WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

Unique UPLC-MS/MS Method of Serotonin Drug Analysis in Human Plasma, Serum and Urine and its Application in Therapeutic Drug Monitoring

'Determination of Serotonin Drug in Human Matrix'

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ABSTRACT:

A reliable and sensitive method for analyzing hormone using liquid chromatography tandem mass spectrometry (LC–MS/MS) is required for research concerning serotonin, which plays a carries messages between nerve cells in your brain (your central nervous system) and throughout your body (your peripheral nervous system). We describe sample processing and analysis methods quantification of serotonin, in plasma, serum, and urine using LC–MS/MS. Method validation consisted of sensitivity, accuracy and precision, serotonin drug was detected and quantified within 6.0 min without endogenous interferences. The correlation coefficient (R^2) was >0.99 for analyte. It improved upon previous methodologies by offering facile and rugged sample preparation with improved chromatographic conditions, which culminated in a highly accurate and precise method for serotonin determination in a wide range of formulas. No carryover was observed in a blank control injected after the highest standard. The method has been successfully verified using authentic case samples that had previously been quantified using different methods. The assay is suitable for clinical utilization and management of patients on these medications.

KEYWORDS: Serotonin; UPLC-MS/MS; human plasma/seraum/urine

Introduction:

Serotonin, also known as 5-hydroxytryptamine (5-HT) (Figure 1), is a monoamine neurotransmitter. It also acts as a hormone. As a neurotransmitter, serotonin carries messages between nerve cells in your central nervous system and your peripheral nervous system [14].

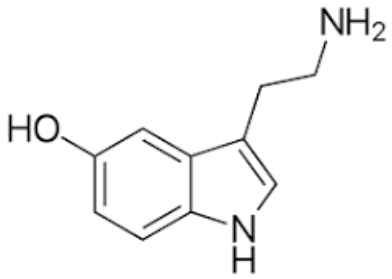


Figure 1. Structure of Serotonin

Acting through several receptor types, it helps regulate the normal functioning of the gastrointestinal tract, cardiovascular system and brain. Serotonin signaling has also been implicated in the etiology of several diseases, including depression, anxiety disorders, hypertension and irritable bowel syndrome. During the 1930s and 1940s, a research group led by I.H. Page at the Cleveland Clinic worked to identify endogenous constrictive factors in blood that might play a role in hypertension [8]. The molecular structure of serotonin was determined in 1949 [20]. Serotonin is found in vertebrates, invertebrates, plants, and bacteria [6, 16, 22, 23 and 26]. Serotonin is readily secreted in patients with carcinoid tumors, especially arising from the mid gut [29] .It is

reported that certain carcinoid tumors could occur at any age in life with an equal gender distribution [7] .In spite of five-year survival rates of patients with a local excision of carcinoid tumors > 95%, the symptoms of these tumors including flushing, wheezing, diarrhea and heart valve symptoms, are atypical, vague and organ-related, which are apt to cause ignorance of these rare tumors and long-term delay in diagnosis[19].

Serotonin is mainly produced by enterochromaffin cells in the gut, and about 9% of the total amount of serotonin in the body is found in the gut [9]. The use of LC coupled with electrospray tandem mass spectrometry has become the very popular technique in bioavailability studies due to the fast, sensitive, and reliable results generated by its use [11]. UPLC has been evaluated as a faster and more efficient analytical tool compared to current HPLC [5]. There are several methods published on the analysis of serotonin and other related compounds in biological fluids. A variety of methods have been developed to measure serotonin including spectrophotometry [2, 25] LC fluorometry [24,30] enzyme immunoassay, and LC electrochemical detection [1, 15], liquid chromatography with an amperometric detection or fluorescence detection [3, 13] and capillary electrophoresis with electro chemiluminescence detection [28]. In addition, liquid chromatography-tandem mass spectrometry (LC-MS/MS), an emerging technique in clinical chemistry with high specificity, high sensitivity, high efficiency and high throughput [4, 10, 12, 15, 18, 29, 21, 27]. Most of methods are higher quantification limit, higher matrix volume, longer chromatographic run time and lengthy sample preparation process which are normally not preferable of samples.

