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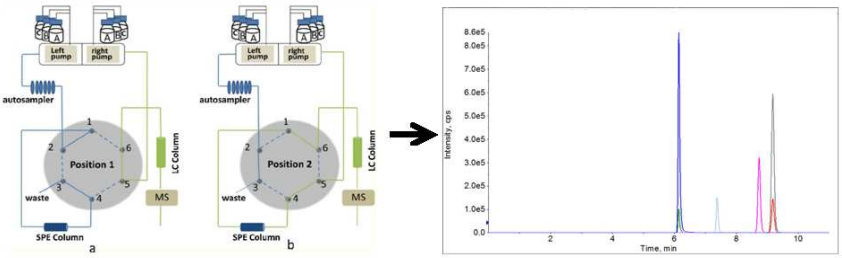

Text:

A simple and automated method for simultaneous determination of olanzapine, fluoxetine and norfluoxetine in human plasma via online SPE-LC-MS/MS method and its application in therapeutic drug monitoring.

A simple and automated online SPE-LC-MS/MS method for simultaneous determination of olanzapine, fluoxetine and norfluoxetine in human plasma and its application in therapeutic drug monitoring

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Abstract

The olanzapine-fluoxetine augmentation strategy has been proved efficacious for treatment-resistant depression, psychotic depression and bipolar depression. To achieve efficient therapeutic drugs monitoring (TDM), we develop an automated online SPE-LC-MS/MS method for the simultaneous quantification of olanzapine, fluoxetine and norfluoxetine in human plasma. After adding internal standard of diphenhydramine and centrifugation, 10μL plasma sample was directly injected into the SPE cartridge. While the analytes were retained on the SPE cartridge, the endogenous materials were washed out by the loading solvent. Following the valve switching, the analytes were eluted from the SPE cartridge to the analytical column by gradient elution. The analytes were quantified using a triple-quadrupole tandem mass spectrometer. Calibration curves were linear over the concentration range of 0.25-50.00 ng/mL for olanzapine, 0.50-100.00 ng/mL for fluoxetine and norfluoxetine. The intra- and inter-day precisions were within 1.17% and 4.63%. And the accuracies were between 95.60% and 101.48%. Mean matrix effect was in the range of 90.35% to 99.89% and mean recovery was in the range of 90.35% to 96.99%. This method has been successfully applied on two Chinese schizophrenia patients. The online SPE-LC-MS/MS method allows sensitive and robust quantification of olanzapine, fluoxetine and norfluxoetine for routine TDM in clinic.

Keywords: Olanzapine; Fluoxetine; Norfluoxetine; Online SPE-LC-MS/MS; Therapeutic drug monitoring

1. Introduction

Olanzapine,2-methyl-4-(4-methyl-1-piperazinyl)-10H-thineno[2,3-b][1,5]-benzodi -azepine, is a second generation antipsychotic approved for the treatment of schizophrenia, bipolar mania and associated agitation [1]. Fluoxetine, N-methyl-γ -[4-(trifluoromethyl)-phenoxy] benzenepropanamine, is a selective inhibitor of serotonin reuptake approved for the treatment of major depressive disorders, bulimia nervosa, obsessive compulsive disorder and panic disorder [2]. The combinatorial therapy of olanzapine and fluoxetine has been proved effective for treatment-resistant depression, psychotic depression and bipolar depression [3-5].

According to AGNP Consensus Guidelines (2011) for TDM in psychiatry, olanzapine is strongly recommended and fluoxetine is recommended for TDM to explain non-response, adverse effect or poor compliance [6]. More importantly, TDM of fluoxetine should include the quantification of norfluoxetine, which is the active metabolite of fluoxetine and contribute significantly to the overall clinical effect of fluoxetine. Thus, a method for the simultaneous quantification of olanzapine, fluoxetine and its active metabolite norfluoxetine in human plasma is of great importance.

Immunoassay and chromatography are two main analytical methods for TDM. TDM basing on immunoassays has some major limitations such as lack of specificity for the parent drug, non-specific binding of the antibody resulting in overestimation and high cost [7]. Chromatography is one of the preferred technologies for TDM of psychoactive drugs because of sufficient precise, accurate and robustness, especially LC-MS/MS methods could be applied to most psychotropic drugs including their metabolites [6]. However, one of the challenges for the application of chromatography technology in TDM is the sample pretreatment. Plasma sample contains proteins, lipids, salts and many other substances which may interfere with the analysis of drugs. To remove biological matrix, sample pretreatment is essential. Protein precipitation, liquid-liquid extraction, membrane filtration and off-line solid phase extraction (SPE) are conventional methods for sample pretreatment [8]. These conventional methods could be time-consuming, labor intensive, error-prone and costly due to the complex manual pretreatment steps [9], thereby resulting into a limited throughput. Furthermore, these conventional methods could not satisfy the TDM requirement of drug analysis within 1 to 2 hours [6]. To simplify sample pretreatment procedure and allow direct injection of plasma into the HPLC system, the fully automated online SPE-HPLC technique has been widely introduced and applied in biological sample analysis to enhance throughput, reduce cost, improve analytical quality and increase efficiency [10-19].

