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Short communication

Validation of a sensitive LC/MS/MS method for simultaneous quantitation of flupentixol and melitracen in human plasma

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Abstract

A sensitive method has been developed and validated, using LC/ESI-MS/MS, for simultaneous quantitation of flupentixol and melitracen—antidepressant drugs, in human plasma. The quantitation of the target compounds was determined in a positive ion mode and multiple reaction monitoring (MRM). The method involved a repeated liquid–liquid extraction with diethyl ether and analytes were chromatographed on a C_8 chromatographic column by elution with acetonitrile–water–formic acid (36:64:1, v/v/v) and analyzed by tandem mass spectrometry. The method was validated over the concentration ranges of 26.1–2090 pg/ml for flupentixol and 0.206–4120 ng/ml for melitracen. The correlation coefficients of both analyst were >0.998 for six sets of calibration curves. The recovery was 60.9–75.1% for flupentixol, melitracen and internal standard. The lower limit of quantitation (LLOQ) detection was 26.1 pg/ml for flupentixol and 0.206 ng/ml for melitracen. Intra- and inter-day precision of the assay at three concentrations were 2.15–5.92% with accuracy of 97.6–103.0% for flupentixol and 0.5–6.36% with accuracy of 98.7–101.7% for melitracen. Stability of compounds was established in a battery of stability studies, i.e., bench-top, autosampler and long-term storage stability as well as freeze/thaw cycles. The method proved to be suitable for bioequivalence study of flupentixol and melitracen in healthy human male volunteers.

Keywords: LC/MS/MS; Flupentixol; Melitracen; Validation

1. Introduction

Antidepressants are clearly beneficial in the treatment of major depression. The use of cyclic antidepressants, such as imipramine, doxepine, chlorimipramine, has been increasingly prevalent for the treatment of depression. However, these drugs are frequently associated with severe neurological side effects including depressed mood, sleep disturbances and autonomic dysregulations [1,2]. The most worrisome side effects of traditional antidepressants are cardiovascular. And flupentixol (the *cis*-isomer is pharmacologically) has antipsychotic effects at higher does. The combination of flupentixol and melitracen is used as antidepressant drug without any serious side effects duo to lower drug dosage (0.5 mg of flupentixol and 10 mg of melitracen per tablet) [3].

Flupentixol in human plasma was detected up to 96 h after oral administration of 0.5 mg, so the LLOQ of flupentixol was especially focused in the investigation. Several analytical methods have been developed to determine flupentixol in biological samples with HPLC with UV or mass spectrometry. The LLOQ of HPLC-UV method [4,5] was 0.5 ng/ml for flupentixol in human serum. The method used relatively large sample amount (1ml), and the analytical time of HPLC-UV for each sample was 30 min, which was too long to pharmacokinetic study. Plasma samples were direct injected into LC-APCI-MS–MS [6] with an ion trap detector system able to on-line sample preparation for flupentixol in human plasma . The LLOQ was 0.523 ng/ml. The LLOQ of the method was 1 ng/ml for flupentixol in human serum for LC-ESI-MS method [7]. The mass detection was in the multiple reaction monitoring (MRM) mode. Weinmann et al. [8] detected the flupentixol in hair by LC–MS–MS in MRM mode. The LLOQ was 0.05 ng/mg flupentixol in hair. Kollroser et al. [9] detected melitracen in human plasma by direct-injection HPLC-APCI-MS–MS with an ion trap detector. The LLOQ of

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melitracen was 10 μg/l. T. Shinozuka et al. [10] analyzed melitracen in human plasma by solid-phase extraction and LC/MS with sonic spray ionization method. The LLOQ of the method was 0.15 μg/ml. However, the sensitivity of these methods is generally inadequate for studies involving low oral doses for topical formulations currently under development. And there has been no report on the simultaneous quantitation of flupentixol and melitracen in human plasma.

In the present investigation, a sensitive LC tandem triple quadrupole mass spectrometer with multiple reaction monitoring (MRM) scan mode was developed with the prime objective of estimating flupentixol and melitracen in human plasma samples from healthy human male volunteers. For sample purification, repeated liquid–liquid extraction (LLE) was employed which offered reasonable recovery of drugs from human plasma matrix. It allows the lower limit of quantitation to 26.1 pg/ml for flupentixol and 0.206 ng/ml for melitracen. The method has been used to successfully support bioequivalence study of two oral formulations of flupentixol–melitracen (0.5–10 mg per tablet).

