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SIMULTANEOUS QUANTIFICATION OF RISPERIDONE AND ESCITALOPRAM IN HUMAN PLASMA BY LC-MS/MS: APPLICATION TO A PHARMACOKINETIC STUDY

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ABSTRACT

A solid phase extraction method was developed for the simultaneous quantification of risperidone and escitalopram in human plasma by a suitable ultra-performance liquid chromatographic / tandem mass spectrometric assay (LC-MS/MS) .Resperidone-D4 was used as an internal standard. The method involves simple isocratic chromatographic conditions at a flow rate of 0.400 mL min^{-1}. Samples were separated on X-terra RP8 column (50 mm × 4.6, 5µm) in a shorter run time of only 2.0 min using a mobile phase mixture of 95:5 v/v (acetonitrile : ammonium acetate) buffer 5mM, pH 5.0±0.05). Calibration plots were linear (r^2 >0.99) over the concentration range of 0.050 to 26 ng mL^{-1} for risperidone and 0.100 to 51 ng mL^{-1} for escitalopram. The overall recoveries for risperidone and escitalopram were 76.88% and 81.14%, respectively. Precision was 2.9%, 5.07% (intra-day) and 4.13%, 6.43% (inter-day) for risperidone and escitalopram, respectively. The validated method was successfully applied to a pharmacokinetic study of human plasma.

KEYWORDS: Antidepressant agent, Depressive disorder, Antipsychotic drugs, Method validation.

INTRODUCTION

Risperidone (RIS) is a benzisoxazole derivative used to treat schizophrenia and other psychoses. Escitalopram oxalate (ESC) is a selective serotonin reuptake inhibitor and novel antidepressant agent, used to treat major general anxiety disorder and depressive disorder in adults and also in panic disorder,[1-4].The chemical structures of risperidone,escitolapram and risperidone-D4 are shown in(Figure 1). According to the Literature survey fewer chromatographic methods were reported for individual determination of escitalopram [5-7] and risperionde [8-13] or in mixtures with their metabolites and other antipsychotic drugs [14-15] in human plasma/serum. No methods for simultaneous quantification of these two co-administrated drugs in human plasma have been reported in the literature so far. This method derives its importance from the fact that risperidone is frequently co-administered with escitalopram in the case of major depression, stress, anxiety and even in generalized anxiety disorders.[16-17] Risperidone is known to be metabolized by the enzyme CYP 2D6, this process interferes with escitalopram, which acts as a moderate inhibitor of the CYP 2D6 enzyme. This interaction leads to the risk of adverse effects due to the increased levels of risperidone in the blood

stream.[18-19] Hence, simultaneous monitoring of risperidone and escitalopram after co-administration of both analytes is of great importance for diagnostic and therapeutic purposes. However, the published methods being developed for the both drugs separately necessitate the treatment of two different methods with subsequent high cost and long run times in comparison to the method reported here (runtime < 2 min). The main objective of the present study is to report a high throughput rapid and sensitive ultra-performance liquid chromatographic / tandem mass spectrometric assay (LC-MS/MS) for simultaneous quantification of risperidone and escitalopram in human plasma. The developed method was successfully applied to a pharmacokinetic study and the main pharmacokinetic parameters for risperidone and escitalopram were determined.

MATERIALS AND METHODS

UPLC operating conditions: Waters Acquity UPLC system consisting of binary solvent delivery capability (Milford, MA, USA was used for the chromatographic separation. The analytical column used was X-terra RP8 (50×4.6 mm, 5-µm particle size) from waters (Milford, MA, USA). The mobile phase composition consisted of a mixture of acetonitrile and 5mM ammonium acetate buffer (95:5, v/v) adjusted to pH 9 ± 0.05. The column oven temperature was maintained at 35°C and the total LC run time was less than 2.0 min at a flow-rate of 0.4 mL min^{-1} under isocratic condition.

Mass spectrometry operating conditions: Quantitation of analytes and the internal standard (IS) was carried out on a Waters Quattro Micro API triple quadrupole mass spectrometer, (Milford, MA, USA), equipped with an ESI interface, and operating in positive ion mode. For the optimization of LC-MS/MS parameters, standard solutions of risperidone, escitalopram and risperidone-D4 (IS) prepared in methanol were infused at a flow-rate of 10 mL min^{-1} using a syringe pump (Harvard Apparatus, Holliston, MA, USA). The dwell time assay was set at 500 ms. Mass Lynx software version 4.2 was used to control all parameters of LC-MS/MS. The optimized ion source parameters were: capillary voltage was 3.50 (kV), cone 35.00 (v), extractor 3.00 (v), RF lens 0.0 (v), source temperature 100 °C, desolvation temperature 400 °C, desolvation gas flow 800 (L h^{-1}), cone gas flow 80 (L h^{-1}) for the analytes and IS.