A number of methods for the determination of these three analytes in biological fluids by LC-MS/MS individually or in combination have been described [20-28] using

conventional method such as protein precipitation, offline solid phase extraction and liquid-liquid extraction for sample pretreatment that were time-consuming, labor intensive and costly. In this paper, we developed a sensitive and automated online SPE-LC-MS/MS method for the simultaneous quantification of olanzapine, fluoxetine and its active metabolite norfluoxetine in human plasma that is easy and convenient to implement in routine TDM. In this method, after adding internal standard and a simple centrifugation procedure, 10μl plasma sample was directly injected into the online SPE-HPLC system. The analytes were trapped on the SPE cartridge while the biological matrix flushed to the waste. Through valve switching, the analytes were eluted and transferred to the analytical column for further separation and quantification. Method validation demonstrated its reliability and robustness. And the lower limit of quantification (LLOQ) is sufficient to quantify the three analytes in human plasma samples for TDM. Moreover, this method has been successfully applied to TDM of two Chinese schizophrenia patients.

2. Experimental

2.1 Materials

Reference standards of olanzapine and fluoxetine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Reference standard of norfluoxetine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Internal standard of diphenhydramine was purchased from J&K scientific Ltd (Beijing, China). Chemical structures of olanzapine, fluoxetine and norfluoxetine are shown in Fig.1. Acetonitrile and menthol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ammonium formate (LC-MS grade) and formic acid (HPLC grade) were purchased from ROE scientic Inc. (Newark, DE, USA). Water used in the experiment was prepared by a Milli-Q50 SP Reagent Water system (Bedford, MA, USA). Blank human plasma samples from healthy volunteers were kindly donated by Tianjin AnDing Hospital. Patients' samples were collected in Tianjin AnDing Hospital. All the blank and Patients' plasma samples were collected under the approval of the ethical committee of Tianjin AnDing Hospital and informed consents were obtained from the volunteers and patients. All plasma samples were

collected in K_2EDTA-treated tubes and stored at -80℃.

2.2 Stock and working solutions

The standard stock solutions of olanzapine, fluoxetine, norfluoxetine and diphenhydramine were prepared separately by dissolving their accurately weighted samples in acetonitrile at the concentration of 1mg/mL. Different volumes of each stock solution were transferred to make combined stock solutions of analytes and then appropriately diluted with acetonitrile: water (50:50, v/v) to make standard working solutions in desired concentrations. All the stock solutions were stored at -20℃ and the work solutions were stored at 4℃.

The calibration standards and quality control samples were prepared by spiking blank human plasma with respective working solutions (19:1, v/v). Calibration standards were made at the concentrations of 0.25, 0.50, 1.00, 2.50, 5.00, 10.00, 25.00, 50.00 ng/mL for olanzapine and 0.50, 1.00, 2.00, 5.00, 10.00, 20.00, 50.00, 100.00 ng/mL for fluoxetine and norfluoxetine. Quality control (QC) samples were prepared at 0.50 ng/mL (low quality control, LQC), 5.00 ng/mL (middle quality control, MQC), 40.00 ng/mL (high quality control, HQC) for olanzapine and 1.00 ng/mL (low quality control, LQC), 10.00 ng/mL (middle quality control, MQC), 80.00 ng/mL (high quality control, HQC) for fluoxetine and norfluoxetine. All samples were stored at 4℃ before LC-MS analysis.

2.3 Sample preparation

A 200 μL volume of plasma sample was transferred to a 1.5 mL Eppendorf tube to which 10μL of internal standard working solution (500 ng/mL diphenhydramine) was added. Then the sample was vortexed for 30 sec. After centrifugation (5min, 12000×g), the sample was transferred into auto sampler vials and 10μL was injected to LC-MS system.

2.4 Instrument

The online SPE and analysis was carried out using the UlitMate 3000×2 Dual-Gradient HPLC system (Sunnyvale, CA, USA) equipped with a SRD-3600 degasser, a DGP-3600SD pump, a WSP-3000TSL analytical autosampler, a TCC-3000RS column compartment and a DAD-3000 diode array detector. The

detection of analytes and internal standard was performed on an API 4000^{+} triple quadrupole mass spectrometer (AB SCIEX, USA) with an electrospray ionization (ESI) source interface in the positive ion mode. Data acquisition was carried out by Analyst 1.6 software (Toronto, Canada).

2.5 Online SPE and HPLC conditions

The online SPE procedure for plasma sample pretreatment was performed on MF Ph-1 SPE cartridge (10mm×4mm, 5μm) . The procedure was consisted of three steps. In the first loading step, 10μL of plasma sample was directly injected into the SPE cartridge with the six-port injector valve in 6-1 position. Through the loading pump (right pump), the mobile phase A (acetonitrile) / the mobile phase B (10mM ammonium formate) solution (1:99, v/v) cleaned up the matrix interferences with the analytes reserved on the SPE cartridge at the flow rate of 1 mL/min for 3 min. Meanwhile, the analytical column was equilibrated through the analytical pump (left pump).

In the second transfer step, the six-port valve was switched to 2-1 position to get the SPE cartridge connected with the analytical column. Through the gradient elution (showed in Table1) by analytical pump, the analytes were transferred from SPE cartridge which was in back-flush mode to analytical column at the flow rate of 1mL/min for 2 min. The mobile phase was consisted of acetonitrile (A) and 10mM ammonium formate with 0.01% formic acid (C).

In the last separation step, the six-port valve was switched back to 6-1 position and the analytes were separated on the Hypersil Gold C18 column (150mm×4.6mm, 5μm) while both columns were kept in 30℃. One fundamental drawback inherent to online SPE is the risk of carryover [29]. To avoid carryover, in the separation step, the loading pump was used to provide gradient elution to wash the SPE cartridge and conditioned with the initial mobile phase prior to the next sample. The online SPE and HPLC conditions are shown in Table 1.

