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Simultaneous quantitative determination of olmesartan and hydrochlorothiazide in human plasma and urine by liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

A specific, sensitive and rapid method based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) was developed for the simultaneous determination of olmesartan (OLM) and hydrochlorothiazide (HCTZ) in human plasma and urine. Solid-phase extraction (SPE) was used to isolate the analytes from biological matrices followed by injection of the extracts onto a C_{18} column with isocratic elution. Detection was carried out on a triple quadrupole tandem mass spectrometer in multiple reaction monitoring (MRM) mode using negative electrospray ionization (ESI). The method was validated over the concentration range of 1.00–1000 ng/mL and 5.00–5000 ng/mL for OLM in human plasma and urine as well as 0.500–200 ng/mL and 25.0–25,000 ng/mL for HCTZ in human plasma and urine, respectively. Inter- and intra-run precision of OLM and HCTZ were less than 15% and the accuracy was within 85–115% for both plasma and urine. The average extraction recoveries were 96.6% and 92.7% for OLM, and 87.2% and 72.1% for HCTZ in human plasma and urine, respectively. The linearity, recovery, matrix effect and stability were validated for OLM/HCTZ in human plasma and urine.

1. Introduction

Olmesartan medoxomil is an anti-hypertensive agent, which is a new potent and selective angiotensin AT1 receptor blocker [1]. It contains a medoxomil ester moiety and is cleaved rapidly by an endogenous esterase to release the active metabolite (olmesartan) [2]. Hydrochlorothiazide is a common diuretic and anti-hypertensive agent which reduces blood volume by increasing the excretion of sodium, chloride and water. The decrease in blood volume, however, causes counter-regulatory stimulation of the rennin–angiotensin–aldosterone system (RAAS) and the sympathetic nervous system [3]. During the therapy procedure of hypertension, combination therapy is needed to achieve adequate control of blood pressure, especially for those with Stage 2 hypertension (systolic blood pressure [SBP] ≥ 160 mmHg or diastolic blood pressure [DBP] ≥ 100 mmHg). Also, it is generally recommended that drugs with complementary mechanisms of action should be used [4]. Based on the different pharmacological mechanisms of the above two drugs and the character of activating the RAAS of HCTZ, olmesartan medoxomil/HCTZ combination tablet was developed recently to treat hypertension [5,6]. Therefore, an analytical method fit for the purpose of application to pharmacokinetics and drug–drug interaction studies of olmesartan medoxomil/HCTZ is required.

Due to the fact that olmesartan medoxomil hydrolyzes rapidly in plasma [7], determination of OLM in plasma is the only choice for the study of pharmacokinetic profile of olmesartan medoxomil. Determination of HCTZ or OLM in biological matrices have been individually achieved using HPLC-UV [8,9] and HPLC-MS/MS [10–12] methods. To date, no method has been reported to determine simultaneously the two analytes in biological matrices. In a drug–drug interaction study [13], the author used different methods to determine them separately (HPLC with fluorescence detection for OLM; HPLC-MS method for HCTZ). Consequently, analysis was rendered tedious and time-consuming, especially for those clinical studies with a considerable sample size. Therefore, a simple and rapid method to quantify simultaneously OLM and HCTZ is warranted.

Herein, a rapid and robust method based on HPLC-MS/MS was developed and fully validated to simultaneously quantify OLM and HCTZ in human plasma and urine.

2. Experimental

2.1. Chemicals

OLM (purity 95.7%) and RNH-6272 (I.S., calculated as 100%) were provided by Daiichi Sankyo Co., Ltd. (Japan); HCTZ (purity 99.4%)

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and hydroflumethiazide (HFTZ) (I.S., calculated as 100%) were provided by Shanghai Sankyo Co., Ltd. (China). Formic acid and aqueous ammonia were all analytical grade and purchased from Beijing Chemical Reagents Company (Beijing, China) and Beijing Chemical Plant (Beijing, China), respectively. Methanol and acetonitrile were both of chromatographic grades and were obtained from Burdick & Jackson Lab (New Jersey, USA). Drug-free human plasma was supplied by Peking Union Medical College Hospital Blood Bank. Drug-free human urine was obtained from six different healthy subjects who were drug free for at least two weeks. Distilled water was prepared by a Milli-Q water purifying system (Millipore, Bedford, USA).

