its mean peak area after extraction (group 2).

2.6.5. Stability

The stability of DHBE regioisomers in human plasma were assessed by analyzing triplicates of QCs at low and high levels, which were exposed to different temperatures and storage conditions. These QCs were analyzed after storage at room temperature for 2.0 h (bench-top), at −70 °C for 40 days and after three freeze–thaw cycles at −70 °C. The stability of the analytes and IS in the injection solvent was determined periodically by re-injecting the processed QCs for up to 24 h (at room temperature) after the initial injection. Samples were considered stable if assay values were within the acceptable limits of accuracy (±15% RE) and precision (15% RSD).

2.7. Application to a pharmacokinetic study

The validated method has been successfully used to analyze DHBE regioisomers concentrations in human plasma from 26 healthy male Chinese volunteers who received an oral dose of 1.0 g DHBE. The pharmacokinetic study was approved by the Medical Ethics Committee of Liaoning University of Traditional Chinese Medicine, Second Affiliated Hospital. Informed consents were obtained from all subjects after explaining the aims and risks of the study. After overnight fasting, subjects were given an oral dose of 1.0 g DHBE capsules (Laphal Laboratories, Cedex, France). Blood samples were collected into heparinized tubes before (0 h) and at 0.08, 0.17, 0.33, 0.50, 0.67, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10 and 12 h after administration. Plasma was separated by centrifugation at 3000 × g for 10 min and stored at −70 °C until analysis. The pharmacokinetic parameters of DHBE regioisomers were calculated by non-compartmental method using DAS 2.0 pharmacokinetic program (Chinese Pharmacology Society).

3. Results and discussion

3.1. Method development

3.1.1. Mass spectra

DHBE regioisomers I, II and III were easily ionized to protonated molecule ion $[M+H]^+$ at m/z 163 in the positive ionization mode. The product ion spectra of DHBE regioisomers I, II and III are shown in Fig. 1. Staccioli et al. reported a GC–MS method for qualitative identification of DHBE regioisomers by the diagnostic fragment ions at m/z 118 and 117 produced from 3-hydroxybutyl chain and 4-hydroxy-2-butyl chain, respectively, in electron ionization (EI) mode [4]. However, these diagnostic ions were not observed in the present study because of the different ionization and fragmentation mechanism between ESI and EI sources. DHBE regioisomers I, II and III produced similar fragments except that the fragment ion at m/z 71 in the spectra of DHBE regioisomers I and III, which might arise from the cleavage of 3-hydroxybutyl chain, was not observed in the product ion spectrum of DHBE regioisomer II (Fig. 1). Fragment ions at m/z 55 with the highest intensity was observed in the product ion spectra of all DHBE regioisomers, which was generated by the cleavage of the ether C—O bond as well as a molecule of water loss. The sensitive and selective mass transition m/z 163 → 55 was chosen to analyze DHBE regioisomers after optimization of collision energy. By a similar method, mass transition m/z 152 → 110 was used to determine IS.

3.1.2. Chromatography

Several reversed-phase C_{18} columns were tested to separate DHBE regioisomers in the study, including Hypersil BDS C_{18} (Elite, China), Venusil ASB C_{18} (Agela, USA), Diamonsil C_{18} (Dikma, China) and Capcell Pak C_{18} MGII column (Shiseido, Japan). Hypersil BDS C_{18} and Venusil ASB C_{18} columns gave wide peaks with poor resolution and low response for the separation of DHBE regioisomers (Fig. 2A and B), although various mobile phases comprised of organic solvents (acetonitrile and methanol) and water with or without typical modifiers and buffers (formic acid and ammonium acetate) were tried. Thin peaks of DHBE regioisomers with good resolution and high response were observed on a Diamonsil C_{18} column using methanol–water (10:90, v/v) as mobile phase at a flow rate of 1 mL/min; however, longer elution time was required for the effective peak separation (Fig. 2C). DEBE regioisomers could be completely separated with high signal intensity on a Capcell Pak C_{18} MGII column in a relatively short time by the same mobile phase (Fig. 2D). Under this condition, DHBE regioisomer II showed single peak at 17.0 min (peak a); DHBE regioisomer III generated two peaks at 26.5 and 28.4 min (peaks b and c); and DHBE regioisomer I produced two peaks at 31.3 and 33.5 min (peaks d and e). This elution sequence was opposite to the result of the GC–MS method developed by Staccioli et al. [4] because of different separation mechanism between HPLC and GC. Staccioli et al. also observed that the regioisomer eluted the fastest showed single peak while

