Research Article

Journal of MASS SPECTROMETRY

Received: 30 September 2011 Revised: 3 November 2011 Accepted: 4 November 2011 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jms.2020

Validated LC–MS/MS method for quantification of agomelatine in human plasma and its application in a pharmacokinetic study

Satish R. Patil,[a,b] Ketan K. Nerurkar,[b] Ashok M. Kalamkar,[b] Vishwas Pukale,[a,b] Kiran V. Mangaonkar[c] and Satish G. Pingale[c]*

An analytical method based on liquid–liquid extraction has been developed and validated for analysis of agomelatine in human plasma. Fluoxetine was used as an internal standard for agomelatine. A Betasil C18 (4.0 × 100 mm, 5 μm) column provided chromatographic separation of analytes followed by detection with mass spectrometry. The method involves simple isocratic chromatographic conditions and mass spectrometric detection in the positive ionization mode using an API-4000 system. The proposed method has been validated with linear range of 0.050–8.000 ng/ml for agomelatine. The intra-run and inter-run precision values are within 12.12% and 9.01%, respectively, for agomelatine at the lower limit of quantification level. The overall recovery for agomelatine and fluoxetine was 67.10% and 72.96%, respectively. This validated method was used successfully for analysis of plasma samples from a pharmacokinetic study. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: liquid–liquid extraction; tandem mass spectrometry; agomelatine; human plasma; pharmacokinetic study

INTRODUCTION

Agomelatine (Fig. 1(a)), an antidepressant drug, which has the chemical name: N-[2-(7-methoxy-1-naphthyl)ethyl] acetamide is rapidly and well (≥80%) absorbed after oral administration. The peak plasma concentration is reached within 1–2 h after administration of agomelatine. Absolute bioavailability is low (approximately 5% at the therapeutic oral dose) and is highly variable because of the first-pass effect and the inter-individual differences of Cytochrome P4501A2 activity.[1]

Agomelatine is a melatonergic agonist (MT1 and MT2 receptors) and 5-HT2C antagonist. Binding studies indicate that it has no effect on monoamine uptake and no affinity for α, β adrenergic, histaminergic, cholinergic, dopaminergic, and benzodiazepine receptors.[2]

The chemical structure of agomelatine is very similar to that of melatonin, where melatonin has an NH group and agomelatine has an HC = CH group. Thus, melatonin contains an indole part, whereas agomelatine has a naphthalene bioisostere instead.[3]

A unique LC–UV method was reported by Xue-jun *et al*. Separation was achieved by using an il-C18 (4.6 × 150 mm, 5 μm) column. The mobile phase consisted of acetonitrile and water (30:70) with a flow rate of 1 ml/min. The limit of quantification (LOQ) was 5000 ng/ml.[4]

As per European Medicines Agency (EMEA) guideline, the lower LOQ should be 1/20 of C_{max} or lower, as pre-dose concentrations should be detectable at 5% of C_{max} or lower,[5] but unfortunately, the method reported by Xue-jun *et al*. failed to quantify LOQ in human plasma.

To the best of our knowledge, no detailed liquid chromatography tandem mass spectrometry (LC–MS/MS) assay for the determination of agomelatine has been described so far. Therefore, the primary aim of the present study was to develop and validate an LC–MS/MS method for the determination of agomelatine in human plasma. Furthermore, the present method was applied in pharmacokinetic study.

EXPERIMENTAL

Chemicals and reagents

The reference standard of agomelatine was obtained from Sigma Aldrich Pvt. Ltd. (Secunderabad, India). The reference standard fluoxetine was provided by Chemieliva Pharma Co. Ltd. (China). Purity of both standards was higher than 99.00%. High-purity water was prepared in-house using a Milli-Q A10 gradient water purification system (Millipore, Bangalore, India). LC-grade methanol was purchased from J. T. Baker Inc. (Phillipsburg, NJ, USA). Acetic acid and ethyl acetate were procured from Merck (Mumbai, India). Drug-free (blank) human plasma containing K3-EDTA was obtained by enrolling healthy volunteers and taking their consent before bleeding. The plasma thus obtained was stored at −20°C prior to use.

