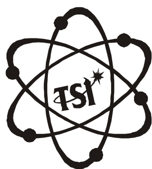

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Development and validation of new RP-HPLC method with UV detection for determination of novel anti-psychotic agent quetiapine in human plasma

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ABSTRACT

A high performance liquid chromatographic method has been developed and validated for estimation of quetiapine, an atypical anti-psychotic agent, in human plasma. Method was developed on the knowledge of previously reported literature and according to the need of improvement required on basis of instrument and experimentation technique(s) available. Separation was achieved by using C18 reversed phase column (Nova-Pack, 5 µm, 4.6mm 250 mm) and a mobile phase comprising of acetonotrile- disodium hydrogen orthophosphate solution (35:65). The UV detector was set at 220 nm and carbamazepine was used as internal standard. A careful pre-treatment procedure of plasma samples was developed using liquid liquid extraction (LLE) involving ethyl acetate-hexane mixture. The limit of quantification was 100 ng/ml and linearity was observed for calibration curves between 103.44 ng/ml to 1403.05 ng/ml. The relative standard deviation (RSD or % CV) for all validation studies was within ±15%. The method proves to be simple, sensitive enough for plasma estimation of quetiapine. The method developed was validated in terms of accuracy, precision, ruggedness, stability and dilution integrity. © 2009 Trade Science Inc. - INDIA

KEYWORDS

Quetiapine;
HPLC-UV method development;
Validation;
Human plasma;
Liquid-liquid extraction;
Stability.

INTRODUCTION

Quetiapine (Seroquel®, Quetiapine fumarate) is a psychotropic agent belonging to chemical class dibenzothiazepine derivatives. The chemical designation is 2-[2-(4-Dibenzo [b, f][1] thiazepin-11-yl-1-piperazinyl) ethoxy]-ethanol fumarate (Figure 1). It is present in tablets as fumarate salt. Its molecular formula is $C_{42}H_{50}N_6O_4S_2.C_4H_4O_4$ and has molecular weight of 883.11. This agent has been evaluated for management of patients with the manifestations of psychotic disorders and has been shown to have efficacy equal to that of traditional anti-psychotics in the short term treatment of schizophrenia and other psychotic syndromes and to have a low propensity for producing extrapyramidal side effects, hyperprolactinemia and agranulocytosis than other neuroleptics[1,2]. Quetiapine

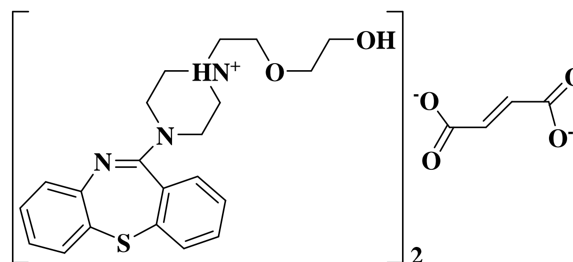

Figure 1: Chemical structure of quetiapine

is available in strengths ranging from 25 to 300 mg and has been shown to be effective over a broad dose range (upto 750 mg/day).

It has been reported that efficacy of Seroquel in schizophrenia and its mood stabilizing properties in bipolar mania and depression are mediated through antagonism at multiple neurotransmitter receptor sites in brain: combination of dopamine type (D1 and D_2) and serotonin type ($5HT_{1A}$ and $5HT_{2A}$), histamine H1 and adrenergic α_1 and α_2 receptors. Quetiapine has no appreciable affinity at cholinergic, muscarinic and benzodiazepine receptors.

In order to quantify quetiapine in biological matrix (plasma) for pharmacokinetic characterization, it is necessary to establish a HPLC-UV method to determine quetiapine. A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. The development of a method of analysis is usually based on prior art or existing literature using the same or quiet similar experimentation. The development of any new or improved method usually tailors existing approaches and instrumentation to the current analyte, as well as to the final need or requirement of the method. Method development usually requires selecting the method requirements deciding on what type of instrumentation to utilize and why. In the development stage, decisions regarding choice of column, internal standard, mobile phase, detectors and method quatitation are considered. So development involves a consideration of all the parameters pertaining to any method[3-4].