2.6 Mass spectrometry conditions

To eliminate matrix effect, a two-position VICI valco was added between the HPLC system and the mass spectrometry. The outlet of analytical column firstly

flowed to waste when the valco was on position B from 0 min to 5.4 min. At 5.4 min the valco switched to position A, the outlet of analytical column directly flowed to the mass spectrometer.

MS/MS acquisition was operated in the ESI positive mode using multiple reaction monitoring (MRM). The parameters were: curtain gas 30 psi, GAS1 50 psi, GAS2 60 psi, ionspray voltage 5500V, ion source temperature 500℃, and CAD gas 5 units. Table 2 shows the declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) for each analyte under MRM acqusion.

2.7 Method validation

The method validation was carried out according to the USFDA Guidance for industry: bioanalytical method validation [30].

2.7.1 Selectivity

The selectivity of the method was estimated by comparing chromatograms of blank human plasma from six sources and the LLOQ samples under the described analytical procedure. Response of the analytes at LLOQ concentration and response of the blank human plasma were compared.

2.7.2 Linearity and LLOQ

The calibration curves of three analytes were obtained using standard plasma samples at eight non-zero concentrations. Least squares linear regression model $y= ax \pm b$ weighted by $1/x^2$ was used to fit calibration curves, in which y is the peak area ratio of analyte to internal standard, a is slope of the calibration curve, b is the y axis intercept and x is the analyte concentration. LLOQ was determined by decreasing the analyte concentrations until the minimal concentrations with a S/N ratio at least 10, an adequate precision less than 20% and an accuracy within 80%-120%.

2.7.3 Accuracy and precision

Intra-batch accuracy and precision were evaluated within a batch while inter-batch accuracy and precision were evaluated by running three batches on three separate days. Each batch was consisted of a set of calibration standard samples and five replicates of quality control samples at LQC, MQC, HQC.

Accuracy was determined by calculating the percentage bias from the nominal concentration. Precision was expressed by calculating the coefficient of variation (% CV) for each replicates. The value of accuracy should be within ±15% of the nominal value while the precision should not exceed 15%.

2.7.4 Extraction recovery and matrix effect

The extraction recovery of the analytes at three QC levels (LQC, MQC and HQC) was determined by comparing the mean peak area response of 5 replicates of spiked plasma samples against the mean peak area response of 5 replicates of quality control samples under online SPE procedure. According to the procedure described by Matuszewski et al. [31], the matrix effect was evaluated at three QC levels by comparing the mean peak area response of 5 replicates of extracted samples (spiked after SPE) against the mean peak area response of 5 replicates of QC samples through regular HPLC analysis.

2.7.5 Stability study and dilution integrity

Stability experiments were carried out to evaluate the analyte stability in plasma samples under different conditions that may occur during sample analysis. Short-term stability, long-term stability, freeze-thaw stability and auto-sampler stability were performed at three QC levels with five replicates for each level.

The dilution integrity study was performed at two times and five times of the highest concentration in the calibration curve. Five replicate samples of half, one-fifth and one-tenth concentration of 2× the highest concentration and one-tenth, one-twentieth and one-fiftieth concentration of 5× the highest concentration were prepared. Their concentrations were calculated by applying the corresponding dilution factor through the calibration curve.

3. Results and discussion

3.1 Method validation

3.1.1 Selectivity

The chromatograms of blank plasma, spiked plasma sample with olanzapine, fluoxetine and norfluoxetine at LLOQ were shown in Fig. 2. The retention time of olanzapine, internal standard, norfluoxetine and fluoxetine were 5.99 min, 7.35 min,

8.67 min, 9.12 min. No interference from endogenous materials was found at the retention time of the analytes or the internal standard indicating that the online-SPE procedure was selective.

3.1.2 Linearity and LLOQ

The assay was linear over the concentration range of the analytes ($r > 0.99$) . Data of linear regression equation of the analytes were listed in Table 3. The LLOQ were 0.25 ng/mL for olanzapine, 0.50 ng/mL for fluoxetine and norfluoxetine. The LLOQ were adequate for TDM since the reference therapeutic plasma concentration were 20 ng/mL-80 ng/mL for olanzapine, 120 ng/mL-500 ng/mL for fluoxetine plus norfluoxetine [6].

3.1.3 Accuracy and precision

The intra-batch and inter-batch accuracy and precision of QC samples were shown in Table 4. The accuracy of the analytes were between 95.60% and 101.15% while the RSD of QC samples were among 1.17% to 4.63%.

3.1.4 Extraction recovery and matrix effect

Since extraction recovery and matrix effect are two important parameters in the development of LC-MS/MS method for TDM, it is essential to evaluate the extraction recovery and matrix effect of the analytes. Results are listed in Table 5. The extraction recovery was within 90.35% to 96.99% with the matrix effect ranging from 90.35% to 99.89%. The above results were within acceptable criteria allowing assay of the analytes in human plasma.

3.1.5 Stability study and dilution integrity

Olanzapine, fluoxetine and norfluoxetine in human plasma were stable at room temperature for 6h and at -80℃ for 30 days. Auto-sampler stability study at 4℃ for 24h and freeze-thaw stability study at -80℃ for 3 cycles were carried out. The resluts showed that the analytes were stable under the above conditions. Data of the stability experiments are listed in Table 6, Table 7 and Table 8 for each analyte.

The mean calculated concentrations adding corresponding dilution factor of one-tenth, one-fifth and half dilution samples were between 92.10% and 100.50% of the nominal values while the value of RSD ranged from 1.51% to 3.82%. Meanwhile,

the mean calculated concentrations adding corresponding dilution factor of one-fiftieth, one-twentieth and one-tenth dilution samples were between 94.20% and 105.72% of the nominal values while the value of RSD ranged from 1.21% to 5.71%. Data of the dilution integrity study were shown in Table 9.