Herein we have reported a simple, and sensitive ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) method for the analysis of serotonin in human plasma, serum and urine matrix by protein precipitation extraction, reversed phase LC and tandem mass detection. Protein precipitation extraction was chosen for the minimum time of sample preparation, because this technique is more feasible and less polluting than the traditional liquid-liquid extraction used in other methods.

MATERIALS & METHODS

Chemicals and reagents.

The working standard of serotonin was procured from Clearsynth (Mumbai, India). High purity water used for the LC-MS/MS was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). HPLC methanol and acetonitrile were purchased from. J. T. Baker (USA). Drug-free (blank) human plasma, serum and urine was obtained from Supratech laboratory (Ahmedabad, India) and was stored at -20°C prior to use. All other regents and solvents were obtained from general commercial suppliers.

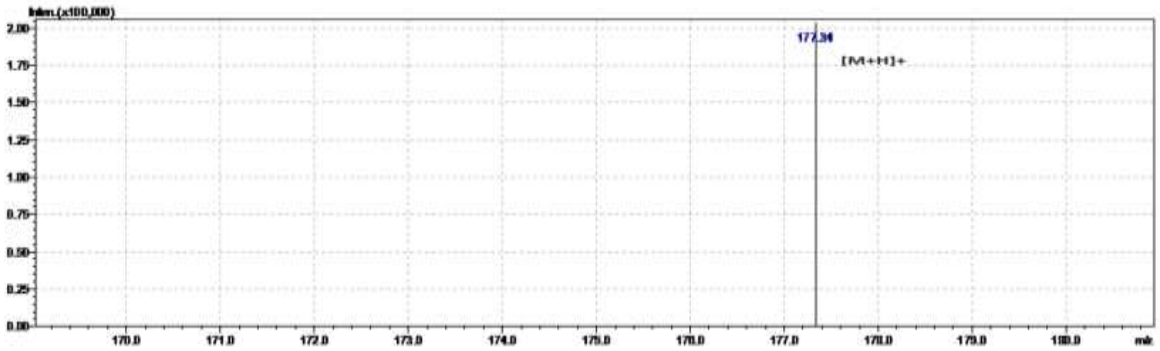
Calibration curves:

The main stock solution of 1mg/mL for serotonin was prepared by dissolving in methanol. Secondary standard solutions were prepared by further diluting stock solution with methanol: water (1:1 *v/v*) to get an intermediate stock solution of 139.6µg/mL for plasma, serum and urine matrix respectively. Working standard solution was prepared from secondary standard solution by dilution with water: methanol (1:1, *v/v*). These diluted working standard solutions were used to prepare the calibration curve and quality control samples. One-point standard calibration curves for serotonin is prepared by spiking 200µL blank plasma/serum/urine with 4µL of appropriate amount of working standard solution. One calibrator curve point 69.825 ng/ml for serotonin. Control samples were prepared at three concentration levels of 19.551 ng/mL (low), 139.650 ng/mL for (medium) & 279.300 ng/mL (high) for serotonin.

Instrument and Chromatographic conditions

Liquid chromatography was performed by a Shimadzu LCMS-8045. Binary Analytical system was consisted of a solvent rack, binary pump, autosampler, column compartment, heated electrospray ESI probe, mass detector, and a PC with Labsolutions 5.109 software. Automated MRM optimization of serotonin standards was carried out using the LabSolutions workstation. The precursors were their protonated ions, $[M+H]^+$ *m/z* 177.34 and major fragment ions observed in each product spectrum was at *m/z* 159.51 for serotonin. The selected fragments of each compound, as precursor ions and product ions to be monitored, are indicated in Figure 2.

