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Original Article

A Novel Bioanalytical Method for Simultaneous Determination of Olanzapine and Samidorphan in Human Plasma Using RP-HPLC

Bonagiri Pavani[1], Narender Malothu[1,*], DSNBK Prasanth[2], Chakravarthi Guntupalli[1]

[1]Department of Pharmacy, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Andhra Pradesh, INDIA.
[2]Department of Pharmacognosy, KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, INDIA.

ABSTRACT

Background: Schizophrenia patients are given with a combined medication of Olanzapine (OLA) and Samidorphan (SAM). A sensitive bioanalytical RP-HPLC technique has to be needed to analyse these drugs in biological samples, hence we have been developed a method for simultaneous determination of OLA and SAM in human plasma. **Materials and Methods:** The separation was conducted on a Zorbax SB-C_{18} column (250 mm x 4.6 mm, 5µ) and an isocratic mobile phase of 10 mM ammonium acetate and acetonitrile (60:40% v/v) at 0.8 mL/min. The quetiapine was employed as Internal Standard (IS). Plasma was treated with phosphoric acid, and then extracted using a HLB cartridge. RP-HPLC coupled with a PDA detector was used for measurement. **Results:** The SAM, OLA, and IS peaks were appeared at 3.894, 9.572, and 2.318 min, respectively. The method has been found to be sensitive up to 2.25 µg/mL for SAM and 1.00 µg/mL for OLA in human plasma matrix. The method was validated for accuracy, precision, recovery, stability and matrix effect. Solid Phase Extraction (SPE) provided clean samples with good and constant mean recovery: 93.32% for SAM, 97.62% for OLA, and 92.78% for IS. The method was linear over 2.25-90 µg/mL and 1-40 µg/mL with $R^2 \geq 0.99$ for both analytes, respectively. Three-month stability investigations were carried out at-20°C, 8°C, and room temperature. **Conclusion:** The developed method was found to be sensitive, accurate, and repeatable, hence it could be applied for routine analysis of these drugs in biological samples.

Keywords: Olanzapine, Samidorphan, Zorbax, Ammonium acetate, Acetonitrile.

Correspondence:
Dr. Narender Malothu
Associate Professor, Department of Pharmacy, K L College of Pharmacy, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur-522 502, Andhra Pradesh, INDIA.
Email: narendermalothu@gmail.com

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INTRODUCTION

Olanzapine (OLA) is a well-known antipsychotic that has benefits like a lower chance of extrapyramidal side effects.[1-3] On the other hand; OLA has some safety and tolerability problems, such as widespread weight gain and metabolic abnormalities. These problems have been linked to serious health complications and may make it harder for patients to stay on OLZ therapy.[4-7] Research done on people with schizophrenia and schizoaffective disorder in the past showed that combining an opioid antagonist and an antipsychotic medicine can help people lose weight.[8] It has been demonstrated that the new opioid system modulator Samidorphan (SAM) acts *in vivo* as a µ-opioid antagonist.[9] SAM is used to treat opioid addiction. SAM binds with a high degree of affinity to human µ-, k-, and δ-opioid receptors. It exerts an antagonistic effect on µ-opioid receptors, although its intrinsic activity is rather weak at δ and k opioid receptors.[10]

Lybalvi is a once-daily oral bilayer tablet that contains a variable dosage of OLA (5 mg, 10 mg, 15 mg, and 20 mg) on one layer and a set dose of SAM (10 mg) on the other. The purpose of Lybalvi is to give OLA demonstrated antipsychotic effectiveness while maintaining a favourable weight and metabolic profile.[11,12] In clinical studies (Phase I and II), combining SAM and OLA led to a reduction in the weight gain that was caused by OLA. The majority of the OLA dosage is metabolised in the liver, with just 7% of the total OLA dose being removed in its original form through the urine.[13] Direct glucuronidation *via* uridine diphosphate glucuronosyltransferase 1A4 and cytochrome P-450 (CYP)-mediated oxidation, largely via CYP1A2, are the two principal metabolic routes for OLA. Both of these mechanisms are mediated by uridine diphosphate glucuronosyltransferase.[14] SAM is mostly gotten rid of from the body by the liver's metabolic process.[15] An earlier clinical trial found that there was no pharmacokinetic drug-drug interaction between OLA and SAM, which is in line with the fact that the two medications have

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independent metabolic pathways.[16] The chemical structures of the OLA and SAM, were shown in Figures 1a and 1b.

