INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Development and validation of a reversed-phase HPLC method for the simultaneous determination of Aniracetam and impurities in the bulk drug

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ABSTRACT

Simultaneous determination of aniracetam and its related impurities [2-pyrrolidinone, 4-Methoxy benzoic acid(*p*-anisic acid), N-anisoyl GABA (4-*p*-anisamidobutyric acid)]were accomplished in the bulk drug, synthesis for drug and drug intermediate and a tablet formulation using a Reversed Phase High Performance liquid chromatography method with UV detection separation was achieved on zorbox Agilent Eclipsc XDB column C_{18}(150mm× 4.6 mm×5µm) as stationary phase with binary gradient mode solvent phase A composed of H_3PO_4 (Ortho phosphoric acid) buffer (pH ≈2, 0.02M) and phase B of acetonitrile. The flow rate of the mobile phase was 1.0 mL/min and the total elution time including the column re-equilibration was approximately 35 min. The UV detection wavelength was 215nm,Injection volume was 10µL and experiments were conducted at 30 °C temperature. The developed method was validated in terms of system suitability, selectivity, linearity, range, precision, accuracy, limits of detection and quantification for the impurity, short term and long term stability of the analysts in the prepared solution and robustness, following the ICH guidelines. Therefore, the proposed method was suitable for the simultaneous determination of aniracetam and its studied related impurity and intermediate.

Keywords: Aniracetam, System suitability, Binary gradient, Precision

1. INTRODUCTION

Aniracetam is chemically1-(4-methoxybenzoyl) pyrrolidin-2-one, Fig.1A, is nootropic and neuroprotective drugs that of drugs that share a pyrrolidone belongs to a Racetams are chemical Class of drugs that share a pyrrolidone nucleus used for improving cerebral insufficiency. It functions of emotional disturbances depressed mood, anxiety, agitation, sleep disorders, sickness, motion and behavior abnormalities (nocturnal delirium wandering) that are associated with cerebral infarction and Alzheimer's and Parkinson's diseases [1–7]. It has been also reported to possess mechanisms for positivity modulation cholinergic and glutaminergic nervous systems, as well as increasing synaptic efficacy and energy metabolism [1].

Several studies on aniracetam determinations, its pharmacokinetics and metabolites in humans and rats have been reported using HPLC and HPLC- MS/MS techniques[8–17], However, there is no method in the literature describing the determination of aniracetam and its related impurities in the bulk drug for synthesis and pharmaceutical formulations. The aim of research paper is development and validation of a simple and reliable RP-HPLC method for simultaneous determination of AN three of its related impurities [2-pyrrolidinine (PD, Fig.1B), 4-methoxy benzoic acid or p-anisic acid (MBA, Fig.1C),4-methoxy benzoylamino butyric acid or 4-anisoyl GABA or 4-p-anisamidobutyric acid(NAG, Fig.1D)],

in the bulk drug, drug synthesis intermediate and in a tablet formulation.

The unique feature and novelty of the proposed method is that first time these three compounds are determined simultaneously. In previous work [8-9] for the determination of AN and its three metabolites PD,MBA,NAG in biological samples[18-19].Two successive methods were proposed, one for AN,MBA,NAG and the other for PD. The difficulty of such a determination is due to the chemical diversity of the analytes, a very polar compound (PD), two acidic substances (MBA and NAG) and hydrophobic components (AN). A Zorbox Eclipsc XDB C_{18} column (150mm × 4.6mm × 5µm) as stationary phase combined with RP and a gradient elution program was chosen to compensate for the extreme compound polarities and online synthesis preparation of drug. The option of ZorboxEclipsc XDB C_{18} column RP-chromatography was not considered in order to avoid the well-known drawbacks of this technique which are the long equilibration time substantial UV detection of the acid base reagents to be washed from the column and the almost impossible use of gradient elution.

The developed method was validated for the analysis of bulk drug and drug intermediate, and in a tablet formulation. The method was intended to be simple, sensitive, accurate, LOQ and LOD, linearity, precision, recovery and accuracy, robustness, solution stability for all studied compound. The increased sensitivity for the three impurities was a demand in the present work because of the very low concentration needed for the validation of the method (<0.05% of the active drug). Synthesis for drug and drug intermediate and check drug and impurities to Aniracetam, 4-Methoxy benzoic acid, N-anisoyl GABA, 2-pyrrolidinone,online % of analysis for (IPC) monitoring in reaction and final drug analysis (Fig.2);its molecular weight is 219.2 g/mol with molecular formula of $C_{12}H_{13}NO_3$its dissociation contents (pKa) are reported. Melting point of compound is 121°C to 122°C.