Materials and chemicals: Risperidone reference standard (potency 99.9%) was procured from Clear Synth laboratories, Mumbai, India Escitalopram reference standard (potency 99.7%) and risperidone-D4 standard (potency 99.5%) were obtained as gifted samples from Hetero labs, Hyderabad, India. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from J.T. Baker, Philipsburg, USA. Hexane (HPLC grade) was procured from Merck, Mumbai, India. Ammonium acetate, orthophosphoric acid (AR grade) and MCX (3CC) cartridges were purchased from Waters Specialties, Mumbai, India. High purity water was prepared with Milli-Q water purification system (Millipore Pvt. Ltd., Bangalore, India). Blank (drug free) human plasma was obtained from Cauvery Diagnostics and Blood Bank, Secunderabad, India and stored at -70 °C until use.

Preparation of stock solutions of analyte (s) and IS 1mg/mL of primary stock solutions of risperidone HCL, escitalopram and risperidone-D4 standard and quality control (QC) samples were prepared separately by dissolving the proper weights in 6 mL of methanol and made up to the mark of 10.0 mL and kept at 2-8°C, the stability of these solutions were approximately stable for seven days. Primary set of working standard solutions of risperidone HCL and escitalopram made in 10 mL with 80% methanol (from primary stock) was diluted with methanol to prepare a series of diluted solutions to plot the calibration curve. Another set of working standard stock solutions of risperidone HCL, escitalopram and IS made in methanol (from primary QC stock) was successively diluted with methanol to prepare a series of QC samples.

Preparation of calibration curves: Calibration curve samples and quality control samples were prepared by spiking 20 µL of appropriate working solution of risperidone HCL and escitalopram into 980 µL of control human plasma. The quality control samples (QC) were prepared at concentrations of 0.050 ng/mL (lower limit of quantification, LLOQ), 0.150 ng mL^{-1} (low quality control, LQC), 11.369 ng mL^{-1} (medium quality control, MQC) and 19.435 ng mL^{-1} (high quality control, HQC) for risperidone, and at concentrations of 0.100 ng mL^{-1} (LLOQ), 0.298 ng mL^{-1} (LQC), 22.699 ng mL^{-1} (MQC) and 38.801 ng mL^{-1} (HQC) for escitalopram. To determine the precision and accuracy, samples were prepared by spiking control human plasma at appropriate concentrations in bulk and 0.500 mL plasma aliquots were distributed into different tubes. All the spiked plasma samples were stored at -70 ± 5°C until analysis.

Sample preparation: A simple solid phase extraction method was followed for extracting the analytes from human plasma. An aliquot of 0.500 mL of human plasma was mixed with 50 µL of IS working solution (0.500 µg mL^{-1} of risperidone-D4). To this, 0.500 mL of 4% ortho phosphoric acid buffer was added and vortexed. Before sample extraction, MCX (3cc, 60mg) cartridges were conditioned with 1.00 mL of methanol followed by 1.00 mL of Milli-Q water followed by 1.00 mL of 4 % ortho phosphoric acid buffer solution. Prepared plasma samples were loaded onto the SPE cartridges and eluted completely under slow vacuum and the cartridges were washed with 1.00 mL of 4% ortho phosphoric acid buffer solution and 1.00 mL of methanol followed by 1.00 mL of n-hexane and was allowed to dry. The analytes and IS were then eluted with 1.00 mL of 5% ammonia in acetonitrile. The eluted samples were injected into the LC-MS/MS system.

METHOD VALIDATION

Selectivity: Selectivity is useful in measuring the analyte response in the presence of its possible impurities or other sample components. Human plasma samples taken from six different lots were analyzed to investigate the potential interferences to evaluate the Selectivity. The retention times corresponding to the responses of the interfering substances or background noises of risperidone and escitalopram were less than 20% of the response of the lowest standard curve point or LLOQ. The responses of the interfering substances or background noise at the retention time of the internal standard were less than 5% of the mean response of internal standard in LLOQ samples.