3.2 Quantification of olanzapine, fluoxetine and norfluoxetine in patients' specimens

The established method has been successfully applied in the TDM of two Chinese patients with schizophrenia. Table 10 showed plasma concentrations of olanzapine, fluoxetine and norfluoxetine of two patients with schizophrenia. The dosage regimen of patient 1 was 20 mg/d and 40 mg/d for olanzapine and fluoxetine while the dosage regimen of patient 2 was 20 mg/d and 20 mg/d for olanzapine and fluoxetine. The specimens were collected immediately before ingestion of morning dosing, representing steady state trough concentration. The olanzapine concentrations were 55.9 ng/mL and 79.0 ng/mL for patient 1 and 2 respectively, falling in the therapeutic reference range of 20-80 ng/mL [6]. The concentrations of fluoxetine plus norfluoxetine were 375 ng/mL and 224 ng/mL for patient 1 and 2 respectively, falling in the therapeutic reference range of 120-500 ng/mL [6]. The ratio of concentrations metabolite : parent drug was 1.23 for patient 1 and 0.85 for patient 2 while reported "normal" ratio ranges were 0.7-1.9 (n=334) [32]. A ratio outside the "normal" ratio range may indicate problems of drug adherence or metabolism abnormalities or a drug-drug interaction [6]. Correspondingly, the symptoms of patient 1 and patient 2 were stable and slightly improved clinically.

3.3 Method development

To achieve the goal of a simple, sensitive and high throughput method, the optimization of online-SPE-HPLC procedure was carried out in three steps.

The first step was SPE procedure including the selection of SPE cartridge, mobile phase composition and proportion, flow rate and t_M (the matrix depletion time) with the aim to find best conditions to eliminate the interference of endogenous substance and concentrate the analytes. SPE cartridge is used for plasma sample pretreatment, so an ideal SPE cartridge should retain all the analytes with endogenous substance eluted by mobile phase. According physicochemical parameters of the analytes shown in

Table 11, olanzapine, fluoxetine and norfluoxetine are all basic and moderate polar compounds. Basing on this, four SPE cartridges including Merck LiChrospher RP-18 ADS, CAPCELL PAK MF Ph-1, Waters Oasis HLB and Waters Oasis MCX were selected.

Peak shape, carryover and cost were taken into account to select the best one among the four SPE cartridges. The carryover was tested by injecting blank plasma sample sequentially right after injecting the highest concentration sample in calibration standard. Then the response of blank plasma at retention time of the analytes was compared with the response of LLOQ sample. The acceptable criteria are within 20% of the response of LLOQ sample [13]. Chromatograms and other characteristics were evaluated as listed in Table 12 and chromatograms of the analytes using different SPE cartridge were shown in Fig. 3.

While RP-18 ADS SPE cartridge exihibited bad trapping efficiency for olanzapine, the other three SPE cartridges were able to retain all analytes. However, serious tailing factor was observed indicating band broadening from Waters Oasis MCX to the analytical column. Waters Oasis HLB and CAPCELL PAK MF Ph-1 gave similar trapping efficiency and sharp peak. Of the two SPE cartridges, Waters Oasis HLB showed larger carryover of norfluoxetine and fluoxetine, being more expensive than CAPCELL PAK MF Ph-1. Considering the carryover and cost, CAPCELL PAK MF Ph-1 was chosen for further development.

Next t_M (matrix depletion time) and flow rate were examined. The amount of endogenous substance at different flow rate and its corresponding t_M were investigated as shown in Fig. 4. According to the results, 1 mL/min was the preferred flow rate. Chromatogram showed that endogenous substance could be eluted within 1 min. However, in order to ensure the elimination of it and prolong SPE cartridges service time, t_M was set for 3 min. Buffer at different pH like formic acid, ammounium formate and ammounium acetate were investigated in combinations with methanol or acetonitrile. Finally, excellent trapping efficiency and endogenous substance elimination were achieved when the MF Ph-1 cartridge was kept at 30℃ and at a flow rate of 1ml/min by isocratic elution of acetonitrile-10mM ammonium

formate buffer (1:99, v/v) for 3 min.

The second step was the optimization of analyte transferring procedure including t_T (transfer time), mobile phase composition and proportion to ensure the target analyte to be completely detected. Buffer at different pH like formic acid, ammounium formate and ammounium acetate were investigated in varying combinations with methanol or acetontrile. Finally, 10mM ammonium formate with 0.01% formic acid was selected as aqueous phase. Different proportions were evaluated as listed in Fig. 5 to find a better one.

Basing on the results, gradient elution from 65% aqueous phase to 55% aqueous phase for 2 min was used to transfer and separate the analytes from SPE cartridge to analytical column.

The last step was the optimization of chromatographic condition including column type, mobile phase composition and proportion to obtain adequate response and sharp peak for the analytes and internal standard. So different columns including InertSustain C18 column (150mm ×4.6mm, 5μm), CAPCELL PAK MG Ⅲ C18 column (150mm ×4.6mm, 5μm) and Hypersil Gold C18 column (150mm ×4.6mm, 5μm) were evaluated. Eventually, Hypersil Gold C18 column was selected for its symmetrical peak shape and good PH stability. Moreover, to get better resolution of norfluoxetine and fluoxetine, gradient elution from 55% aqueous phase to 40% aqueous phase for 1 min was started at 8.5 min.