2. MATERIALS AND METHODS

2.1 Instrumentation and software

The HPLC system of Agilent HPLC 1100 Series, Variable Wavelength Detector (VWD), The Diole Array Detector (DAD) Microprocessor, quaternary pump, Agilent Technologies international sarl,1100 series, auto sample, micro auto sample, preparative auto sample, Thermostatic column compartment used for this entire study and chromatographic separation was achieved on Eclipse XDB C_{18} (150 mm×4.6 mm×5µm) column as stationary phase with binary gradient mode.

2.1 Chemicals and reagents

Acetonitrile (HPLC Grade), Ortho phosphoric acid (85 %, AR), Hydrochloride acid (AR), Sodium hydroxide (AR), Hydrogen Peroxide (AR) and Methanol (HPLC) were purchase from SD-Fine Chemical Ltd., India. Milli-Q water used for the experiments.

2.2 Standards and Sample Materials

The pharmaceutical grade Aniracetam (99.9%), 4-Methoxy benzoic acid (99.9%), N-anisoyl GABA (99.9%) and 2-pyrrolidinone (99.9%) were gifted by ShriHari Ohm Chemicals &Pharma Company, India. Other chemicals used for the analysis (AR grade) were procured from Spectrochem & SD Fine Ltd., Ahmadabad, India.

2.3Details of Method:

Chromatographic conditions are given in Table 1.

2.4 Preparation of solutions

2.4.1 Diluted standard solution:

Accurately weighted 500 mg of Aniracetam is transferred to 100 mL volumetric flask and diluent is added to desire volume. The concentration of sample is 5000ppm.

2.4.2 Impurity stock solution

(1). 25 mg of 4-Methoxy benzoic acid is accurately weighted and transferred in 50 mL volumetric flask and diluent is added to desire volume. Concentration of the sample is 500 ppm.

(2). 25 mg of N-anisoyl GABA is accurately weighted and transferred to 50 mL volumetric flask and diluent is added to desire volume. Concentration of the sample is 500 ppm.

(3). 25 mg of 2-Pyrrolidinone is accurately weighted and transferred to 50 mL volumetric flask, and diluent is added to desire volume. Concentration of the sample is 500 ppm.

2.4.3 Standard stock solution

500 mg of Aniracetam(reference/working standard) is accurately weighted and transferred in to 100 mL volumetric flask, and diluent isadded.5.0 mL impurity(1 stock solution), 1.5 mL impurity(3 stock solutions) and 0.5 mL impurity (2 stock solutions) aresonicatedfor 10 min, and make up to 100 mL with diluent. The concentration of aniracetam is 5000 ppm, 4-Methoxy benzoic acid is 25 ppm, 2-Pyrrolidinone is 7.5 ppm and N-anisoylGABA is 2.5 ppm.

2.4.4 Resolution Solution

10.0 mL Standard stock solution is taken in to 100 mL volumetric flask, and makes up to 100mL with diluent. This solution is sonicated for 10 min.The sample concentration of aniracetam is 500 ppm, 4-Methoxy benzoic acid is 2.5 ppm, 2-Pyrrolidinon is 0.75 ppm and N-anisoylGABA is0.25 ppm.

2.5 Method Validation

Validation of the developed method for determination of aniracetam and three impurities is performed according to ICH guidelines with standards bulk drug and powder[20, 21]. Thus, system suitability along with method selectivity, specificity, linearity, range, precision (repeatability) and intermediate precision, accuracy, limits of detection and quantification of the three impurities, short team and long term stability of the analysts in the prepared solutions and robustness have been demonstrated.

2.5.1 System suitability

The system suitability solution (diluted in diluent and contained each compound at a concentration level of 0.5mg/mL) was prepared as a mixed standard solution of AN and the three impurities (Fig.3).

2.5.2 Selectivity

Selectivity of a method can be defined as the absence of any interference at retention times of peaks of interest and is normally evaluated by observing the chromatograms of blank sample and spiked with the API (Active Pharmaceutical Ingredient) in the presence of all impurities in the diluent the bulk drug and the formulation in this work due to the lack of an appropriate placebo solution the verification using a complete series of the excipients solutions of each excipient were prepared diluted in diluent and injected in the HPLC (Fig.3).