2. Experimental

2.1. Chemicals and reagents

Flupentixol, melitracen and internal standard (IS) (carbamazepine and mosapride) were provided by Keride pharma, Chendu province, China (all purity ≥ 99.6%). Acetonitrile (HPLC grade) was purchased from Fisherchemicals, USA. Formic acid and diethyl ether (analytical grade) were purchased from Hongxing chemical factory, Beijing, China. Distilled water, prepared from demineralized water, was used throughout the study. Blank human plasma was obtained from the affiliated hospital of the Academy of Military Medical Sciences, China.

2.2. Calibration standards and quality control (QC)

Standard stock solution and IS stock solutions of mosapride (IS of flupentixol) and carbamazepine (IS of melitracen) were prepared from solid powders and dissolved into mobile phase. Standard stock solutions (Flupentixol 2.09 mg/ml, Melitracen 4.12 mg/ml) and IS stock solution (mosapride 2.06 mg/ml, carbamazepine 1.8 mg/ml) were stored at approximately 4 °C. Standard curve and QC samples were prepared by diluting stock solutions with blank human plasma. The concentrations of standard curve and QC samples are summarized in Table 1.

2.3. Instrumentation

2.3.1. Chromatographic conditions

An Agilent 1100 system (Wilmington, DE, USA) consisting of a vacuum degasser, a binary pump, a column oven and an autosampler was used for solvent and sample delivery. Chromatography was carried out using a 150 mm × 4.6 mm, particle size 5 μm, Agilent Zorbax Eclipse XDB-C_8 column with a flow rate of 1.0 ml/min. The column was exposed at room temperature. Mobile phase consisted of acetonitrile–water–formic acid (36:64:1, v/v/v).

2.3.2. Mass spectrometric conditions

The analytes and IS were detected using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Ontario, Canada) equipped with a Turbo spray interface. Zero-air was used as source gas while ultra high pure (UHP) nitrogen was used as both curtain and collision gases. The mass spectrometer was operated in a positive ion multiple reaction monitoring (MRM) mode. The MRM transitions for analytes and IS were shown on Table 2. The ion source parameters were set as follows: collision gas = 6 p.s.i., curtain gas = 40 p.s.i., gas1 = 60 p.s.i., gas2 = 50 p.s.i., ionspray voltage = 5500 V, temperature = 400 °C. The declustering potential (DP), collision energy (CE) and other compound parameters for the analytes and IS were optimized individually and were shown on Table 2.

2.4. Sample preparation

Prior to assay, frozen human plasma samples were thawed at ambient temperature and centrifuged at 3000 rpm for 5 min at 4 °C to precipitate solids. In the following order, 0.04 ml of IS work solution (0.625 ng/ml mosapride and 18 ng/ml carbamazepine) was added into each glass tubes except for blank plasma. 0.5 ml of standards, QCs, study samples and blank plasma were transferred into the glass tubes. After vortex for 30 s, 0.2 ml of 0.2 M Na_3PO_4 aqueous solution was added to each tube and then followed by another 30 s mixing. After 3 ml diethyl ether was transferred inward, the tubes were stopper well and shaken vigorously for approximately 3 min. Following centrifugation at 3000 rpm for 10 min, upper organic phase was transferred out. Another 3 ml diethyl ether was added to aqueous layer and the same extraction procedure was repeated. The organic layers from two extraction procedures were mixed together and evaporated to dryness under a slightly heated stream of nitrogen at approximately 30 °C. The residue was reconstituted with 0.2 ml mobile phase. 0.05 ml sample was injected to LC/MS/MS.

2.5. Data acquisition and analysis

Data acquisition was performed using Analyst 1.4 software (Applied Biosystems-SCIEX). Calibration curves were constructed using the peak area ratios of analytes to IS by weighted

Table 1
Standard solution concentrations

Standard and QCs	Flupentixol (pg/ml)	Melitracen (ng/ml)
Standard 1	26.1	0.206
Standard 2	52.5	2.06
Standard 3	104	20.6
Standard 4	209	51.5
Standard 5	261	103
Standard 6	522	206
Standard 7	1040	2060
Standard 8	2090	4120
QC1	26.1	0.206
QC2	261	103
QC3	2090	4120

Table 2
Optimized parameters for MRM analysis of flupentixol, melitracen, mosapride and carbamazepine

Compound	Transition (*m/z*)	Dwell time (ms)	DP (V)	CE (eV)	EP (V)	CXP (V)
Flupentixol	435.2/305.2	100	68	42	10	10
Melitracen	292.3/247.2	100	60	26	10	10
Mosapride (IS)	422.2/198.2	100	55	32	10	10
Carbamazepine (IS)	237.2/194.2	100	52	28	10	10

least square linear regression. Test samples and quality control samples were then interpolated from the calibration curves to obtain the concentrations of the respective analytes.