[A]



[B]

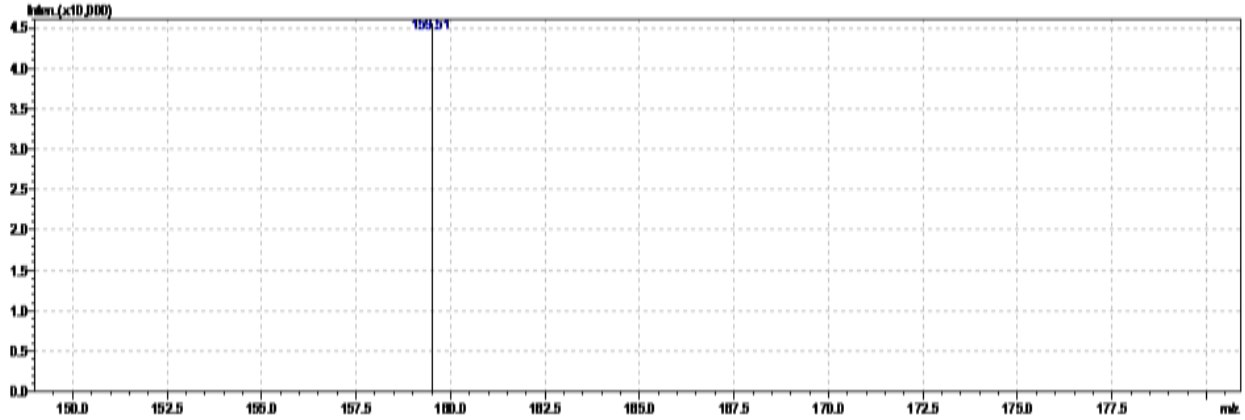


Figure 2**.** Electrospray positive ion mass spectra of the precursor ion of [A] and product ion mass spectra for [B]

The analysis temperature, nebulizer gas, and ESI temperature were investigated to optimize the specificity and sensitivity of ions detection.

LC separation was carried out on Nexera X3 UPLC system (Shimadzu, Japan). Separation was performed with a reversed-phase Shim pack GIST C18, 4.6*75mm, 3µm column maintained at 40˚C. The mobile phase was a gradient of 5% methanol (solvent A) and 0.1% formic acid (solvent B), with a constant flow rate of 0.3 mL/min (Table 1).

Time (min)	Flow (ml/min)	Conc. A (%)	Conc. B (%)
0.00	0.300	5.0	95.0
0.20	0.300	5.0	95.0
0.50	0.300	50.0	50.0
3.00	0.300	50.0	50.0
3.10	0.300	90.0	10.0
4.00	0.300	90.0	10.0
4.10	0.300	5.0	95.0
6.00	0.300	5.0	95.0

Table 1. Chromatographic conditions (gradient).

Mobile phase was used as weak wash and strong wash solvent to avoid any carry over from previous injection. The auto-sampler was maintained at 10˚C and the injection volume was 5µL. Total run time for each sample analysis was 6.0min.

Analysis of Plasma/Serum

An aliquot of 200µL of (CC, QC) or unknown samples were transferred to a 2ml capacity of polypropylene disposable micro centrifuge tube. Then 300µL of Acetonitrile was added in each tube. The samples were vortexed before centrifuged (4˚C) at 13000 rpm for 10 min. The organic supernatant layer was transferred to an auto sampler vial and injects 5 µL of sample for LCMS/MS system.

Analysis of Urine

An aliquot of 50µL of (CC, QC) or unknown samples were transferred to a 2ml capacity of polypropylene disposable micro centrifuge tube. Then 300µL of reconstituted solution [Methanol: HPLC grade water (01:01, V/V) with 0.1% formic acid] was added in each tube. The samples were vortexed before centrifuged (4˚C) at 13000 rpm for 10 min. The organic supernatant layer was transferred to an auto sampler vial and injects 5 µL of sample for LCMS/MS system.