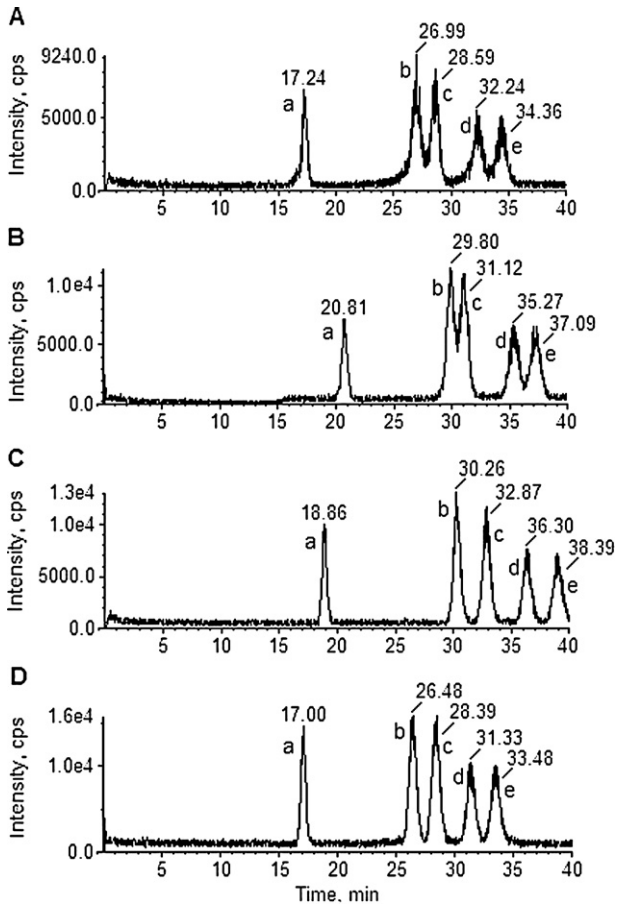

Fig. 2. Typical chromatograms of DHBE regioisomers acquired at different columns to evaluate peak separation resolution and signal response. Panel (A): separation of DHBE regioisomers on a Hypersil BDS C_{18} column (150 mm × 4.6 mm, 5 μm, Elite, China) with methanol–water (6:94, v/v) as mobile phase; panel (B): separation of DHBE regioisomers on a Venusil ASB-C_{18} column (150 mm × 4.6 mm, 5 μm, Agela, USA) with methanol–water (6:94, v/v) as mobile phase; panel (C): separation of DHBE regioisomers on a Diamonsil C_{18} column (150 mm × 4.6 mm, 5 μm, Dikma, China) with methanol–water (10:90, v/v) as mobile phase; panel (D): separation of DHBE regioisomers on a Capcell Pak C_{18} MGII column (150 mm × 4.6 mm, 5 μm, Shiseido, Japan) with methanol–water (10:90, v/v) as mobile phase. The concentrations of regioisomers I, II and III were 400, 100 and 200 ng/mL, respectively.

the other two regioisomers showed two peaks each [4]. Our result suggested that five isomers of DHBE (one is regioisomer II, two are the diastereomeric forms of regioisomer I, and the other two are the diastereomeric forms of regioisomer III) could be separated under the present chromatographic condition. The five isomers were referred as isomer a, b, c, d and e, respectively, according to their elution sequence.

