Development and Validation of an LC-MS/MS Bioanalytical Approach for the Quantitative Analysis of Agomelatine in Human Plasma

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Abstract

An LC-MS/MS Bioanalytical approach has been established to develop a sensitive and quick method for quantifying Agomelatine in human plasma. As an internal standard, Agomelatine d6 was utilized. Ethyl acetate was used to extract agomelatine and internal standard from a plasma sample by liquid-liquid extraction.The upper layer was centrifuged, evaporated, and reconstituted using methanol:5mM ammonium acetate (80:20, v/v) to serve as the mobile phase. Inertsil C18 (50 x 4.6 mm, 5 m) GL Sciences (make) column was used in the method. Multiple reaction monitoring (MS/MS) was used to identify Agomelatine and Agomelatine d6 in human plasma without significant interference from the matrix. The protonated precursor ion of agomelatine ([M+H]+) was detected at m/z 244.80, while the corresponding product ion was detected at m/z 185.10. The protonated precursor ion ([M+H]+) at m/z 250.00 and the matching product ion at m/z 188.10 were both generated by the internal standard. Over a concentration range of 0.0503-8.0055 ng/mL, the analyte calibration curves were linear (R2 0.9956, n=4). After 14 hours on a laboratory bench, 73 hours in an injector, four freeze-thaw cycles, and seven days at -70°C±5°C, Agomelatine was found to be stable in plasma. The developed method was validated in accordance with USFDA guidelines, and theresults were found to be acceptable; this

suggests that the developed method can be used for routine and pharmacokinetic research of Agomelatine in human plasma.

Keywords: *Agomelatine; HPLC-MS/MS; Method Validation; Human Plasma; Stability studies*

1. Introduction:

Agomelatine (AML) (Figure 1) is chemically N-[2-(7-methoxynaphthalen-1-yl)ethyl]acetamide [1]. AML shares many structural similarities with melatonin. Using an animal model of depression, researchers found that AML acts as a powerful agonist at melatonin receptors while also being an antagonist at serotonin-2C (5-HT2C) receptors. In 2005, Servier Laboratories Ltd. presented AML to the European Medicines Agency (EMA) after developing it in Europe. On July 27, 2006, the CHMP (Committee for Human Use in Medical Products) advised against granting marketing permission. The main worry was that the effectiveness had not been shown. Servier sold Novartis the rights to produce AML in the United States in 2006. In October of 2011, work ceased on the project's intended target market in the United States. In Australia, you can get it marketed as Valdoxan[2, 3].Antidepressants can be used to help a wide range of mental health problems, such as severe depression, anxiety, chronic pain, and drug abuse. Antidepressants often cause side effects like dry mouth, weight gain, dizziness, headaches, sexual problems, and dulling of emotions [4]. There is a growing interest in studying AML's pre-clinical and clinical data, including how it works, how much to take, and any side effects [5–8].

A review of the literature provides some approaches for determining AML. HE et al. [9] used an HPLC approach using a PlastilC18 column and UV detection to determine AML. AML in tablets was determined byYujing et al.,using an HPLC technique with UV detection [10]. For separation of AML contaminants, an HPLC technique with diode array detection was described by Liua et al., with gradient elution mode [11]. As a stability indicator for determining AML in human plasma and tablets, an HPLC technique with fluorescence detection [12] was also devised by El- Shaheny et al. Another LC-FD for AML analysis in plasma and saliva has been reported bySaracino et al[13]. Two LC-MS/MS techniques for quantifying AML in human plasma were developed by Patil et al., &Wang et al [14, 15]. For the quantitative determination of AML in bulk and pharmaceutical dosage form, a stability indicating HPTLC approach was established by Joshi et al.,[16]. Stability-indicating chromatographic techniques for determining AML in drug substance and drug product were also developed byRizk et al.,[17]. Abdelrahman et al. [18]

devised a combined HPLC and HPTLC technique for the estimation of AML. In the present study, we describe a new, rapid, and cost-effective LC–MS/MS method for estimating AML.

2. Materials and Methods

Chemicals and reagents

Hetero laboratories in Hyderabad, India provided the pure standard of agomelatine (AML). Clear-synth laboratories limited, Mumbai, India, provided the pure standard of agomelatine d6 (IS). We sourced HPLC-grade reagents, including methanol, acetonitrile, ammonium acetate, and ethyl acetate, from Merck specialties in Mumbai, India. Human plasma was prepared using K2 EDTA and received from a recognized blood bank in Secunderabad, India.

HPLC and MS operating conditions

The API 4000 LC-MS/MS system was utilized alongside a Shimadzu HPLC. It used a positive ionization mode and had Atmospheric Pressure ionization. Inertsil C18 (50 x 4.6 mm, 5 µm), GL Sciences (make), column was used for separation using methanol: 5mM (80: 20 v/v) as the mobile phase. The isocratic flow rate was 0.8 ml/min. The temperature in the column was kept at room temperature. The following table details the analytical and IS source parameters that were kept constant. For AML and IS, the ions were detected in MRM mode by following transition pairs (precursor to product ion) between m/z 244.00 and 185.10. To analyse the data, we utilized Analyst 1.6.1 software.

Standard solutions, calibration standards and quality control (QC) sample

Agomelatine working standard, precisely weighed at roughly 5.0000 mg, was transferred to a 5 mL clean glass volumetric flask, dissolved in methanol, and the volume was made up with the same solvent to yield a 1 mg/mL solution. Adjusted the solution concentration to reflect its true strength and weight. The stock solution could be kept in the fridge between 2 and 8 °C for up to 7 days.Diluent for spiking plasma was used to prepare stock solutions to acceptable quantities for calibration curve (CC), quality control (QC)samples. AML concentrations of 0.0503, 0.1006, 0.2011, 0.4023, 0.8046, 1.6091, 3.2182, 4.8033, 6.4044, and 8.0055 ng/mL were diluted with blank plasma samples to provide human plasma calibration standards. AML quality control (QC) samples had working solutions at 0.0525, 0.1522, 1.6025, 4.0061, and 7.0283 ng/mL. The stock solution (1.0 mg/mL) was diluted to form an IS working standard solution

(100 ng/mL). Stock, operating, calibration, and quality controls were stored at -20 degrees Celsius immediately.

Sample preparation

For thorough mixing, the samples were thawed at room temperature and then vortexed. Pipetting 250 µL of plasma into 15 mL glass stoppered tubes, adding 25 µL of 100.0000 ng/mL Agomelatine D6 dilution, and then vortexing the mixture (except for the blank plasma samples, which received 25 L of diluent). To the above solution five mL of ethyl acetate were added, and the mixture was shaken at 200 revolutions per minute for 20 minutes. For 10 minutes at 4 °C, samples were centrifuged at 4000 rpm. After transferring the organic supernatant layer (4.0 mL) to labelled glass test tubes, they were heated in a moderate stream of nitrogen at 45 °C until completely dry. After dissolving the samples in 500 µL of mobile phase, they were injected into the system.