2.6. Method validation

Analytical method validation was performed in accordance to the recommendations published by the FDA [11].

Specificity was ascertained by analyzing six blank human plasma samples without adding IS to determine the interference with the analytes. Matrix effects for analytes were evaluated by comparing the peak areas of analyte in extracted samples of blank plasma from six different drug free volunteers spiked with known concentrations with the corresponding peak areas obtained by direct injection of standard solutions. Matrix effects for the IS were also investigated. The interference between flupentixol and melitracen was observed. The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met: The analyte response at the lowest limit of quantitation should be at least five times the response compared to blank response. Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was the concentration with signal-to-noise ratio of 3. Six sets of calibration curves ranging from 26.1 to 2090 pg/ml for flupentixol, from 0.206 to 4120 ng/ml for melitracen, respectively, were constructed by plotting the peak area ratios of analyte/IS versus analyte concentrations in blank human plasma. The intra-day precision and accuracy were estimated by analyzing six replicates at three different QC levels. The inter-assay precision was determined by analyzing three level QC samples on six different runs. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (R.S.D.). The extraction recoveries of flupentixol and melitracen at three QC levels were determined by comparing peak area of the analytes obtained from plasma samples with the analytes spiked before extraction to those spiked after the extraction. QC samples prepared to test stability were subjected to short-term (12 h) at room temperature, 48 h autosampler at 18 °C, 48 h refrigeration at 4 °C, two freeze-thaw cycles at −70 ± 5 °C, and long-term at −70 ± 5 °C for 30 days stability tests.

2.7. Application of the assay

The method described in this paper was applied to a bioequivalence study of two oral formulation of flupentixol 0.5 mg–melitracen 10 mg tablet of Keride pharma, Chendu province, China (test formulation, lot no. 060101) versus that of Lundberk pharma, Denmark (standard reference formulation, lot no. 2076964).

Eighteen healthy male volunteers were selected for the study. The study followed a single dose, two-way randomized crossover design with a 2-week washout period between doses. Blood samples were collected at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 11, 15, 24, 36, 48, 96 h post-dosing. Samples were stored at −70 °C until analyzed. The bioequivalence of the two formulations was assessed according FDA guidelines [11].

3. Results and discussion

3.1. Bio-analytical method validation

3.1.1. MS/MS optimization

Flupentixol and melitracen are both weak bases, therefore, the mass spectrometer was operated in the positive ion multiple reaction monitoring mode in the LC–MS/MS analysis. ESI spectra revealed higher signals for *m/z* 435.2 (flupentixol) and *m/z* 292.3 (melitracen). The full scan spectra were dominated by protonated molecules $[M+H]^+$. The product ion mass spectrums of two protonated molecular ions are showed in Fig. 1 in which the most abundant ions were observed at *m/z* 305.2 (flupentixol) and *m/z* 247.2 (melitracen). By monitoring the product ions, a highly sensitive assay for flupentixol and melitracen was developed. Additional tuning of ESI source and collision-induced dissociation (CID) parameters onto the transition *m/z* 435.2 → 305.2 (flupentixol) and *m/z* 295.3 → 247.2 further improved the sensitivity.

Although, the stable isotope labeled compounds of the analytes would be the ideal IS, structurally related compounds were commercially available with similar chromatographic behaviors, mass spectrometric behaviors and extraction characteristics. Therefore, mosapride was chosen as IS for flupentixol and carbamazepine as IS for melitracen. In the positive ESI mode, mosapride and carbamazepine predominately formed the protonated molecule $[M+H]^+$ in full scan spectrum. To determine mosapride and carbamazepine using the MRM mode, full scan and product ion spectra of mosapride and carbamazepine were investigated. Fig. 1 shows product ion spectrum of $[M+H]^+$ ions of mosapride and carbamazepine. The major fragment ion at *m/z* 422.2 → 198.2 (mosapride) and *m/z* 237.2 → 194.2 (carbamazepine) were chosen in the MRM.