Although good resolution and sensitivity were obtained on Capcell Pak C_{18} MGII column with methanol–water (10:90, v/v) as mobile phase, the analytical time (40 min) was too long for bioanalysis. To shorten the analytical time, gradient elution with methanol (A) and water (B) was performed. After optimization, the gradient was controlled as follows: 0–5 min, 20% A, 5–10 min, 15% A, 10–12 min, 15–20% A, 12–16.5 min, 20% A. Five DHBE isomers (a–e) were completely separated within 16.5 min.

3.1.3. Extraction

Extraction process with evaporation was not suitable for the sample preparation of DHBE due to the volatility of DHBE. Protein precipitation without concentration of the supernatant was a good choice for the extraction of DHBE from plasma. Initial choice of methanol for plasma protein precipitation was instantly rejected due to poor peak shapes resulting from the different composition between the sample and the mobile phase. When 10% $HClO_4$ was chosen for deproteinization, symmetrical peak shape was obtained in chromatography. Therefore, 10% $HClO_4$ was used as protein precipitation agent to extract DHBE isomers from plasma.

3.2. Method validation

3.2.1. Selectivity

The selectivity was assessed by comparing the chromatograms of blank plasma samples from six different subjects with the corresponding spiked plasma. Fig. 3 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with DHBE regioisomers at the LLOQ and a plasma sample from healthy volunteer 3.0 h after an oral administration. DHBE isomers a, b, c, d, e and IS eluted at 8.6, 13.3, 14.1, 14.6, 15.4 and 5.9 min, respectively. No significant interferences from endogenous substances with DHBE isomers or IS were detected.

3.2.2. Linearity and sensitivity

DHBE regioisomer III was separated as two diastereomeric forms, isomers b and c, under the present chromatographic condition. The peak areas of isomers b and c were equal, thus the concentrations of isomers b and c were the same, which were the half of the concentration of regioisomer III in the stock and working solutions (Figs. 2 and 3). Similarly, regioisomer I was separated as isomers d and e with the same peak area, so the concentrations of isomers d and e were half the concentration of regioisomer I in the stock and working solutions. Because regioisomer II only showed one peak in the present method, which was referred as isomer a, the concentration of isomer a equaled to the concentration of regioisomer II. According to the concentrations of DHBE isomers in the working solutions, the plasma calibration curves were constructed over the concentration range of 5.0–200 ng/mL for DHBE isomers a, b and c, and 10.0–400 ng/mL for DHBE isomers d and e. The ULOQ was set at 200 ng/mL for DHBE isomers a, b and c, and 400 ng/mL for DHBE isomers d and e because ionization saturation of isomers a, b and c was observed at 250 ng/mL whereas ionization saturation of isomers d and e was observed at 500 ng/mL using ESI source. Excellent linearity was obtained in the standard curve concentration ranges with a correlation coefficient (r) greater than 0.993. All back-calculated standard concentrations were within 15% deviation from the nominal value, except at the LLOQ, for which the maximum acceptable deviation was set at 20%. The LLOQ was confirmed to be 5.0 ng/mL for DHBE isomers a, b and c, and 10.0 ng/mL for DHBE isomers d and e. The precision and accuracy values corresponding to LLOQ are shown in Table 1.

3.2.3. Precision and accuracy

The method showed good precision and accuracy. Table 1 summarizes the results for intra- and inter-day precision and accuracy for DHBE isomers measured by QCs. In this assay, the intra- and inter-day precisions were measured to be below 13.1% and 13.7%, respectively, with relative errors from −7.3% to 2.8%. These values were within the acceptable range, and the method was thus judged to be suitably accurate and precise.