2.5.3 Linearity and range

Standard calibration curves were prepared as discussed in section 2.4.1 (for aniracetam) and 2.4.2 to 2.4.4 (for impurities).The data of peak area of each analyte versus corresponding concentration were treated by linear least square regression analysis at six complete calibration curves were constructed in six consecutive days for each compound (Fig. 4).

2.5.4 Precision and accuracy

The precision of the determination of aniracetam and impurities was studied with respect to both repeatability and intermediate precision by one-way for six consecutive days using the daily calibration curves five concentration levels were used for the (low, medium, high) were used and the prepared samples were analyzed in duplicate. The repeatability and intermediate precision were expressed as the % relative standard deviation (% RSD)of the analyst concentration. The accuracy of the method for all analytes was expressed as intra and inter-assay accuracy, and was obtained using the data of the previous precision experiments from the relative error of the mean concentration and the theoretical concentration of each day independently. The inter assay accuracy was determined from the relative error of the mean concentration at each level for the six day as a whole over the theoretical concentration (Fig.5).

2.5.5 Recovery

Recovery studies of AN and its three impurities were performed, both in bulk drug and in the powder formulation, using the standard addition method in particular, for the estimation of the recovery of AN in powder a level of four solution was prepared the first solution was a powder test solution prepared in such a way to contain a nominal concentration 0.5mg/mL of AN and impurities. The other three solution with appropriate aliquots of the stock solution of AN and level 75%, 100%, 125%, LOQ add amounts AN powder for the recovery studies on the impurities a similar procedure was followed. The only difference was that the concentration of AN in this level of solution was the method nominal concentration of the powder test solution 0.5mg/mL in this solution four standard additions of all impurities were performed (75%,100%,125%,LOQ).

2.5.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection is the lowest concentration of the analyte in a sample that can be detected, but not necessarily quantities, under the stated experiment conditions. The limit of quantification is the lowest concentration of the analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions (Fig.6).

Study design:

Determine the LOD and LOQ for all impurities and AN and as unknown. Inject solution as per the following sequence. Evaluate the data and draw a linearity plot from the level, which detected to 150.0% of specification limit.

Calculation:

LOD and LOQ are calculated by using the formula given in ICH guideline.

Precision at LOQIf the signal to noise ratio for the data of LOD and LOQ observed using above calculation is satisfactory (signal to noise for LOQ ≥10 and for LOD ≥3), and then precision of LOQ and

LOD shall be done by injecting six replicate of LOQ solution and duplicate injections of LOD solution.
Acceptance criteria:
System suitability parameter should comply, mention the LOD and LOQ level in % for AN and all impurities, % RSD for response of six injections at LOQ level should not be more than 10.0, Signal to noise ratio for LOQ should be ≥10 and LOD should be ≥3.

2.5.7Stability

The stability of the studied compounds was estimated based on the studied comparison of the peak area of the analytes at t_0 h over those after t_h h fist short term stability of aniracetam for 24 h and 48 h was examined in stock solutions prepared in diluents at room temperature and 5°C temperature and measured in standard solutions of AN and all impurities. Finally the long term stability of the stock solution of AN and all impurities in diluents (ACN:Water,50:50) are observed at same RT.

2.5.8 Robustness

The purpose of this study was to evaluate the influence of small changes in the operating conditions on certain responses of the method, affecting both the quality of the separation and the quantification of the analytes. Three major parameters were individually altered the flow rate. Evolution of the robustness of proposed method was approached by recording the retention time. The relative retention time of each imparity was calculating by dividing their t_R by the t_R of AN.