3.1.2. Chromatography

The chromatographic conditions were investigated to optimize sensitivity, speed, and peak shape. Acetonitrile was chosen

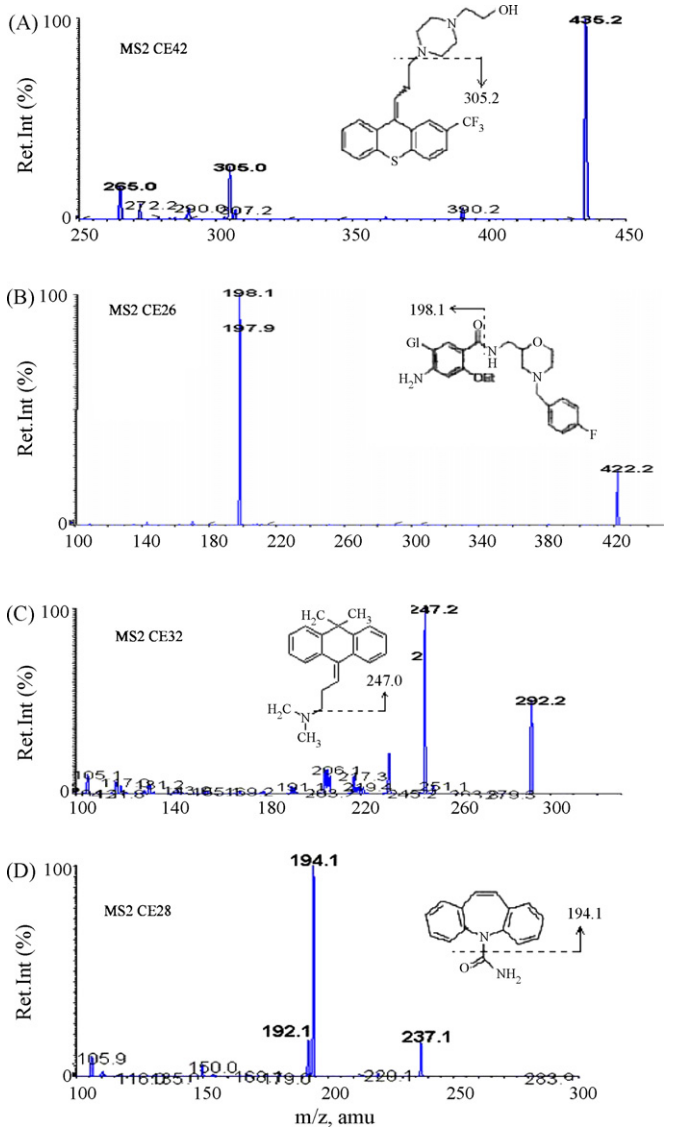

Fig. 1. Positive ESI mass spectrum of flupentixol (A), mosapride (B), melitrancen (C) and carbamazepine (D).

as the organic solvent because it provided a higher sensitivity and lower background noise than methanol. The compositions of mobile phase were optimized by flow injection analyses with carrying percentages of acetonitrile. It was found that organic solvent content about 36% in HPLC system decreased the background noise and provided rapid separation and stable MS signal throughout the analytical run, allowing an enhancement of sensitivity. But when the percentage of acetonitrile was increased to 36%, the reproducibility decreased. It was also found that the presence of a low amount of formic acid in the mobile phase could improve the sensitivity by promoting the ionization of the analytes. To achieve symmetrical peak shapes, a short chromatographic analysis time, and to eliminate the matrix effect, a mobile phase consisting of acetonitrile–water–formic acid (36:64:1, v/v/v) was used in the experiment. Under the optimum conditions, analytes and IS were free of interference from endogenous substances.

3.1.3. Sample preparation

Due to the complex nature of plasma, a sample pre-treatment is often needed to remove protein and potential interferences prior to LC–MS/MS analysis. Currently, the most widely employed biological sample preparation methodologies are protein precipitation (PPT) [12,13], solid phase extraction (SPE) [14,15], and liquid–liquid extraction (LLE) [16,17]. As the PPT procedure has the advantages of simplicity and universality for drug molecules in plasma, our initial approach of developing an assay for flupentixol and melitracen in plasma was based on PPT with methanol and acetonitrile. However, this technique resulted in strong interferences from the sample matrix and low recoveries of both analytes and IS. LLE was adopted in the end because this technique cannot only purify but also concentrate the sample. Ethyl acetate, *n*-hexane, diethyl ether, *n*-hexane:isopropanol (95:5, v/v), ethyl acetate:*n*-hexane (1:1, v/v), ethyl acetate:*n*-hexane (2:1, v/v), ethyl acetate:*n*-hexane (1:2, v/v), were all tested, and finally diethyl ether was adopted because of its high extraction efficiency and less interference. Since both analytes are weak bases, they are present as free bases at pH > 10. Na_3PO_4 solution was added into the plasma samples to accelerate the drugs dissociation from the plasma and reduce interference from endogenous substance which were of acid nature. In the present experiment, it was found that the recovery of analytes was not reasonable with extraction in the plastic tubes or extraction only one time. The reason may be that the analytes were prone to adhere to the plastic tubes. Therefore, a sample preparation method of repeated liquid–liquid extraction in glass tubes was adopted.