Calibration curve and quality control samples

Two separate stock solutions of agomelatine were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as the subject sample analysis. The stock solutions of agomelatine and fluoxetine were

* *Correspondence to: Satish Pingale, Analytical Chemistry Research Laboratory, Mithibai College, Vile Parle (W), Mumbai 400056, India. E-mail: sgpingale_pk@yahoo.co.in*

a *Department of pharmacology, SVKMS NMIMS University, Vile Parle (W), Mumbai 400056, India*

b *Drug Monitoring Research Institute, Rabale 400701, Navi Mumbai, India*

c *Analytical Chemistry Research Laboratory, Mithibai College of Arts, Chauhan Institute of Science & Amrutben Jivanlal College of Commerce and Economics, Vile Parle (W), Mumbai 400056, India*

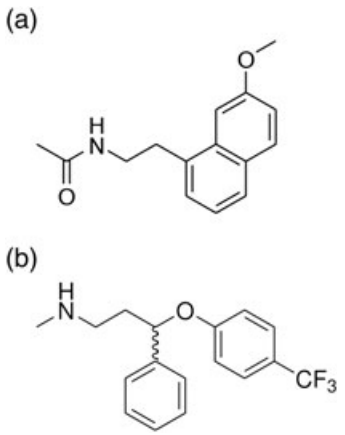

Figure 1. (a) Agomelatine and (b) Fluoxetine.

prepared in methanol at free base concentration of 1000 μg/ml. Primary dilutions and working standard solutions were prepared from stock solutions using water: methanol (40:60, v/v) solvent mixture. These working standard solutions were used to prepare the calibration curve and quality control samples. Blank human plasma was screened prior to spiking to ensure that it was free of endogenous interference at retention times of agomelatine and internal standard fluoxetine. A nine-point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of agomelatine. Calibration samples were made at concentrations of 0.050, 0.101, 0.403, 0.807, 1.614, 3.228, 4.841, 6.455, and 8.069 ng/ml, and quality control samples were made at concentrations of 0.153, 2.017, 4.033, and 6.856 ng/ml for agomelatine.

Plasma sample preparation

A 0.5-ml aliquot of human plasma sample was mixed with 25 μl of internal standard working solution (8 μg/ml of fluoxetine). To this, 2 ml of ethyl acetate was added and vortexed for 5 min. It was then centrifuged for 5 min at 3500 rpm, and the upper organic layer was transferred into evaporation tubes. The sample was then evaporated under nitrogen at 45°C and reconstituted with 0.5 ml of mobile phase. From this solution, 10 μl was injected into the LC–MS/MS system through an autosampler.

Liquid chromatography and mass spectrometric conditions

Chromatographic separation was carried out on a Shimadzu LC (Kyoto, Japan) with A Betasil C18 (4.0 × 100 mm, 5 μm) purchased from Chromatopak, Thermo Scientific (India). A mobile phase consisting of methanol and 0.1% acetic acid in 2 mM ammonium acetate solution in the volume ratio of 80:20 v/v was delivered with splitter at a flow rate of 1.2 ml/min. The total run time for each sample analysis was 3.0 min. Mass spectra were obtained using an API-4000 from Applied Biosystems, Canada, equipped with electrospray ionization source. The mass spectrometer was operated in the multiple reaction monitoring mode. Electrospray ionization was used for sample introduction and ionization in the positive ion mode. Source-dependent parameters optimized were gas 1 (nebulizer gas): 30 psi; gas 2 (heater gas): 30 psi; ion spray voltage: 5500 V; temperature: 500°C. The compound-dependent parameters such as the declustering potential, entrance potential, collision energy, and cell exit potential were optimized during tuning as 55, 10, 20, 11 80, 10, 25, and 11 eV for agomelatine and fluoxetine, respectively. The collision activated dissociation gas was set at 4 psi, whereas the curtain gas was set at 30 psi using nitrogen. Quadrupole 1 and quadrupole 3 were both maintained at unit resolution, and dwell time was set at 200 ms for agomelatine and fluoxetine. The mass transitions were selected as *m/z* 244.1 → 185.0 for agomelatine and *m/z* 310.0 → 44.0 for fluoxetine. The product ion spectra for agomelatine and fluoxetine are represented in Figs 2 and 3, respectively.