3.2.4. Dilution integrity

The mean back-calculated concentrations for 1/5 and 1/20 dilution samples of DHBE isomers were within 90.8–100.9% of their nominal. The precision for 1/5 and 1/20 dilution samples were 9.0% and 8.6% for DHBE isomer a, respectively; 7.7% and 7.8% for DHBE isomer b, respectively; 7.9% and 7.5% for DHBE isomer c, respectively; 8.5% and 9.4% for DHBE isomer d, respectively; 7.8% and 8.2% for DHBE isomer e, respectively.

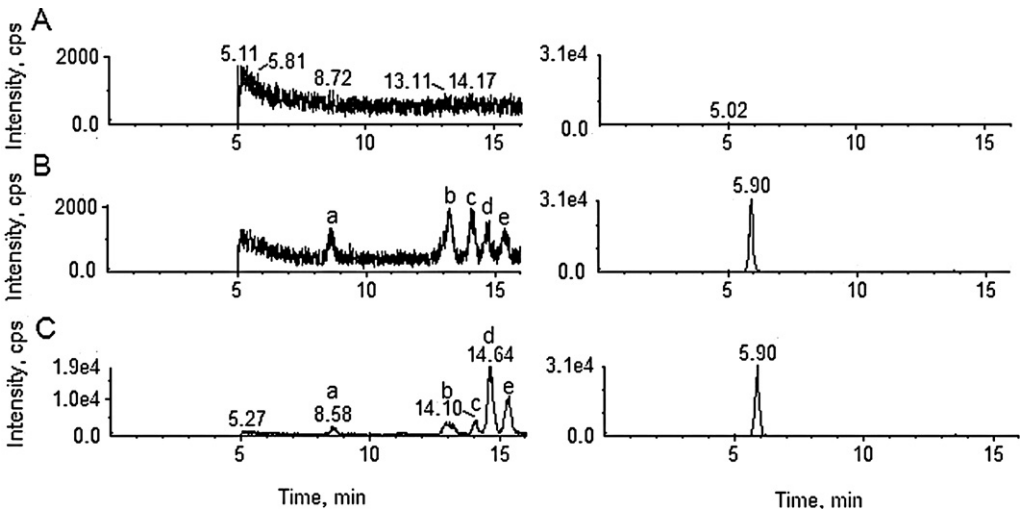

Fig. 3. Representative MRM chromatograms for DHBE isomers a, b, c, d, e (left) and acetaminophen (IS, right) in human plasma samples. Panel (A): a blank plasma sample; panel (B): a blank plasma sample spiked with DHBE regioisomers at the LLOQ; panel (C): a human plasma sample collected at 2 h after drug administration, in which the concentrations of isomers a, b, c, d and e were 12.3, 17.2, 12.3, 237.5 and 126.0 ng/mL, respectively.

3.2.5. Recovery

As shown in Table 2, the recoveries of DHBE isomers a, b, c, d and e extracted from plasma at the low, middle and high concentrations (10.0, 40.0 and 160 ng/mL for isomers a, b and c; 20.0, 80.0 and 320 ng/mL for isomers d and e) were in the range from 72.4% to 89.4%. The recovery of IS at 80.0 ng/mL was 53.3 ± 4.5% (n = 18). This low recovery was mainly caused by the partial transference during QC samples preparation as described in Section 2.4. In addition, adsorption of IS with precipitation and the drift of signal intensity of mass spectrometer might also contribute to its low recovery.

3.2.6. Matrix effect

The matrix effects of DHBE isomers a, b, c, d and e from six different human plasma samples at three concentrations (10.0, 40.0 and 160 ng/mL for isomers a, b and c; 20.0, 80.0 and 320 ng/mL for isomers d and e) were in the range of 93.4–114.5% with RSD values below 13.8%. The matrix effect of IS (80.0 ng/mL in plasma) was 137.2% and the RSD value was 4.9%. These results suggested that the effect of matrix on the determination of DHBE isomers could be ignored. Although there was an extent to the matrix enhancement for IS, the inter-subject variability of matrix effect of IS was less than 15%, which was within the acceptable range. The matrix enhancement for IS did not influence the accurate determination of DHBE isomers in human plasma.

