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Short communication

Simultaneous quantitation of hydrochlorothiazide and metoprolol in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A rapid and sensitive method for the simultaneous quantitation of hydrochlorothiazide (HCT) and metoprolol (MET) in human plasma based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed and validated. MS/MS detection involved switching the electrospray ionization (ESI) mode during chromatography from negative to detect HCT and its internal standard (I.S.) 5-bromouracil to positive to detect MET and its I.S. tramadol. Sample preparation by liquid–liquid extraction with diethyl ether–dichloromethane (60:40, v/v) was followed by chromatography on a Venusil MP-C18 column using methanol–ammonium acetate (10 mM)–formic acid (pH 3.4) (50:50:0.05, v/v/v) at a flow rate of 0.8 mL/min. The method was linear in the concentration range 3–1000 ng/mL for both HCT and MET using 100 μL human plasma. Intra- and inter-day precisions (as relative standard deviation) for HCT were 2.9–3.9% and 3.9–4.7%, respectively and for MET were 2.4–4.1% and 4.7–6.2%, respectively. Accuracies (as relative error) were ±3.8% and ±2.6% for HCT and MET, respectively. The assay was successfully applied to a pharmacokinetic study involving a single oral dose of a combination tablet (25 mg HCT, 50 mg MET) in healthy volunteers.

1. Introduction

Hydrochlorothiazide (HCT) is one of the oldest thiazide diuretics used to treat hypertension. It is often prescribed in combination with other antihypertensive drugs such as β-blockers, angiotensin-converting enzyme inhibitors, or angiotensin II receptor blockers [1]. A combination dosage form of HCT with the β_1-selective (cardioselective) β-blocker, metoprolol (MET) [2], is more effective than either drug alone in the management of hypertension and is effective in patients unresponsive to monotherapy with either agent [3,4]. Despite widespread use, a method for the simultaneous determination of HCT and MET suitable for application to clinical pharmacokinetic (PK) studies has not been reported.

For the determination of MET in human plasma, several methods based on high performance liquid chromatography (HPLC) with UV [5] and fluorescence [6] detection, gas chromatography-mass spectrometry [7] and LC–MS/MS [8] have been published. Similarly, a number of methods for the determination of HCT in human plasma have been developed based on HPLC with UV or diode array detection [9,10] and LC–MS/MS [11,12]. In terms of the combination, the main problem is that HCT (an acidic drug) is ionized efficiently only in the negative ion mode whereas MET (a basic drug) has much higher ionization efficiency in the positive ion mode. This demands a method involving switching of the ionization mode during a chromatographic run and the use of an internal standard (I.S.) for each analyte.

In this article, a rapid and sensitive LC–MS/MS method for the simultaneous quantitation of HCT and MET in human plasma is described. The method combines simple sample preparation with good specificity and sensitivity and a short run time. The method has been successfully applied to a PK study of a combination tablet containing HCT 25 mg and MET 50 mg.

2. Experimental

2.1. Chemicals and reagents

Hydrochlorothiazide and metoprolol tartrate (purity > 99.0%) as well as 5-bromouracil (BRU) and tramadol hydrochloride (TRA) (purity > 99.5%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tablets containing both drugs were supplied by TaiHua Pharmaceutical Co., Ltd. (Harbin, China). Methanol (HPLC-grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were of analytical grade and used without further purification. Distilled water, prepared from demineralized water, was

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used throughout the study. Heparinized blank (drug-free) human plasma was obtained from the Changchun Blood Donor Service (Changchun, China).

2.2. Instrumentation

HPLC was carried out on an Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) consisting of a vacuum degasser, a binary pump and an autosampler. An API 4000 triple-quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) equipped with a Turbo IonSpray ionization (ESI) source was used for mass analysis and detection. Data acquisition and integration were controlled by Applied Biosystems Analyst Software (Applied Biosystems/MDS SCIEX, version 1.3).