Calibration curve: Linearity was determined for the concentration range of 0.050 to 26 ng mL^{-1} for risperidone and 0.100 to 51 ng mL^{-1} for escitalopram by weighted linear regression ($1/X^2$) of the analytes/IS peak area ratios based on four independent calibration curves prepared on two different days using eight points each.

Precision and accuracy: The intra-assay precision and accuracy were proposed by analyzing six replicates at four different QC levels, that is, LLOQ, LQC, MQC, and HQC. The inter-assay precision and accuracy were estimated by analyzing six replicates at four different QC levels on four different runs.

Matrix effect and process efficiency: The relative matrix effect, recovery and process efficiency were assessed for the tested analytes with neat, post-extracted and extracted QC samples (extracted and processed blank plasma samples of six different volunteer matrix lots containing EDTA as anticoagulant were spiked by aqueous solutions). These parameters were analyzed in five different standard levels.

Dilution integrity: The aim of the dilution integrity test was to validate the dilution test of real-time analysis of subject samples on higher analytes concentrations above the ULOQ level. Dilution integrity experiment was carried out at double the ULOQ concentration for the analytes. Half and quarter concentrations of six replicates each were prepared by dilution with blank plasma for 2 times and 4 times and by applying the dilution factors 2 and 4 their concentrations were calculated.

Stability experiments: By injecting the replicate preparations of processed samples up to 24h(in the auto sampler at 10°C), the stability of risperidone, escitalopram and IS in the injection solvent were determined periodically. Six replicates of samples kept at a temperature below 10°C for 24 h at low and high concentrations were analyzed to evaluate the wet and dry extract stability successfully. Bench top stability was assessed for six replicates of risperidone and escitalopram samples (5.22 h), room temperature (~25 °C) at two concentration levels (LQC and HQC). The Freeze-thaw stability of risperidone and escitalopram in human plasma following four cycles was also assessed. The samples were kept at -70°C between freeze/thaw cycles and the samples were thawed by allowing them to stand (unassisted) at room temperature for ~1.5 h. The long-term stability of analytes during freezing of human plasma was also evaluated. The acceptable limits of accuracy (± 15%) and precision ≤15% (%CV) for the assay values to consider the samples as stable according to the US FDA Guidelines for bio analytical validation.[23]

RESULTS AND DISCUSSION

Method development: By infusing the standard solutions of the tested compounds of suitable concentrations mass spectrometry conditions were optimized. Even though the tuning was done in both positive and negative ion modes, due to the basic nature of analytes the intensity obtained in the positive ion mode seems to be higher for both the analytes and their respective internal standards (IS). Ions were detected in the multiple reaction monitoring mode (MRM), by monitoring the transition pairs of m/z 411.37→191.31 for risperidone, m/z 325.26 →108.91 for escitalopram, and m/z 415.34 → 195.34 for risperidone-D4. Mass lynx software (version 4.2) was used to process the

analyzed data. Mass spectra of risperidone and escitalopram
are shown in Figure-2.The typical chromatograms for human plasma samples spiked with risperidone, escitolapram and IS at LLOQ and HQC are shown in Figure 3 and Figure 4.
The chromatographic parameters optimized include varying mobile phases, column types, and flow-rates, among others. Buffers like formic acid, ammonium acetate and ammonium formate varying combinations with methanol or acetonitrile on different columns (Symmetry C8, Symmetry C18, Hypersil Gold C18, X-terra RP8, and Inertsil-15 ODS) were tried initially to separate the analytes. Using a hit and trail procedure, a mixture consisting of acetonitrile and 5mM ammonium acetate buffer (95:5, v/v) adjusted to pH 9 ± 0.05 was found to be optimum on a X-terra RP8 (50×4.6 mm, 5-µ m) at a flow rate of 0.400 mL min^{-1}.

Selectivity, sensitivity and matrix effect: Inspecting the chromatograms derived from processed blank and spiked plasma samples showed that no significant interferences from indigenous components in the human plasma [20]. The respective lower limits of quantization's (LLOQs) were calculated to be 0.050 ng mL^{-1} and 0.100 ng mL^{-1} for risperidone and escitalopram, respectively. No significant matrix effect was observed for the analyses at LQC and HQC. Table 1 shows the results for comparative data of the absolute matrix effect, relative recovery and process efficiency for risperidone, escitalopram and IS.