3.2.7. Stability

Table 3 contains the results of investigations into the stability of DHBE isomers under the various conditions tested throughout the validation process. The tests were designed to cover anticipated conditions which might be encountered during sample handling and processing. The results indicated the stability of DHBE isomers in plasma after storage at room temperature for 2.0 h (bench-top), at −70 °C for 40 days, after three freeze–thaw cycles at −70 °C and in processed samples at room temperature for 24 h.

Table 1
Precision and accuracy of the LC–MS/MS method to determine DHBE regioisomers in human plasma (in three consecutive days, six replicates for each day).

Isomers	Concentration (ng/mL)		Precision (RSD%)		Accuracy
	Added	Measured	Intra-day	Inter-day	(RE%)
a	5.0	5.1 ± 0.6	6.2	9.2	1.1
	10.0	10.1 ± 1.0	10.7	10.5	0.8
	40.0	40.1 ± 3.6	9.5	4.1	0.3
	160	152.0 ± 14.2	8.2	13.4	−5.0
b	5.0	4.9 ± 0.6	5.4	9.3	−2.4
	10.0	9.6 ± 0.9	8.9	12.3	−4.0
	40.0	40.0 ± 0.7	9.8	3.6	−0.1
	160	151.2 ± 13.3	7.6	11.7	−5.5
c	5.0	5.0 ± 0.4	3.3	8.7	0.8
	10.0	9.9 ± 1.0	9.5	13.7	−1.4
	40.0	40.1 ± 3.9	10.3	4.5	0.3
	160	152.3 ± 16.2	9.4	13.6	−4.8
d	10.0	10.3 ± 1.6	8.1	3.3	2.8
	20.0	20.4 ± 2.5	13.1	4.3	2.0
	80.0	81.8 ± 6.9	8.7	7.1	2.2
	320	296.5 ± 29.9	9.1	9.3	−7.3
e	10.0	10.0 ± 1.1	5.2	8.0	0.0
	20.0	20.1 ± 2.5	12.3	13.2	0.7
	80.0	80.8 ± 8.2	10.2	10.2	0.9
	320	298.1 ± 29.3	8.3	12.1	−6.9

Table 2
Recovery for DHBE isomers and acetaminophen (IS).

Analytes	Added concentration (ng/mL)	Recovery (%)
DHBE isomer a (n = 6)	10.0	86.6 ± 5.8
	40.0	89.4 ± 5.2
	160	88.6 ± 7.9
DHBE isomer b (n = 6)	10.0	85.1 ± 3.1
	40.0	82.3 ± 4.5
	160	79.5 ± 3.0
DHBE isomer c (n = 6)	10.0	80.5 ± 6.6
	40.0	78.1 ± 4.1
	160	75.9 ± 4.3
DHBE isomer d (n = 6)	20.0	72.4 ± 4.0
	80.0	79.0 ± 4.1
	320	76.2 ± 3.2
DHBE isomer e (n = 6)	20.0	83.6 ± 5.3
	80.0	75.2 ± 5.8
	320	76.1 ± 3.7
Acetaminophen (IS, n = 18)	80.0	53.3 ± 4.5

Table 3
Stability of DHBE isomers under various storages in plasma ($n = 3$).