2.3. LC–MS/MS conditions

Chromatography was performed on a Venusil MP-C18 column (100 mm × 4.6 mm, 5 μm) maintained at 35 °C using a mobile phase of methanol–ammonium acetate (10 mM)–formic acid (pH 3.4) (50:50:0.05, v/v/v) at a flow rate of 0.8 mL/min. The column eluant was split 1:1 (v/v) so that approximately 0.4 mL/min entered the mass spectrometer. The electrospray interface heater was set to on mode and IonSpray Voltage was set at −3500 V for negative ionization and 4000 V for positive ionization. Curtain gas, Gas 1 (GS1) and Gas 2 (GS2) (all nitrogen) were set to 10, 50 and 40 units, respectively, and the source temperature was 500 °C. MS parameters were optimized to obtain maximum sensitivity at unit resolution. Precursor to product ion transitions subject to multiple reaction monitoring (MRM) with associated declustering potentials (DPs, eV) and collision energies (CEs, eV) were: in the negative ion mode HCT m/z 296.1 → 269.0, DP −65, CE −27; BRU m/z 188.8 → 78.8, DP −38, CE −55; in the positive ion mode MET m/z 268.3 → 116.2, DP 66, CE 26; TRA m/z 264.3 → 58.2, DP 27, CE 41. The pause time was set at 10 ms and the dwell time at 200 ms.

2.4. Preparation of standard and quality control (QC) solutions

All solutions were prepared in methanol–water (50:50, v/v) and kept refrigerated at 4 °C when not in use. Stock solutions of HCT (1 mg/mL) and MET (1 mg/mL) were used to prepare mixed standard solutions containing 3, 10, 30, 100, 300, and 1000 ng/mL of both HCT and MET. Low, medium and high QC solutions containing 9, 100, and 800 ng/mL of both HCT and MET were prepared independently in the same way. A working I.S. solution containing 2 μg/mL BRU and 1 ng/mL TRA was also prepared.

2.5. Sample preparation

An aliquot of plasma (100 μL) was placed in a 10 mL glass tube followed by 50 μL I.S. solution and 100 μL methanol–water (50:50, v/v) (or a standard or QC solution of HCT and MET). The mixture was vortexed for 30 s and then subject to liquid–liquid extraction (LLE) with 3.0 mL diethyl ether–dichloromethane (60:40, v/v). After shaking for 10 min and centrifuging at 3500 × g for 5 min, the organic phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL mobile phase and 20 μL injected into the LC–MS/MS system.

2.6. Assay validation

Assay validation was performed according to FDA guidelines [13]. Linearity was assessed by construction of three independent