At present some methods in literature have been reported for quantitative estimation of quetiapine and to the best of our knowledge most of them are based on liquid chromatography[5]. In some methods the estimation range is narrow (e.g. Barrett et al.[6]: 1.0-382.2 ng/ml; Davis et al.[7]: 2.50 to 500 ng/ml; Mandrioli et al.[8]: 4-400 ng/ml; Sachse et al.[9]: between 10 and 50 ng/ml) as compare to the present method (100 to 1400 ng/ml approx.). In certain other available methods extraction techniques like solid phase (SPE) using HLB[5] or MCX[10] cartridges are employed that are uneconomical on lab scale as compare to LLE technique, which is comparably economical and less laborious. Some researchers used instrumentation such as mass spectrometry detectors[11] are no doubt more sensitive but the cost of analysis and availability are limiting factors at the present level of research.

In the present study attempt has been made for development and validation of a new HPLC-UV method (103.44 ng/ml to 1403.05 ng/ml) based on LLE techniques for quetiapine determination in plasma. The current method is sensitive and specific and has been validated in terms of stability (long term, short term, bench top, in-injector, stock solution), dilution integrity, ruggedness as well, in addition to accuracy and precision of the method[12-13]. The method employs liquid-liquid extraction technique for analyte recovery. The method was developed and validated at the Department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratories Limited, Gurgaon.

EXPERIMENTAL

Chemicals and reagents

Quetiapine working standard (purity 99.7%), Carbamazepine internal standard (IS, purity 100%) and control human plasma was kindly provided by Ranbaxy labs. Other reagents and chemicals {acetonitrile, methanol, tert-butyl methyl ether (TBME), Disodium hydrogen orthophosphate (S.D.fine chemicals ltd, Mumbai, India), ethyl acetate, hexane and phosphoric acid (Qualigens fine Chemicals, India)} used were of HPLC or AR grade. Ultrapure water was obtained by means of a MilliQ apparatus by Millipore (Milford, USA) for whole experimental work.

Method development

Selection of column

On the basis of the physicochemical properties of the drug, reverse phase chromatography with C-18 column was preferred. Various C-18 columns of different manufacturers like RP Select B (Lichrosphere), Discovery, Nucleosil, Bondapak, Zorbax SB and Hypersil BDS were tried. Symmetrical peaks, desired retention time and better resolution were obtained when Novapack C18 column (250 mm×4.6 mm, 5μm) was used

Selection of internal standard

Various compounds were tried including indinavir, clomipramine, amitryptyline, olanzapine, oxcarbazine

etc. Carbamazepine was selected as IS because of its desired retention time (6.4 min), good extraction recovery (47 %) and quantification at the λ_{max} of the analyte. The peaks of analyte and IS were well separated and distinguishable. It was selected on basis of parameters like similar solubility and pKa (determined using software CHEMSKETCH) as that of analyte and on basis of the literature reviewed.

Selection of mobile phase

Starting with simple combination of methanol-water, various systems (e.g. varying composition of methanol-water , acetonitrile -water, Acetate or Formate or Phosphate buffer with methanol, acetonitrile etc.) were tried but the desired elution and resolution along with retention time was obtained with acetonotrile- disodium hydrogen orthophosphate solution (35:65). Quetiapine was eluted at 10.2 minutes using this mobile phase.

Selection of sample processing method

Protein precipitation and liquid liquid extraction (LLE) were tried as sample processing methods because of economy and ease of technique at laboratory scale. Two samples each of blank plasma, lower limit of quantification (LLOQ- standard A) and upper limit of quantification (ULOQ-standard H) were processed by different methods like protein precipitation by acetonitrile, methanol, perchloric acid and trichloroacetic acid.