Method Validation

Industrial criteria for bioanalytical technique validation were used to validate the methods [19].

Selectivity& System Suitability

Human plasma samples from six different lots were analyzed to determine the method's selectivity by examining possible interferences in the LC peak region for AML and IS. Six successive injections were made at the beginning of the method validation and at the beginning of each day as part of a system suitability experiment using an aqueous standard corresponding to MQC mixture of AML and IS. The percent CV for system appropriateness must be acceptable.

System performance & carryover effect

One extracted LLOQ sample with an internal standard was created and injected at the start of each analytical batch for a method validation experiment on system performance. To test the auto sampler's carryover effect, reconstitution solution (mobile phase), aqueous standard equivalent to highest standard in the CC (Aq. CC1), aqueous standard equivalent to lower limit of quantification (Aq. LLOQ), standard blank, extracted standard equivalent to CC1 and LLOQ, and standard blank were injected in a specific order.

Specificity

The accuracy of the new method was checked by screening a standard blank, which was wasn't altered with AML. Seven normal plasmas with K_2EDTA anticoagulant, one lipidemic

plasma with K_2EDTA anticoagulant, one haemolyzed plasma with K_2EDTA anticoagulant, and one heparin plasma were used in this investigation.

Calibration curve

By comparing the peak areas of the AML/IS transition pair to the nominal concentration of calibration standards, calibration curves were obtained. Calibration curves for AML were generated using concentrations of 0.0503, 0.1006, 0.2011, 0.4023, 0.8046, 1.6091, 3.2182, 4.8033, 6.4044, and 8.0055 ng/mL; those for LLOQ QC, LQC, MQC1, MQC2, and HQC were generated using concentrations of 0.0525, 0.1522, 1.6025, 4.0061, and 7.0283 ng/mL. Each calibration curve was tested using a blank sample (without IS) and a zero sample (with IS). Each computed standard concentration was accepted at a 15% departure from the nominal value, except for the LLOQ, where a 20% deviation was considered acceptable.

Precision and accuracy

To calculate the intra-assay precision and accuracy, six replicates of human plasma samples containing AML at the LLOQ and three QC levels were analysed. Inter-assay precision and accuracy were evaluated across all four levels on separate days. Multiple batches and individual checks were performed on the parameter. A 15% tolerance band around nominal values and a 15% tolerance band around the relative standard deviation (RSD) were used to evaluate the quality of the data.

Recovery& Matrix effect

AML and IS extraction efficiency from human plasma was assessed by comparing the analytes extracted from triplicate QC samples at LQC, MQC, and HQC with those from the post-extracted plasma reference sample at equivalent quantities. By comparing the responses of the post-extracted plasma standard QC samples (n=4) to the response of analytes from aqueous samples at equivalent concentrations, we were able to assess the effect of plasma constituents over the ionization of analytes and IS. The matrix effect was calculated using the same analyte concentrations as those used in the recovery study.

Dilution integrity

Spiking the highest standard concentration (14.0566) by a factor of 1.75 yielded 24 replicates of dilution integrity samples. Dilution integrity samples were divided into 12 groups and diluted twice for six groups and four times for the remaining two groups. Accuracy and precision (PA-2) calculations relied on the analysis of quality control samples coupled with

processed calibration curve standards (undiluted) covering the same concentration range. Adjusting for the appropriate dilution factor, the concentrations of the quality control samples were determined. The agomelatine results are satisfactory for both 2- and 4-fold dilutions. Precision of 15% and accuracy of 100% are required for the six replicates, respectively.

Ruggedness

Three distinct lots of measured precision and accuracy were used to determine the robustness of the system. Each batch was tested with a unique combination of equipment and personnel: one column, one analyst, and three pieces of equipment.

Stability experiments

Replicate preparations of processed samples were re-injected into the auto-sampler up to 73 hours after the initial injection to estimate the stability of AML and IS in the injection solvent. To evaluate the consistency of the analytes at later time points, we compared their peak-areas to those of the IS acquired during the first cycle. Three concentrations were tested in six duplicates to see how stable the analyte was in the plasma after 14 hours and zero minutes at room temperature (bench top). Analyses of QC samples kept at -20 degrees Celsius for at least seven days were used to determine the freezer stability of the analytes in plasma. Quality control samples were spiked with analytes and then frozen and thawed four times (at -20 degrees Celsius and -70 degrees Celsius) to test the stability of analytes in plasma. The procedures outlined above were applied to the samples. Assay values were considered stable if they fell within the allowable ranges for accuracy (i.e., 15% SD) and precision (i.e., 15% RSD).

3. Results

Sample preparation and chromatographic conditions

The process of preparing samples is essential in bioanalysis. The liquid-liquid extraction method has been tested with a variety of solvents, including ethyl acetate, diethyl ether, dichloromethane, and n-hexane. Estimating AML in human plasma samples has also been attempted using a precipitation approach with methanol and acetonitrile. AML was separated using methanol: 2mM ammonium acetate in the ratio of 80:20, v/v on Inertsil C18, 50 x 4.6 mm, 5 µm column at 0.8 ml/min. AML d6 was chosen as the internal standard due to its close association with the analyte. The analyte was extracted from plasma using liquid liquid extraction (LLE) with methanol and ethyl acetate as extraction buffer and solvents, respectively. We found ideal chromatograms with minimal matrix effects. The entire process takes less than

three minutes to run. For both AML and the in-house standard AML d6, the retention time was determined to be 1.55 minutes.

Protonated precursor [M+H]+ ions at m/z 244.00 to m/z 185.10 for AML and m/z 250.00 to m/z 188.10 for IS were the most abundant ions in their respective Q1 full-scan mass spectra. The parent (precursor) ion generated was a (M+H+) ion, and the ionisation of AML and the internal standard was successful in the positive mode.

Method Validation

Selectivity & system suitability

Figures 2 and 3 show example chromatograms of blank plasma isolated from screened plasma batches; both Agomelatine and the internal standard show no significant interference from endogenous components at their mass transitions. Acceptance criteria state that the coefficient of variation (CV) of the system must be less than or equal to 4.00%, and measurements showed that the CV ranged from 1.23 to 2.16 percent for AML and IS retention time and 0.89 to 2.81 percent for area ratio.

Sensitivity& carryover effect

Agomelatine in human plasma has an LLOQ of 0.0503 ng/mL, which is the lowest concentration at which a valid assay can be performed. At this concentration, Agomelatine was found to have a precision of 8.27% and an accuracy of 102.65%. In consecutive injections of reconstitution solution or Std BL following Aq CC1 or CC1, the area of the peak at the retention time of AML should not exceed 20.00% of area in Aq LLOQ or LLOQ. Area of peak at retention time of IS in standards blank samples or reconstitution solution (mobile phase) was found to be less than 5.00% of area in Aq LLOQ or LLOQ after Aq CC1 or CC1.