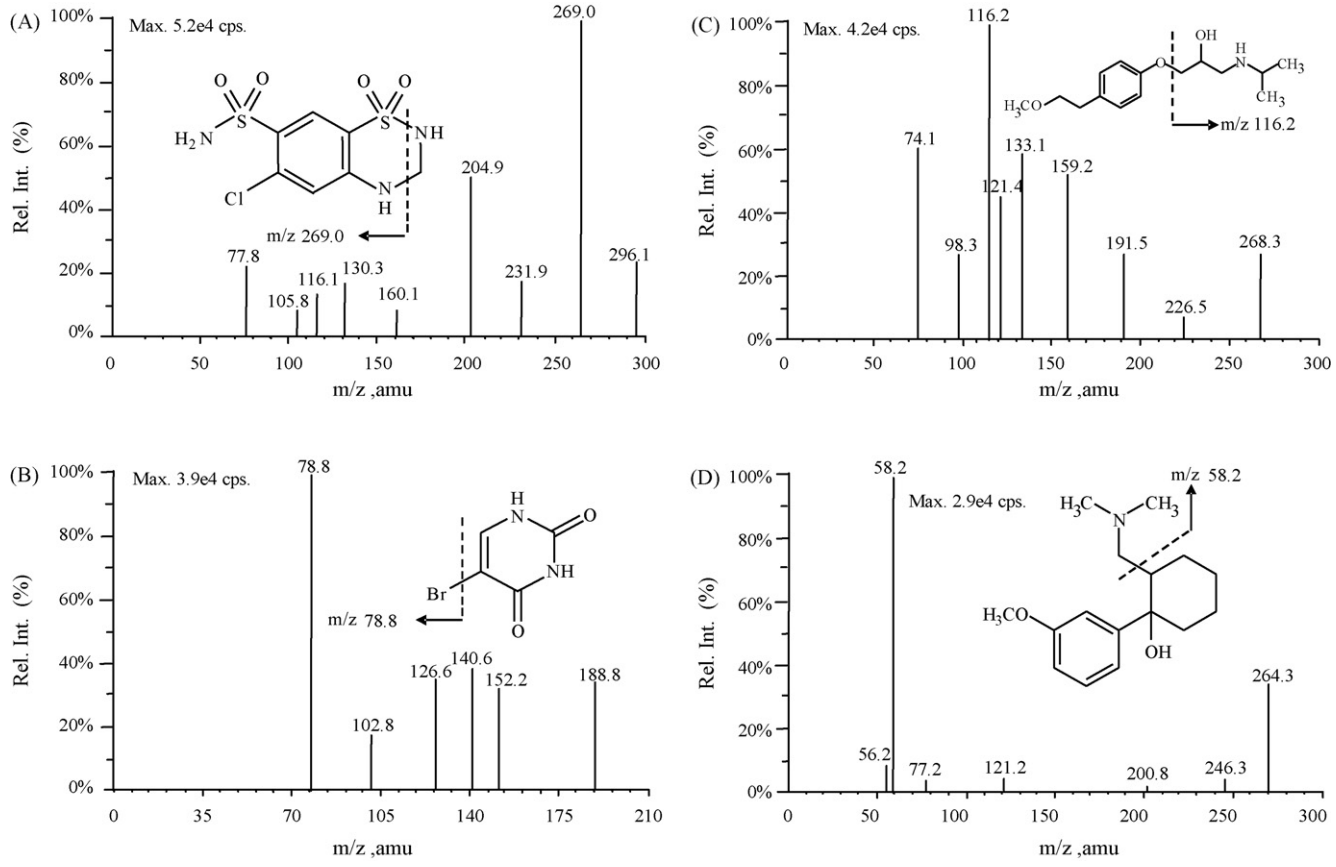

Fig. 1. Full-scan product ion mass spectra of $[M-H]^-$ ions of (A) HCT and (B) BRU and of $[M+H]^+$ ions of (C) MET and (D) TRA.

calibration curves based on peak area ratios of analyte-I.S. for six spiked plasma samples (3–1000 ng/mL) on three separate days. Calibration curves were analyzed by weighted linear regression ($1/x^2$). Accuracy [as relative error (R.E.)] and intra- and inter-day precision [as relative standard deviation (R.S.D.)] were assessed by assay of six replicate QC samples (9, 100 and 800 ng/mL) on three different days. The lower limit of quantitation (LLOQ) was defined as the lowest concentration with signal/noise >5, precision ≤ 20% and accuracy ±20%. Recovery was calculated by comparing peak areas of QC samples with those prepared by spiking the upper organic layer of extracted blank plasma with the corresponding QC solutions.

Matrix effects of HCT and MET were evaluated by comparing the peak areas of post-extraction blank plasma spiked at concentrations of QC samples with the areas obtained by direct injection of corresponding standard solutions. Matrix effects for the I.S. BRU and TRA were evaluated in the similar way. Stability in human plasma was assessed after three freeze–thaw cycles, storage for 30 days at −20 °C and storage at room temperature for 6 h. Stability in mobile phase on storage in plastic autosampler vials under autosampler conditions for 12 h was also assessed.