The data acquisition was ascertained by Analyst 1.4.2 software. For quantification, the peak area ratios of the target ions of the analyte to those of the internal standard were compared with weighted ($1/x^2$) least squares calibration curves in which the peak area ratios of the calibration standards were plotted *versus* their concentrations.

Validation

A thorough and complete method validation of agomelatine in human plasma was carried out, following US Food and Drug Administration (USFDA) guidelines.[6] The method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, dilution integrity, partial volume, matrix effect, re-injection reproducibility, and stability. Selectivity was performed by analyzing the human blank plasma samples from ten different sources (or donors) with an additional haemolysed group and lipemic group to test for interference at the retention times of analytes. The intra-run (within a day) and inter-run (between days ($n = 3$)) accuracy was determined by replicate analysis of quality control

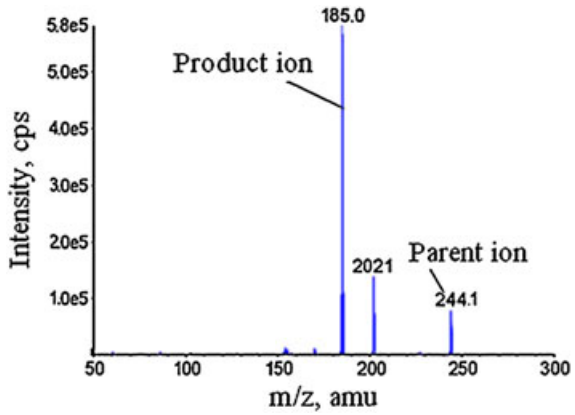

Figure 2. Product ion mass spectrum of agomelatine.

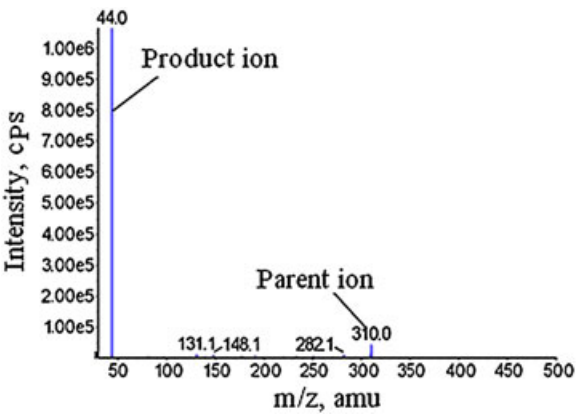

Figure 3. Product ion mass spectrum of fluoxetine.

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samples ($n = 6$) at lower limit of quantification (LLOQ), low quality control (LQC), middle quality control (M1QC), medium quality control (MQC), high quality control (HQC), and upper limit of quantification (ULOQ) levels. The percent coefficient of variation (%CV) should be less than 15, and accuracy [percent relative error (%RE)] should be within 15, except LLOQ, where it should be within 20%.

Accuracy is defined as the %RE and was calculated using the formula $\%RE = ((E - T)/T) \times 100$; where E is the experimentally determined concentration and T is the theoretical concentration. Assay precision was calculated by using the formula $\%CV = (SD/M)\ (100)$; where M is the mean of the experimentally determined concentrations and SD is the standard deviation of M. The %change was calculated by using the formula $\%change = (S/F\text{-}1) \times 100$; where S is the mean concentration of stability samples and F is the mean concentration of freshly prepared samples.

The extraction efficiencies of agomelatine and fluoxetine were determined by analysis of six replicates at each quality control concentration level for agomelatine and at one concentration for the internal standard fluoxetine. The percent recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of unextracted standards (spiked into extracted matrix of same lot).

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above ULOQ, which may be encountered during real subject sample analysis. Dilution integrity experiment was carried out at 1.8 times the ULOQ concentration. Six replicates each of 1/2 and 1/4 concentrations were prepared, and their concentrations were calculated by applying the dilution factors 2 and 4 against the freshly prepared calibration curve.