Specificity

The selected blank samples across all batches were determined to be free of any substantial endogenous interferences. At the AML internal standard retention time, no significant interferences were detected in the batches. Standard CC and QC samples were prepared from these batches.

Linearity

Figures 9 show that the optimized approach yields linear results over a concentration range of 4.063 to 8000 ng/ml. The best fit for AML was seen to be a straight line when the data points were plotted using the 1/x2 weighing method. According to the parameters, the obtained

value of the correlation coefficient (r) was greater than 0.99. The results showed that the approach was linear over the specified range (Table 2).

Recovery

Complete extraction of analyte and internal standard (non-extracted samples) was achieved by processing 18 blank plasma samples and spiking them with six sets of (12.5 µL of drug and 25µL of IS) LQC, MQC2, and HQC final dilutions and internal standard. There were a total of six samples of each type (LQC, MQC2, and HQC) that were injected (extracted). Agomelatine samples that had been extracted were put up against LQC, MQC2, and HQC samples that had not been extracted. The response of the internal standard was compared between the extracted samples and the whole quality control sample set of eighteen (1-18). The response of the internal standard was compared between the extracted samples and the whole quality control sample set of eighteen (1-18). Agomelatine was recovered on average at a rate of 43.08%, with a confidence interval of 1.44%-13.13%. Agomelatine d6 was recovered by an average of 46.81 percent, with a precision of 7.63 percent to 8.23 percent. (Table 3)

Precision and accuracy

The percent coefficient of variation (CV) and the percentage of accuracy were calculated for both the inter-batch and intra-batch experiments. LLOQ QC, LQC, MQC1, MQC2, and HQC all had within-batch precisions between 2.51% and 7.10%, 0.35% and 0.66%, 0.21% and 0.37%, and 0.24% and 0.34%, respectively. The percentage of correct products produced within a batch varied from 94.79% to 99.30% for LLOQ QC to 97.85% to 99.30% for LQC to 97.94% to 98.32% for MQC1 to 98.58% for MQC2 and from 98.81% to 99.62% for HQC. LLOQ QC, LQC, MQC1, MQC2, and HQC all had intraday precisions of 3.79 percent, 1.68 percent, 0.44 percent, 0.29 percent, and 0.34 percent, respectively. LLOQ QC, LQC, MQC1, MQC2, and HQC all had intraday accuracies of 99.13%, 98.46%, 97.95%, and 98.64%, respectively. The percentage of variation between batches was 5.31 percentage points for LLOQ QC, 1.82% for LQC, 0.54% for MQC1, 0.58% for MQC2, and 0.44% for HQC. Accuracy between batches was 97.01% for LLOQ QC, 98.07% for LQC, 98.07% for MQC1, 98.97% for MQC2, and 99.19% for HQC. LQC precision was 5.47 percent, MQC1 precision was 0.9 percent, MQC2 precision was 0.6 percent, and HQC precision was 0.89% within the batch. LQC accuracy was 102.06%, MQC1 accuracy was 97.51%, MQC2 accuracy was 97.51%, and HQC accuracy was 96.81% within the batch (Figure 4-8). (Table 4).

Matrix effect& Dilution integrity

There was no discernible matrix effect for either the low (LQC) or high (HQC) amounts of Agomelatine across all eight batches. IS normalized factor was 1.006 for LQC and 0.999 for HQC, while precision for IS normalized matrix factor was 1.23% and 0.60%, respectively. The AML results show that both the 2- and 4-fold dilutions are of sufficient quality. For a dilution factor of 2, the precision and accuracy of AML were 0.39 percent and 99.33 percent, respectively. For a dilution factor of 4, AML precision and accuracy were 0.31% and 92.37%.

Ruggedness

For precision and accuracy batch (PA-3), a different column of the same make was used, as well as a different analyst and different solutions.LLOQ QC, LQC, MQC1, MQC2, and HQC all had precisions of 7.10%, 1.68%, 0.66%, 0.37%, and 0.24% within their batches, respectively. LLOQ QC, LQC, MQC1, MQC2, and HQC all have within-batch accuracies of 94.79%, 101.10%, 98.32%, and 99.64%, respectively (Table 5).

Stability studies

Conditions such as temperature, time, etc. were altered experimentally to conduct stability tests. The analyte shows no significant change in precision and % recoveries after being stored in the injector (for 73 hours), on the bench (for 15 hours), and in the freezer (for at least 7 days) at -70 °C 15 °C. The findings of four cycles of freeze-thaw stability testing showed that AML was stable. According to the results (Table 6), the plasma samples under study can be frozen and thawed without compromising the analyte's dependability.

4. Discussion

Using LLE and HPLC-API/MS/MS, we have developed a fast and sensitive approach for quantifying AML in human plasma. When conditions are ideal, AML and IS are more sensitive to positive ions than negative mode. After experimenting with a variety of organic solvents for chromatographic optimization, we settled on methanol because to its superior sensitivity and resolution for AML and IS analysis. Methanol: 2mM ammonium acetate (80:20, v/v) was utilized as the mobile phase. Ammonium acetate was used, and the chosen drug now has greater ion intensities and peak symmetry. The sensitivity of AML and IS was maximized under these settings.The main issue in bio-analytical studies is co-elution, which occurs when a large number of endogenous products elute at the same time as the analytes of interest. This influences the analyte's ionization efficiency, which in turn leads to decreased reproducibility and accuracy and

ultimately failure to reach the bounded limits. Considering this, we were very careful while picking out and fine-tuning the extraction solvent for the analyte. Matrix analyses showing low values show that the analyte is being effectively extracted with few byproducts.

5. Conclusion

The detection of AML in human plasma has been made possible by a newly developed and validated HPLC-MS/MS approach. The method's precision and accuracy are within the range needed for bio-analytical studies. The method can be applied to pharmacokinetic research because of its low limit of quantification. With a total run time of only 2.5 minutes, the approach can analyze roughly 24 samples per hour with ease. Human volunteers were subjected to bioequivalence experiments while fasting and after a meal using the proposed assay method.

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Conflicts of interest

The authors declare no conflicts of interest.