2.2. Calibration standards (CS) and quality control (QC) samples in human plasma and urine

Stock solutions of OLM and HCTZ for CS and QC were prepared separately in methanol:water (1:1, v/v). Stock solutions of OLM and HFTZ were prepared in methanol. The concentrations of OLM and HCTZ stock solutions were both of 1.00 mg/mL. They were further diluted with methanol to yield working solutions at several concentration levels.

CS and QC samples in plasma and urine were prepared by diluting corresponding working solutions with drug-free human plasma and urine, respectively. The final calibration curve range of OLM and HCTZ were as follows: 1.00–1000 ng/mL and 0.500–200 ng/mL in plasma; 5.00–5000 ng/mL and 25.0–25,000 ng/mL in urine, respectively. The concentrations of QC sample of OLM and HCTZ were as follows: 2.50, 25.0 and 800 ng/mL and 1.00, 10.0 and 160 ng/mL in plasma; 10.0, 200 and 4000 ng/mL and 50.0, 1000 and 20,000 ng/mL in urine. The internal standard working solution was prepared in mobile phase. All of plasma and urine samples were stored at −30 °C and were prepared in subdued red light.

2.3. Extraction procedure

Calibration standard, QC, and clinical plasma and urine samples were purified with solid-phase extraction (SPE) using QUADRA 3 automation (TOMTEC Inc., Hamden, USA) in subdued red light. 0.2 mL of I.S. working solution and 0.2 mL of 2% formic acid solution were added to 0.2 mL of plasma sequentially. Following vortex, the mixture was loaded to Waters Oasis MCX SPE 96-well plate (10 mg) which was pretreated with 0.5 mL of 2% aqueous ammonia in methanol first and followed by 0.5 mL of 2% formic acid solution. SPE columns were washed with 0.3 mL of 2% formic acid solution, 0.3 mL of water, and 0.3 mL of 25% methanol solution in water sequentially. The column was vacuumed to dryness and the analytes were eluted twice with 0.1 mL of 85% methanol solution in water with 2% aqueous ammonia. The eluate was collected and evaporated to near-dryness (until approximately 50 μL liquid remained) under a nitrogen stream at ambient temperature, and then mixed with 0.15 mL of acetonitrile:water (1:1, v/v).

0.2 mL of internal standard (I.S.) working solution and 0.4 mL of 2% formic acid solution were added to 50 μL of urine sample. Preparation steps were the same as for plasma sample preparation.

2.4. Liquid chromatography/mass spectrometry

Plasma and urine samples were analyzed using a model 20A HPLC system (Shimadzu Co., Kyoto, Japan) coupled with API 4000 tandem MS (Applied Biosystems, CA, USA) equipped with electrospray interface. Analysis of OLM and HCTZ in plasma and urine samples was carried out on an XTerra MS C_{18} column (2.1 mm × 50 mm, 3.5 μm) protected by an XTerra MS C_{18} guard column (2.1 mm × 10 mm, 3.5 μm) at ambient temperature. The mobile phase was composed of acetonitrile/0.05% formic acid/methanol (60/36/4, v/v/v). The flow rate was 0.2 mL/min during 0–1.2 min and was changed into 0.35 mL/min during 1.2–2.0 min. The injection volumes were 10 μL and 5 μL for plasma and urine sample, respectively.