2.7. PK study

After a 12 h fast, 10 healthy male volunteers received a single combination tablet containing HCT (25 mg) and MET (50 mg) with 200 mL water. Blood samples (4 mL) were collected by venepuncture into heparinized tubes prior to the dose and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 9, 10, 12 and 24 h after the dose. Following centrifugation (3000 × *g* for 10 min), plasma samples were stored in polypropylene tubes at −20 °C and analyzed within 1 month. Non-compartmental PK parameters were calculated using TopFit version 2.0 [14].

3. Results and discussion

3.1. LC–MS/MS conditions

BRU was chosen as the I.S. for HCT since, being an acid with a similar pK_a value (8.0 vs 8.8 for HCT), it was expected to give similar recovery on LLE and MS/MS response in the negative ion mode. TRA was chosen as the I.S. for MET for the same expecta-

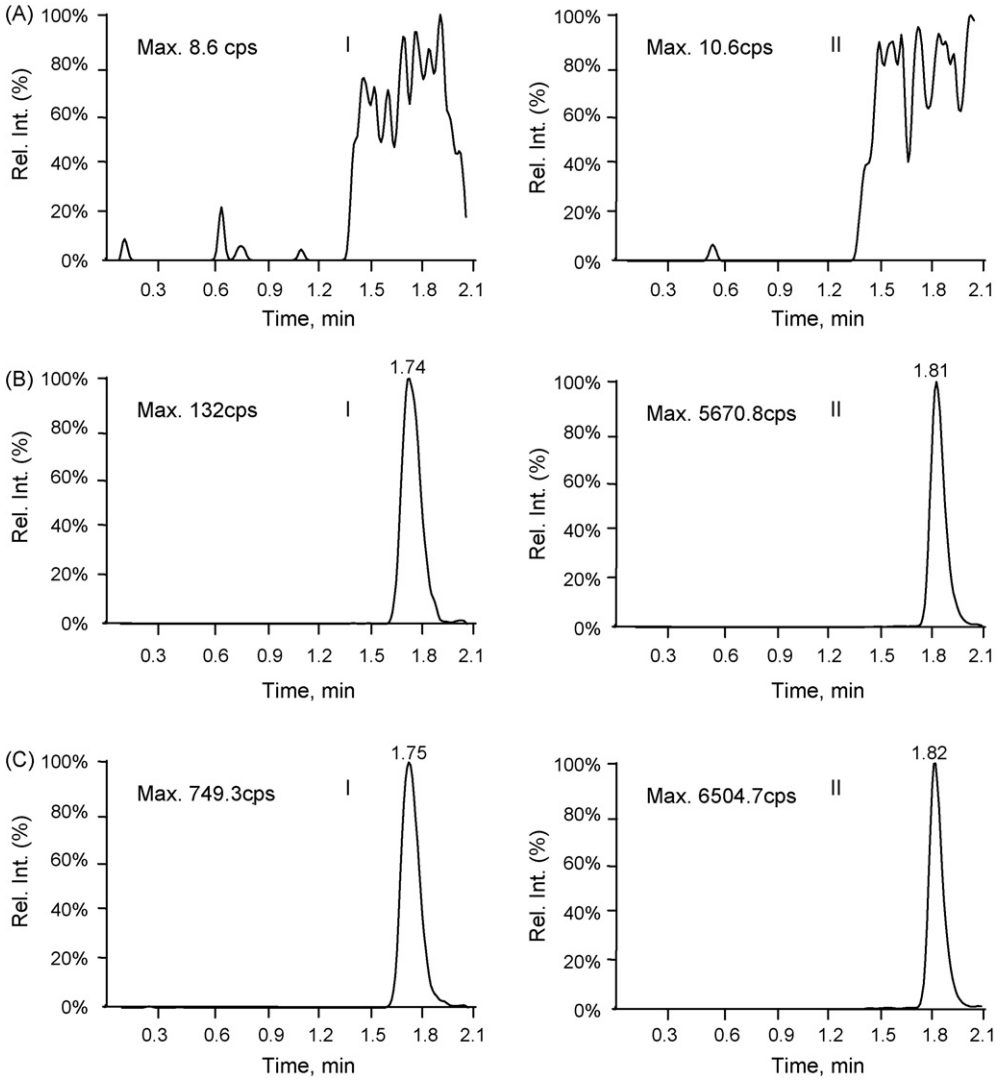

Fig. 2. Typical MRM chromatograms of (I) HCT and (II) BRU (2 μg/mL) in human plasma: (A) blank plasma; (B) blank plasma spiked with 3.0 ng/mL HCT; and (C) a plasma sample from a human volunteer 1.5 h after oral administration of a combination tablet containing HCT 25 mg and MET 50 mg.

tion of a base (pKa 9.4 vs 9.7 for MET) in the positive ion mode. Full-scan mass spectra of each analyte and I.S. were acquired in both positive and negative ion modes for both ESI and atmospheric pressure chemical ionization (APCI) by direct infusion of the respective standard and working solutions. ESI provided better ionization than APCI. MET and TRA ionized in both positive and negative ion modes but had much higher ionization efficiencies in the positive ion mode. HCT and BRU ionized in the negative ion mode only as previously observed for HCT [7,12]. The product ion mass spectra of the $[M-H]^-$ ions of HCT and BRU and the $[M+H]^+$ ions of MET and TRA are shown in Fig. 1.