Result and Discussion

We have developed a new and fast analytical method that allows the quantification of serotonin in biological fluids such as plasma, serum and urine. Quantitative analysis of compounds with high selectivity and sensitivity by LC-MS/MS uses the technique of selected multiple reaction monitoring

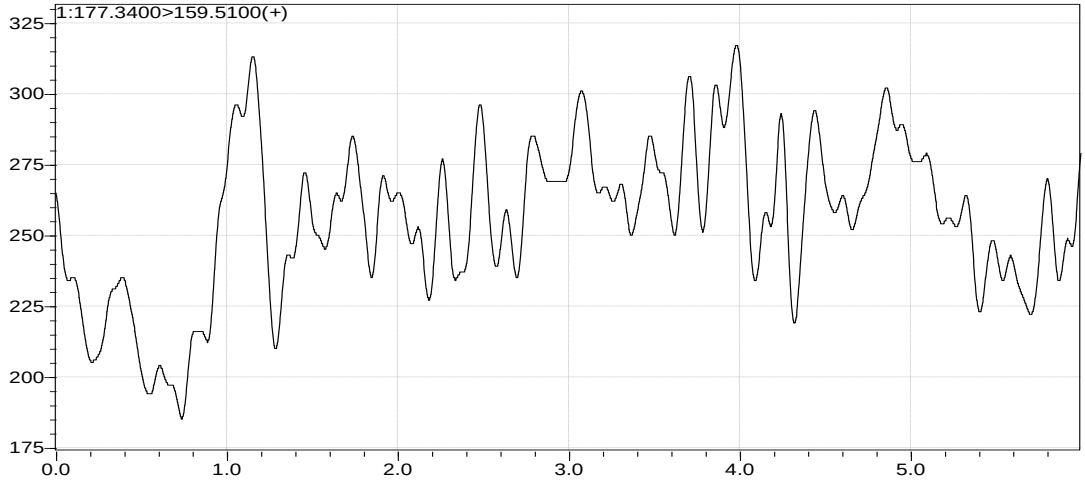
(MRM). In this mode only a selected MS/MS or collision induced dissociation (CID) transition needs to be monitored. This is now considered the best analytical approach for accurate and highly selective quantitative measurement of drugs in complex matrices such as biological samples. Using a both sample preparation as simple protein precipitation method, the total sample preparation time for this LC-MS/MS was approximately 20 min. Chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and increase the signal of analytes, as well as run time 6.5 min per injection. Formic acid buffer in the mobile phase improved the detection of the analytes. It was found that mixture of methanol-formic acid could achieve this purpose and was finally adopted as the mobile phase. The use of small particles of stationery phase allowed UPLC to push the limits of peak capacity, speed of analysis and this met the requirement for a high sample throughput.

Specificity and Selectivity

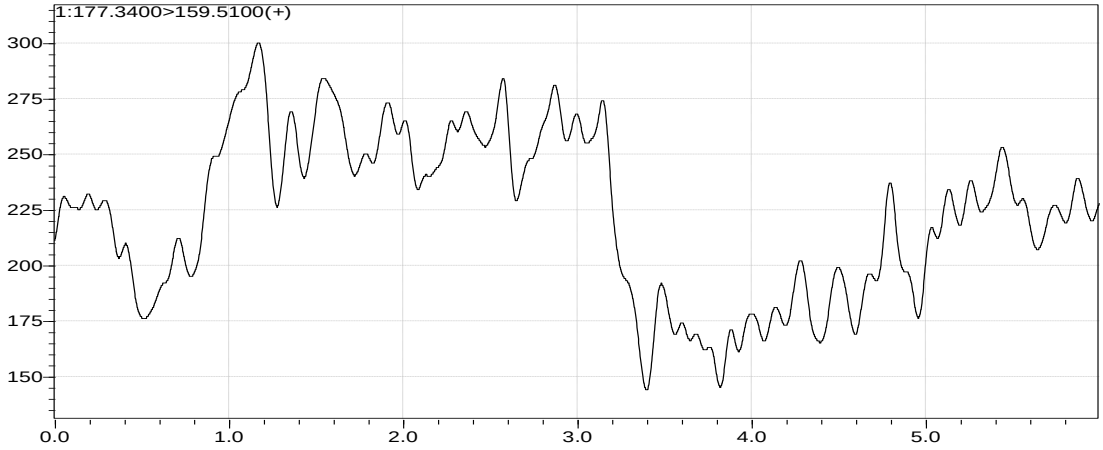
Selectivity and specificity of the method was verified by analysing a pool (at least 10 individual donors) of analyte-free matrix with lower limit of quantification (LLOQ) samples.