3.1.4. Specificity

No significant interfering peaks were observed at the retention times of flupentixol, melitracen and IS in blank plasma extraction which are shown in Fig. 2A. No matrix effect for analytes (flupentixol R.S.D. = 3.8%, melitracen R.S.D. = 7.8%) and IS (mosapride R.S.D. = 5.2%, carbamazepine R.S.D. = 6.3%) was observed for six different plasma pools indicating that no undetected co-eluting compounds that could influence the ionization of the analytes. There was no interference between flupentixol and melitracen from Fig. 2B.

3.1.5. Limit of quantitation, linearity

For this method, the lower limit of quantitation (LLQQ) was 26.1 pg/ml for flupentixol, with an S/N ration of 8 and 0.206 ng/ml for melitracen with an S/N ration of 40.6. Representative chromatograms of LLOQ samples were shown in Fig. 2B. The LOD was 19.5 pg/ml for flupentixol and 0.05 ng/ml for melitracen. The coefficient of determination ($r2$) during the validation ($n = 6$) was both >0.998 for flupentixol and melitracen. The calibration range was from 26.1 to 2090 pg/ml ($Y = 18.4 \pm 0.02C + 0.121 \pm 0.01$) for flupentixol and 0.206–4120 ng/ml ($Y = 1.55 \pm 0.1C + 0.0819 \pm 0.04$) for melitracen.

3.1.6. Recovery from plasma matrix

The mean extraction recoveries of flupentixol and melitracen were more than 60.9 and 72.9%, respectively. To IS,