Precision and accuracy: The reliability of the method was tested by spiking the analytes into blank human plasma samples at their LLOQs (0.050 ng mL^{-1} and 0.100 ng mL^{-1} for risperidone and escitalopram, respectively) and HQCs (19.435 ng mL^{-1} and 38.801 ng mL^{-1} for risperidone and escitalopram, respectively). The precision at LLOQ concentrations were found to be 7.10% and 12.69 % for risperidone and escitalopram, respectively. Overall method precision (%CV) was 2.9%, 5.07% (intra-day) and 4.13%, 6.43% (inter-day) for risperidone and escitalopram, respectively, detailed results are presented in Table 2. Accuracy presented as mean recovery was 76.88% ± 8.79, for risperidone, and 81.14% ± 8.20 for escitalopram.

Linearity and dilution integrity: The assay was linear over the concentration range of 0.050 to 26 ng mL^{-1} for risperidone and 0.100 to 51 ng mL^{-1} for escitalopram. Using the $1/X^2$ model, values for correlation coefficient (r^2) were calculated as ≥0.99 for both analytes and IS. The mean back calculated concentrations for 1/2 and 1/4 dilution samples were within 85-115% of their nominal values. The coefficient of variation (%CV) for 1/2 and 1/4 dilution samples were 3.58%, 0.92 % and 2.44 %, 2.95 % for risperidone and escitalopram, respectively. The % nominal recovery values for 1/2 and 1/4 dilution samples were 97.05 %, 97.60 % for risperidone, and 98.19 %, 99.01% for escitalopram.

Stability studies: The long term stability of the analytes in human plasma stored for a period of 22 days at -70 ± 5 °C when compared to zero day stability showed a promising stability behavior. The results of bench-top samples (5.22 h), auto-sampler samples (24 h), wet extract stability samples (24 h), dry extract stability samples (24 h) and freeze-thaw stability samples (4 cycles) are presented in Table 3.

Pharmacokinetic study: The present validated method was applied to a pharmacokinetic study for risperidone (1 mg) and escitalopram (20 mg) in human plasma samples collected from local healthy male volunteers (n=12). In a selected range of time intervals, the blood samples (post dose) were collected in K_2EDTA vacutainer tubes. The plasma from these samples was centrifuged at 4000 rpm, 2–8^0 C for 20 min and was stored at -20^0 C until analysis. Post analysis, the pharmacokinetic parameters were computed using Win Nolin software, Version 5.2 (Pharsight Corporation; Mountain View, CA). If not more than 33% of the QC samples were greater than +15% of the nominal concentration then the analytical runs were within the acceptable limits. The maximum concentration in plasma (C_{max}), time of C_{max} (T_{max}), area under the plasma concentration-time curve from zero hour to the last measurable concentration (AUC_{0-t}) and area under the plasma concentration-time curve from zero hour to infinity ($AUC_{0-\alpha}$) for risperidone were 5.71 ±2.35 ng mL^{-1}, 1.25 ±0.34 h, 0.64 ±29.41 ng.h mL^{-1} and 35.34 ±39.16 ng.h mL^{-1}, respectively, and for escitalopram 37.12 ±7.21 ng mL, 5.00 ±2.75 h, 1421.471 ±255.57 ng.h $mL^{-1}$and 2033.08 ± 436.39 ng.h mL^{-1}, respectively. The means of plasma concentration versus time profile of risperidone and escitalopram are shown in Fig. 5.

CONCLUSION

In summary, an accurate, selective, and high-throughput LC-MS/MS assay has been developed for simultaneous quantification of risperidone and escitalopram in human plasma. The cost-effective measures, simplicity of the assay using SPE and sample turnover rate of about 2.0 min per sample makes the present method an efficient procedure in

high-throughput bioanalysis [21-22] suitable for routine measurement of risperidone and escitalopram in both formulations and diagnostic samples. The method was successfully applied to study pharmacokinetic parameters using a tablet containing 1 mg of risperidone and 20 mg of escitalopram as an oral dose in healthy human volunteers under fasting conditions.