3. RESULTS AND DISCUSSION

3.1Optimization of chromatographic conditions

The development of HPLC method for the determination of drugs has received great attention in analytical research because of their importance in quality control. The technique is unique, versatile, universal and basic, and well utilized by the researchers because of its ease of operation. The main objective of method development is to determine the drug and drug intermediate content of synthesis of drugs. The presence of the polar compound PD among the other constituents that necessitated an almost aqueous mobile phase led to the selection of Eclipse XDB C_{18} (150 mm × 4.6 mm × 5µm) or equivalent column for the development of the proposed method. The highly polar nature of PD combined with the acidic nature of MBA and NAG and the relatively non polar behavior of AN demanded extensive optimization of the pH of the mobile phase and the need of and a gradient elution. The selection of the pH of the mobile phase was very critical for pH<2,the peaks of the acidic substances MBA and NAG would move to the right, closer to the aniracetam peak for pH>2, these two substances would co elute and move to the left of the chromatogram. Finally, a linear gradient program with an initial mobile phase consisted of 0.02M ortho phosphoric acid (H_3PO_4, pH=2.0) was considered as the optimal for the adequate retention of PD and acceptable separation of the five compounds. The wavelength variation of the detector during the analysis was based on the maximum wavelength (λ_{max}) absorbance of the relevant compounds. PD absorbed below 220 nm. While MBA, NAG, and AN have their λ_{max}at 250,252,285 and 280nm, respectively. Thus the detector was set at 215nm at the beginning of the elution to achieve high enough sensitivity for PD detection, while keeping the background noise as low as possible. Then the wavelength was switched to 250nm for maximum sensitivity of MBA, and NAG, while for the pair of compounds. The 280 nm was selected in order to favor the sensitivity of the impurity over that of AN, finally the wavelength was set at the initial value of 215nm in order prepare the system for the next injection. The optimal wavelength Scheme along with details is given in section 2.3. A typical chromatogram of the separation of the five analytes under these conditions is presented in Fig 2.

3.2 Method Validation.

3.2.1System Suitability

The system suitability test is very important because it ensures the validity of the analytical procedure. In present work, a mixed standard, consists of AN and the three impurities (described in section 2.5.1) was the system suitability solution used daily for this purpose. Thus the consistency of the retention time of AN (t_R), the area of peaks, the relative retention times (Rt_R), and resolution (R_S) between adjacent peaks were the critical parameters examined every day. During the validation of the method, the critical parameters of RS met the acceptance criteria (RS ≥1.5) and assured for good separation quantification [18–20].

3.2.2 Selectivity

Under the optimized chromatographic conditions in all studies, the obtained resolution between the adjacent peaks of AN and the three impurities in the way they eluted (PD, MBA, NAG, AN) was satisfactory (R_S>3.2).The critical resolution between PD and early eluted peaks at dead volume was always greater than 3.2, verifying reliable quantification of PD. Typical values of the following

chromatographic parameters $t_{R,}$ $R_{tR,}$ R_S obtained with the system suitability solution is shown in Fig.3.
In addition, Selectivity in the memodrin powder was tested by running individual solutions containing only the excipients of the formulation where it was found that there were no additional peaks coming from the excipients in the chromatogram (Fig.3).

3.2.3 Linearity and range

Table 2 presents the analytical parameters of typical standard calibration curves of AN and its three related impurities in diluent. All calibration curves for AN presented coefficient of determination R^2>0.9999, while for the impurities R^2 was greater than 0.995, as required [21-22]. A lack-of-fit test was performed for all calibration curves and the calculated R^2-values of the representative curves included in Table 2, were the following at 96% confidence level for AN correlation 0.99981,PD correlation 0.99555, MBA correlation 0.99992, NAG correlation 0.99914, system suitability parameter was comply. The correlation coefficient would not be less than 0.995. Y- Intercept ≤25% referred to the calculated response of the x-value corresponding to the concentration of the specification limit. Representative Linearity and range results in memodrin tablets are included in Fig.4 and Table2.

3.2.4 Precision and accuracy

The repeatability and intermediate precision were expressed as the % relative standard deviation (% RSD) of each analyte concentration using AN one way. The results are presented in Table 3.The signal to noise ratio for the data LOD and LOQ observed using above calculation is satisfactory then precision of the LOQ and LOD was done by injecting six replicate of LOQ solution (Fig.5) and duplicate injections of LOD solution. The signal to noise ratio for the data 100 % level observed using above calculation, is satisfactory then precision of the 100 % level was done by injecting six replicate of 100 % level solution(Fig.5).Above calculation is satisfactory then precision of % RSD for response of six injections at LOQ and 100 %level was not be more than 10.0%.Intermediate precision study expresses within- laboratory precision on different day, by a different analyst and on different instrument using same lot of sample as specified precision. Representative precision results in memodrin tablets are included in Table 3; system suitability parameter was complying.
Accuracy study:
The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The true value is that result which was observed in the absence of error. Accuracy may often be expressed as present recovery by assay of known, added amount of analyte. Accuracy is a measure of the exactness of the analytical method that is true for all practical purpose.
Study design:
Accuracy of the test method was be demonstrated by spiking all impurities and AN in a test sample at LOQ, 75%, 100%,125% level of target concentration. Three sets were prepared for each level. Inject this solution into the chromatographic system and record the area of impurity peak at each level Calculate the % recovery. Recovery studies were performed by the standard addition method in the bulk drug and in the powder formulation. The slopes of the standard calibration curves and those of the standard addition method were statistically same [23]. Recovery values of AN and its four impurities have been found in the following ranges, AN 95.11% to 98.11%, PD 91.47% to 98.1%,MBA 93.58% to 101.78%, NAG 96.52% to 100.78%. Representative recovery results in memodrin tablets are included in Table 2 and Table 3.System suitability parameter was comply, and % RSD obtained from three sets was be NMT 10.0.Aceeptabble for recovery LOQ level(90.9% to 96.0%) and 75%,100%,125% level (95% to 102%).Acceptable criteria for recovery study. Accuracy of the test method was to be demonstrated by spiking all impurities and AN in a test sample at 100% Level Representative Retention time (min). Related RT and Resolution, results in memodrin tablets are included inTable2.