4. Conclusions

This paper describes an online SPE-LC-MS/MS method for the simultaneous quantification of olanzapine, fluoxetine and norfluoxetine in human plasma. The method used online solid phase extraction technique to automate plasma sample pretreatment which lead to an easy use, fully automated procedure of TDM. The total run time including sample pretreatment and compound analysis was 11 min which could enable high-throughput plasma sample analysis. The rapid turnaround time of

this TDM method make it even more attractive in case of suspected intoxications. This method has been successfully applied to the therapeutic drug monitoring in two Chinese schizophrenia patients. The integration of sample pretreatment automation using online SPE with chromatography technique could provide opportunities to enable easy to use, efficient, sensitive and high quality methods for TDM.

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Table legends

Figure legends

Table1. Online-SPE and HPLC conditions

Loading pump				Analytical pump				Valve switching	
Time (min)	Flow rate (mL/min)	A (%)	B (%)	Time (min)	Flow rate (mL/min)	A (%)	C (%)	Time (min)	Position
0	1	1	99	0	1	35	65	0	6-1
4	1	1	99	3	1	35	65	3	2-1
6	1	90	10	5	1	45	55	5	6-1
8	1	90	10	8.5	1	45	55		
9	1	1	99	9.5	1	60	40		
11	1	1	99	10	1	60	40		
				10.5	1	35	65		
				11	1	35	65		

A, acetonitrile ; B, 10mM ammonium formate ; C, 10mM ammonium formate with 0.01% formic acid

Table 2. MS/MS parameters for analytes

Analyte	Q1 mass (*m/z*)	Q3 mass (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)
Olanzapine	313.0	256.2	70	10	30	17
	313.0	213.0	60	10	40	20
Fluoxetine	310.3	44.1	40	10	40	8
	310.3	147.9	40	10	12	10
Norfluoxetine	296.0	134.1	40	10	9	25
Diphenhydramine	256.2	167.2	60	10	30	15

Table 3. Data of linear regression equation of the analytes

Analytes	Linear range(ng/ml)	LLOQ (ng/ml)	Slope	Intercept	r
Olanzapine	0.25-50.00	0.25	0.125	-0.000434	0.9998
Fluoxetine	0.50-100.00	0.50	0.09	-0.00215	0.9998
Norfluoxetine	0.50-100.00	0.50	0.0516	-0.000297	0.9998

Table4. Intra-batch and inter-batch precision and accuracy

Analyte	Nominal Concentration (ng/ml)	Intra-batch				Inter-batch			
		N	Mean concentration found(ng/ml)	Accuracy (%)	CV (%)	N	Mean concentration found(ng/ml)	Accuracy (%)	CV (%)
Olanzapine	0.50	5	0.50	100.40	3.09	15	0.50	100.50	3.31
	5.00	5	4.78	95.60	1.17	15	4.86	97.28	3.64
	40.00	5	39.74	99.35	1.59	15	39.44	98.60	3.98
Fluoxetine	1.00	5	1.01	101.48	2.50	15	1.01	100.74	3.67
	10.00	5	9.63	96.30	1.72	15	9.67	96.67	2.38
	80.00	5	79.02	98.78	1.34	15	80.92	101.15	3.42
Norfluoxetine	1.00	5	1.00	100.26	1.79	15	1.01	100.94	4.63
	10.00	5	9.95	99.46	2.69	15	9.70	96.96	3.42
	80.00	5	79.54	99.43	1.30	15	79.23	99.04	2.65

Table 5. Matrix effect and extraction recovery

Analyte	Nominal concentration (ng/ml)	Matrix effect (%)			Extraction recovery (%)		
		Mean	SD (%)	CV (%)	Mean	SD (%)	CV (%)
Olanzapine	0.50	99.89	3.42	3.42	94.50	1.48	1.56
	5.00	90.35	3.19	3.53	93.76	1.71	1.82
	40.00	99.84	0.88	0.88	95.12	1.36	1.42
Fluoxetine	1.00	97.69	1.08	1.11	90.35	1.65	1.82
	10.00	96.03	1.37	1.43	91.48	1.12	1.22
	80.00	96.33	1.60	1.66	90.86	0.52	0.57
Norfluoxetine	1.00	91.25	3.06	3.36	91.84	1.40	1.53
	10.00	93.47	3.10	3.31	92.42	3.52	3.81
	80.00	91.15	2.09	2.29	96.99	0.85	0.88

Table 6. Stability study for olanzapine under different conditions

Stability	Storage conditions	Level	Mean comparison samples (ng/ml)	Mean stability samples (ng/ml)	Accuracy (%)	CV (%)
Short-term	Room temperature for 6h	LQC	0.4978	0.4606	92.53	5.08
		MQC	4.79	4.54	94.78	5.98
		HQC	38.90	36.18	93.01	5.25
Auto-sampler	At 4℃ for 24h	LQC	0.4948	0.4676	94.50	5.73
		MQC	4.71	4.64	98.51	3.01
		HQC	40.10	38.26	95.41	4.41
Freeze-thaw	At -80℃ for 3 cycles	LQC	0.5002	0.4682	93.60	2.13
		MQC	4.78	4.97	104.02	2.07
		HQC	39.74	42.72	107.50	3.01
Long-term	At -80℃ for 30 days	LQC	0.4948	0.4750	96.00	1.85
		MQC	4.71	4.36	92.61	3.59
		HQC	40.10	38.44	95.86	3.20