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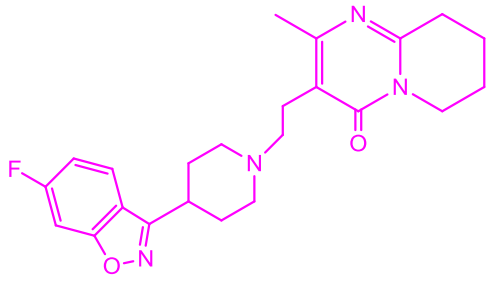
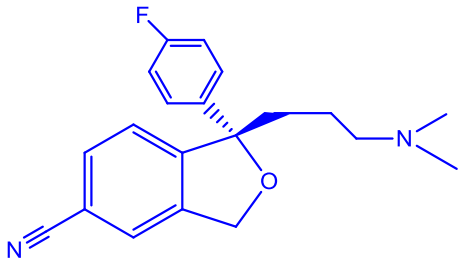
Conflicts of Interest: The authors declare no conflicts of interest.

(A)



(B)



(C)

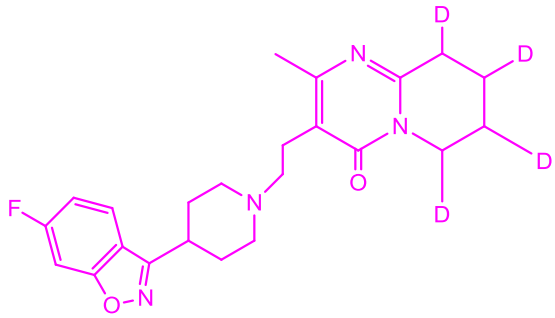


Figure 1. Chemical structures of (A) risperidone (B) escitalopram and (c) risperidone-D4