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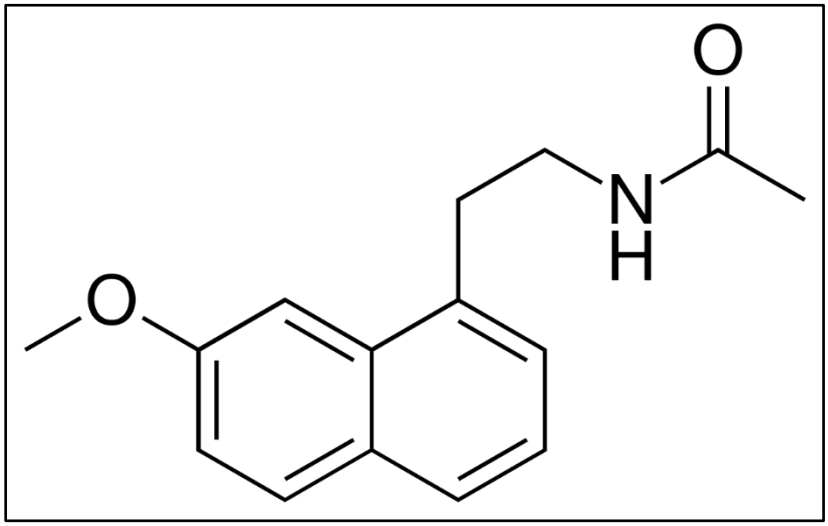