Detection was performed with an ionizing voltage of −3500 V. Ion source temperature was set at 350 °C with ultrahigh-purity nitrogen as curtain gas (10 p.s.i.), nebulizer and auxiliary gas were both of 40 p.s.i. Other mass-dependent parameters such as declustering potential (DP), entrance potential (EP), focusing potential (FP) and collision energy (CE) for each compound were determined in negative mode using standard solutions. Multiple reaction monitoring (MRM) was carried out using nitrogen as collision gas (6 p.s.i.) and with a dwell time of 200 ms for each transition. OLM, RNH-6272, HCTZ and HFTZ were detected by monitoring the transitions m/z 445.1 → 148.8, 459.1 → 162.9, 295.9 → 268.8 and 329.9 → 238.9 with collision energies of −46, −48, −28 and −36 eV, respectively.

2.5. Method validation

The method was validated for selectivity, matrix effect, precision, accuracy, linearity, sensitivity, recovery, and stability according to the US Food and Drug Administration (FDA) [14] and Chinese State Food and Drug Administration (SFDA) guidelines [15] for the validation of bioanalytical method.

The selectivity of this method was investigated by analyzing six individual human blank plasma and urine samples. Each blank sample was tested for interference using the present analytical method and was compared with spiked sample whose concentration of the analyte was at the LLOQ in plasma or urine.

Calibration standard samples in human plasma and urine were prepared for three separate batches. Intra- and inter-batch precision and accuracy were evaluated by measurement of OLM and HCTZ in plasma and urine in five replicates of QC samples at three different concentrations for three separate batches.

The extraction recoveries of OLM and HCTZ at three concentration levels were calculated as the ratio of calculated concentrations of QC samples spiked with and without extraction at the same concentration level. The extraction recoveries of RNH-6272 and HFTZ at the working concentration level were measured by comparing peak area of biological sample spiked before and after extraction.

The matrix effect was investigated by comparing the calculated OLM/HCTZ concentration in spiked QC samples in six biological matrices separately from different drug-free volunteers (A) and in mobile phase (B) at different concentration levels. Similarly, the matrix effect on the I.S. was measured from their peak areas at the working concentration level. Matrix effect was defined as the concentration or peak ratio (A/B × 100). During the preparation of QCs or blank samples at same concentration level, each individual's biological matrix was used only once. The inter-subject variability of matrix effect at every concentration level should be less than 15% for acceptable performance [16].

The stabilities of OLM and HCTZ in biological matrix and working solutions at different storage conditions were evaluated as follows and the results were expressed as percentage recoveries (concentration of sample under different storage condition/theory concentration). The stabilities of OLM and HCTZ working solutions were tested for 6 h at ambient temperature. The stabilities of OLM and HCTZ in plasma and urine sample at different concentrations were examined under different study conditions; i.e. standing at ambient temperature for 24 h (Bench-top stability) and storing at −30 °C for at least 2 months (Long-term stability). The stabilities of OLM and HCTZ in plasma and urine extracts were also tested by sitting samples at ambient temperature for 24 h (Autosam-

pler stability). Freeze/thaw stability was determined after freezing (−30 °C) and thawing QC samples for three cycles.

3. Results and discussion

3.1. HPLC-MS/MS optimization

Previous articles revealed that OLM and HCTZ were ionized in positive and negative ion mode, respectively [10–12]. Because we also found the higher signals for OLM in positive ion mode, the method was originally designed to detect OLM and HCTZ with switching between positive/negative (+/−) ionization, which needed at least 0.3 min for the separation interval between the peaks of OLM and HCTZ. After optimizing the electronic parameters (such as IS, DP, CE and EP) in the tandem MS (using an API 3000, Applied Biosystems, CA, USA), a HPLC-MS/MS method with +/− mode switching was developed. After we tried Thermo, Atlantis and XTerra C_{18} columns with various mobile phases (methanol, acetonitrile, formic and acetic acid, ammonium acetate and aqueous ammonia), XTerra column was selected because of full separation between OLM and HCTZ, and the highest sensitivity with weak basic (0.002% aqueous ammonia)-acetonitrile mobile phase system. However, the sensitivity was still unsatisfactory. Therefore, based on the presence of a carboxy group in OLM, we decided to try negative mode for both of them, using a more sensitive tandem mass spectrometer (API 4000), which removed the separation problem and met sensitivity requirement simultaneously. The product ion mass spectra of these compounds are depicted in Fig. 1 where $[M-H]^-$ of each compound was selected as precursor ion, and the most abundant fragment ion was chosen as the product ion in the MRM acquisition with the optimized collision energy for each compound.