As shown in Figure 3 which should show no interfering matrix signals for the MRM transition at the expected retention times of the analytes.

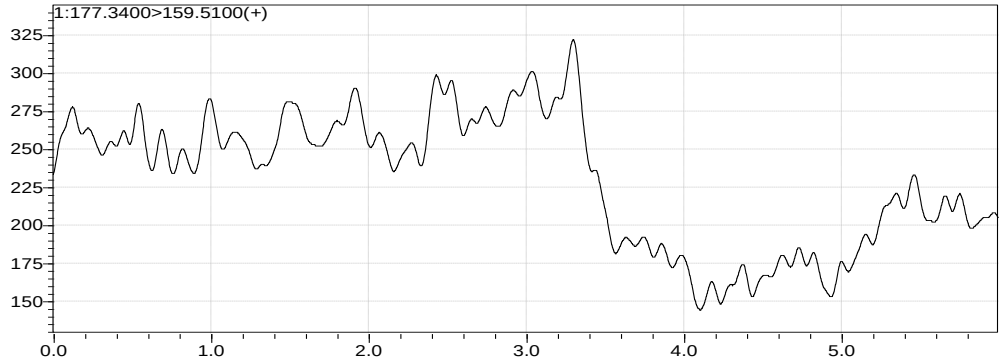
(Fig – 3A) Extracted Blank Sample:



(Fig 3A1) Urine Matrix

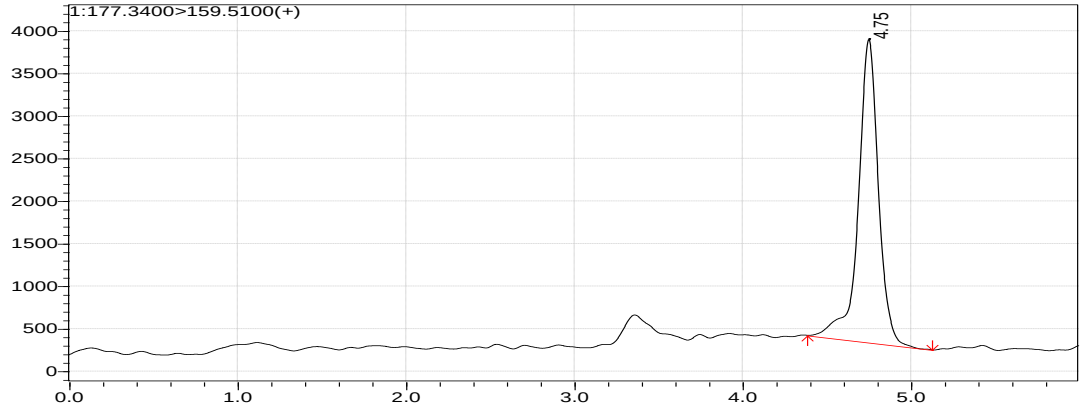


(Fig 3A2) Plasma Matrix

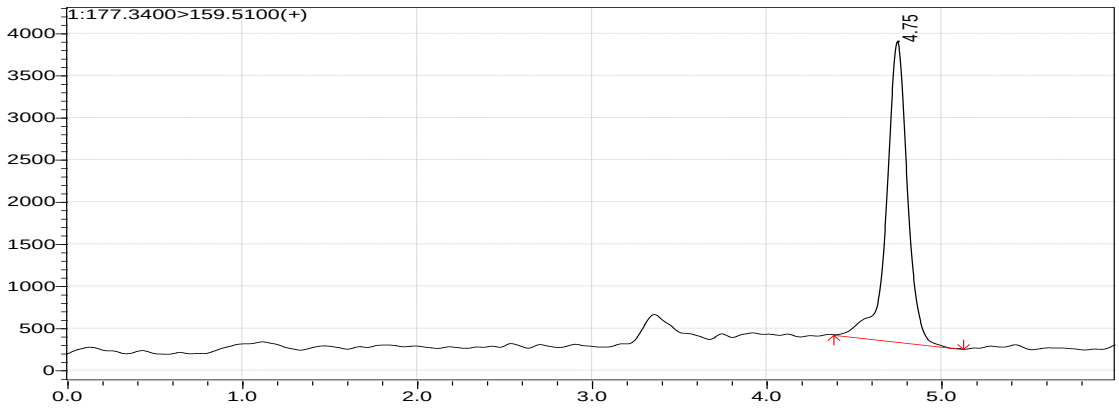


(Fig 3A3) Serum Matrix

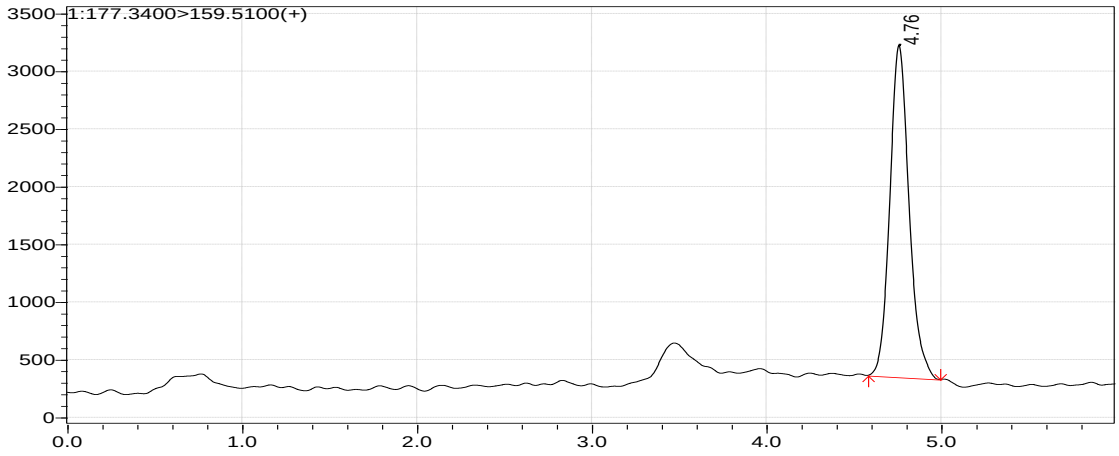
(Fig – 3B) **Extracted Lower Limit of Quantification Plasma Sample.**



(Fig 3B1) Urine Matrix



(Fig 3B2) Plasma Matrix



(Fig 3B3) Serum Matrix

Figure 3: Representative chromatograms of (A) Extracted blank plasma sample; (B) Extracted lower limit of quantification plasma sample.

Linearity and lower limit of detection (LOD)

The linearity of the target compound peak area versus the calculated concentration was verified in human plasma, serum and urine using a 1/x2 weighted linear regression and the linearity was conducted by external calibration, for it is a better way using the specific standard serotonin to quantitative themselves. The correlation coefficient (r^2) was > 0.99 for all of the target compounds in human matrix. Hence, the method exhibited good linearity. LOD was determined by repeated analyses of spiked samples at decreasing concentrations. Five different sources of matrix samples were spiked at decreasing concentrations and were processed and analyzed by proposed extraction procedure.

Drug	**Serotonin**
Calibration point (ng/mL)	69.825
Quality Control (ng/mL)	-
Level-1	19.551
Level-2	139.650
Level-3	279.300
LLOQ (ng/mL)	19.551
LOD (ng/mL) for Plasma	6.517
LOD (ng/mL) for Serum	6.517
LOD (ng/mL) for Urine	4.877

Table 2. Calibration range, LLOQ and LOD for Serotonin drug in human plasma, serum and urine.