In optimizing the response of the selected precursor/product ion combinations, it was found that MET had at least 50 times higher ion abundance than HCT at the same concentration. Therefore, for simultaneous quantitation of the two analytes, it was necessary to optimize the overall experimental conditions based mainly on the response of HCT. Although this means some of the source parameters are not optimum for MET, the assay retains good sensitivity for MET because of its high ionization response. The I.S. behaved similarly to their respective analytes.

Evaluation of mobile phases containing various combinations of acetonitrile, methanol, 10 mM ammonium acetate and water showed that methanol produced stronger signals than acetonitrile with no solvent-clustered ions. The inclusion of 10 mM ammonium acetate enhanced MS response without increasing matrix effects and produced more symmetrical peaks. Peak shape was also improved through the use of 0.1% formic acid to adjust the mobile phase pH and by increasing the flow rate. A number of C18 columns were evaluated and Venusil MP-C18 gave the best chromatography with a flow rate of 0.8 mL/min. Under these conditions, HCT and BRU co-eluted at 1.74–1.82 min and MET and TRA co-eluted at 2.44–2.46 min compared to a t_0 of approximately 1.1 min (Figs. 2 and 3). The cycle time of the assay was 3.0 min and included a switch from the negative to the positive ion mode at 2.1 min.

3.2. Sample preparation

Sample preparation by simple protein precipitation and LLE were evaluated. Protein precipitation gave poor peak shape and significant matrix effects. LLE with diethyl ether, hexane, ethyl acetate, dichloromethane and their combinations was evaluated. A mixture of diethyl ether and dichloromethane (60:40, v/v) at a relative volume of 3 mL to 100 μL plasma gave efficient extraction of both analytes and I.S. with minimal interference and without the need for pH adjustment despite the difference in acid–base properties of the analytes and I.S.