Figure 1. Chemical structure of Agomelatine

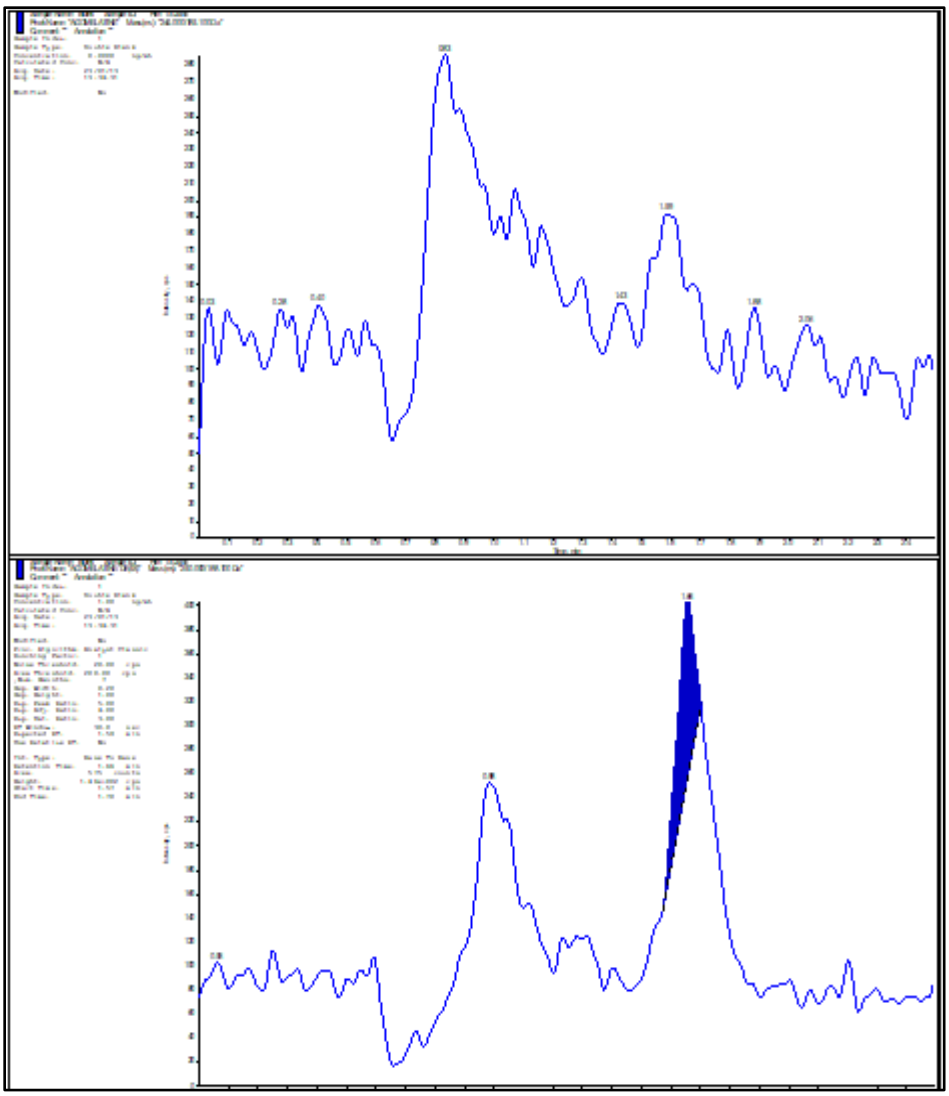

Figure 2. Chromatogram of standard blank withAgomelatine

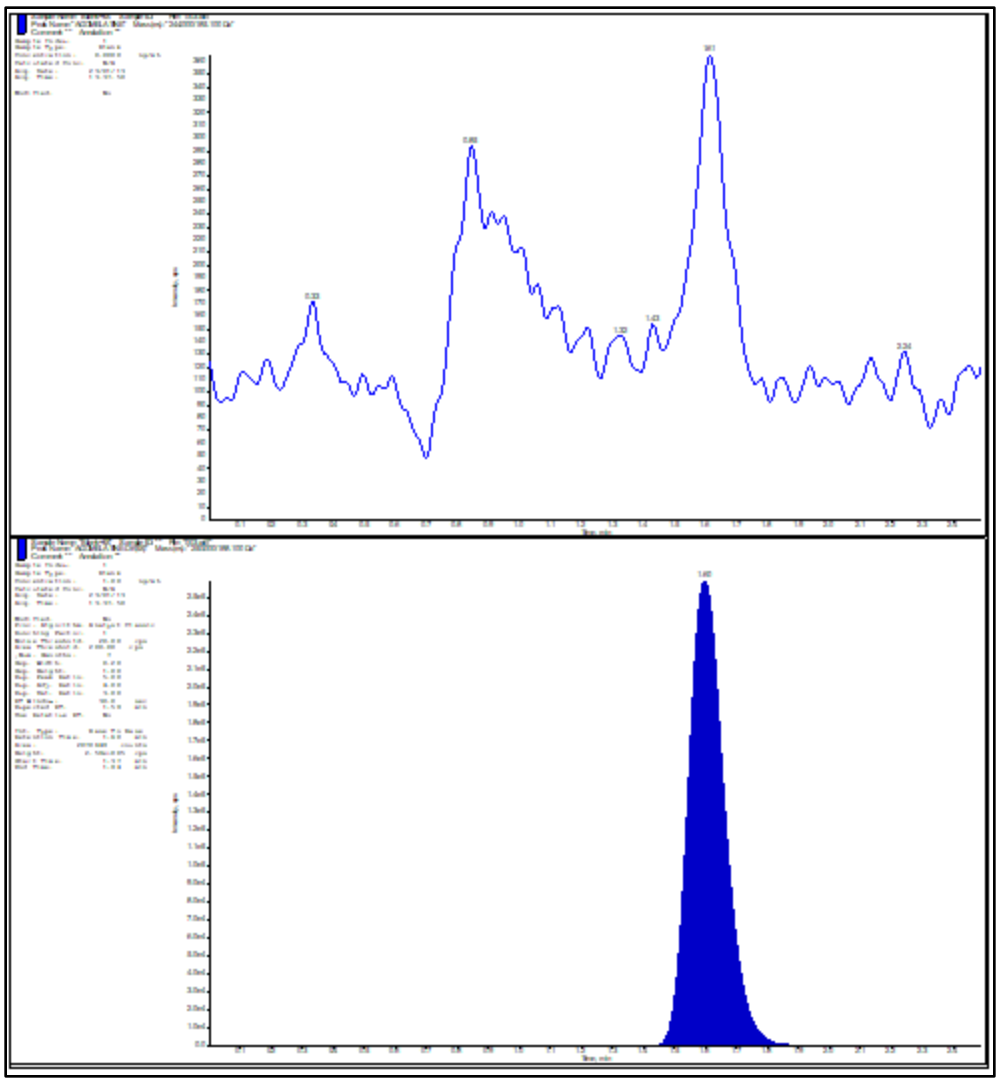

Figure 3. Chromatogram of standard blankwithAgomelatine IS

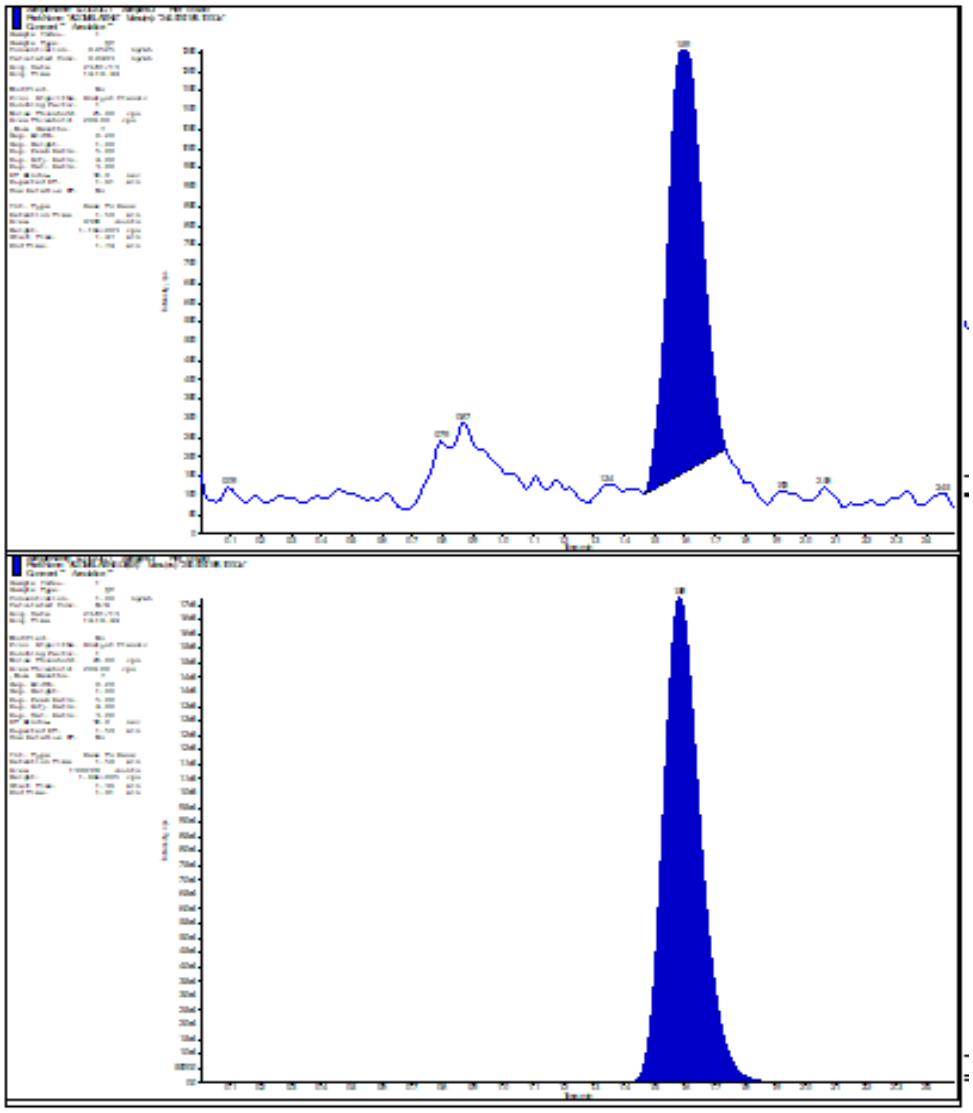