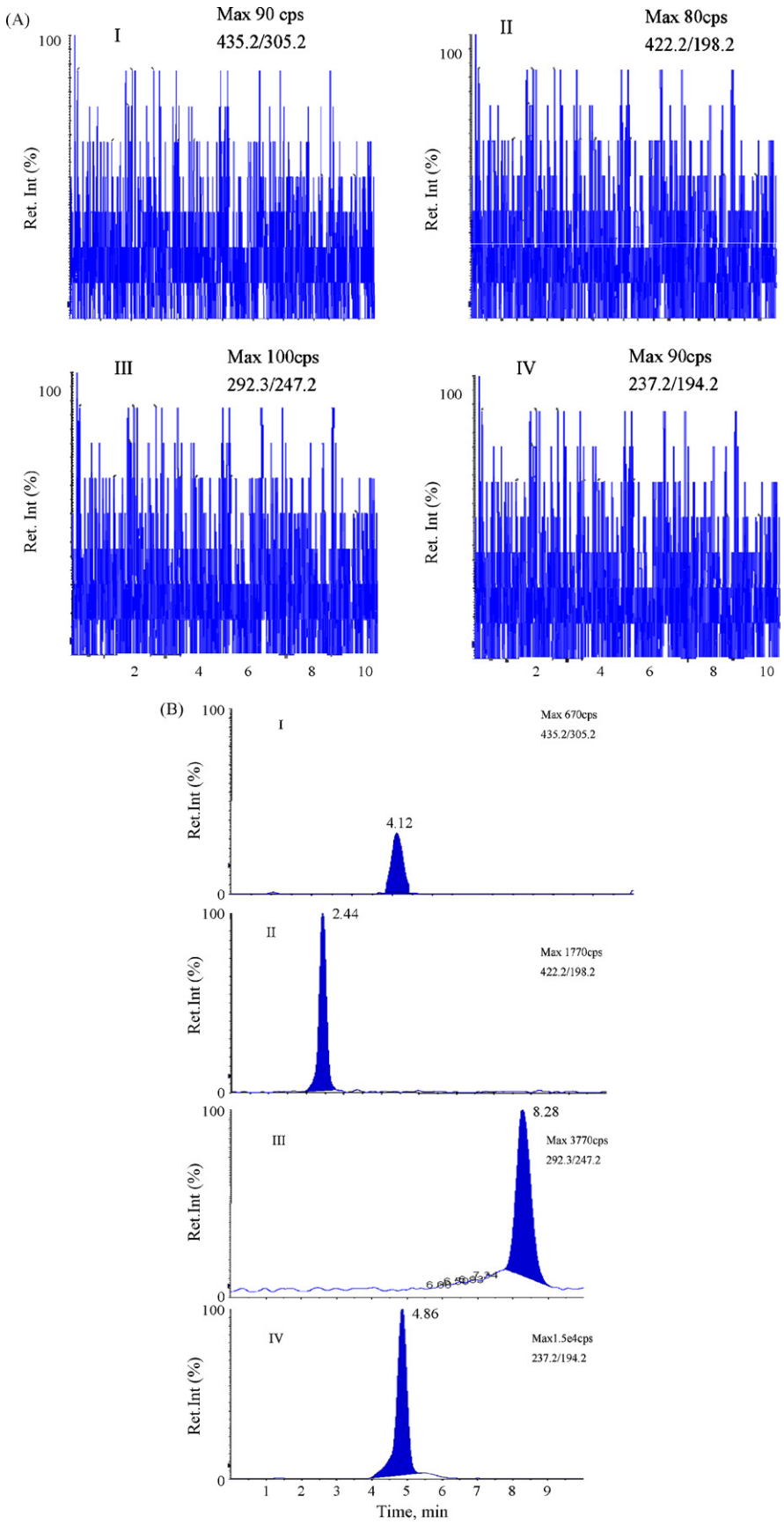

Fig. 2. Representative chromatogram for subject sample (I) flupentixol, (II) mosapride (IS for flupentixol), (III) melitracen, (IV) carbamazepine (IS for melitracen). (A) Blank plasma sample; (B) plasma sample spiked with flupentixol at LLOQ (26.1 pg/ml), melitracen at LLOQ (0.206 ng/ml) and IS; (C) plasma sample 5.5 h after oral dose of 0.5 mg flupentixol–10 mg melitracen.