Same process was followed repeatedly using LLE. Protein precipitation was excluded as extraction method due to presence of endogenous peaks which interfered with the RT of analyte. Extraction efficiency of different LLE solvents including TBME, ethyl acetate, diethyl ether, dichloromethane was tried. Maximal yield of recoveries of analyte and internal standard were obtained using the mixture of ethyl acetate- hexane (80:20 v/v) combination. They provide significant outcomes as recoveries of analyte and IS were maximal with this combination. The possible reason being the analyte has polar nature and hence extracted easily with the help of polar solvent, ethyl acetate in combination with hexane.

Standard solution

The primary stock solutions of quetiapine (1mg/ml) and carbamazepine (1mg/ml) were prepared in methanol and serially diluted to working solutions with methanol-water (50:50) using MS-EXCEL software. All the stock and working solutions were stored at -10^{0}C.

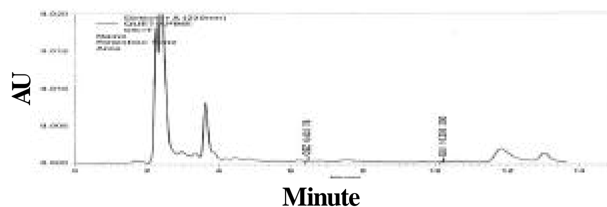

Figure 2: Chromatogram of extracted blank plasma sample

TABLE 1: CC and QC standards used for validation studies

ID of spiked sample	Spiked concentration in plasma (ng/ml)
Standard A	103.444
Standard B	206.889
Standard C	344.814
Standard D	574.691
Standard E	718.363
Standard F	897.964
Standard G	1122.442
Standard H	1403.053
LOQQC	104.177
LQC	231.504
MQC	526.145
HQC	1052.290

Working solutions

The calibration curve (CC or non zero) standards along with quality control (QC) standards were used for validation studies. The CC range (6-8 non zero standards covering the entire range) was selected from expected *in-vivo* concentration profile (derived through literature)[6]. The highest level (ULOQ, standard H-1403.053ng/ml) being twice the expected maximum plasma concentration and lowest level (LLOQ, standard A-103.444 ng/ml) being approximately 10% of expected maximum plasma concentration, rest CC levels being in between the range. The upper and lower levels are to be spiked in duplicate. The CC consists of a standard blank (blank processed without analyte and without IS figure 2) and a standard zero (blank processed with IS-Figure 3a) samples in duplicate also. A minimum of 3 (or 4) samples of QC concentrations covering entire range are to be used which helps to assess the integrity and validity of the results of the known samples analysed in an individual batch. The volume of spiked working solution sample being less than or equal to 2% of total volume of plasma spiked (e.g. 20µl for 500µl plasma). The CC and QC standards used for