Precision and accuracy

The accuracy and precision of the assay were assessed on 3 different days (between-run), and on the one day (within-run) using 6 replicate high, medium, and low control samples. Concentrations were measured using a freshly prepared calibrator sample. The criteria for acceptability of the data included accuracy within ±15% deviation from the nominal values and a precision of within ±15% relative standard deviation**.** The results for intra-day and inter-day precision and accuracy for serotonin in plasma, serum and urine quality control samples are summarized in Table 3.

Analytes/Matrix	Concentration added (ng/mL)		**Intra-day precision(n=6)**		**Inter-day precision (n=6)**	
			Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Serotonin/Plasma Matrix	Level-1	19.551	3.9	105.8	4.5	103.8
	Level-2	139.650	3.5	104.1	2.9	102.6
	Level-3	279.300	1.8	103.8	2.5	101.1

Serotonin/Serum Matrix	Level-1	19.551	4.7	99.2	4.2	97.6
	Level-2	139.650	2.8	102.9	2.9	102.4
	Level-3	279.300	2.7	104.1	4.4	101.6
Serotonin/Urine Matrix	Level-1	19.551	2.1	96.7	4.0	98.3
	Level-2	139.650	3.5	102.8	3.5	102.1
	Level-3	279.300	4.2	99.3	3.4	101.6

Table 3. Precision an accuracy of the method for determining Serotonin drug concentration in matrix samples.

Matrix Effect

The matrix effect was evaluated by analyzing low and high control samples. Average matrix factor values (matrix factor = response of post-spiked concentrations/response of neat concentrations). Matrix effect was not observed at analytes retention times.

Carryover effect

Carryover effects must be evaluated during assay validation intended for confirmation and/or quantitation. Carryover effect was assessed by injecting the processed blank sample just after the highest calibrator of CC in triplicate. Carryover in the blank samples following the highest calibration standard should not be greater than 20% of the analyte response at the LLOQ.

Stability

The stability of each analyte in serum was assessed by analyzing two concentration levels of QC samples with six determinations under different conditions, including kept at bench top stability, the results allowed us to conclude that analytes are stable for at least 08 hrs at room temperature in serum samples. The auto sampler stability was conducted by reanalyzing the extracted QC samples kept under auto sampler conditions (10°C) for 48 hrs. The stability experiments were performed exhaustively to evaluate the serotonin in plasma, serum and urine sample under different temperature and timing conditions was evaluated as follows and the results of the stability studies are enumerated in Table 4.

Analytes/ Matrix	**Concentration added (µg/mL)**		**Mean calculated comparison sample concentration for BT**	**Mean calculated stability sample concentration for BT**	**Mean percentage change for BT**	**Mean calculated comparison sample concentration for ASS**	**Mean calculated stability sample concentration for ASS**	**Mean percentage change for ASS**
Serotonin/ Plasma Matrix	Level-1	19.551	19.810	19.493	-1.6	18.675	19.025	1.9
	Level-3	279.300	289.606	296.397	2.3	287.769	284.379	-1.2
Serotonin/ Serum Matrix	Level-1	19.551	18.887	19.517	3.3	18.791	19.712	4.9
	Level-3	279.300	280.448	287.078	2.4	284.502	289.302	1.7
Serotonin/ Urine Matrix	Level-1	19.551	18.627	19.264	3.4	19.162	18.491	-3.5
	Level-3	279.300	286.705	288.258	0.5	287.445	283.436	-1.4

Stability: after 8 h at bench top (BT); after 46 h in autosampler (ASS) at 10°C.

Table 4. Stability samples result for Serotonin drug concentration in matrix samples.

CONCLUSION

A sensitive and selective LC-MS/MS method with a simple extraction procedure was established for quantitation of serotonin. The method performance of analysis and sample preparation was evaluated. The method also has the advantage of using PPT to obtain clean and consistent extracts with minimum matrix interference. Because of the relative short chromatographic run time and straight forward sample pre-treatment procedure, the method is easy to

follow and this validated method was successfully applied therapeutic drug monitoring of this serotonin in regular hospitals and reference laboratories for better therapeutic outcome.

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