3.2.7 Stability

At the beginning of the method development, it was observed that aniracetam stock solutions prepared in diluent (Acetonitrile : Water; 50:50) seemed to be very unstable, and additional peaks appeared in the chromatograms. Therefore, based on this observation, a stability study on AN stock solution in diluent at RT and 5^{o}Cfor 24 hrs,48hrs followed at which they were stable(Fig.5).Although no significant degradation of AN, PD and MBA were produced receptivity at a very small rate, capable of elevating their concentration. In the case of MBA, where the hydrolysis was faster the concentration exceeded the acceptance limits at RT Specifically was % MBA and % NAG of API standard solution of diluent A seemed to be very stable.
The stability of individual standard solution of impurities in diluent was also examined. This study showed that PD, NAG, and could be considered as stable at RT for 48hrs. However, the standard solution of MBA was unstable with a tremendous peak are reduction after a period of 48 hrs at RT. It is

as stable as at 5 °C. It should be noted were that zero level concentrations of impurities in figment unstable quantities much below the LOD level. Representative Linearity and range results in memodrin tablets are included in Table 4.

3.2.8 Robustness

The robustness of the method was evaluated by analyzing standards and best solutions at the methanol nominal concentration of AN (≈100% level) in the presence of all impurities at 0.1 % of the parent drug peak. The parameters altered when the flow rate was (±10%) 0.9 or1.1mL/min instead of 1.0 mL/min, pH was(±0.1 pH unit)1.9 or 2.1 instead of 2.0and the different column. Representative Linearity and range results in memodrin tablets are included in Table 4.

Evaluation of the results was based mainly on the most important parameter of the separation e.g. resolution the method proved to be robust since resolution (R_S≥2.0) met the acceptance limit(R_S≥1.5) for all compounds of interest.

Table 1
Details of Method: Chromatographic condition.

Column	Eclipse XDB C_{18} (150 mm × 4.6 mm × 5µm) or equivalent
Buffer	Buffer,0.02 M Ortho phosphoric acid (H_3PO_4) in water, pH was to 2.0, filtered through 0.45µm membrane
Mobile phase-A	Buffer
Mobile phase-B	Acetonitrile
Diluent	Water:Acetonitrile ,50:50 (V/V)
Column oven temp.	30°C
Detector	UV –Vis. Detector
Detection	UV at 215 nm
Injection volume	10.0 µL
Run time	30.0 minutes
Post time	5.0 minutes
Flow rate	1.0 mL/ min
Gradient Programme	

Time(min)	Mobile Phase-A %	Mobile Phase-B %
0.01	80	20
10	70	30
20	30	70
30	30	70

Table 2
Linearity, t_R(retention time), Coefficient of determination(R^2), Y-intercept,LOD and LOQ data for proposed Method of AN and MBA, NAG and PD

Substance	Range (mg/mL)	t_R, (retention time)	Rt_R, (relative time)	Coefficient of determination (R^2)	Y-intercept	LOQ (mg/mL)	LOD (mg/mL)
AN	0.0025-0.75	10.11	1	0.99981	359.650	0.019925	0.00655
MBA	0.0000125-0.000375	8.51	0.84	0.99992	-0.2522	0.0000916	3.02×10^{-6}
NAG	0.00000125-0.000375	0.51	0.51	0.99914	-0.9386	9.96×10^{-6}	3.28×10^{-6}
PD	0.00000375-0.001125	1.58	0.16	0.99555	-0.1276	2.98×10^{-5}	9.86×10^{-6}