According to a survey of the relevant literature, only a few analytical techniques that employ a variety of approaches have been published for the estimation of OLA and SAM both separately and in combination with other analytes. Some of the techniques that were applied include LC/MS,[17,18] LC-MS/MS,[19-22] RP-HPLC,[23-29] spectrophotometry,[23,30-35] and voltammetry.[30-36] There is no well-established bioanalytical technique that can determine SAM in conjunction with OLA. It is necessary to develop a new RP-HPLC bioanalytical technique that is cost-effective, accurate, and quick for simultaneous estimation of both drugs in human plasma. This method could be used to study the bioavailability of drugs in different dosage forms, which is needed because the global demand for these drugs is growing.

MATERIALS AND METHODS

Chemicals and solvents

The API of OLA and SAM were procured from Dr. Reddy's Laboratories in Hyderabad, India, while Quetiapine (Internal Standard) was obtained from Spectrum Laboratories, Hyderabad, India. During the analysis, Millipore Milli-Q system Type II HPLC water was employed. The HPLC quality acetonitrile was purchased from Merck, India. All other chemicals were acquired from SD Fine Chemicals, Mumbai, India, and were analytical grade. The human K_2-EDTA plasma was procured from Bruhada blood bank, Kadapa, India.

Instrumentation

A RP-HPLC system manufactured by Waters LC (Waters, Milford, Massachusetts, United States) was utilised. This system was equipped with a quaternary gradient system (controller 600), inline degasser (Waters, model AF), PDA detector (Waters, model 2998), and auto sampler (Waters, model 717 plus). To process the data, Empower Pro was utilised (Waters, Milford, MA, USA).

Chromatographic conditions

The technique was employed on a Zorbax SB-C_{18} column (250 x 4.6 mm, 5 µ) that was maintained at room temperature. An isocratic mobile phase consisting of ammonium acetate (10 mM) and acetonitrile (60:40 v/v) was pumped to the system at a flow rate of 0.8 mL/min. The isobestic wavelength of detection for analytes was 270 nm. To prepare working standards of OLA and SAM, the mobile phase was utilised as a diluent.

Stock and working standards solutions

About 25 mg of each OLA and SAM were dissolved in separate 25 mL volumetric flasks to make the stock solutions using acetonitrile. Then after the concentrations of OLA and SAM solutions were adjusted to have final concentration of 500 µg/mL. From this the calibration standards prepared to achieve the desired final concentrations of 2.25, 4.5, 9, 20.25, 33, 45, 76.5 and 90 µg/mL for SAM and 1, 2, 4, 9, 14.5, 20, 34, and 40 µg/mL for OLA. Quality control (QC) samples were prepared for SAM as HQC: 76.5 µg/mL, MQC: 45 µg/mL; LQC: 6.75 µg/mL; and LLOQQC: 1.13 µg/mL. Similarly, for OLA the HQC, MQC, LQC and LLOQQC were made to 0.5, 3, 20 and 34 µg/mL, respectively. Further, from the stock solution (2.5 mg/mL) of IS the working standard solution of IS (30 µg/mL) was prepared. All the solutions were kept 2-8°C.

Extraction protocol for samples

After being stored in a deep freezer at -70°C, human plasma control samples were removed and allowed to defrost at room temperature for 30-45 min. A vortex mixer was used to produce the samples with a suitable amount of a whirl before pipetting them. With the use of a micropipette, plasma (0.75 mL) was transferred into an Eppendorf micro-tube. After adding 50 µL each of OLA, SAM, and IS working solutions, the tubes were vortexed to mix the contents. In the same tube, the 0.1 % phosphoric acid (20 µL) and water (0.5 mL) was added, and the mixture was vortexed again to mix the ingredients. After conditioning the HLB