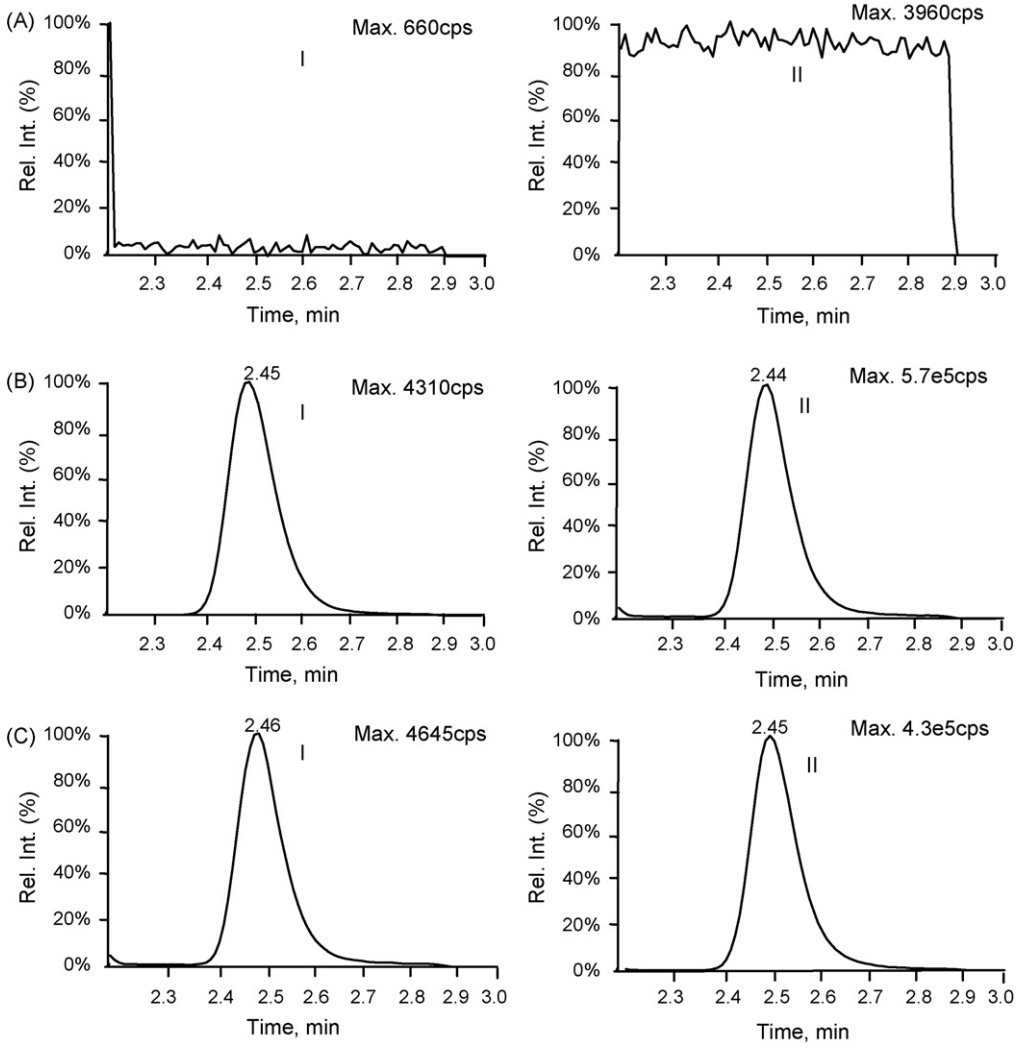

Fig. 3. Typical MRM chromatograms of (I) MET and (II) TRA (1 ng/mL) in human plasma: (A) blank plasma; (B) blank plasma spiked with 3.0 ng/mL MET; and (C) a plasma sample from a human volunteer 1.5 h after oral administration of a combination tablet containing HCT 25 mg and MET 50 mg.

Table 1
Accuracy and precision for the analysis of HCT and MET in human plasma (data are based on assay of 6 replicates on three different days).

	Concentration (ng/mL)		R.S.D. (%)		R.E. (%)
	Spiked	Mean calculated	Intra-day	Inter-day	
HCT	9.00	8.66	3.4	4.2	−3.8
	100	97.4	2.9	3.9	−2.6
	800	787	3.9	4.7	−1.6
MET	9.00	9.03	3.4	4.7	0.4
	100	102.6	2.4	6.2	2.6
	800	777	4.1	5.6	−2.1

3.3. Assay validation

3.3.1. Selectivity and matrix effects

Figs. 2 and 3 show typical MRM chromatograms of a blank plasma sample, a plasma sample spiked with HCT and MET at the LLOQ (3 ng/mL for both analytes) and a plasma sample from a healthy volunteer 1.5 h after the oral administration of the combination tablet. There was no significant interference from endogenous substances observed at the retention times of the analytes or I.S.