Table 3
Accuracy and Recovery and Precision data for proposed method of AN and MBA, NAG and PD

Substance	Level of %	Recovery Data		Accuracy Data		Precision Data			
		Mean %	RSD %	Mean %	RSD %	SD %		RSD %	
						1 Day	2 Day	1 Day	2 Day
AN	125	100.59	0.995	100.20	0.992				
	100	98.17	0.99	99.36	0.983	1.72	5.30	0.009	0.030
	75	97.86	0.968	97.65	0.966				
	LOQ	96.12	0.952	96.05	0.952	2.934	2.934	0.415	0.41
MBA	125			101.81	1.007				
	100			100.19	0.991	0.16	0.428	0.182	0.484
	75			96.29	0.953				
	LOQ			94..91	0.951	0.147	0.147	4.97	7.04
NAG	125			101.62	1.006				
	100			97.56	0.966				
	75			94.67	0.937				
	LOQ			90.86	0.908	0.065	0.067	6.94	7.134
PD	125			98.09	0.971				
	100			95.75	0.947				
	75			95.16	0.942				
	LOQ			90.91	0.888	0.210	0.210	7.04	7.04

Table 4
Stability and robustness data for proposed method of AN and MBA, NAG and PD

Substance	Stability at RT for 24 hrs and 48 hrs of area (LOQ Level)	Stability at RT for 24 hrs and 48 hrs of area (LOQ Level)	Stability at 5°C for 24 hrs and 48 hrs of area (LOQ Level)	Stability at 5°C for 24 hrs and 48 hrs of area (LOQ Level)	Robustness Mobile phase pH of 100 % level of area	Robustness Mobile phase pH of 100 % level of area	Robustness Flow rate of 100 % level of area of $t_R$difference	Robustness Flow rate of 100 % level of area of $t_R$difference
	SD %	RSD %	SD %	RSD %	1.9 pH/2.1 pH	1.9 pH/2.1 pH	1.1/ mL	0.9/mL
					SD%	RSD%	Difference of t_R	Difference of t_R
AN	1.10	0.133	0.58	0.070	3.28/3.28	0.46/0.46	0.01	0.01
MBA	1.63	17.36	0.38	3.612	0.165/.0.165	5.56/5.56	0.01	0.02
NAG	0.07	7.76	0.0035	3.083	0.06/0.06	6.41/6.41	0.005	0.019
PD	0.89	9.33	0.47	4.87	0.234/0.234	7.52/7.52	0.026	0.002

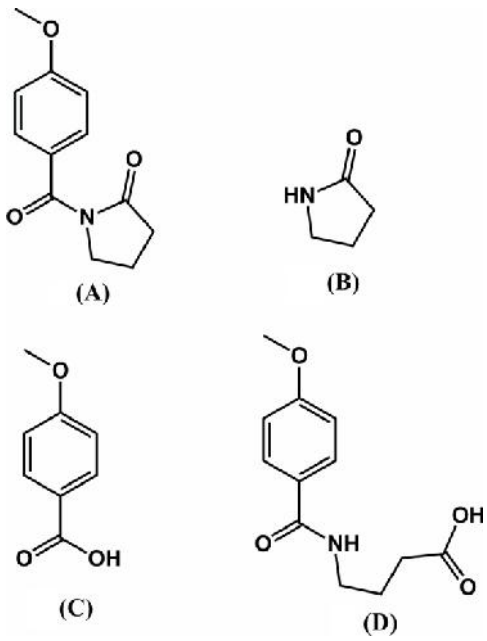

Figure 1
(A)Structure of the Aniracetam,chemically1-(4-methoxybenzoyl) pyrrolidin-2-one (AN), (B)Structure of the Aniracetam related impurity 2-pyrrolidinine (PD),(C)Structure of the Aniracetam related impurities4-methoxy benzoic acid (MBA), (D)Structure of the Aniracetam related impurity4-p-anisamidobutyric acid (NAG).