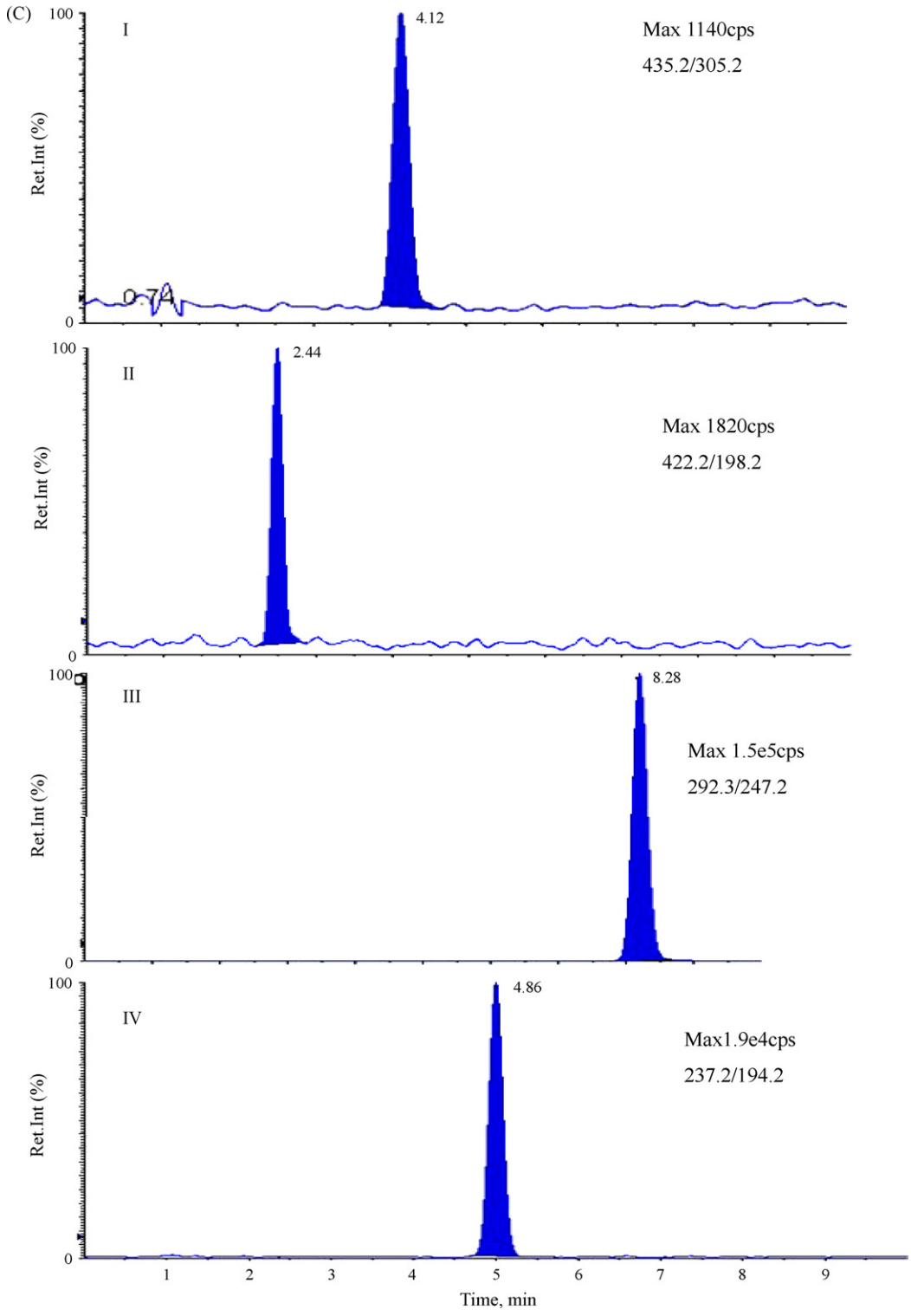

Fig. 2. (*Continued*).

the mean recoveries for mosapride and carbamazepine were 75.7 and 71.7%, respectively. The results are indicated in Table 3.

3.1.7. Accuracy and precision

Data for intra- and inter-day precision and accuracy of the assay were summarized in Table 4. The intra-day accuracy ranged between 99.6 and 101.6% with a precision of 2.15–5.74%, the inter-day accuracy between 99.0 and 101.2% with a precision of 2.52–5.92% for flupentixol. And the intra-day accuracy ranged between 97.6 and 103.0% with a precision of 0.5–4.8%, the inter-day accuracy between 98.7 and 101.9% with a precision of 4.15–6.36% for melitracen.

Table 3
Extraction recoveries of flupentixol–melitracen and IS from human plasma[a]

	QC1		QC2		QC3		IS	
	Flupentixol	Melitracen	Flupentixol	Melitracen	Flupentixol	Melitracen	Mosapride	Carbamazepine
Concentration	26.1 pg/ml	0.206 ng/ml	261 pg/ml	103 ng/ml	2090 pg/ml	4120 ng/ml	0.265 ng/ml	18 ng/ml
Peak area[b] (A)	1030	24200	7170	1320000	27600	3.13e7	738	5380
Peak area[c] (B)	651.4	18180	4368	955600	18080	2.38e7	558.8	3826
Recovery[d] (%)	63.2	75.1	60.9	72.9	65.5	76.1	75.7	71.1
CV (%)	2.49	7.90	2.65	5.5	3.0	3.0	3.67	2.53

[a] $n = 5$.
[b] Standard spiked after extraction.
[c] Standard spiked before extraction.
[d] Extraction recovery (%) expressed as the ratio of the mean peak area of the analytes spiked into plasma before extraction (B) to the mean peak area of the analytes spiked into plasma after extraction (A).

Table 4
Intra- day and inter-day accuracy and precision of flupentixol and melitracen in human plasma

	QC1		QC2		QC3	
	Flupentixol 26.1 pg/ml	Melitracen 0.206 ng/ml	Flupentixol 261 pg/ml	Melitracen 103 ng/ml	Flupentixol 2090 pg/ml	Melitracen 4120 ng/ml
Intra-day ($n = 6$)						
Mean concentration founded	26.5	0.205	271	100	2175	4243
CV (%)	2.15	0.50	5.53	3.83	5.74	4.80
Accuracy (%)	101.6	99.7	99.6	97.6	101.1	103.0
Inter-day ($n = 6$)						
Mean concentration founded	27.0	0.204	262	101.7	2130	4168
CV (%)	2.52	4.15	3.88	3.59	5.92	6.36
Accuracy (%)	99.0	98.7	101.2	101.7	100.2	101.9