In studies of matrix effects, peak area ratios for low, medium, and high QC samples were 97.3 ± 1.6%, 94.5 ± 0.8%, and 96.1 ± 2.4% respectively for HCT and 93.5 ± 2.2%, 96.3 ± 1.9%, and 92.8 ± 1.5% respectively for MET. The results for BRU and TRA were 98.7 ± 1.8% and 97.7 ± 1.0%, respectively. The results indicate that ion suppression or enhancement due to the plasma matrix was consistent and would not interfere with the quantitation of analytes.

3.3.2. Linearity and LLOQ

The assay was linear over the concentration range 3–1000 ng/mL for both HCT and MET. Typical equations of calibration curves are as follows:

$$\text{HCT}: y = 0.00385x + 0.00155 \quad r = 0.9980$$

$$\text{MET}: y = 0.00246x + 0.00106 \quad r = 0.9983$$

where y represents the analyte I.S. peak area ratio and x represents the plasma concentration of the analyte. LLOQ was 3 ng/mL for both HCT and MET and was adequate for clinical PK studies following oral administration of therapeutic doses.

3.3.3. Precision and accuracy

Table 1 summarizes the accuracy and precision data. The intra- and inter-day precisions for HCT sand MET were ≤4.7% and ≤6.2% at each QC level. The accuracy was ±3.8% for HCT and ±2.6% for MET.

3.3.4. Recovery and stability

The mean recoveries of HCT in low, medium and high QC samples were 93.5 ± 4.9%, 97.2 ± 2.7% and 96.3 ± 3.3%, respectively and of MET were 81.5 ± 4.9%, 84.4 ± 1.9% and 84.3 ± 0.4%, respectively. Mean recoveries of BRU and TRA were 94.9 ± 3.1% and 97.8 ± 2.5%, respectively. The data show that the simple LLE procedure efficiently extracts all four compounds from human plasma.

Table 2 summarizes stability data and shows there were no stability-related issues that might cause problems in application of the assay to PK studies.

3.4. PK study

The validated method was successfully applied to quantitate the concentrations of HCT and MET in human plasma samples after oral

Table 2
Stability data of HCT and MET in human plasma and processed QC samples ($n = 3$).

Storage conditions	Drug	Concentration (ng/mL)		R.S.D. (%)	R.E. (%)
		Spiked	Mean calculated ± SD		
Room temperature for 6 h	HCT	9.00	8.76 ± 0.16	1.8	−2.6
		100	97.7 ± 1.7	1.7	−2.3
		800	788 ± 7	0.9	−1.5
	MET	9.00	8.56 ± 0.29	3.4	−4.9
		100	95.6 ± 1.9	2.0	−4.4
		800	776 ± 21	2.7	−3.0
Freezing for 30 days at −20 °C	HCT	9.00	8.72 ± 0.14	1.6	−3.2
		100	93.8 ± 3.5	3.8	−6.2
		800	732 ± 30	4.2	−8.5
	MET	9.00	8.48 ± 0.24	2.9	−5.7
		100	91.5 ± 2.0	2.2	−8.5
		800	743 ± 35	4.8	−7.2
Three freeze/thaw cycles	HCT	9.00	8.75 ± 0.18	2.0	−2.8
		100	95.6 ± 3.2	3.4	−4.4
		800	779 ± 8.0	1.0	−2.7
	MET	9.00	8.12 ± 0.78	9.6	−9.8
		100	92.6 ± 2.6	2.8	−7.4
		800	747 ± 45	6.0	−6.6
Processed QC samples under autosampler conditions	HCT	9.00	8.92 ± 0.12	1.4	−0.9
		100	98.5 ± 1.3	1.3	−1.5
		800	793 ± 4	0.5	−0.9
	MET	9.00	8.58 ± 0.26	3.0	−4.6
		100	95.9 ± 4.0	4.1	−4.1
		800	778 ± 20	2.6	−2.7