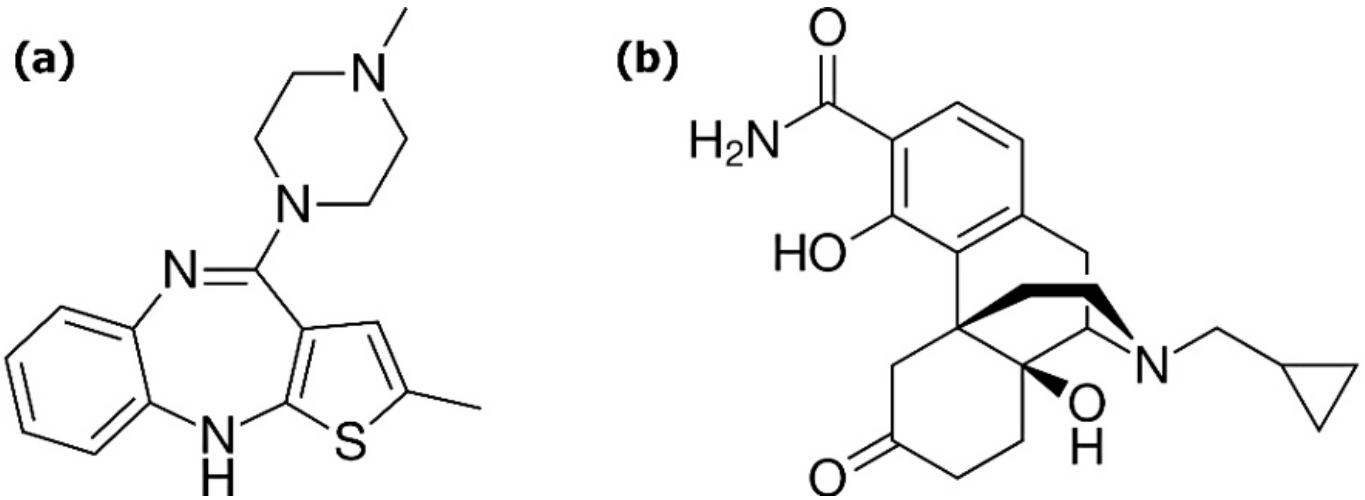

Figure 1: Chemical structures of (a) OLA (b) SAM.

cartridge with each 1 mL of acetonitrile and aqueous acetic acid at a concentration of 2% (v/v), then these samples were inserted into the cartridge. The sample-loaded cartridge was washed twice with 2 mL water, 1 mL acetonitrile (5%), and then once more with 1 mL water. The aqueous component was then vacuumed for 5 min. With 0.5 mL of acetonitrile, OLA, SAM, and IS were eluted, and 10 µL of the eluate was immediately fed into the HPLC.

Method Validation

The validation procedure was validated in the accordance with the standards set out by the USFDA.[37]

Selectivity

By comparing the signals in six distinct plasma lots (four were K_2EDTA and one was each lipidemic and hemolyzed) with the analyte and IS signals at the LLOQ level, interference owing to endogenous plasma matrix components was investigated. At this concentration level, the reference noise should be around 20% of the analyte response.

Calibration curve and LLOQ

A series of eight (8) calibration curve standards, 2.25, 4.5, 9, 20.25, 32.63, 45, 76.5 and 90 µg/mL for SAM and 1, 2, 4, 9, 14.5, 2, 34 and 40 µg/mL for OLA were prepared to test the method's linearity. Validation of each calibration curve was accomplished through the use of a 1/x2 weighted least squares regression analysis of standard plots related with an eight-point standard curve. Resulted standard curve was selected to encompass the patients' clinically relevant concentration range. The curve has been verified; at least six of the eight calibration standards must have a Coefficient of Variation (CV) of less than 15%. All calibration curves were checked to have a better correlation coefficient (R^2= 0.99). The lowest concentration that appeared on the calibration curve was chosen to serve as the LLOQ. In general, the CV and accuracy error of any single calibration standard should be less than 15%, while the LLOQ should have a CV and accuracy error that is less than 20%. In addition, it is necessary for the response of the LLOQ of the analyte to be at least five times greater than the response of the blank.

Precision and Accuracy

Precision and accuracy are key components in the evaluation of repeatability. Six repeat samples from each LLOQ, as well as low, medium, and HQC samples were required to be collected in order to assess the precision and accuracy of the method. Plasma samples were analysed the same day to establish the test's intraday precision and accuracy. Six distinct batches were assessed to determine the precision and accuracy over different days. By calculating the percentage (%) difference between the theoretical value and the value attained by using the equation, the degree of accuracy was ascertained:

$$\text{Accuracy}\ (\%) = \frac{(\text{Measured Concentration} - \text{Theoretical Concentation})}{(\text{Theoretical Concentation})} \text{X}100\%$$

In terms of accuracy and precision, the % deviation of each concentration level from the nominal concentration should be less than 15%, with the exception of LLOQ, which should not exceed 20%.

Recovery from plasma

The extraction (recovery) efficiency of the method was assessed as the ratio between the mean analyte concentrations in plasma after extraction of the HQC, MQC, and LQC samples and the corresponding amounts directly dissolved in the elution solution. Comparing the mean concentration of six plasma samples to an identical concentration of reference IS solution allowed to estimate the IS recovery. Analyte recovery must not be 100%, but rather consistent, accurate, and repeatable, in accordance with FDA regulations.

Matrix effect

By comparing the mean peak areas of samples of IS spiked with blank plasma to those of samples of a specific analyte concentration, it was possible to identify the matrix effect. A 1000 µL final elution solution can be added to the same amount of analyte and IS to compare this reaction. In order to evaluate the matrix influence on the HPLC technique, six different chromatographically screened human plasma lots were used. Each batch of plasma was injected in triplicate with sample concentrations made to correspond to LQC and HQC at each level. The matrix effect was proven if the % CV did not exceed 15% of all Calibration curve standards (CCs).