Table 7. Stability study for fluoxetine under different conditions

Stability	Storage conditions	Level	Mean comparison samples (ng/ml)	Mean stability samples (ng/ml)	Accuracy (%)	CV (%)
Short-term	Room temperature for 6h	LQC	0.9966	0.9648	96.81	4.07
		MQC	9.88	9.45	95.64	2.71
		HQC	78.44	74.10	94.47	2.43
Auto-sampler	At 4℃ for 24h	LQC	1.0400	1.0100	97.31	3.12
		MQC	9.94	9.80	98.59	2.74
		HQC	79.18	75.98	95.96	2.72
Freeze-thaw	At -80℃ for 3 cycles	LQC	1.0148	0.9686	95.45	4.32
		MQC	9.63	9.04	93.87	1.30
		HQC	79.02	75.56	95.62	2.22
Long-term	At -80℃ for 30 days	LQC	1.0400	0.9832	94.54	7.31
		MQC	9.94	9.61	96.66	4.35
		HQC	79.18	77.10	97.37	2.59

Table 8. Stability study for norfluoxetine under different conditions

Stability	Storage conditions	Level	Mean comparison samples (ng/ml)	Mean stability samples (ng/ml)	Accuracy (%)	CV(%)
Short-term	Room temperature for 6h	LQC	0.9512	0.8942	94.01	2.90
		MQC	9.71	9.40	96.80	3.43
		HQC	75.22	72.02	95.75	2.71
Auto-sampler	At 4℃ for 24h	LQC	0.9644	0.9918	102.84	3.74
		MQC	10.44	9.97	95.49	3.57
		HQC	80.56	78.68	97.67	1.57
Freeze-thaw	At -80℃ for 3 cycles	LQC	1.0640	0.9692	91.09	3.29
		MQC	9.94	9.12	91.75	2.83
		HQC	79.54	75.40	94.80	2.73
Long-term	At -80℃ for 30 days	LQC	0.9644	0.9254	95.96	3.50
		MQC	10.44	9.74	93.30	3.33
		HQC	80.56	76.18	94.56	3.43

Table 9. Dilution integrity study of analytes

Analyte	N	Dilution integrity	Nominal Concentration (ng/ml)	Mean concentration found(ng/ml)	Accuracy (%)	CV (%)
Olanzapine	5	10	100.00	95.64	95.64	1.71
	5	5	100.00	98.38	98.38	1.59
	5	2	100.00	94.50	94.50	2.92
	5	50	250.00	252.60	101.04	5.71
	5	20	250.00	253.80	101.52	4.56
	5	10	250.00	238.40	95.36	2.85
Fluoxetine	5	10	200.00	194.60	97.30	3.12
	5	5	200.00	201.00	100.50	2.79
	5	2	200.00	192.40	96.20	1.63
	5	50	500.00	496.00	99.20	1.32
	5	20	500.00	508.00	101.60	2.91
	5	10	500.00	471.00	94.20	1.21
Norfluoxetine	5	10	200.00	190.80	95.40	3.82
	5	5	200.00	195.40	97.70	2.86
	5	2	200.00	184.20	92.10	1.51
	5	50	500.00	528.60	105.72	1.54
	5	20	500.00	511.60	102.32	2.84
	5	10	500.00	488.20	97.64	5.64

Table 10. Olanzapine, fluoxetine and norfluoxetine levels in schizophrenia patients' specimens

Patient	Age	Sex	Smoker	OLA level (ng/mL)	FLX level (ng/mL)	NOR level (ng/mL)	Ratios of conc. NOR: FLX
1	18	M	No	55.9	168.0	207.0	1.23
2	24	F	No	79.0	121.0	103.0	0.85

OLA: olanzapine ; FLX: fluoxetine ; NOR: norfluoxetine ; Conc. :concentration

Table 11. Physicochemical parameters of the analytes

Analyte	log P	H acceptors	H donors	pKa
Olanzapine	3.61	4	1	7.24/14.17
Fluoxetine	4.09	2	1	9.8
(S)-Norfluoxetine	3.8	2	1	9.77
Diphenhydramine	3.65	2	0	8.87

Table 12. Characteristics of the four SPE cartridges

SPE cartridge	Chromatogram	Peak Shape	Carryover
RP-18 ADS	Fig.3 (a)	Symmetry peak	Not available
MF Ph-1	Fig.3 (b)	Symmetry peak	Within acceptable criteria
Waters HLB	Fig.3 (c)	Symmetry peak	Within acceptable criteria
Waters MCX	Fig.3 (d)	Tailing peak	Within acceptable criteria