The partial volume experiment was performed on MQC concentration level to validate the method for application in case of insufficient volume of plasma in real subject samples. Six replicates each of half and quarter volume of total volume of plasma required for processing were prepared, and their concentrations were calculated by applying the dilution factors 2 and 4 against the freshly prepared calibration curve.

The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) constitutes an important and integral part of validation for quantitative LC–MS/MS method for supporting pharmacokinetics studies. It was performed by processing ten different lots of normal, controlled plasma samples in quadruplet ($n = 4$). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The %CV for ten values at each level was calculated by taking the mean value obtained by injecting the post-extracted samples prepared in duplicate from each plasma lot, which should be less than ten.

To check re-injection reproducibility, we initially injected LQC and HQC samples, turned off the system, and restarted the system after 2 h. The same samples were then re-injected, and original values were compared with re-injected values with respect to %change, which should be less than 10.

As a part of the method validation, stability was evaluated in stock solutions and in plasma under different conditions, maintaining the same conditions, which occurred during study sample handling and analysis. Stock solution stability was performed by comparing area response of analyte and internal standard in stability sample, with the area response of sample prepared from fresh stock solutions. Stability studies in plasma were performed at LQC and HQC concentration level using six replicates at each level. The analyte was considered stable if the %change is less than 15, as per US FDA guidelines.[6] The stability of spiked human plasma samples stored at room temperature (bench top stability) was evaluated for 12 h. The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after storing in the autosampler at 10°C for 24 h. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen at −20°C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration level were used for the freeze–thaw stability evaluation. For long-term stability evaluation, the concentrations obtained after 90 days were compared with initial concentrations.

Application of method

The validated method has been successfully used to analyze agomelatine concentrations in 24 human volunteers under fasting conditions after administration of a single tablet containing 25 mg agomelatine as an oral dose. The study design was a randomized, two-period, two-sequence, two-treatment single dose, open label, pharmacokinetic study using Valdoxan ® manufactured by Servier Lab, France, as the reference formulation. The study was conducted after a signed consent of the volunteers, according to current Good Clinical Practice (GCP) guidelines and an approval by an authorized ethics committee.

There were a total of 17 blood collection time points per period including the pre-dose sample. The blood samples were collected in separate vacutainers containing K3-EDTA as an anticoagulant. The plasma from these samples was separated by centrifugation at 3500 rpm within the range of 2–8°C. The plasma samples thus obtained were stored at −20°C till analysis. Post analysis, the pharmacokinetic parameters were computed using WinNonlin® software version 5.2 and 90% confidence interval was computed using SAS® software version 9.2.

RESULTS AND DISCUSSION

Method development

During method development, different options were evaluated to optimize detection parameters, chromatography, and sample extraction.

Mass spectra

Electrospray ionization (ESI) provided greater response over atmospheric pressure chemical ionization mode, so was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at both polarities at a flow rate of 10 µl/min. As agomelatine contains basic >NH functionality, the signal intensity increased in the positive ion mode. The predominant peaks in the primary ESI mass spectra of agomelatine and fluoxetine correspond to the MH^+ ions at *m/z* 244.1 and 310.0, respectively. The product ions of agomelatine and fluoxetine scanned in quadrupole 3 after a collision with nitrogen in quadrupole 2 had an *m/z* of 185.0 and 44.0, respectively.

Chromatography

Initially, a mobile phase consisting of ammonium acetate, having different molarities and acetonitrile in varying combinations was tried, but produced poor peak shape. The mobile phase containing

ammonium formate of different molarities and acetonitrile in varying combinations was tried, but produced poor peak shape and very low response, which was insufficient to quantify LOQ. Because agomelatine is basic in nature, a mobile phase, 0.1% formic acid in water combined with methanol and 0.1% formic acid in acetonitrile in varying combinations were tried. It gave better response, but a poor peak shape was observed. Finally, the best signal was obtained for agomelatine and fluoxetine using a mobile phase containing 0.1% acetic acid in water in combination with methanol (20:80 v/v), as there was almost a twofold increase in its area count as compared with the mobile phase containing 0.1% formic acid in water solution in combination with acetonitrile. Moreover, a marked improvement in the peak shape of agomelatine and fluoxetine was also observed using this mobile phase combination.