Stability

The analyte stock solutions were tested for stability over a period of 7 days at 8°C and 7 hr at room temperature. The difference between the mean peak areas of the freshly prepared solutions and the stability solutions was expressed as a mean% change. Six replicates of each concentration were tested at the workbench (at room temperature for 6 hr), during a freeze-thaw cycle (at -20°C for 24 hr), during short-term stability (6 hr at 8°C and 7 hr at room temperature), and during long-term stability (at -20°C) in the study of each analyte. As long as the change is less than 15%, the analyte is considered stable, according USFDA regulations. Under a variety of temperature and time conditions, the analytes' laboratory stability, short-term stability, and freeze-thaw stability were all assessed. The level of stability was expressed as a % of the mean difference between the estimated concentration and the actual concentration following the computation of the concentrations for both the stability samples and the freshly processed samples. At a temperature of -20°C, the long-term stability was tested for 30 days before being treated and put up

against freshly made solutions. The % change was calculated using following formula,

$$\% \text{ Change} = \frac{\text{Mean Stability Sample} - \text{Mean Comparision Sample}}{\text{Mean Comparision Sample}} \text{X100}$$

RESULTS AND DISCUSSION

Sample preparation is one of the crucial fundamental procedures that must be carried out in the process of establishing a bioanalytical approach. The sample must be prepared quickly, simply, and uncomplicatedly in order to produce the maximum analyte recovery while utilising the fewest reagents and solvents. Solid Phase Extraction (SPE) was used to improve the recovery when protein precipitation procedure failed to recover the analytes to a satisfactory level. The HLB cartridge was employed for extraction. It had previously been conditioned with 1 mL of acetonitrile and 1 mL of 2% (v/v) aqueous acetic acid. Following that, acetonitrile and water were used to load and wash the sample. The aqueous component was then removed by vacuuming for five minutes. The OLA, SAM, and IS were eluted with 0.5 mL of acetonitrile, and 10 µL of the eluate was immediately put into the HPLC.

Because of its reduced plasma volume and injection volume requirements, the current methodology has gained more acceptability. Furthermore, neither the analyte recovery nor sensitivity were impacted by the addition of IS in any manner. There was no external or endogenous plasma matrix influence.

In the process of optimization of method, the clozapine, loxapine, and quetiapine were all tried as IS, in which quetiapine was finally chosen as the IS due to its compatible physio-chemical properties including pKa 6.8 and log P 2.81. Furthermore, a tiny amount of quetiapine was enough to provide a potent signal in PDA. All of the chromatographic conditions were adjusted and optimized to produce a high level of sensitivity, peak symmetry, as well as a short chromatographic run time for the analyte and the IS. Thermos C_{18} Column (150 x 4.6 mm, 5 µ), Gemini C_8 Column (250 x 4.6 mm, 5 µ), and Luna C_8 Column (150 x 4.6 mm, 5 µ) columns were utilised with a variety of mobile phases in this experiment. As a result of its superior separation and detection capabilities, a Zorbax SB-C_{18} Column (250 x 4.6 mm, 5 µ) was ultimately chosen. In order to develop a sensitive, accurate, and precise RP-HPLC method for the simultaneous quantification of SAM and OLA in human plasma, a number of systematic trials were carried out to optimise the mobile phase. Different organic solvents were used to examine the solubility of the drugs; water, ethanol, acetonitrile, and DMSO showed the greatest solubility. Methanol and ammonium acetate buffer were used in equal proportions for the initial trial. Both analytes recorded with a very significant matrix effect and were not eluted. The methanol content in the ratio was optimised by a number of trials, however they were unsuccessful. Methanol was then replaced with acetonitrile, and ammonium acetate was utilised as the counter buffer in the mobile phase to balance the non-polar end. However, the IS peak was eluted in less than 2 min, which is regarded a void peak. As a result, the ammonium acetate buffer concentration was increased to 60%, resulting in a perfect chromatogram that eluted for both the analytes. This column was operated at a flow rate of 0.8 mL/min with an isocratic mobile phase that was composed of 10 mM ammonium acetate and acetonitrile in a 60:40 v/v ratio, in a column oven temperature of 30±2°C. A larger number of different investigations were carried out on the same column because to the small injection volume of 10 µL that helped prevent the column from being overloaded with analytes. Retention times for SAM, OLA, and IS were 3.894, 9.572, and 2.318 min, respectively, for the entire trial, which took 13 min to accomplish. In Figures 2 (a-f), the chromatograms for Blank, the IS peak, HQC, MQC, LQC, and LLOQ are displayed in order from left to right.