Table 5
Summary of stability of flupentixol and melitracen in human plasma

	QC1		QC2		QC3	
	Flupentixol 26.1 pg/ml	Melitracen 0.206 ng/ml	Flupentixol 261 pg/ml	Melitracen 103 ng/ml	Flupentixol 2090 pg/ml	Melitracen 4120 ng/ml
Room temperature (12 h)						
Mean concentration founded ($n = 5$)	25.6	0.200	242	97.1	1938	4090
CV (%)	3.14	0.006	2.27	8.13	4.49	3.52
Bias (%)	−1.92	−2.91	−7.28	−5.73	−7.27	−0.73
Autosampler (48 h)						
Mean concentration founded ($n = 5$)	26.9	0.205	277.6	103.2	2122	4112
CV (%)	4.48	0.815	3.10	6.69	4.18	1.67
Bias (%)	3.07	−0.49	6.36	0.19	1.53	−0.19
4 °C (48 h)						
Mean concentration founded ($n = 5$)	25.6	0.20	261	102.11	2039	4066
CV (%)	7.31	4.39	5.21	7.57	7.7	2.88
Bias (%)	−1.92	−2.91	0.00	−0.86	−2.44	−1.31
Freeze-thaw						
Mean concentration founded ($n = 5$)	25.9	0.203	261	102.64	2047	4095
CV (%)	4.7	2.63	4.22	8.43	7.84	3.03
Bias (%)	−0.77	−1.46	0.00	−0.35	−2.06	−0.61
Long-term (30 day)						
Mean concentration founded ($n = 5$)	26.8	0.195	274	99.7	2070	4270
CV (%)	8.52	5.11	5.81	4.23	3.90	7.68
Bias (%)	2.68	−5.34	4.98	−3.2	−0.96	3.64

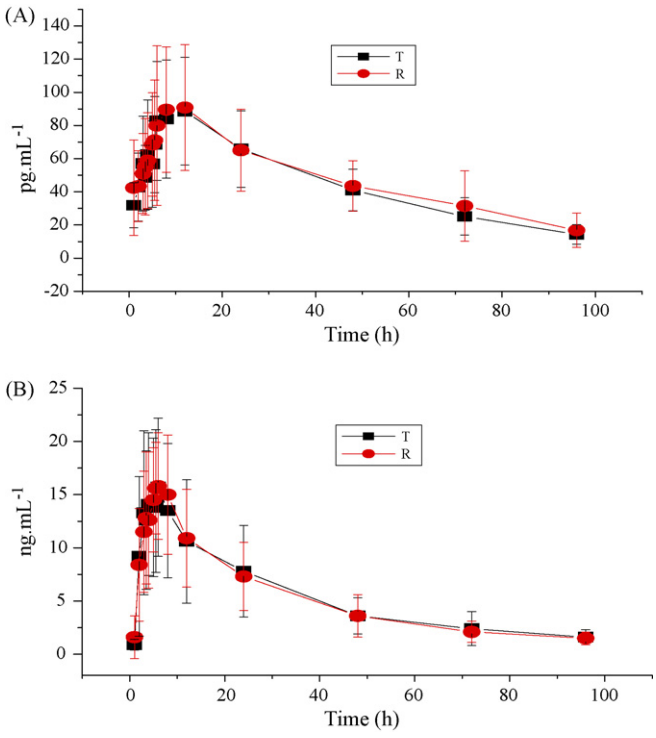

Fig. 3. Concentration–time profile for 18 subjects after 0.5 mg dose flupentixol (A) and 10 mg dose of melitracen (B). T: test formulation, R: reference formulation.

3.1.8. Stability

The stability of flupentixol and melitracen was evaluated under the conditions described in Table 5. The samples were stable under these conditions.

3.2. Application of the developed LC–MS/MS method

The method described in this paper was applied to a bioequivalence study that generated over 640 human plasma samples. Comparison of peak area ratios from the unknown samples with those from calibration curve allowed quantitation of the assayed samples. The concentration versus time profiles for flupentixol and melitracen were presented in Fig. 3. After oral administration of the flupentixol–melitracen tables to the volunteers, the observed analytes peak plasma concentration (C_{max}) values and the time values taken to be achieve (T_{max}) were similar to those reported in the literature and equivalent between the formulations. In addition, the calculated 90% CIs for mean C_{max}, AUC_{last} and $AUC_{0-\infty}$ Keride/Lundberk individual ratios were within the 80–125% interval defined by the US FDA.

4. Conclusion

A sensitive LC/MS/MS method for simultaneous quantitation of flupentixol and melitracen in human plasma was validated for human plasma. The method involved a sample preparation by repeated liquid–liquid extraction with adequate recovery. This method provides superior sensitivity with the lower limit of quantitation as low as 26.1 pg/ml for flupentixol. The method was rugged and was successfully applied to bioequivalence study of flupentixol 0.5 mg–melitracen 10 mg tablet. This method appears to be the first truly direct method for simultaneous quantitation of flupentixol and melitracen in human plasma to be reported.

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