Short-length columns, such as Inertsil C18 (50 mm × 4.6 mm, 5 μm), HyPURITY C18 (50 mm × 4.6 mm, 5 μm), HyPURITY Advance (50 mm × 4.0 mm, 5 μm), and Betasil (100 mm × 4.0 mm, 5 μm) column were tried during the method development. Inertsil C18 and HyPURITY C18 columns gave a poor peak shape, and the response was low. HyPURITY Advance gave better peak shape, but the response was low. The best signal was obtained using the Betasil (100 mm × 4.0 mm, 5 μm) column. It gave satisfactory peak shapes for all the analytes, and a flow rate of 1.2 ml/min reduced the run time to 3 min. Introducing such a high flow directly into the ionization source affects evaporation of solvents, which further causes improper ionization and reduces response, so a splitter was utilized to control direct flow in the ionization source. The column oven temperature was kept at a constant temperature of about 25°C.

Extraction

Prior to LC injection, the co-extracted proteins should be removed from the prepared solution.

Initially, protein precipitation method using acetonitrile and methanol as a precipitation agent was tried for sample preparation, but the extraction efficiency was very low in precipitation method as compared with the present method. Also, when samples were not clean, they caused deposition of matrix on curtain plate of the mass spectrometer and needed cleaning of the curtain plate frequently.

Several organic solvents were employed to extract analytes from the plasma sample. Out of the tested solvents, (ethyl acetate, chloroform, hexane, dichloromethane, and methyl tertiary butyl ether) ethyl acetate yields better results as compared with the other solvents.

It was difficult to find a compound that could ideally mirror the analytes to serve as a good internal standard (IS). Several compounds were investigated to find a suitable IS, and finally, fluoxetine, although belonging to a different class of compounds, was found most appropriate for the present purpose. There was no significant effect of IS on analyte recovery, sensitivity, or ion suppression. The results of method validation using fluoxetine as the IS were acceptable in this study on the basis of the FDA guidelines. High recovery and selectivity were observed in the liquid–liquid extraction method.

These optimized detection parameters, chromatographic conditions, and extraction procedure resulted in reduced analysis time with accurate and precise detection of agomelatine in human plasma.

Method validation

Selectivity and sensitivity

Representative chromatograms obtained from blank plasma, plasma spiked with LLOQ, and real subject sample for agomelatine and fluoxetine are shown in Fig. 4. The mean %interference observed at the retention time of analytes between ten different lots of human plasma including haemolysed and lipemic plasma containing K3-EDTA as the anticoagulant calculated was 1.51 and 0.02 for agomelatine and fluoxetine, respectively, which was within acceptance criteria. Six replicates of extracted samples at the LLOQ level in one of the plasma sample having least interference at the retention time of agomelatine was prepared and analyzed. The %CV of the area ratios of these six replicates of samples was 6.38 for agomelatine, confirming that interference does not affect the quantification at LLOQ level. Utilization of selected product ions for each compound enhanced mass spectrometric