Selectivity

No interference peaks were detected at the retention time of the sample of SAM, OLA, and IS that was isolated from human plasma, which was shown in Figures 2(c-f). In general, these interference peaks might be attributable to either endogenous or exogenous components. When the analytes were evaluated on blank plasma, there was a response of less than 2%. A typical retention time of 3.894, 9.572, and 2.318 min was observed for SAM, OLA, and IS, respectively.

Calibration and LLOQ

It has been demonstrated that the linear calibration curves for SAM and OLA comprise the ranges of 2.25-90 µg/mL and 1-40 µg/mL, respectively. SAM had the lowest detectable concentration of 1.13 µg/mL, and OLA had the lowest detectable concentration of 0.50 µg/mL. The coefficient of correlation (R^2) for each of the six calibration curves was greater than 0.99, which was the threshold for statistical significance. The current bioanalytical technique provided results with a wide range of linearity and a lower limit of quantitation. The obtained mean concentrations are shown in Table 1, which also includes the calibration curve.

Precision and accuracy

For SAM, the method's precision ranged from 0.43 to 7.93% of CV, while its intraday accuracy ranged from 93.40 to 105.46%. On the other hand, OLA had an average accuracy range of 87.01 to 115.29% and a precision range of 1.90% to 12.63% of CV. For SAM, the mean precision ranged from 1.70 to 12.11% of CV, and the inter-day accuracy ranged from 92.21 to 107.78%. The mean precision ranged from 5.24 to 12.17% of CV, whereas the mean accuracy for OLA ranged from 88.60 to 114.03%. The approach demonstrated acceptable levels of repeatability and reproducibility, according to the results. Table 2 contains the details on accuracy and precision.