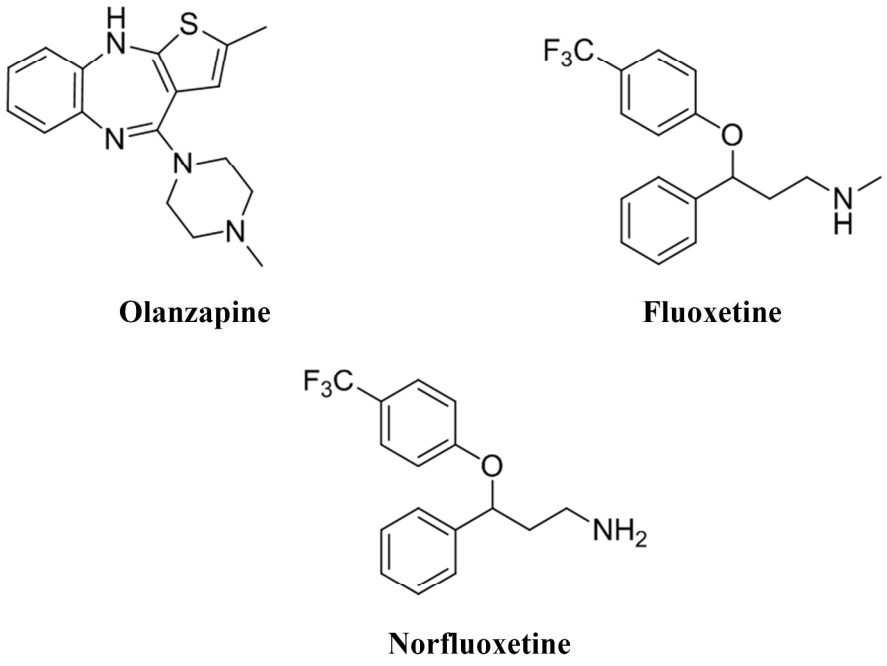

Figure 1. Chemical structures of olanzapine, fluoxetine and norfluoxetine
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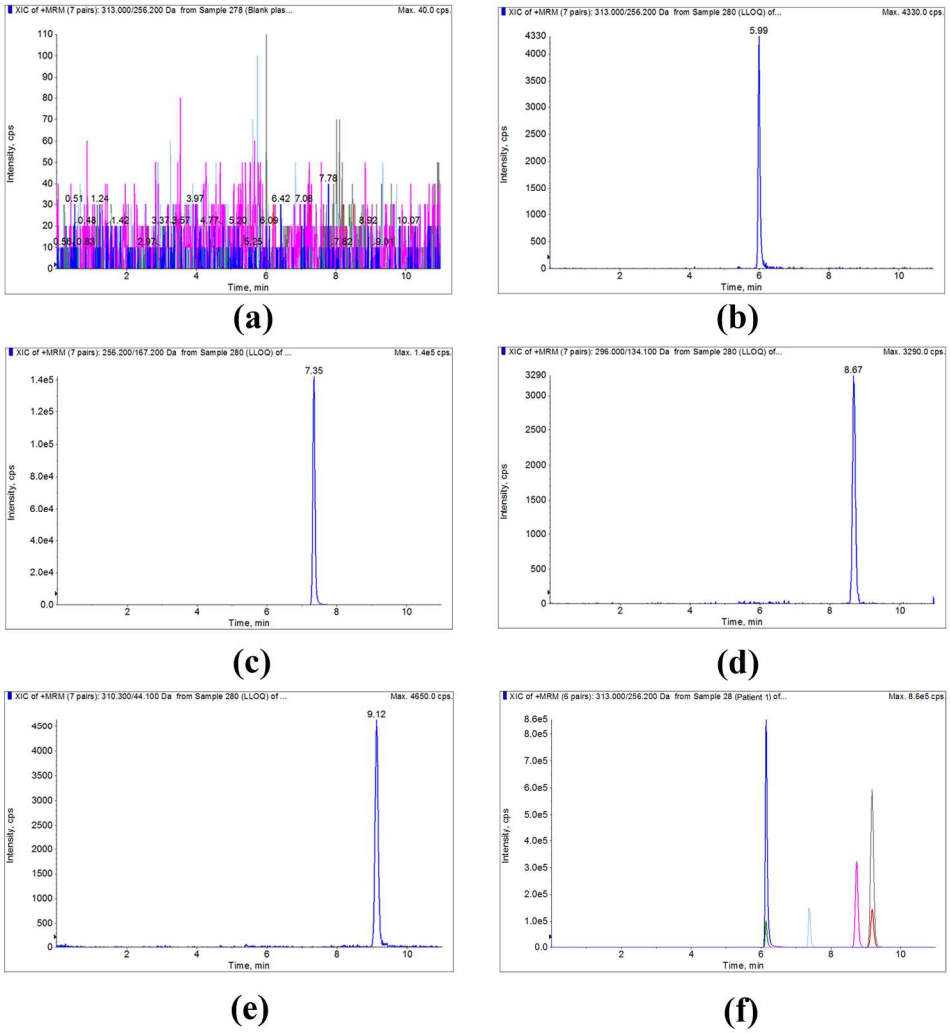

Figure 2. (a) Chromatogram of blank plasma, (b) Chromatogram of olanzapine at LLOQ, (c) Chromatogram of internal standard at LLOQ, (d) Chromatogram of norfluoxetine at LLOQ, (e) Chromatogram of fluoxetine at LLOQ, (f) Chromatogram of patient plasma sample.
210x224mm (300 x 300 DPI)