After optimizing the parameters such as ionizing voltage, DP and EP to obtain de-protonated molecular ion $[M-H]^-$, we initially developed an HPLC separation on the XTerra column with a weakly basic-acetonitrile mobile phase system. Unfortunately, the poor shape of OLM observed suggested to us a mis-match between mobile phase and injection solution, which contained a little elution solution un-evaporated during SPE procedure. After that, a weak acid mobile phase was tried and selected since it yielded a good peak shape.

3.2. Extraction procedure optimization

Based on the structural characteristics of OLM and HCTZ containing secondary amines, an Oasis WCX SPE cartridge was selected to yield specified retention with OLM and HCTZ. Owing to the great difference of physical and chemical features between OLM and HCTZ (log P were 3.7 and 0.11; pKa_1 were 13.9 and 8.9), an careful and systemic development for SPE method has to be proceeded to retain firstly and elute OLM and HCTZ simultaneously. Two sets of eluting solutions (with and without aqueous ammonia) containing different composition were used to optimize rinsing and elution programs of SPE method and the peak area-composition% in different elution solution of OLM and HCTZ after elution are depicted with in Fig. 2. It was found that if the samples were washed with methanol/water (25/75, v/v) and eluted using 2% aqueous ammonia in methanol/water (85/15, v/v) during the SPE procedure, the highest and most robust extraction efficiency would be obtained. We modified the elution dryness procedure from full dryness to near 50 μL of residue (which mainly consisted of water) in order to save time because it is hard and time-consuming to evaporate water in slim and long 96-well collection tubes.

Direct dilution was first used to prepare urine samples because they are cleaner and have a lower sensitivity requirement than