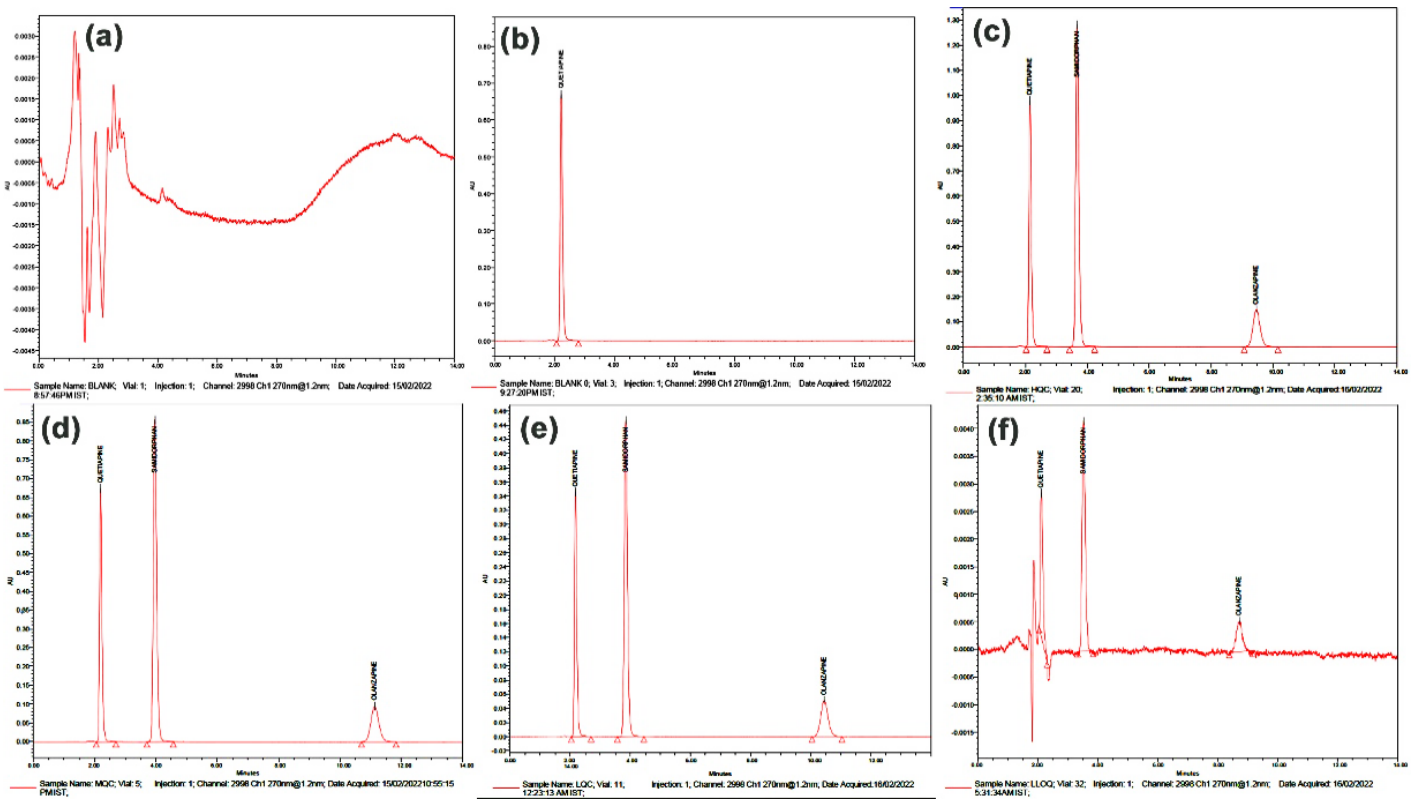

Figure 2: Representative chromatograms of (a) Blank; (b) IS; (c) HQC; (d) MQC; (e) LQC; (f) LLOQ.

Table 1: **Calibration data of SAM and OLA.**

SAM											
STD ID	**STD-1**	**STD-2**	**STD-3**	**STD-4**	**STD-5**	**STD-6**	**STD-7**	**STD-8**	**Slope**	**Intercept**	**R^2**
Nominal Concentration (µg/mL)	**2.250**	**4.500**	**9.000**	**20.250**	**32.625**	**45.000**	**76.500**	**90.000**			
Back Calculated Concentration (µg/mL)											
Mean	2.2535	4.4305	8.8895	20.1970	32.5325	44.8375	76.4360	89.5855	0.9973	-0.0205	0.9991
SD	0.01061	0.10112	0.15768	0.03111	0.07000	0.07707	0.05940	0.43487			
% CV	0.47	2.28	1.77	0.15	0.22	0.17	0.08	0.49			
% Mean Accuracy	100.16	98.46	98.77	99.74	99.72	99.64	99.92	99.54			
OLA											
STD ID	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8	Slope	Intercept	R^2
Nominal Concentration (µg/mL)	1.000	2.000	4.000	9.000	14.500	20.000	34.000	40.000			
Back Calculated Concentration (µg/mL)											
Mean	0.4026	0.9479	3.7189	10.0644	15.0038	19.8642	32.8676	39.5857	0.9914	-0.1167	0.9991
SD	0.12221	0.36923	0.21791	0.39233	0.02652	0.34059	0.52526	0.13388			
% CV	30.36	38.95	5.86	3.90	0.18	1.71	1.60	0.34			
% Mean Accuracy	40.26	47.40	92.97	111.83	103.47	99.32	96.67	98.96			

Table 2: Precision and accuracy data of SAM and OLA.

Analytes	SAM				OLA			
QC ID	HQC	MQC	LQC	LLOQ QC	HQC	MQC	LQC	LLOQ QC
Nominal Concentration (µg/mL)	76.500	45.000	6.750	1.125	34.000	20.000	3.000	0.500
Calculated Concentration (µg/mL)								
Within the batch precision and accuracy								
Mean	72.6098	43.2320	6.3044	1.1864	29.5830	19.0029	3.4586	0.5340
SD	0.46410	0.18391	0.50019	0.01888	0.56185	0.65465	0.30925	0.06744
% CV	0.64	0.43	7.93	1.59	1.90	3.45	8.94	12.63
% Mean Accuracy	94.91	96.07	93.40	105.46	87.01	95.01	115.29	106.79
Between Batch Precision and Accuracy								
Mean	74.2660	43.8884	6.2241	1.2126	31.5881	17.7192	3.4208	0.5337
SD	1.68863	0.74574	0.75344	0.06323	1.65587	1.42676	0.41622	0.05344
% CV	2.27	1.70	12.11	5.21	5.24	8.05	12.17	10.01
% Mean Accuracy	97.08	97.53	92.21	107.78	92.91	88.60	114.03	106.74

Table 3: Results of recovery.

Replicate No.	SAM						OLA						ISTD	
	HQC		MQC		LQC		HQC		MQC		LQC			
	AR	ER	AR	ER	AR	ER	AR	ER	AR	ER	AR	ER	AR	ER
Mean*	76.40	75.60	43.30	42.50	7.00	5.80	35.50	33.80	21.40	20.90	15.20	15.20	19.40	18.00
SD	0.50	0.50	0.70	0.20	0.20	1.00	1.10	0.40	0.40	0.30	0.10	0.60	0.70	0.80
% CV	0.65	0.66	1.62	0.47	2.86	17.24	3.10	1.18	1.87	1.44	0.66	3.95	3.61	4.44
% Mean Recovery	98.95		98.15		82.86		95.21		97.66		100.00		92.78	
Overall% Mean recovery	93.32						97.62						92.78	
Overall SD	9.07						2.40						4.03	
Overall% CV	9.72						2.45						4.34	

AR: Aqueous Response; ER: Extracted Response; *Average of 6 replicates.