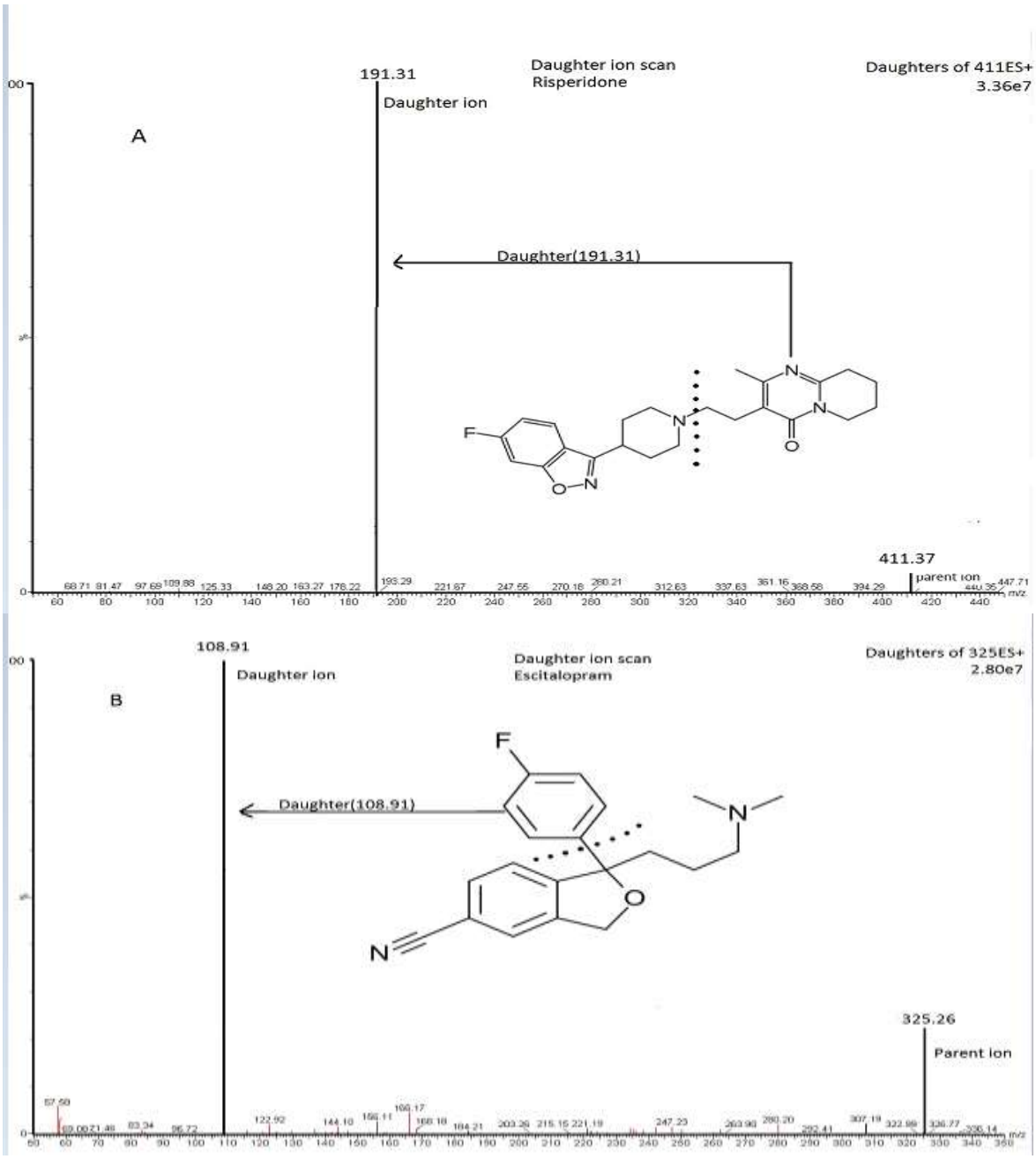


Figure 2. The production mass spectra of risperidone and escitalopram

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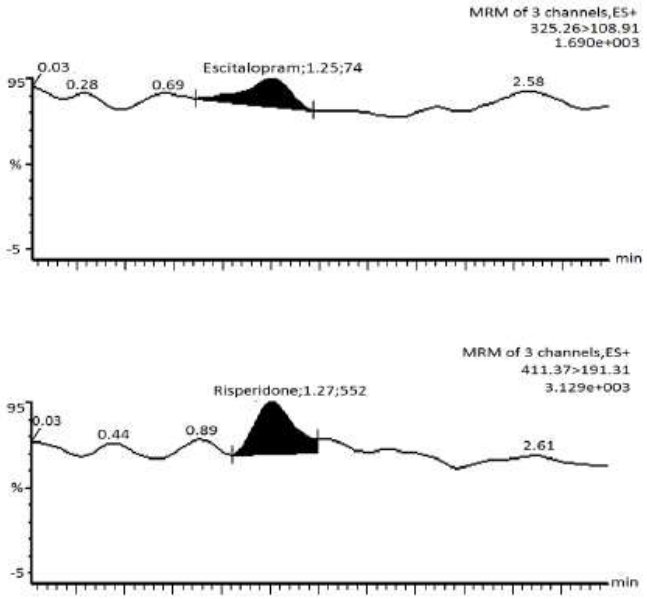

Figure 3. The typical chromatograms for human plasma samples of blank samples spiked with risperidone and escitalopram at LLOQ levels

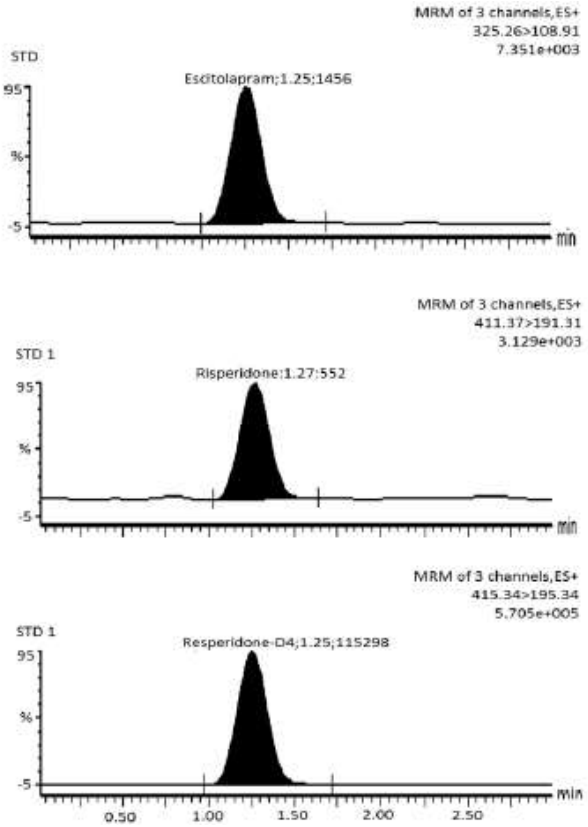

Figure 4. The typical chromatograms for human plasma samples of a blank sample spiked with risperidone,escitalopram and IS at HQC levels.