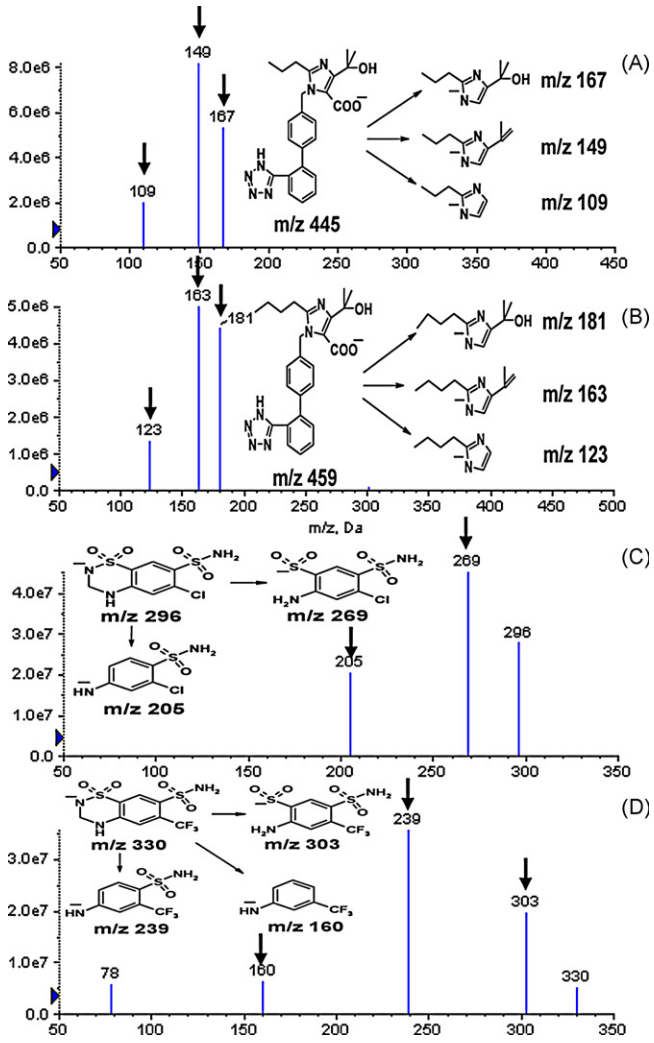

Fig. 1. Product ion spectrum of OLM (A), RNH-6272 (B), HCTZ (C) and HFTZ (D), and their proposed fragmentation pathways (arrow: main fragment ion).

plasma. However, interference was found in blank samples. Hence, a similar SPE procedure to that developed for plasma was used to purify OLM and HCTZ in urine samples. Finally, typical MRM chromatograms obtained from blank sample, LLOQ sample and a subject's sample in plasma and urine after dosing of 20/12.5 mg Olmesartan Medoxomil/HCTZ tablet are presented in Figs. 3 and 4, respectively.

3.3. Olmesartan method optimization

A method for the determination of OLM has been developed and validated using SPE followed by HPLC-MS/MS method with a LLOQ of 0.2 ng/mL in plasma [12]. In this article, similar procedures were used for the simultaneous determination of OLM and HCTZ in biological matrices. However, the additional HCTZ urged us to change some elements of the previous method, such as SPE (HLB vs MCX Cartridge), HPLC (Hypersil vs XTerra) and MS/MS (positive vs negative ionization).

3.4. Validation

3.4.1. Selectivity and sensitivity

No endogenous source of interference was observed at the retention times of the analytes at approximate 0.9 min. Typical chromatograms obtained from blank plasma, LLOQ plasma

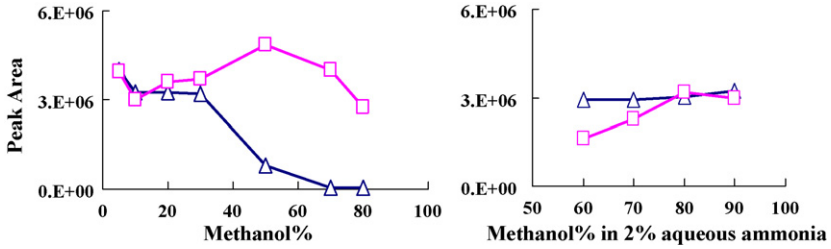

Fig. 2. The peak area of OLM (square) and HCTZ (triangle)-methanol% in water (A) and peak area of OLM (square) and HCTZ (triangle)-methanol% in 2% aqueous ammonia (B) curve.