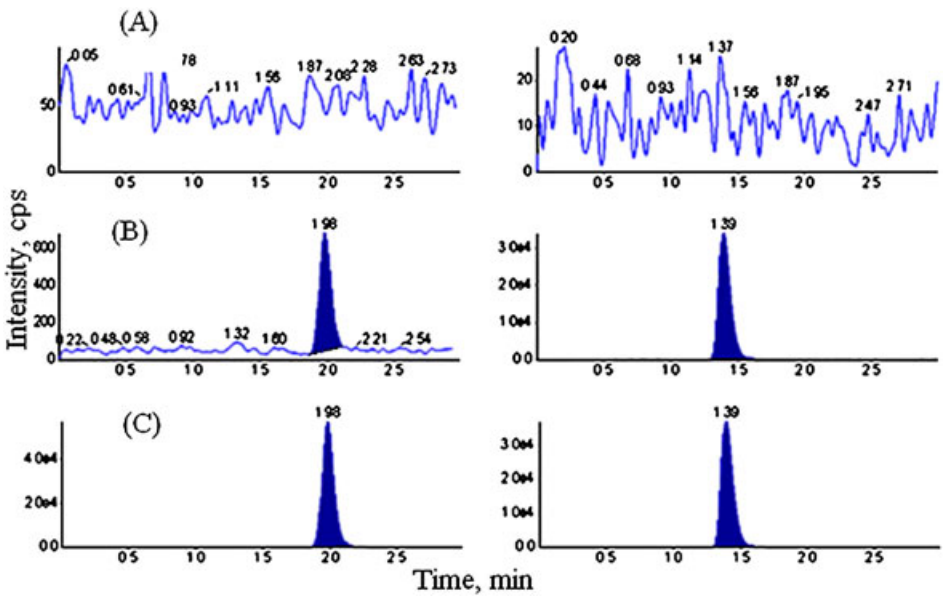

Figure 4. Representative chromatograms of agomelatine (left) and fluoxetine (right) in human plasma. (a) Blank plasma, (b) LLOQ, and (c) real subject sample.

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selectivity. The product ions of *m/z* 244.1 and 310.0 were concluded to be specific for agomelatine and fluoxetine. The sensitivity was determined by analyzing six replicates of LLOQ samples along with one calibration curve set. The precision (%CV) was found to be 3.53, and accuracy (%RE) was found to be −11.98, which indicates that the method is sensitive at the LLOQ level (0.050 ng/ml).

The LLOQ for agomelatine was 0.050 ng/ml. The intra-run precision and intra-run accuracy (%RE) of the LLOQ plasma samples containing agomelatine was 12.12 and 4.04, respectively. The mean C_{max} of agomelatine obtained for test and reference formulations were 4.37 and 4.50 ng/ml, respectively. As per EMEA guideline, the lower LOQ should be 1/20 of C_{max} or lower, as pre-dose concentrations should be detectable at 5% of C_{max}.[5] So for agomelatine, the LLOQ was easily quantified using the present method. All the values obtained below 0.050 ng/ml for agomelatine were excluded from statistical analysis, as they were below the LLOQ values validated for agomelatine.

Table 1. Recovery for agomelatine and fluoxetine

Analytes	Level	A	B	%recovery	%CV
Agomelatine	LQC	21542	13482	62.71	5.83
	M1QC	224399	150323	66.96	5.97
	MQC	449616	306080	68.10	3.93
	HQC	704513	497833	70.65	1.40
Fluoxetine	LQC	215604	155078	72.01	6.33
	M1QC	212643	157702	74.12	4.56
	MQC	208553	149525	71.72	4.03
	HQC	196996	145655	73.99	4.88

A, Mean area response of post-extracted sample; B, Mean area response of extracted sample; CV, coefficient of variation; LQC, low quality control; M1QC, middle quality control; MQC, medium quality control; HQC, high quality control.

Mean recovery was found to be 67.10% for agomelatine and 72.96% for fluoxetine.

Linearity, precision and accuracy, recovery

The peak area ratios of calibration standards were proportional to the concentration of agomelatine in each assay over the nominal concentration range of 0.050–8.069 ng/ml. The calibration curves appeared linear and were well described by least-squares linear regression lines. As compared with the $1/x$ weighing factor, a weighing factor of $1/x^2$ properly achieved the homogeneity of variance and was chosen to achieve homogeneity of variance. The correlation coefficient was ≥0.9992 for agomelatine. The observed mean back-calculated concentration, accuracy (%RE), and precision (%CV) of four linearities analyzed during method validation are, 0.049, 0.112, 0.383, 0.806, 1.596, 3.172, 4.968, 6.456, 8.226; 2.45, 6.79, 2.54, 1.03, 0.82, 1.72, 1.04, 1.08, 1.92 and −2.07, 10.59, 4.91, 0.10, 1.14, 1.73, −2.62, −0.01, 1.95, respectively.

The deviation of the back-calculated values from the nominal standard concentrations was less than 15%. This validated linearity range justify the concentration observed during real sample analysis.