Recovery

The mean peak areas of three freshly produced, extraction free samples of varied concentrations were compared to the mean peak areas of the collected samples to determine the % of recovery. To assess the mean% of recoveries, Recovery was determined by comparing the area under the curve (AUC) of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentrations of OLA and SAM as in the QC samples. The % of recovery was determined by comparing the mean peak areas of the collected samples with the mean peak areas of three freshly prepared, extraction-free samples of varied concentrations. The HQC, MQC, and LQC have average recovery rates of 98.95%, 98.15%, and 82.86% for SAM, respectively. The MQC, LQC and HQC for OLA were found correspondingly as 95.21%, 97.66%, and 100.00%. Table 3 presents the recovery's results. In these studies about 92.78% of the IS was recovered. It has been stated that the recovery % in analysis methods should be at least 80%. In general, the development of a bioanalytical technique for the purpose of recovery is not viewed as problematic if the method obeys sensitivity and precision, but it is problematic if it does not.

Table 4: Results of Matrix effect.

Parameter	SAM		OLA	
	HQC	LQC	HQC	LQC
	Nominal concentration (µg/mL)		Nominal concentration (µg/mL)	
	76.500	6.750	34.000	3.000
	Calculated concentration (µg/mL)	Calculated concentration (µg/mL)	Calculated concentration (µg/mL)	Calculated concentration (µg/mL)
Mean*	75.6348	6.7550	34.2688	3.1622
% Mean Accuracy	98.87	100.07	100.79	105.41
SD	1.5651	0.4858	1.0294	0.1487
% CV	2.07	7.19	3.00	4.70

Note: *Average of 6 plasma batches.

Table 5: Stability data of SAM at Low and High QC levels.

Stability	QC Level	Mean Measured concentrations (µg/mL); (*n*=6)		% Change	%CV	% Mean stability*
		Comparison sample	Stability sample			
Benchtop	HQC	77.64	80.49	3.67	1.19	103.67
	LQC	6.65	6.92	4.08	4.34	104.08
Freeze-thaw	HQC	77.45	77.79	0.45	0.64	100.45
	LQC	6.70	7.25	8.11	2.77	108.11
Auto sampler	HQC	77.61	75.55	2.66	0.75	97.34
	LQC	6.70	6.68	0.33	1.56	99.67
Short term	HQC	76.38	76.30	-0.11	0.14	99.89
	LQC	6.66	7.11	6.79	4.09	106.79
Long term	HQC	74.91	74.35	-0.75	1.21	99.25
	LQC	6.40	6.72	5.04	11.84	105.04

Note: * % Stability = % mean change in the concentration of the stability samples when compared to the freshly spiked samples.

Matrix Effect

Six distinct batches of human plasma were used to establish LQC and HQC concentrations of SAM and OLA, which were then chromatographically examined to determine the effect of the matrix. The matrix effect data were shown in Table 4. The calculated % mean accuracy showed that the plasma lots selected have no matrix influence.

Stability

Bench top stability

A six-hour benchtop stability test of plasma samples of HQC and LQC concentrations was conducted. Freshly prepared samples were compared to analyze stability. Based on the % mean stability for HQC and LQC was found as 103.67 and 104.08% for SAM and 100.96 and 90.78% for OLA, respectively.

Freeze-thaw stability

The stability of frozen samples was evaluated by subjecting them to three cycles of freezing and thawing; following this, the samples were kept at a temperature of -20°C. Freeze-thaw stability % mean values for SAM were 100.45 and 108.11% for HQC and LQC, respectively, whereas OLA shown 107.17 and 106.82% for HQC and LQC, respectively.

Auto sampler stability

In an auto sampler maintained at 10°C, HQC and LQC samples were stored. A comparison of the samples injected at zero hours and those injected at stability time was undertaken for the assessment of auto sampler stability. In terms of % mean stability, the % mean for HQC and LQC for SAM was found as 97.34 and

Table 6: Stability data of OLA at Low and High QC levels.