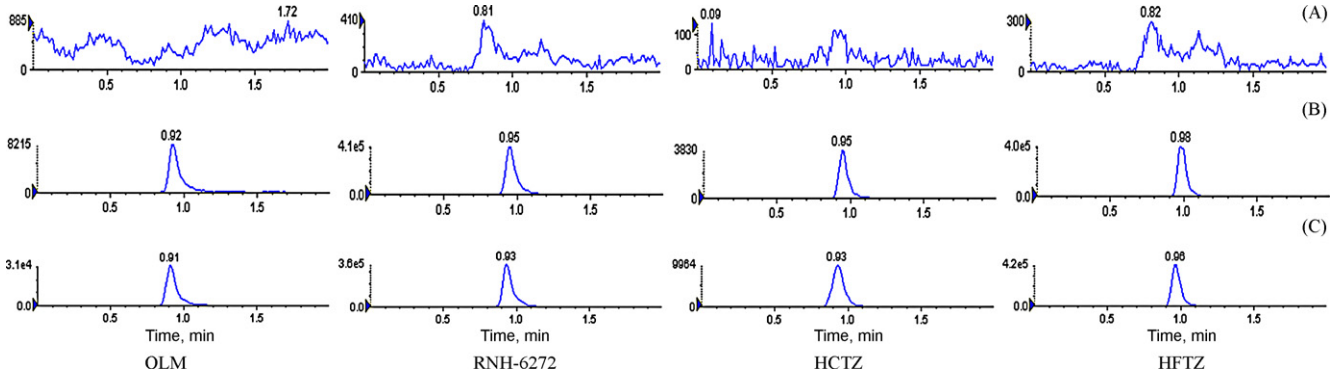

Fig. 3. Typical MRM chromatograms obtained from blank plasma (A), LLOQ plasma sample (B) and a subject's plasma sample (C) collected at 24 h post-dose of 20/12.5 mg olmesartan medoxomil/HCTZ tablet.

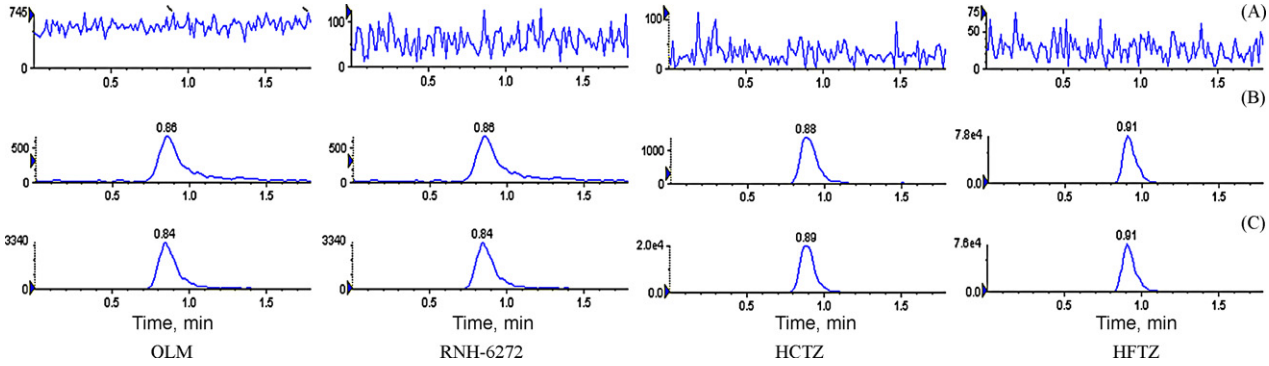

Fig. 4. Typical MRM chromatograms obtained from blank urine (A), LLOQ urine sample (B) and a subject's urine sample (C) dosing 20/12.5 mg olmesartan medoxomil/HCTZ tablet.

sample and a subject's plasma sample collected 24 h post-dose of 20/12.5 mg olmesartan medoxomil/HCTZ tablet are presented in Fig. 3. Representative chromatograms of blank urine, LLOQ urine sample and a subject's urine sample dosing 20/12.5 mg olmesartan medoxomil/HCTZ tablet are presented in Fig. 4.

3.4.2. Linearity

The calibration curve of OLM/HCTZ in plasma and urine are both regressed using linear equation with a weighting factor of $1/x^2$ except for OLM in plasma, for which quadratic regression was used because of some saturation. Coefficient of correlation of all calibration curves are more than 0.99.

Table 1
Results of extraction recovery and matrix effect of OLM, RNH-6272, HCTZ and HFTZ in human plasma and urine ($n = 6$).

	OLM			RNH-6272	HCTZ			HFTZ
	QCL	QCM	QCH		QCL	QCM	QCH	
Human plasma								
Nominal concentration (ng/mL)	2.50	25.0	800	40.0	1.00	10.0	160	10.0
Recovery% (CV%)	88.3 (6.1)	96.8 (4.3)	104.6 (3.9)	94.2 (7.2)	86.1 (5.1)	82.7 (11.0)	92.8 (8.3)	78.2 (14.6)
Matrix effect (CV%)	7.8	3.9	8.3	9.3	4.3	13.8	7.1	8.4
Human urine								
Nominal concentration (ng/mL)	10.0	200	4000	20.0	50.0	1000	20000	100
Recovery% (CV%)	101.4 (13.9)	83.1 (3.9)	93.5 (7.8)	45.6 (8.7)	77.8 (11.7)	64.2 (12.1)	74.4 (9.4)	55.1 (5.4)
Matrix effect (CV%)	6.8	1.6	6.8	2.8	6.9	11.2	5.3	4.9

Table 2
Accuracy and inter, intra-precision for the detection of OLM and HCTZ in human plasma.