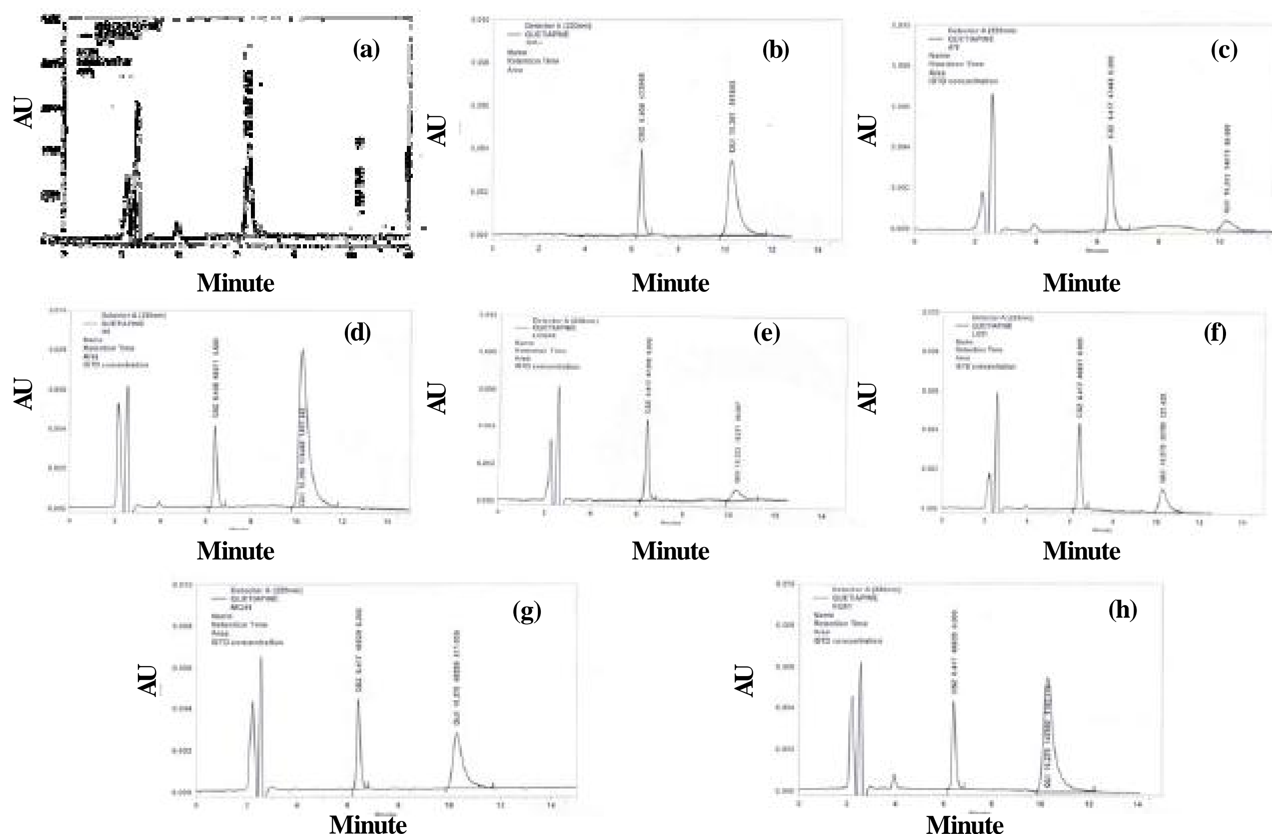

Figure 3 (a-h) : Different chromatograms: (a) Extracted blank plasma with internal standard, (b) A mixture of Drug and IS (Aqueous mixture), (c) Extracted standard (Std. A) sample (LLOQ), (d) Extracted Standard (Std. H) sample (ULOQ), (e) Extracted Limit of quantitative quality control, (f) Extracted lower quality control (LQC) sample (LOQQC)sample, (g) Extracted middle quality control (MQC) sample, (h) Extracted higher quality control (HQC) sample

validation studies are enlisted in TABLE 1.

Plasma extraction and sample preparation

After addition of internal standard, (50 µL, 4.0µg/ml IS: carbamazepine) to 0.5 ml aliquot of analyte (quetiapine) spiked plasma samples in stoppered tubes, the samples were vortex-mixtured for 60 seconds and 5.0 ml extracting solvent mixture (ethyl acetate-hexane, 80:20) was added to all tubes. The samples were then placed on reciprocating shaker for 30 min and after that subjected to centrifugation for 7 minutes at 4000 rpm. After centrifugation, the organic phase was removed into glass tubes and was subjected to dryness under stream of nitrogen. The residue was reconstituted in mobile phase and transferred entirely into 1 ml auto sampler vials and a volume of 50 µl was injected into the HPLC column for analysis.

Instrumentation

Shimadzu, Japan HPLC system with LC-10 Atvp pump, SCL 10 Avp system controller, SIL 10Advp auto injector, CTO 10 Avp column oven and 10 Avp UV detector. Branson-5510 sonicator, Eppendorf refrigerated centrifuge 5810R, Turbo vap LV nitrogen evaporator dryer, Cyberscan-2500 pH-meter, Finnpipette Tripette-brand Micropipettes, RS-02 INFROS-HT Reciprocating Shaker, BRAND Repeater, SPINIX Vortex shaker, SARTORIOUS Micro balance etc.