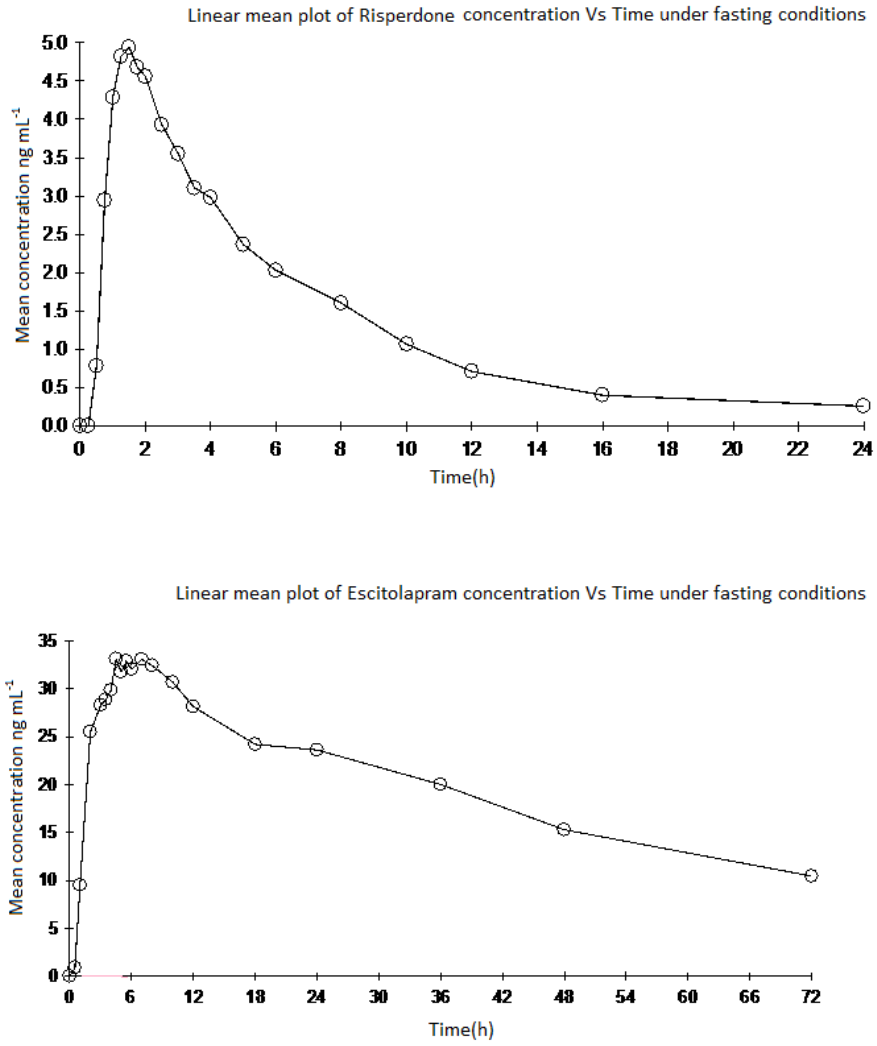

Figure. 5. Means of plasma concentration versus time profile of risperidone and escitalopram

TABLE 1. Comparative data of absolute matrix effect, relative recovery and process efficiency for risperidone and escitalopram spiked to blank samples

Analyte	A[a] (%CV)	B[b] (%CV)	C[c] (%CV)	Absolute matrix effect (%ME) [d]	Relative recovery (%RE) [e]	Process efficiency (%PE) [f]
STD 1						
RIS	755 (7.25)	825 (8.32)	555 (3.17)	109.16	67.31	73.48
ESC	1787 (7.02)	1956 (3.05)	1497(3.36)	109.46	76.50	83.74
RIS-D4	161896 (5.81)	176487 (3.53)	119568 (1.85)	109.01	67.75	73.85
STD 3						