	OLM				HCTZ			
	LLOQ	QCL	QCM	QCH	LLOQ	QCL	QCM	QCH
Nominal concentration (ng/mL)	1.00	2.50	25.0	800	0.500	1.00	10.0	160
Intra-batch ($N = 5$)								
Mean	0.957	2.37	26.7	899	0.482	0.970	11.0	155
CV (%)	11.1	2.4	3.9	6.8	8.5	3.4	4.3	3.7
RE (%)	−4.3	−5.2	6.7	12.4	−3.6	−3	9.6	−3.2
Inter-batch ($N = 3$)								
Mean	ND	2.46	25.1	851	ND	0.990	10.1	155
CV (%)	ND	5.3	5.7	6.0	ND	5.3	7.4	3.1
RE (%)	ND	−1.7	0.5	6.3	ND	−0.6	0.9	−3.3

ND: Not done.

Table 3
Accuracy and inter, intra-precision for the detection of OLM and HCTZ in human urine.

	OLM				HCTZ			
	LLOQ	QCL	QCM	QCH	LLOQ	QCL	QCM	QCH
Nominal concentration (ng/mL)	5.00	10.0	200	4000	25.0	50.0	1000	20000
Intra-batch ($N = 5$)								
Mean	5.43	10.4	190	3950	25.5	51.1	1020	20300
CV (%)	9.5	11.3	3.1	2.7	4.6	6.3	3.8	4.8
RE (%)	8.6	3.7	−4.9	−1.3	1.9	2.2	1.8	1.3
Inter-batch ($N = 3$)								
Mean	ND	10.3	190	3910	ND	51.0	996	20000
CV (%)	ND	10.9	4.9	3.0	ND	5.6	5.2	3.7
RE (%)	ND	2.6	−5.0	−2.3	ND	2.0	−0.4	0.0

ND: Not done.

3.4.3. Recovery

The absolute recoveries observed for the sample preparation method from plasma and urine are shown in Table 1 (value and CV%, $n = 5$). Recoveries are more than 64.2% at different concentrations for both OLM and HCTZ in plasma or urine with little variability. The recoveries of RNH-6272 and HFTZ in plasma are more than 78.2%, which are similar to those of OLM and HCTZ. The recoveries of them in urine are only 45.6% and 55.1%, which are lower than those of OLM and HLTZ in urine. The difference in recovery could be caused by no systemic SPE method development for urine samples. However, the variance of recoveries of RNH-6272 and HFTZ in urine samples are small (less than 8.7%), and almost all variance of ratios of OLM/RNH-6272 and HCTZ/HFTZ in urine QC samples (Table 2) are smaller than the variance of recoveries of internal standard in urine (8.7%) are observed, which means correction function of RNH-6272/HFTZ as internal standard for analysis still work well.

3.4.4. Matrix effect

Matrix effects and inter-subject variability data from plasma and urine of individual subjects who not receiving OLM/HCTZ are summarized in Table 1. Our results of the inter-subject variability were calculated as up to 13.8% in both plasma and urine. It indicates that the analytical method was reasonably free from effects of endogenous substances in human plasma and urine.

3.4.5. Precision, accuracy and LLOQ

Five quality control samples at each concentration level were processed and calculated for three batches to provide precision

Table 4
Stability results of OLM and HCTZ ($N = 5$).