Stability	QC Level	Mean Measured concentrations (µg/mL); (*n*=6)		% Change	%CV	% Mean stability*
		Comparison sample	Stability sample			
Benchtop	HQC	34.16	34.49	0.96	3.44	100.96
	LQC	3.27	2.97	-9.22	4.10	90.78
Freeze-thaw	HQC	32.03	34.33	7.17	2.03	107.17
	LQC	3.15	3.37	6.82	5.22	106.82
Auto sampler	HQC	32.60	34.85	6.91	1.19	106.91
	LQC	3.35	3.51	4.84	10.27	104.84
Short term	HQC	33.38	34.67	3.88	0.76	103.88
	LQC	3.16	3.21	1.53	10.25	101.53
Long term	HQC	34.36	34.32	-0.11	2.67	99.89
	LQC	2.97	3.09	4.06	7.14	104.06

Note: * % Stability = %mean change in the concentration of the stability samples when compared to the freshly spiked samples.

99.67%, respectively, and whereas OLA was shown 106.91% and 104.84%, respectively.

Short term stability of drugs in plasma

During six and a half hours, post-extracted HQC and LQC samples were stored at ambient temperature for determining drug concentrations. The samples were compared against freshly prepared samples to determine stability. During the last six and a half hours of testing, the % mean stability of HQC and LQC was 99.89 and 106.79% for SAM, respectively. For OLA HQC and LQC were found to be 103.88 and 101.53% respectively. The IS % mean stability was 99.45%.

Long term stability of drug in plasma

A 30-day long term stability test of the spiked QC samples (HQC and LQC) was performed below -20°C and below -50°C. The stability of the freshly spiked QC samples was evaluated by comparing them to samples that were frozen for about 15 and 30 min and then thawed. HQC and LQC had stable % mean values for SAM were 99.25 and 105.04%, respectively. In contrast, the overall HQC and LQC for OLA were 99.89 and 104.06%. The IS% mean stability was 93.81%. SAM and OLA stability results were shown in Tables 5 and 6.

CONCLUSION

The proposed HPLC approach has a high linear range and excellent selectivity for the quick, sensitive, and repeatable detection of OLA and SAM in human plasma. In validation it met every criterion with a very high level of accuracy and precision in compliance with the norms and recommendations provided by the USFDA. The absence of matrix effects has been established. The results of the stability assessment for OLA and SAM were found to be stable in plasma under varied storage conditions and throughout sample preparation. This novel method is more appropriate and pertinent to represent the clinical pharmacokinetics and bioequivalence assay investigations of simultaneous estimation of OLA and SAM since it has a lower LLOQ and uses less plasma than earlier approaches.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

RP-HPLC: Reverse Phase-High Performance Liquid Chromatography; **PDA:** Photodiode Array Detector; **USFDA:** United States Food and Drug Administration; **HQC:** High Quality Control; **LOQ:** Low Quality Control; **MQC:** Medium Quality Control; **LLOQ:** Lower Limit of Quantification; **ULOQ:** Upper Limit of Quantification; **K_2EDTA:** Di Potassium Ethylene Diamine Tetra Acetic Acid.

SUMMARY

The developed HPLC method was optimized for the simultaneous estimation of Olanzapine (OLA) and Samidorphan (SAM) in human plasma. Quetiapine was used as internal standard (IS) due to its structural resemblance. The analytes were extracted from plasma using a HLB cartridge with mixture of acetonitrile

(1 mL) and aqueous acetic acid (1 mL) at a concentration of 2% (v/v). The separation of OLA and SAM was achieved with 10 mM ammonium acetate and acetonitrile (60:40% v/v) at 0.8 mL/min flow rate on Zorbax SB-C18 Column (250 mm x 4.6 mm, 5 µ). Furthermore, the method was validated in accordance with USFDA criterion. The LLOQ for OLA and SAM plasma determined using the suggested approach was1.13 µg/mL and 0.50 µg/mL, respectively. The retention times of SAM, OLA, and IS were found at 3.894, 9.572, and 2.318 min, respectively. The method has a found sensitive up to 2.25 µg/mL for SAM and 1 µg/mL for OLA in human plasma matrix. Solid phase extraction provided clean samples with good and constant mean recovery: 93.32% for SAM, 97.62% for OLA, and 92.78% for IS. The method was linear over 2.25-90 µg/mL and 1-40 µg/mL with $R^2 \geq 0.99$ for both analytes, respectively. At three QC levels (HQC, MQC, and LQC), the accuracy and precision were assessed. Drug variation in plasma using the proposed approach showed % CV less than 12.11% for SAM and 12.17% for OLA, indicating that the method was accurate. The mean % CV obtained for the drugs spiked in plasma for stability studies were less than 2.8% for SAM and 4.7% for OLA. Therefore the proposed method could be considered as valid for routine analysis drugs in biological matrices.

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