Storage condition	Isomers	Concentration (ng/mL)		RSD (%)	RE (%)
		Added	Measured		
Room temperature for 2 h (bench-top)	a	10.0	9.5 ± 1.2	12.4	−5.2
		160	154.4 ± 1.5	1.0	−3.5
	b	10.0	9.2 ± 1.0	10.7	−8.1
		160	154.6 ± 1.1	0.7	−3.4
	c	10.0	9.5 ± 0.8	8.8	−5.2
		160	163.8 ± 0.9	0.6	2.4
	d	20.0	19.5 ± 2.1	10.7	−2.6
		320	321.5 ± 3.9	1.2	0.5
	e	20.0	18.5 ± 2.2	11.7	−7.7
		320	318.6 ± 2.4	0.8	−0.5
Frozen (−70 °C) for 40 days	a	10.0	10.0 ± 0.5	4.6	−0.4
		160	173.1 ± 7.9	4.6	8.2
	b	10.0	9.7 ± 0.3	3.4	−2.6
		160	176.9 ± 6.6	3.8	10.6
	c	10.0	10.1 ± 0.5	4.6	0.6
		160	169.6 ± 8.5	5.0	6.0
	d	20.0	20.0 ± 1.1	5.6	0.0
		320	327.7 ± 12.0	3.7	2.4
	e	20.0	21.4 ± 0.7	3.3	6.9
		320	319.5 ± 13.6	4.3	−0.2
Three freeze–thaw cycles at −70 °C	a	10.0	9.8 ± 0.0	0.4	−1.9
		160	165.4 ± 1.4	0.9	3.4
	b	10.0	10.2 ± 0.8	7.9	2.4
		160	169.9 ± 2.1	1.3	6.2
	c	10.0	10.2 ± 0.4	3.8	2.4
		160	161.6 ± 4.8	3.0	1.0
	d	20.0	19.3 ± 1.4	7.2	−3.6
		320	306.1 ± 4.1	1.3	−4.3
	e	20.0	21.1 ± 1.6	7.5	5.7
		320	305.7 ± 10.0	3.3	−4.5
Post-pretreatment at room temperature for 24 h	a	10.0	10.8 ± 0.8	7.4	7.7
		160	177.1 ± 7.3	4.1	10.7
	b	10.0	11.3 ± 0.5	4.8	12.5
		160	187.2 ± 4.8	2.6	14.2
	c	10.0	10.9 ± 0.9	8.3	9.4
		160	175.2 ± 6.1	3.5	9.5
	d	20.0	22.2 ± 0.3	1.5	11.1
		320	328.9 ± 15.3	4.7	2.8
	e	20.0	22.7 ± 0.9	4.1	13.7
		320	326.8 ± 13.3	4.1	2.1

3.3. Application to a pharmacokinetic study

The validated LC–MS/MS method was successfully applied to a pharmacokinetic study of DHBE isomers after administration of 1.0 g DHBE to 26 volunteers. The mean plasma concentration–time curves of DHBE isomers are shown in Fig. 4 and the main pharmacokinetic parameters of DHBE isomers are presented in Table 4.

Marked inter-subject variability was observed in the pharmacokinetic profile of each DHBE isomers after drug administration (Fig. 4). The values of C_{max} of DHBE isomers a, b and c were higher than 1000 ng/mL in some volunteers, while were less than the LLOQ in other volunteers. The individual differences in the C_{max} of isomers d and e were not as obvious as those of isomers a, b and c, while great differences were found in the T_{max} of these two isomers. The peak concentrations of both isomer d and e were arrived from 0.17 to 4.0 h. This high variability in T_{max} gave rise to plateaus in their mean plasma concentration–time curves (Fig. 4D and E). In addition, the pharmacokinetic profiles of isomers a, b and c differed greatly from those of isomers d and e. Isomers a, b and c were eliminated so rapidly that their plasma concentrations were below the LLOQ at 4 h after drug administration. Isomers d and e showed a relatively slow elimination with an elimination half-life ($t_{1/2}$) of 3.0 and 2.3, respectively. Remarkable higher values of AUC for isomers d and e were obtained due to the slow elimination, although their C_{max} were obviously lower than those of isomers a, b and c (Table 4).

Table 4
Pharmacokinetic parameters of DHBE isomers after an oral administration of 1.0 g DHBE.