Chromatographic conditions

The HPLC analysis was performed as per the method developed using Nova-Pack reversed phase (C18, 5 µm, 4.6mm × 250 mm) column with column oven temperature set at 40^0C and detection was done using UV spectrophotometer set at 220 nm. The flow rate of mobile phase [acetonotrile-disodium hydrogen orthophosphate solution (35:65)] was 1.0 ml/min and Acetonitrile-Water (50:50) was used as the rinsing solution. The injection volume was 50 µl and carbamazepine was used

as the internal standard. The retention time for quetiapine and carbamazepine was 10.2 and 6.4 minutes, respectively.

Method validation

Validation involves documenting, though the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications[14]. The acceptability of analytical data corresponds directly to the criteria used to validate the method. The following validation parameters were estimated with the respective method developed: System suitability, Precision, Accuracy, Recovery, Selectivity, Ruggedness, Dilution integrity, Stability-Stock solution. Stability, bench top, freeze thaw, in-injector, long term, short term or aqueous mix stability[15].

Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system. Stability procedures were used to evaluate the stability of the analytes during sample collection and handling, after long term (frozen at the intended storage temperature, for 50 days) and short term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process (in-injector- putting the processed samples in the auto sampler and injected after 96 hours along with freshly spiked calibration standards). The procedure also included an evaluation of analyte stability in stock solution. The stability was determined at lower and higher quality control samples by evaluating 6 replicate samples at each level.

RESULTS AND DISCUSSION

Method development

In the present study attempt has been made for development and validation of a new HPLC-UV method for quetiapine determination in the human plasma. To develop a precise, accurate and reproducible method, various mobile phases (e.g. varying composition of methanol-water , acetonitrile -water, Acetate or Formate or Phosphate buffer with methanol, acetonitrile etc.), stationary phases and sample preparation methods were tried and the optimized chromatographic conditions were found appropriate for the quantitative determination of quetiapine in plasma. The current method is sensitive and specific and has been validated in terms of stability (long term, short term, bench top, in-injector, stock solution), dilution integrity and ruggedness as well. In addition to accuracy and precision method employs liquid - liquid extraction technique for analyte recovery. The CC and QC standards used for validation studies are enlisted in TABLE 1. The CC consists of a standard blank (blank processed without analyte and without IS -Figure 2) and a standard zero (blank processed with IS-Figure 3-a) samples in duplicate also.

Optimized chromatographic conditions

The column used was Novapack C18 (4mm id, 250mm length 5μm particle size) as it provide symmetrical peaks, desired retention time and better resolution (Figure 3b). The chromatographic separations were accomplished using mobile phase comprised of acetonitrile-disodium hydrogen orthophosphate solution (35:65). In order to resolve quetiapine peaks the mixture was tried in different ratios and finally, the ratio (35:65) proved to be better in terms of resolution and peak shape along with the desired elution and retention time (10.2 min) (Figure 3c-3h).

Carbamazepine was selected as internal standard because of its desired retention time (6.4 min), extraction recovery and quantitative measurement at the λ_{max} of the analyte. The peaks of quetiapine and carbamazepine were well separated and distinguishable with the selected column and mobile phase in the optimized chromatographic conditions (Figure 3b).

Liquid liquid extraction was chosen as the sample processing method because maximal yield of recoveries of analyte and internal standard were obtained. The optimum wavelength was set at 220 nm at which much better response of the drug was obtained. The retention time was 10.2 min for quetiapine. The mobile phase was pumped at a flow rate of 1ml/min, column temperature was set at 40^0C and the injection volume was 50μl.