Figure 4. Chromatogram of LLOQ Standard of Agomelatine

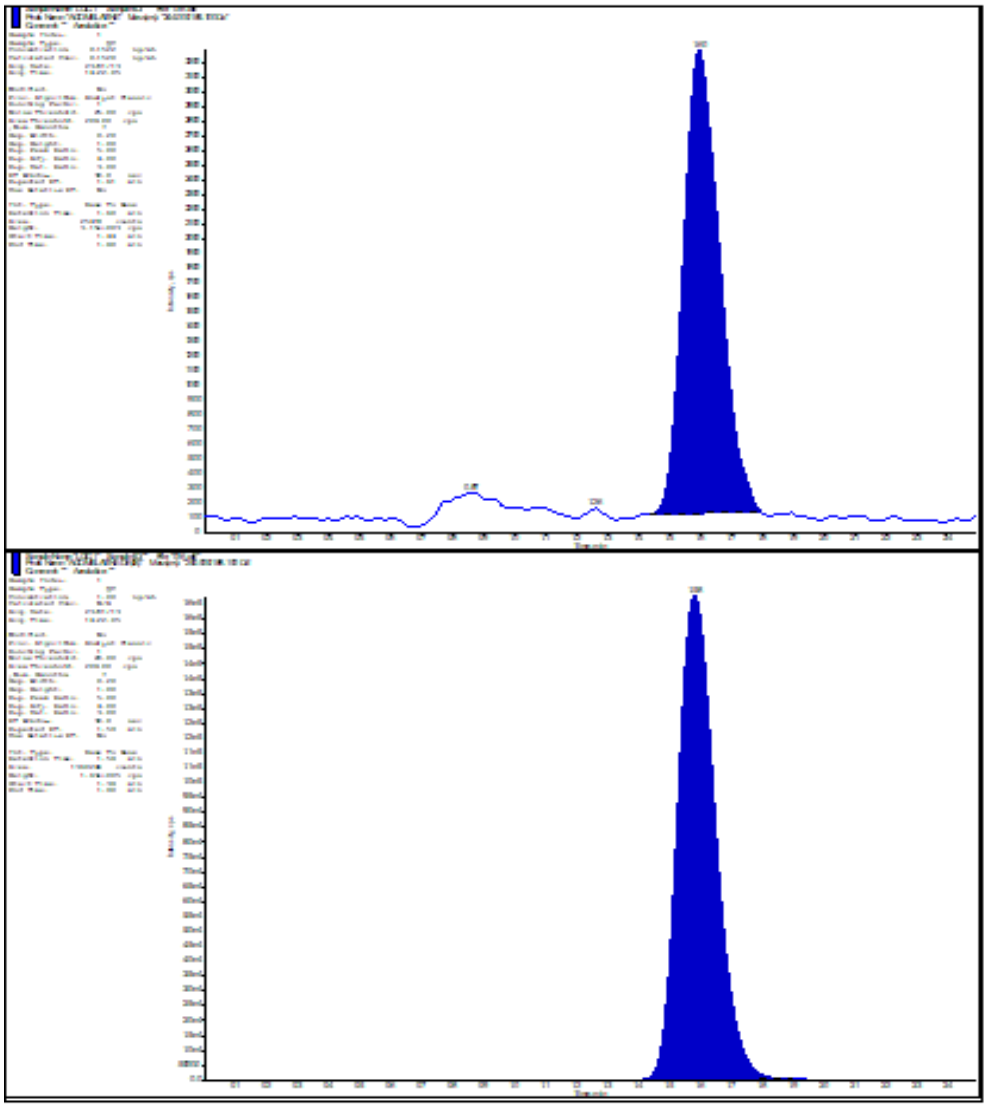

Figure 5. Chromatogram of LQC standard of Agomelatine

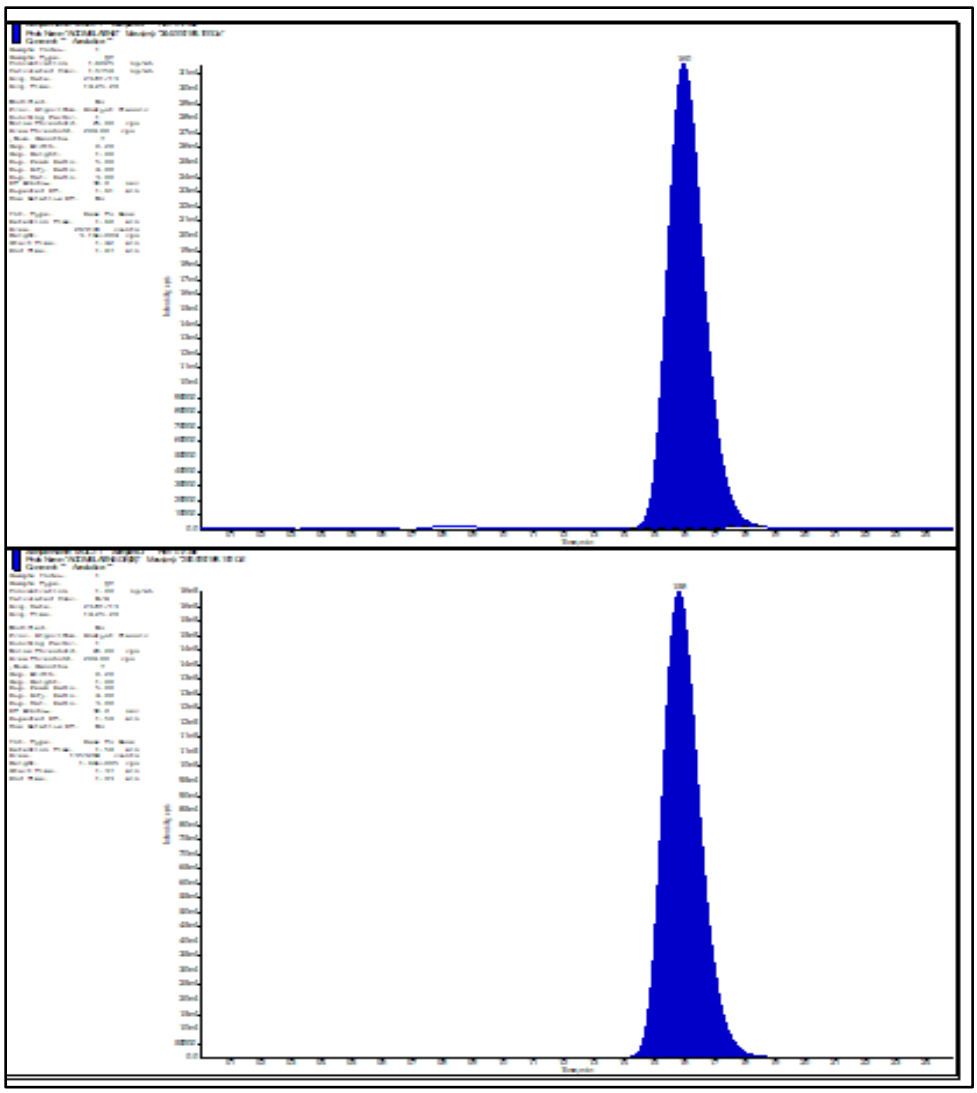

Figure 6. Chromatogram of MQC1 standard of Agomelatine

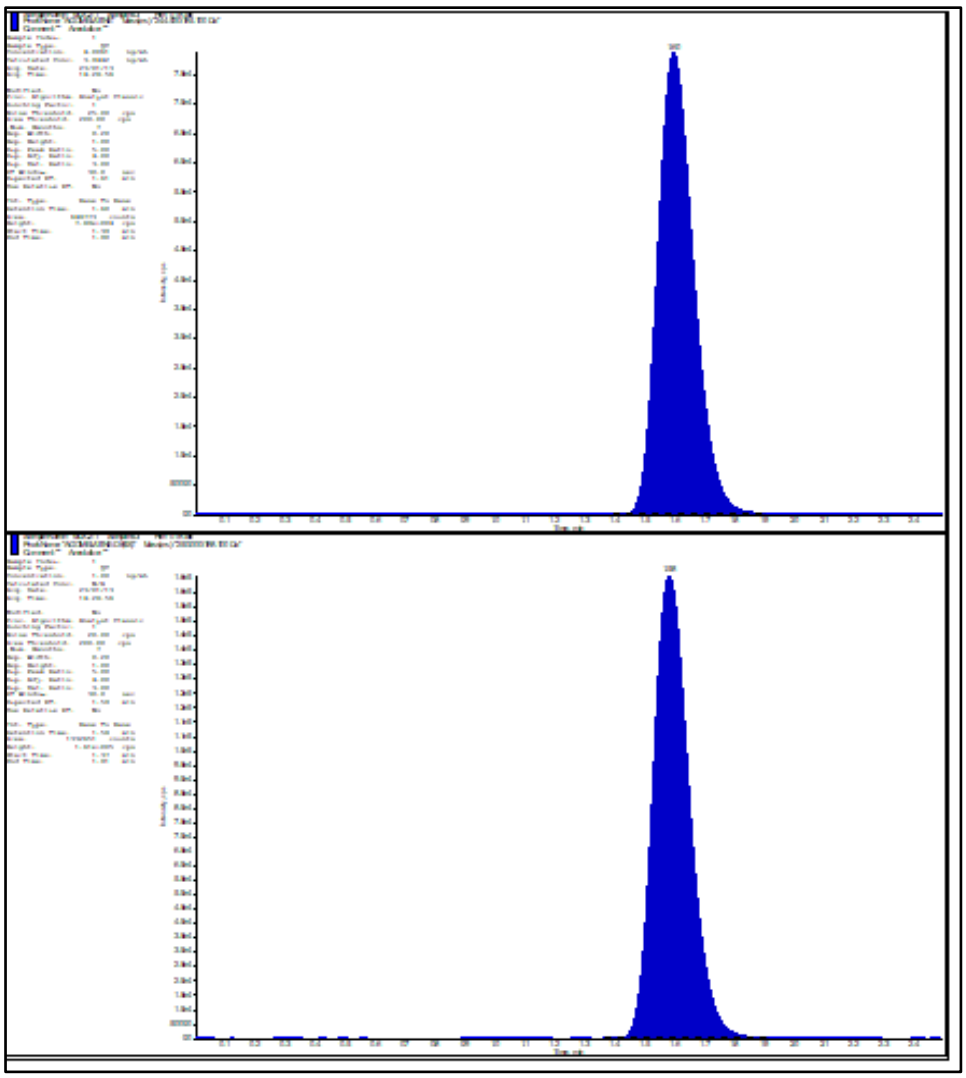