The inter-run precision and accuracy were determined by pooling all individual assay results of replicate ($n = 6$) quality control over three separate batch runs analyzed on three different days. The inter-run precision (%CV) was 9.01, 5.14, 2.23, 12.46, 4.40, and 3.32 for LLOQ, LQC, M1QC, MQC, HQC, and ULOQ level, respectively, for agomelatine. Inter-run accuracy (%RE) was 5.63, −6.82, −6.93, −6.04, −4.35, and −2.46 for LLOQ, LQC, M1QC, MQC, HQC, and ULOQ level, respectively, for agomelatine. The intra-run precision and accuracy were determined by pooling all individual assay results of replicate ($n = 6$) quality control of two separate batch runs analyzed on the same day. The intra-run precision (%CV) was 12.12, 4.15, 5.25, 5.46, 5.32, and 5.17 for LLOQ, LQC, M1QC, MQC, HQC, and ULOQ level, respectively, for agomelatine. Intra-run accuracy (%RE) was 4.04, −4.21, −9.56, −6.66, −6.59, and −3.15 for LLOQ, LQC, M1QC, MQC, HQC, and ULOQ level, respectively, for agomelatine.

Six post extracted replicates (samples spiked in extracted matrix of same lot) at low, medium, middle, and high quality control concentration levels for agomelatine were prepared for recovery determination, and the areas obtained were compared *versus* the areas obtained for extracted samples (shown in Table 1) of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery for agomelatine was 67.10% with a precision of 6.10%, and the mean recovery for fluoxetine was 72.96% with a precision of 4.93%. This indicates that the extraction efficiency for the agomelatine as well as fluoxetine was consistent and reproducible.

Dilution integrity and partial volume

The mean back-calculated concentrations for 1/2 and 1/4 dilution samples were within 85–115% of their nominal. The %CV for 1/2 and 1/4 dilution samples were 6.02 and 7.30, respectively. The mean back-calculated concentrations for half and quarter partial volume samples were within 85–115% of their nominal. The %CV for half and quarter partial volume samples were 1.47 and 2.32, respectively.

Matrix effect, re-injection reproducibility, and stabilities

The assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS/MS for supporting pharmacokinetics studies. The results found were well within the acceptable limits as the %CV of the area ratios of post-spiked recovery samples at LQC and HQC levels were 3.81 and 1.05, respectively, which were within 10 for agomelatine. Hence, minor suppression of analyte signal because of endogenous matrix interferences did not affect the quantification of agomelatine. Also, a matrix-effect experiment by the post-infusion method was conducted during method development to check ion suppression or enhancement at agomelatine and fluoxetine retention times. It confirms that there is no ion suppression or enhancement at agomelatine and fluoxetine retention times.

Re-injection reproducibility exercise was performed to check whether the instrument performance remains unchanged after hardware deactivation because of any instrument failure during real subject sample analysis. Percent change was less than −2.90 for LQC and HQC level concentration; hence the batch can be re-injected in case of instrument failure during real subject sample analysis.

Stock solution stability was performed to check stability of agomelatine and fluoxetine in stock solutions prepared in methanol and stored at 2–8°C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 16 days. The %change for agomelatine and fluoxetine were 0.80 and 1.45, respectively, which indicates that stock solutions were stable for at least 16 days.

Bench top, dry extract, and autosampler stability for agomelatine was investigated at LQC and HQC levels. The results revealed that agomelatine was stable in plasma for at least 12 h at room temperature and 24 h in an autosampler at 10°C. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples

Table 2. Stability results for agomelatine

Stability	Level	A	%CV	B	%CV	%change
Autosampler	LQC	0.153	2.37	0.154	3.09	−1.29
(24 h, 10°C)	HQC	6.856	6.50	6.861	4.44	−4.45
Bench top	LQC	0.153	3.44	0.154	3.09	−4.89
(12 h at Room temp.)	HQC	6.856	−3.69	6.861	4.44	−3.69
Dry extract	LQC	0.153	1.36	0.154	3.09	2.27
(24 h)	HQC	6.856	5.32	6.861	4.44	−2.79
Re-injection	LQC	0.153	2.09	0.153	4.78	0.58
(2 h,)	HQC	6.856	1.38	6.856	1.67	−2.90
3rd freeze–thaw cycle	LQC	0.153	3.25	0.154	3.09	2.25
	HQC	6.856	5.53	6.861	4.44	−3.53
Long term	LQC	0.153	4.38	0.153	3.078	−5.43
(90 days, −20°C)	HQC	6.856	0.84	6.856	2.24	−2.57

A, comparison sample concentration (ng/ml); B, stability sample concentration (ng/ml); CV, coefficient of variation; h, hours; temp., temperature; LQC, low quality control; HQC, high quality control.