Validation study

The method was subjected to complete validation for linearity, precision, accuracy, system suitability, di-

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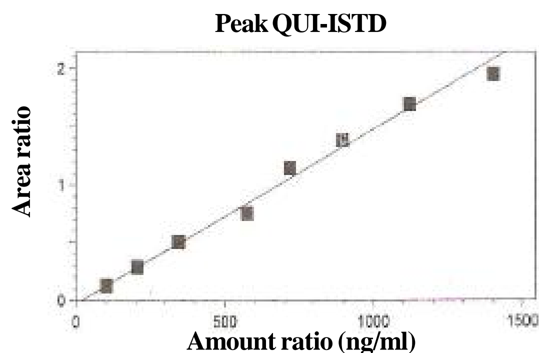

Figure 4: Calibration curve of quetiapine

TABLE 2: The validation results of the HPLC method

Validation parameters	Observations
Linearity range (ng/ml)	103.44 to 1403.05
Minimum quantifiable (ng/ml)	103.44
Total accuracy (%)	89.5 to 103.8
Total precision (% CV)	2.3 to 13.1
% Recovery (mean)	
-Analyte	55.2
-I.S.	47.09
Ruggedness	
- % CV	2.0 to 7.0
-% Nominal	92.3 to 100.7
Dilution integrity (Mean % nominal)	98.4
Stability (Mean)	
Stock solution (S.S.)	
- Analyte	95.4
- I.S	98.8
Bench top (B.T.)	99.3
Freeze thaw (F.T.)	91.9
In- injector (I.Inj.)	87.55
Long term (L.T.)	86.77
Short term or aqueous solution stability (S.T.)	
- Analyte	96.1
- I.S.	100.5

TABLE 3: Blank plasma screening (Selectivity)

Quetiapine method validation-selectivity						
S. no.	Interference at RT of analyte	LOQ area	% LOQ	Interference at RT of IS	IS area	% IS
1	0	15716	0.0	0	47331	0.00
2	0	14896	0.0	0	47355	0.00
3	0	14221	0.0	0	47055	0.00
4	0	14001	0.0	0	47050	0.00
5	0	15271	0.0	0	47329	0.00
6	0	15702	0.0	0	47345	0.00
-	Mean	14967.8	-	-	47244.2	-
-	SD	733.26	-	-	148.78	-
-	%CV	4.9	-	-	0.3	-

Value represent as Mean ± SD (n=6)

lution integrity and all the validation parameters and results were within the acceptance limit as specified by Centre for Drug Evaluation and Research (CDER)[12]. The validation results of the HPLC method are summarized in TABLE 2

Linearity determined by a weighted least square regression analysis of standard plot associated with an eight-point standard curve. The calibration curve was plotted against concentration ratio vs. area ratios and was found to be linear in concentration range 103.44 ng/ml to 1403.05 ng/ml as shown in figure 4. Best fit calibration lines of peak area ratios of drug and IS versus concentration of calibration standards were determined by weighted least square regression analysis with a regression factor of $1/X^2$. The r^2 were consistently greater than 0.99 during the course of validation.

System suitability (S.S.) was tested by calculating the percent coefficient of variation (% CV) of peak areas and retention time of six injections of aqueous mixture of analyte and internal standard. % CV of areas was found less than 2% and that of retention time was found less than 5%, which is within the acceptable range. System suitability test of one more system, to be used for ruggedness was done in the same manner as above and the system was passed the system suitability test.

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Six lots of blank plasma were evaluated for any interfering peak at the retention time of analyte or IS at the lower limit of quantification i.e.LLOQ-103.44 ng/ml (TABLE 3) and chromatogram shown in figure 2. Thus the method was verified by checking the interference of any endogenous compounds in human plasma at RT of analyte and I.S. Hence, method seems to be selective enough for the validation study.

Accuracy of the method was determined by replicate analysis of six sets of samples at high, middle and low quality control concentration and comparing the difference between the spiked value (nominal) and that actually found. Accuracy was expressed as % nominal concentration. Within batch accuracy of the method was found in the range of 89.5% to 103.8% for quetiapine and total accuracy of the method was in the range of 92.9 to 102.5.