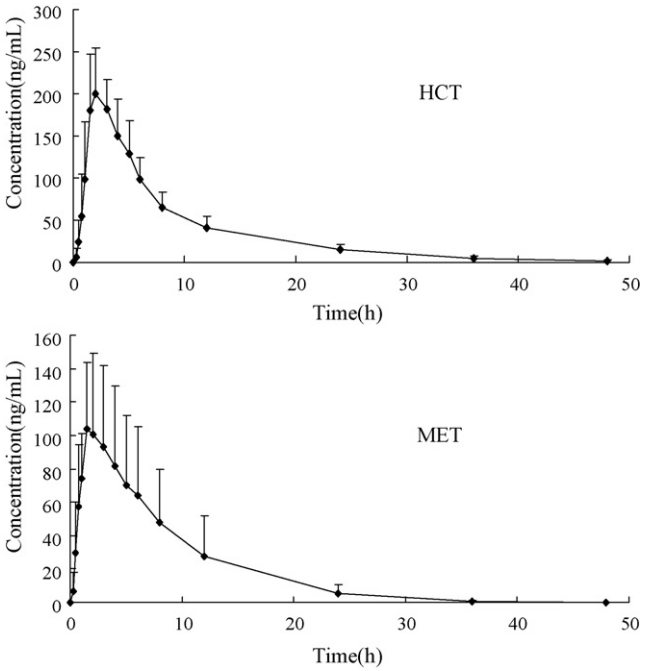

Fig. 4. Mean plasma concentration–time curves for HCT and MET in healthy volunteers after a single oral administration of a tablet containing 25 mg HCT and 50 mg MET (data are means ± SD, $n = 10$).

Table 3
PK parameters for HCT and MET in healthy volunteers after a single oral administration of a tablet containing 25 mg HCT and 50 mg MET (data are means ± SD, $n = 10$).

Parameters	HCT	MET
T_{max} (h)	2.30 ± 0.80	1.63 ± 0.62
C_{max} (ng/mL)	216 ± 54	117 ± 43
$AUC_{0-\infty}$ (ng ×h/mL)	1690 ± 402	957 ± 617
$t_{1/2}$ (h)	7.93 ± 1.21	4.42 ± 1.25

administration of a tablet containing 25 mg HCT and 50 mg MET. The plasma concentration–time profiles are illustrated in Fig. 4 and corresponding PK parameters are summarized in Table 3. For HCT, the time to reach maximum plasma concentration (T_{max}) and the elimination half-life ($t_{1/2}$) were similar to values previously determined for HCT given alone as a single oral 12 mg dose [12] and as an oral 25 mg dose at steady state [15]. In the latter case, the maximum plasma concentration (C_{max}) was similar to our value and the AUC_{0-12} was consistent with our value of $AUC_{0-\infty}$. For MET, T_{max} and $t_{1/2}$ were similar to values for MET given alone as a single oral 100 mg dose to a group of CYP2D6 poor metabolisers [16]. However, our values of C_{max} and $AUC_{0-\infty}$ are lower than predicted based on linear PK possibly reflecting a greater proportion of faster metabolisers in our healthy volunteers. Our PK results do not reveal any evidence for a drug–drug interaction between HCT and MET consistent with the results of previous studies [4,17].

4. Conclusions

A simple and sensitive LC–MS/MS method for the simultaneous quantitation of HCT and MET in human plasma has been developed and validated. Despite using an internal standard for each analyte, the method retains the advantages of a simple sample preparation step and a short run time through switching the ionization mode during a chromatographic run. The method has been successfully applied to a clinical PK study of HCT and MET administered in a combination tablet.

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