Mean (RE%, CV%)	OLM			HCTZ		
Stock solution[a]						
Ambient for 6 h	(−2.9, 0.9)			(−5.6, 1.3)		
−30 °C for 58 day	(1.7, 1.3)			(−0.3, 2.3)		
Human plasma	QCL	QCM	QCH	QCL	QCM	QCH
Nominal concentration (ng/mL)	2.50	25.0	800	1.00	10.0	160
Bench-top stability (ambient for 24 h)	2.39 (−4.3, 7.4)	25.0 (0.0, 2.2)	790 (−1.3, 1.2)	0.956 (−4.4, 4.9)	10.4 (3.8, 2.5)	153 (−4.6, 3.0)
Autosampler stability (10 °C for 24 h)	2.52 (0.7, 3.5)	26.5 (6.1, 3.7)	792 (−1.1, 3.8)	0.983 (−1.7, 2.7)	10.3 (2.5, 2.6)	150 (−6.0, 1.2)
Freeze–thraw stability (3 cycles)	2.57 (2.7, 7.0)	24.4 (−2.5, 1.5)	820 (2.5, 0.9)	0.987 (−1.3, 8.8)	10.4 (3.8, 3.4)	158 (−1.4, 1.1)
Long-term stability (30 °C for 73 days)	2.55 (1.8, 10.2)	22.5 (−9.9, 4.5)	834 (4.2, 5.4)	0.962 (−3.8, 12.3)	9.6 (−4.2, 5.7)	170 (6.5, 4.9)
Human urine	QCL	QCM	QCH	QCL	QCM	QCH
Nominal concentration (ng/mL)	10.0	200	4000	50.0	1000	20000
Bench-top stability (ambient for 24 h)	8.80 (−12.0, 3.5)	200 (0.1, 2.6)	3908 (−2.3, 5.2)	47.0 (−6.0, 2.3)	991 (−0.9, 1.5)	19100 (−4.5, 3.9)
Autosampler stability (10 °C for 24 h)	9.36 (−6.4, 5.8)	196 (−1.9, 2.4)	3712 (−7.2, 2.9)	48.8 (−2.5, 5.0)	1021 (2.1, 2.3)	18320 (−8.4, 2.8)
Freeze–thraw stability (3 cycles)	10.0 (0.1, 9.8)	202 (0.9, 2.2)	4018 (0.5, 4.5)	52.3 (4.6, 6.0)	1044 (4.4, 3.1)	19680 (−1.6, 7.7)
Long-term stability (30 °C for 113 days)	9.19 (−8.1, 9.9)	197 (−1.3, 1.5)	4178 (4.5, 5.8)	44.7 (−10.6, 4.0)	1080 (8.0, 2.4)	21040 (5.2, 7.1)

[a] Showed as (RE%, CV%).

(CV%) and accuracy of this method. Five LLOQ samples were also analyzed to provide further precision and accuracy. The intra- and inter-day precision and accuracy data for OLM/HCTZ in plasma and urine are summarized in Tables 2 and 3, respectively. The results shows that the intra- and inter-precision were less than 15% for both compounds and intra- and inter-accuracy were within the range of ±15%.

3.4.6. Stability

The stability tests of the analytes were designed to cover expected conditions concerning the handling of clinical samples. The stabilities of the analytes in human plasma and urine were investigated under various storage and processing conditions. The results are summarized in Table 4. The results indicate that OLM and HCTZ were stable for the entire period of the experiment.

3.4.7. Carryover test

The MRM chromatograms of double blank (free HCTZ, OLM and internal standards) analyzed by following the upper limit of quantification samples had showed that there was no carryover.

4. Conclusion

A sensitive and selective HPLC-MS/MS method using a SPE sample preparation procedure has been developed and validated for the determination of OLM and HCTZ in plasma and urine. The extraction procedure and HPLC-MS/MS conditions were optimized in order to improve the sensitivity and robustness of the method. The procedure was fully validated to meet the requirements for sensitivity, accuracy and precision from State Food and Drug Administration and GLP Guidelines for Industry.

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