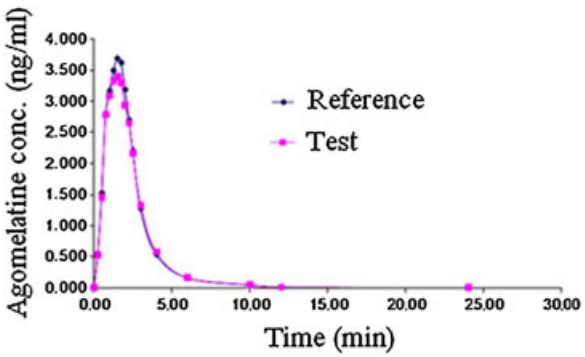

Figure 5. Mean agomelatine concentration *versus* time profile.

spiked with agomelatine at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that agomelatine was stable in matrix up to 90 days at a storage temperature of −20°C. The results obtained from all these stability studies are shown in Table 2.

Application

The validated method has been successfully used to quantify agomelatine concentrations in 24 human volunteers, under fasting conditions after administration of a single tablet containing 25 mg agomelatine as an oral dose. The study was carried out after approval from an independent ethics committee and obtaining signed approval from the volunteers.

The pharmacokinetic parameters evaluated were C_{max} (maximum observed drug concentration during the study), AUC_{0-t} (area under the plasma concentration–time curve measured to the last quantifiable concentration, using the trapezoidal rule), AUC_{0-inf} (AUC_{0-t} plus additional area extrapolated to infinity, calculated using the formula $AUC_{0-t} + Ct/Kel$, where C_t is the last measurable drug concentration), T_{max} (time to observe maximum drug concentration), Kel (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration *versus* time curve, using the method of least squares regression), $T_{1/2}$ (terminal half-life as determined by quotient 0.693/Kel), Vd (volume of distribution), and clearance. The mean C_{max} data obtained justified the linearity range selected.

The mean C_{max} observed for agomelatine for test and reference formulations were 4.37 and 4.50 ng/ml, respectively. The 90% confidence intervals of the ratios of means C_{max}, AUC_{0-t} and AUC_{0-inf} all falls within the acceptance range of 0.8–1.25, demonstrating the pharmacokinetic study of the two formulations of agomelatine. Steady-state volume of distribution is about 33 l. Clearance is high (about 1000 ml/min) and essentially metabolic. The mean concentration *versus* time profile of agomelatine in human plasma from 24 subjects that are receiving a single oral dose of 25 mg agomelatine tablet as test and reference is shown in Fig.5.

CONCLUSION

The developed LC–MS/MS assay for agomelatine is rapid, selective, and suitable for routine measurement of subject samples. This study reports for the first time a high throughput liquid–liquid extraction method for extraction of agomelatine in human plasma using LC–MS/MS. Hence, this method has been successfully applied to the pharmacokinetic study of a tablet containing 25 mg agomelatine as an oral dose in 24 healthy human volunteers under fasting conditions.

A simple, specific, rapid, and sensitive analytical method for the determination of agomelatine in human plasma has been developed. As there are no reported articles in the public domain citing estimation of agomelatine from human plasma, this present research work becomes unique for quantification of agomelatine. The present method provided excellent specificity and linearity with an LOQ of 0.050 ng/ml for agomelatine, which is sufficient to give data for calculation of the required pharmacokinetic data and establish bioequivalence. The other major advantage of this validated method is the runtime of 3 min, which allows the quantitation of over 150 plasma samples per day.

Acknowledgements

The authors are indebted to Mrs. Harini Chouta and the directors of Drug Monitoring Research Institute, India) for their continuous support and help.

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