Figure 7. Chromatogram of MQC2 standard of Agomelatine

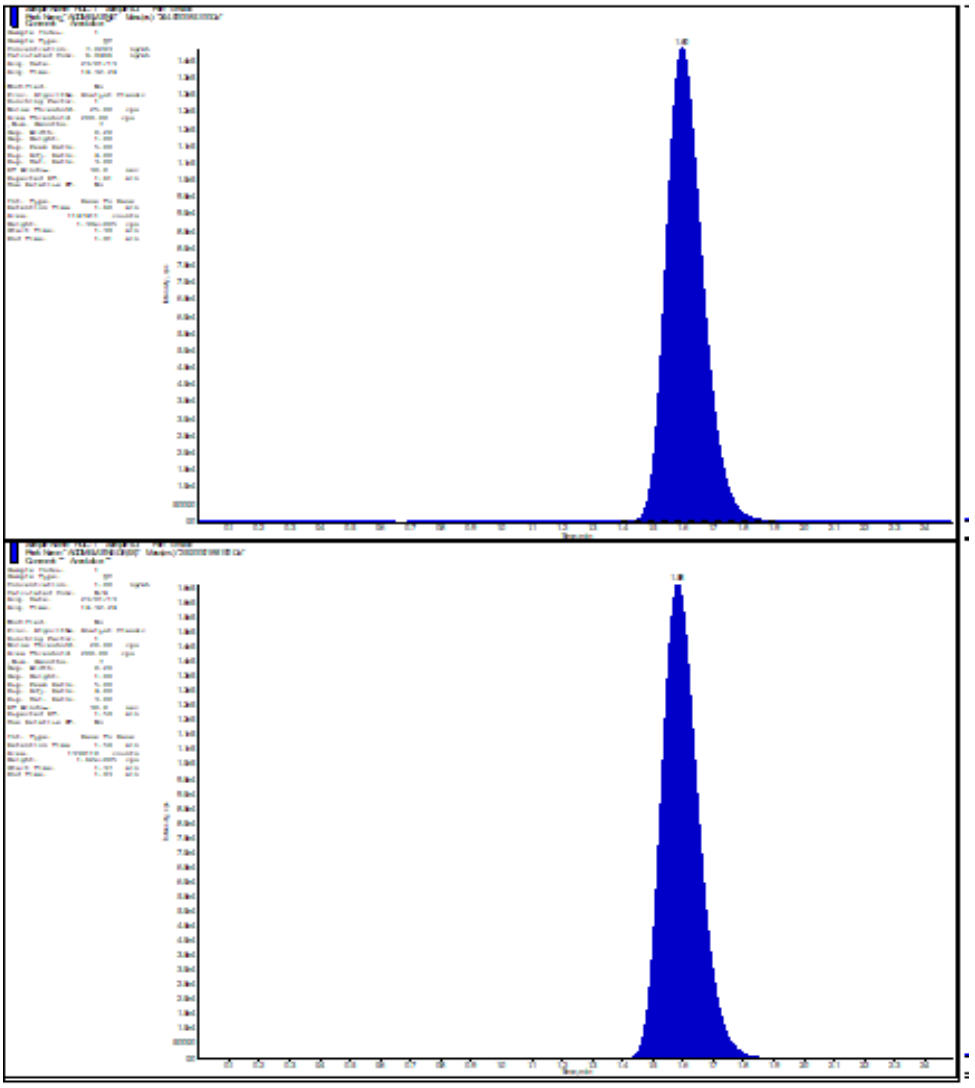

Figure 8. Chromatogram of HQC standard of Agomelatine

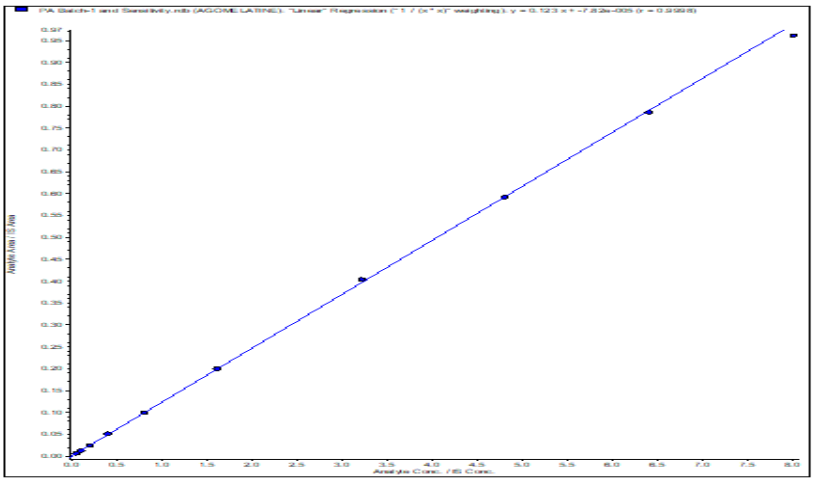

Figure 9. Representative Calibration Curve for Regression Analysis of Agomelatine