% Relative accuracy = Measured value/ Mean expressed as %age of actual or true value × 100

TABLE 4: Recovery of drug from human plasma

Quetiapine-method validation	
Recovery of ouetiapine in human plasma	
QC	Percent recovery
LQC	52.4
MQC	57.6
HQC	55.5
Mean	55.2
S.D.(±)	2.62
C.V. (%)	4.7

TABLE 5: Recovery of internal standard from human plasma

Quetiapine-method validation		
Recovery of carbamezapine in human plasma		
IS S.no.	Extracted samples	Unextracted samples
1	46821	102386
2	46715	98282
3	46772	98708
4	46728	100162
5	46829	98664
6	46750	98982
Mean	46769.2	99530.7
S.D (+/-)	47.48	1538.81
C.V. (%)	0.1	1.55
% Recovery	47.0	

Value represent as Mean ± SD (n=6)

The precision of the method is based on with-in day repeatability was determined by replicate analysis of six sets each of high, middle and low quality control samples. The reproducibility (day to day variation) of the method was validated using similar six sets of high, middle and low quality control samples on different days. Coefficient of variation (% CV) were calculated from the ratios of standard deviation (SD) to the mean and expressed as percentage. Within batch /intrabatch precision of the method were in the range of 2.3 to 13.1 % and between batch precision of the method were in the range of 0.7 to 3.6 (TABLE 2).

The analytical recovery of analyte (quetiapine) and internal standard (carbamazepine) was estimated by comparing the peak areas of extracted samples at three conc. levels i.e. LQC (231.504), MQC (526.145) and HQC (1052.290) with the response of extracted blank samples to which analyte and internal standard has been added at the same nominal concentration (TABLE 4 and 5).

Stability studies

The summarized stability data of quetiapine in different condition of storage like bench top, freeze thaw, in-injector, long term and short term and stock solutions stability is provided in TABLE 2. Stability was found 86.5% to 100.5% in different storage conditions. Stability data indicates drug to be stable at different conditions of storage.

Dilution integrity

Dilution Integrity was assessed by assaying six replicates of QC samples spiked with approximately two times of 90% concentration of ULOQ and diluted by factor of two and four prior to extraction. The samples were processed and analysed against freshly spiked calibration standards (**TABLE 6**).

Batch acceptance criteria

Matrix- based standard calibration samples

75% or a minimum of six standards, when back calculated (including ULOQ) should fall within ± 15% except for LLOQ, when it should be ±20% of the nominal value. Values falling outside these limits can be discarded, provided they do not change the established model.

Quality control samples

Quality control samples replicated (at least once) at a minimum of three concentrations (one within 3 X of the LLOQ (low QC), one in the mid range (middle QC), and one approaching the high end of the range (high QC) should be incorporated into each run. The results of the QC samples provide the basis of accepting or rejecting the run. At least 67% (four out of six) of the QC samples should be within ±15% of their respective nominal (theoretical) values; 33% of the QC samples (not all replicates at the same concentration) can be outside the±15% of the nominal value.

CONCLUSION

At present various other powerful analytical techniques like GC-MS, HPLC-MS etc. are emerging for determination of drugs and metabolites in biological fluids but HPLC-UV technique is an economical technique and can be easily operated at low cost research level and still the technique of interest at industrial scale also. The present method is better than other available literature in being developed for a wide range (103.44 ng/ml to 1403.05 ng/ml) of quetiapine in plasma using liquid -

liquid extraction technique instead of costlier techniques like solid phase extraction using HLB or MCX cartridges and using a lower fraction of organic solvent in mobile phase not seen normally in cases with HPLC-MS methods. Moreover, present method is validated in terms of various stability parameters, dilution integrity of samples also, in addition to accuracy – precision only. The yield values and relative standard deviation (expressed as % CV) of quetiapine were within limits specified by CDER. In conclusion, the HPLC-UV method described in this report was sensitive and specific enough for determination of quetiapine in human plasma.

Abbreviations

HPLC: High performance liquid chromatography, CC: Calibration curve, QC: Quality control, IS: Internal standard, SS: System suitability, ULOQ: Upper limit of quantification, LLOQ: Lower limit of quantification, CV: Coefficient of variation, LOQQC: Limit of quantification quality control, LQC: Lower quality control, MQC: Middle quality control, HQC: Higher quality control

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