DHBE isomers	Parameters				
	$t_{1/2}$ (h)	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-t} (ng h/mL)	$AUC_{0-\infty}$ (ng h/mL)
a	0.4 ± 0.1	462.5 ± 662.5	0.4 ± 0.1	132.7 ± 166.5	132.7 ± 166.5
b	0.5 ± 0.1	474.0 ± 679.9	0.4 ± 0.2	169.8 ± 205.6	169.8 ± 205.6
c	0.5 ± 0.4	431.1 ± 656.7	0.4 ± 0.2	142.5 ± 189.4	142.5 ± 189.4
d	3.0 ± 0.8	320.7 ± 119.9	1.3 ± 1.1	1560 ± 518	1683 ± 547
e	2.3 ± 1.3	160.0 ± 81.5	1.5 ± 1.2	591.7 ± 168.0	632.5 ± 188.8

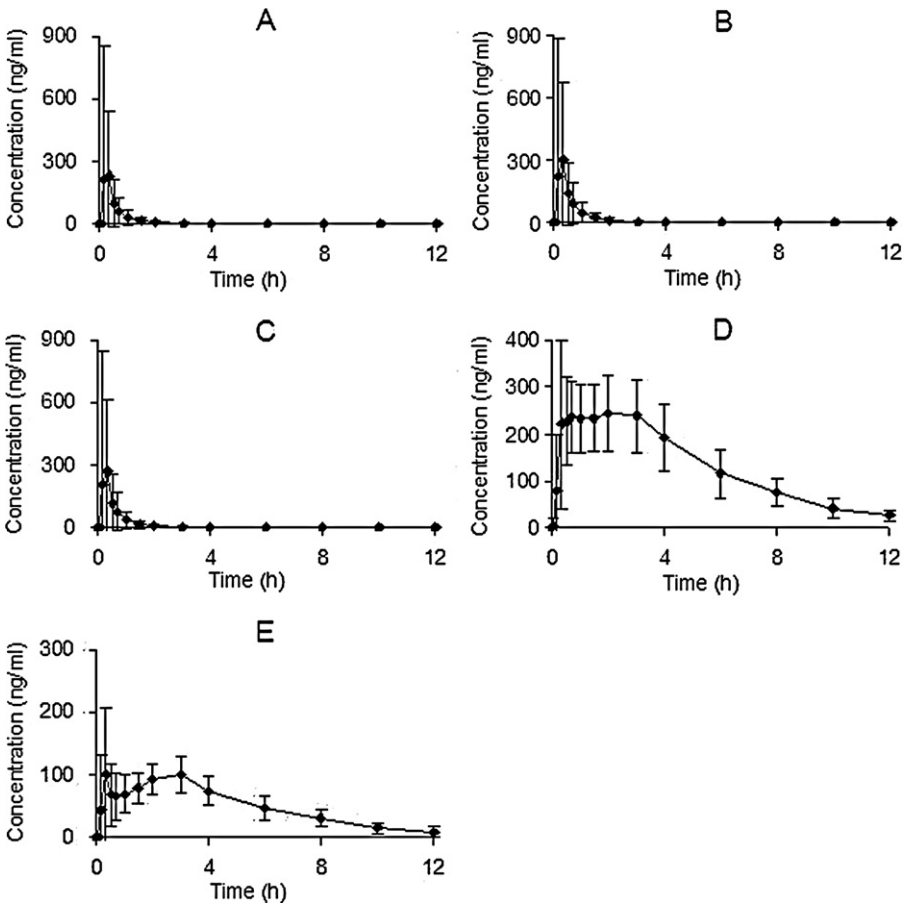

Fig. 4. Mean plasma concentration–time curve of DHBE isomers a (A), b (B), c (C), d (D), e (E) in 26 healthy Chinese volunteers after an oral administration of 1.0 g DHBE capsules. The error bars are standard deviations of the mean.

4. Conclusion

To the best of our knowledge, this is the first LC–MS/MS method for the quantification of DHBE regioisomers in human plasma. The developed method provides simple and rapid sample processing technique, high sensitivity and selectivity. The method was fully validated and successfully applied to a pharmacokinetic study of DHBE regioisomers in humans.

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