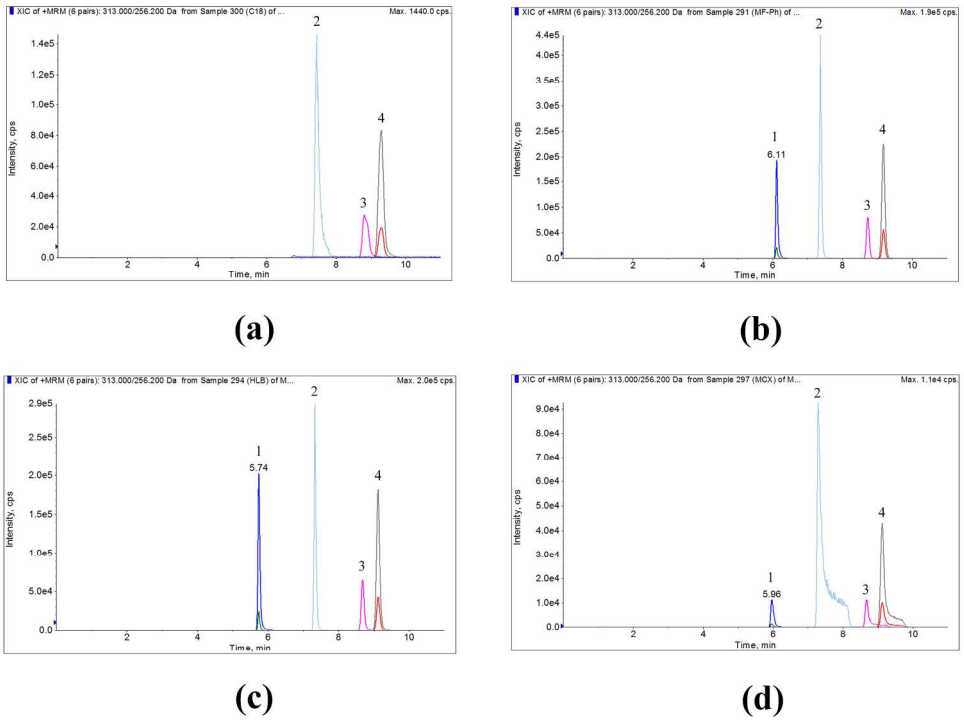

Figure 3. Chromatograms of the analytes using different SPE cartridge: (a) Merck LiChrospher RP-18 ADS, (b) CAPCELL PAK MF Ph-1, (c) Waters Oasis HLB, (d) Waters Oasis MCX (1. Olanzapine, 2. Diphenhydramine, 3. Norfluoxetine, 4. Fluoxetine).
209x158mm (300 x 300 DPI)

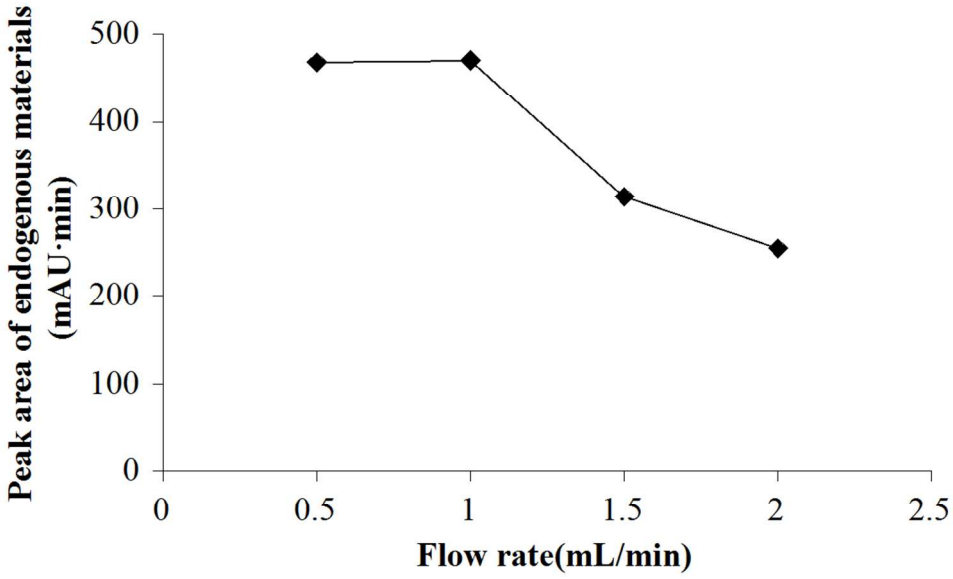

Figure 4. Effect of the flow rate of loading solvent on the peak area of endougenous materials
315x194mm (300 x 300 DPI)

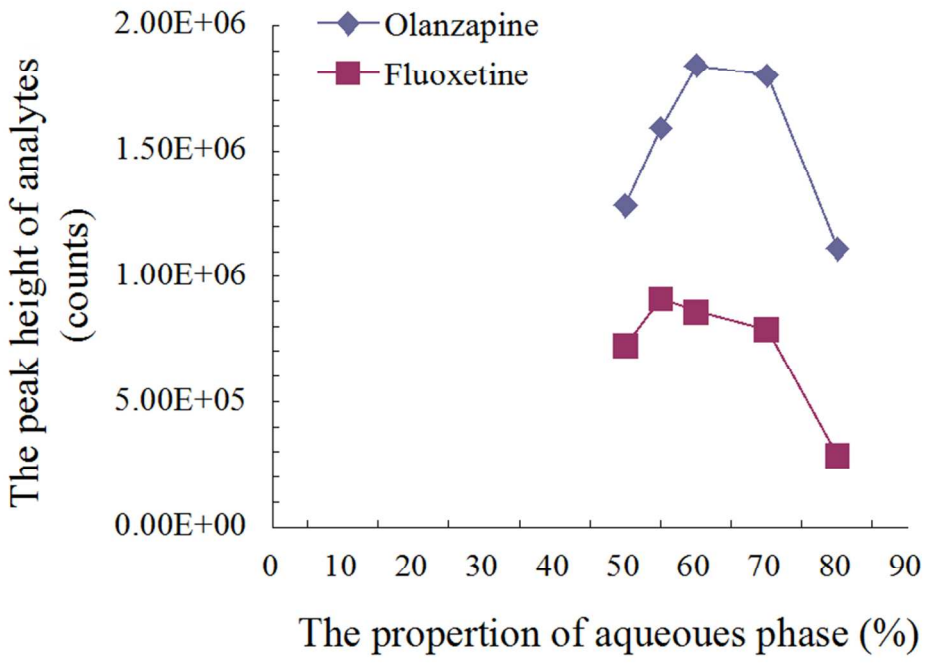

Figure 5. Effect of aueous phase proportion on the peak area of olanzapine and fluoxetine
229x159mm (300 x 300 DPI)