Table 1. Optimized operating conditions of LC-MS/MS method

Parameter	Agomelatine	Agomelatine D6
Ionization mode	Positive	Positive
Detection (m/z)	m/z – 244.0 (parent) and 185.10 (product)	m/z – 250.00 (parent) and 188.10 (product)
Dwell time (msec)	200	200
Ion Spray Voltage (IS)	5500.00 V	5500.00 V
Temperature (TEM^{0}C)	500.00	500.00
Curtain Gas (CUR)	30.00 V	30.00 V
Collision Gas (CAD)	9.00 V	9.00 V
Nebulizer Gas (GS1)	45.00 psi	45.00 psi
Auxillary Gas (GS2)	40.00 psi	40.00 psi
Collision Energy (CE)	22.00 V	24.00 V
Collision Cell Exit Potential (CXP)	12.00 V	12.00 V
Declustering Potential (DP)	78.00 V	77.00 V
Entrance Potential (EP)	10.00 V	10.00 V

Table 2. Back calculated concentrations for calibration curve standardsof AML

STD ID	Conc. (µg/mL)	N	Mean	SD	%CV	Recovery%
STD 1	0.0503	6	0.05090	0.000458	0.90	101.19
STD 2	0.1006	6	0.09800	0.001992	2.03	97.42
STD 3	0.2011	6	0.19930	0.002200	1.10	99.10
STD 4	0.4023	6	0.41033	0.004092	1.00	102.00
STD 5	0.8046	6	0.80873	0.005279	0.65	100.51
STD 6	1.6091	6	1.61923	0.003004	0.19	100.63
STD 7	3.2182	6	3.26120	0.009737	0.30	101.34
STD 8	4.8033	6	4.81373	0.017081	0.35	100.22
STD 9	6.4044	6	6.40440	0.040328	0.63	100.00
STD 10	8.0055	6	7.81047	0.020422	0.26	97.56

Table 3. Recovery of AML QC samples

Analyte	ID	QC sample (ng/mL) n=6	% Recovery ±%CV
AML	LQC	0.152	46.85±13.07
	MQC	1.6025	40.18±4.23
	HQC	7.0283	42.20±5.29
AML-IS	----	200	46.81±7.63

Table 4. Intra and inter-day precision of AML QC samples

Measured concentration (ng/mL)				
Intra-day variation (Six replicates at each concentration)				
Theoretical Concentration (nm/ml)	AML			
	Mean	SD	% CV	Accuracy (%)
LLOQ	0.0525	0.004273	8.27	102.65
LQC	0.1522	0.124002	1.23	97.94
MQC1	1.6025	0.006001	0.60	99.07
MQC2	4.0061	0.002613	5.03	98.95
HQC	7.0283	0.005162	0.35	98.69
Inter-day variation				
LLOQ	0.0525	0.008429	0.21	98.81
LQC	0.1522	0.019408	0.28	97.85
MQC1	1.6025	0.001311	2.51	99.30
MQC2	4.0061	0.003535	7.10	98.58
HQC	7.0283	0.002552	1.68	99.13

RSD: Relative standard deviation (SD X 100/Mean)

Table 5a. Ruggedness results of AML

Nominal Concentration (ng/mL)									
STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
0.0503	**0.1006**	**0.2011**	**0.4023**	**0.8046**	**1.6091**	**3.2182**	**4.8033**	**6.4044**	**8.0055**
0.0508	0.0991	0.1971	0.4091	0.8081	1.6174	3.2572	4.8166	6.4461	7.8234
100.97	**98.54**	**97.99**	**101.69**	**100.44**	**100.51**	**101.21**	**100.28**	**100.65**	**97.73**

Table 5b. Ruggedness results of AML

	Nominal Concentration (ng/mL)									
	LLOQ QC		**LQC**		**MQC1**		**MQC2**		**HQC**	
	0.0525	**% Accuracy**	**0.1522**	**% Accuracy**	**1.6025**	**% Accuracy**	**4.0061**	**% Accuracy**	**7.0283**	**% Accuracy**
A	0.0514	97.97	0.1549	101.77	1.5739	98.22	3.9815	99.39	6.9950	99.53
B	0.0488	92.99	0.1532	100.64	1.5588	97.27	3.9734	99.18	7.0179	99.85
C	0.0523	99.64	0.1537	100.98	1.5888	99.14	4.0018	99.89	7.0040	99.65
D	0.0543	103.35	0.1500	98.58	1.5711	98.04	3.9856	99.49	6.9734	99.22
E	0.0469	89.28	0.1539	101.12	1.5782	98.49	4.0136	100.19	7.0008	99.61
F	0.0449	85.53	0.1484	97.53	1.5829	98.78	3.9941	99.70	7.0199	99.88
Mean	**0.04977**		**0.15235**		**1.57562**		**3.99167**		**7.00183**	
S.D.	**0.003535**		**0.002552**		**0.010389**		**0.014584**		**0.017003**	
C.V.%	**7.10**		**1.68**		**0.66**		**0.37**		**0.24**	
% Nominal	**94.79**		**100.10**		**98.32**		**99.64**		**99.62**	
N	**6**		**6**		**6**		**6**		**6**	

Table 6. Stability data of AML quality controls in human plasma

Drug	Nominal conc. (ng/mL)	Stability	Mean ± SD[a] (n=6) (ng/mL)	Accuracy (%)[b]	Precision(%) % C.V.
AML LQC	0.1522	0.00 h (for all)	0.1512±1.325	97.36	1.35
	0.1522	14.00 h (bench-top)	0.1503±0.254	99.12	0.65
	0.1522	33.00 h (short term)	0.1519±1.325	96.35	0.32
	0.1522	68.00 h (wet extract)	0.1509±2.125	98.32	6.25
	0.1522	70.00 h (dry extract)	0.1513±0.259	100.32	3.25
	0.1522	73.00 h (in injector)	0.1509±1.021	99.12	5.62
	0.1522	07 days (long term)	0.1513±0.321	98.98	2.25
	0.1522	Freeze-thaw stability (4 cycles at-70±5 ˚C)	0.1503±0.598	99.15	3.25
AML HQC	7.0283	0.00 h (for all)	7.0212±1.231	98.32	3.25
	7.0283	14.00 h (bench-top)	7.0235±2.032	99.32	1.65
	7.0283	33.00 h (short term)	7.0215±0.621	96.32	2.65
	7.0283	68.00 h (wet extract)	7.0229±0.325	98.32	6.25
	7.0283	70.00 h (dry extract)	7.0231±2.314	100.95	1.35
	7.0283	73.00 h (in injector)	7.0261±1.325	99.65	2.35
	7.0283	07 days (long term)	7.0235±1.624	96.21	1.65
	7.0283	Freeze-thaw stability (4 cycles at-70±5 ˚C)	7.0216±3.261	101.65	5.32

[a]Back-calculated plasma concentration; [b](Mean assayed conc. At 0 h) X 100