RIS	5478 (3.62)	5995 (7.43)	4747 (2.84)	109.44	79.17	86.65
ESC	16249 (3.21)	17359 (5.76)	15473 (3.04)	106.83	89.14	95.23
RIS -D4	165082 (3.94)	179668 (6.57)	146634 (1.93)	108.84	81.61	88.83
STD 5						
RIS	60543 (3.74)	57861 (2.40)	44137 (5.77)	95.57	76.28	72.90
ESC	162774 (3.67)	156606 (2.13)	117049 (5.56)	96.21	74.74	71.91
RIS -D4	181307 (3.02)	173004 (2.98)	117416 (6.56)	95.42	67.87	64.76
STD 6						
RIS	88135 (2.55)	82118 (2.25)	62076 (2.60)	93.17	75.59	70.43
ESC	227733 (2.67)	208445 (2.17)	162294 (3.14)	91.53	77.86	71.26
RIS -D4	169266 (3.22)	155881 (1.86)	113390 (1.94)	92.09	72.74	66.99
STD 8						
RIS	122030 (1.35)	124531 (2.75)	107156 (2.62)	102.05	86.05	87.81
ESC	285734 (2.09)	291095 (2.42)	254638 (1.28)	101.88	87.48	89.12
RIS -D4	142720 (1.82)	145694 (2.55)	122364 (4.90)	102.08	83.99	85.74

[a] Mean area response of six replicate samples prepared in Mobile phase neat samples), [b] Mean area response of six replicate samples prepared by spiking in post extracted blank, [c] Mean area response of six replicate samples prepared by spiking in plasma before extraction,[d] %Matrix effect: Post extracted mean response/Aqueous (Neat) mean response × 100, [e] %Recovery: Extracted mean response / Post extracted mean response × 100, [f] %Process efficiency: Extracted mean response / Aqueous Mean response × 100.

TABLE 2. Intra- and inter-day precision and accuracy data risperidone and escitalopram (six replicates at each level)

Level	Quality control samples (ng/mL)	Intra-batch				Inter-batch			
		N	Mean conc. observed (ng/mL)	% Nominal Conc.	% CV	*N*	Mean conc. observed (ng/mL)	% Nominal Conc.	% CV
Risperidone									
LLOQC	0.050	6	0.056	112.17	7.10	24	0.053	106.83	7.47
LQC	0.150		0.149	99.33	2.72		0.101	101.42	3.93
MQC	11.369		11.591	101.95	1.06		11.545	101.55	2.73
HQC	19.435		19.852	102.14	0.70		19.567	100.68	2.38
Escitalopram									
LLOQC	0.100	6	0.114	113.87	12.69	24	0.107	106.64	10.72
LQC	0.298		0.310	104.14	5.83		0.292	98.07	6.68
MQC	22.699		23.917	105.36	1.24		22.886	100.82	5.18
HQC	38.801		38.771	99.92	0.54		38.427	99.04	3.13

Table 3. Stability data of risperidone and escitalopram under various conditions (n = 6)

Condition	Risperidone			Escitalopram		
Storage condition	Nominal conc[1].(ng/mL)	Mean calculated conc. (ng/mL) ± SD	%Mean accuracy	Nominal conc. (ng/mL)	Mean calculated conc. (ng/mL) ± SD	% Mean accuracy
Bench top stability (5.22 hours)						
LQC	0.150	0.151 ± 0.0046	100.67	0.298	0.302 ± 0.0330	101.45
HQC	19.435	19.498 ± 0.1990	100.33	38.801	36.525±0.1881	94.13
Dry extract stability (24.00 Hours)						
LQC	0.150	0.140 ± 0.0105	93.22	0.298	0.295 ± 0.0108	99.05
HQC	19.435	19.809 ± 0.0867	101.92	38.801	36.358 ± 0.4477	93.70
Wet extract stability (24.00 Hours)						
LQC	0.150	0.145 ± 0.0034	96.78	0.298	`1	98.66
HQC	19.435	20.004 ± 0.1906	102.93	38.801	36.151 ± 0.3225	93.17
Auto sampler stability (24.00 Hours)						
LQC	0.150	0.150 ± 0.0086	100.11	0.298	0.291 ± 0.0098	97.48
HQC	19.435	18.781 ± 1.1229	96.63	38.801	40.180 ± 4.3256	103.55
Freeze & thaw stability (Three cycles)						
LQC	0.150	0.143 ± 0.0109	95.33	0.298	0.310 ± 0.0244	103.86
HQC	19.435	19.440 ± 0.2258	100.03	38.801	36.323 ± 0.3053	93.61
Long term stability in plasma at -70°C (22.00 days)						
LQC	0.150	0.151 ± 0.0093	100.89	0.298	0.300 ± 0.0172	100.62
HQC	19.435	18.650 ± 0.8890	95.96	38.801	37.863 ± 2.5497	97.58

[1]: Conc= concentration

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