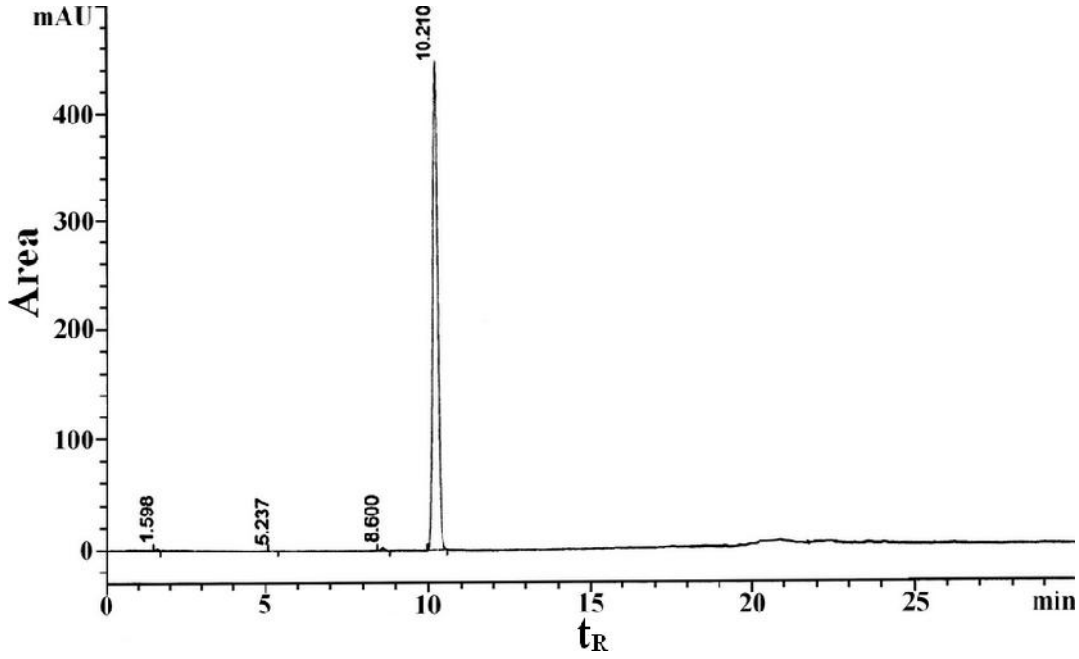

Figure 2
Chromatograph for method development of AN and MBA,NAG,PD.

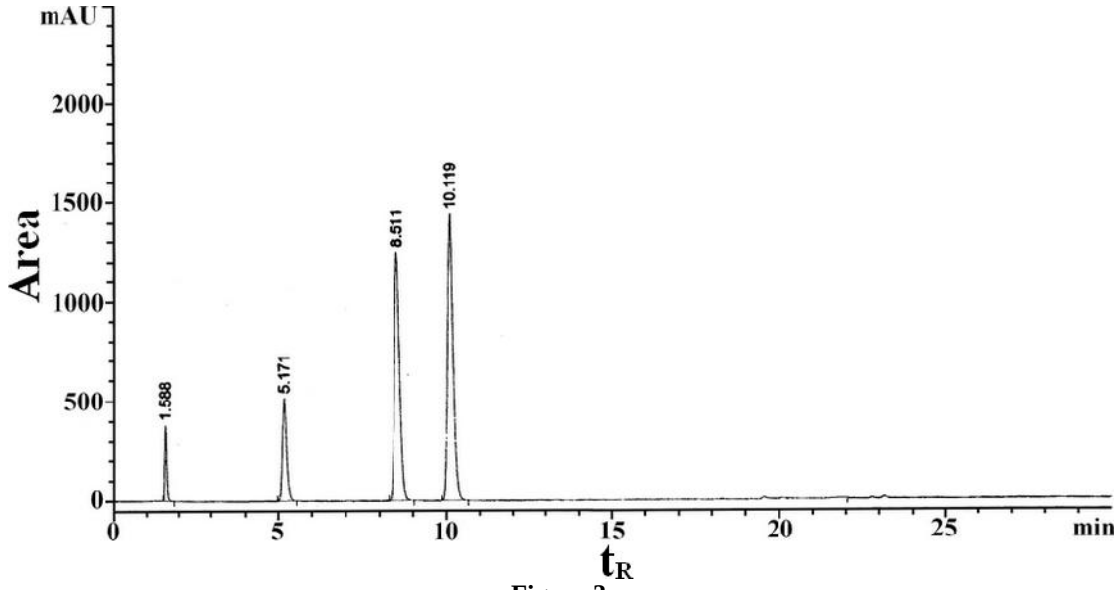

Figure 3
Chromatograph for system suitability and method validation (Linearity, LOQ and LOD) of AN and MBA, NAG, PD.

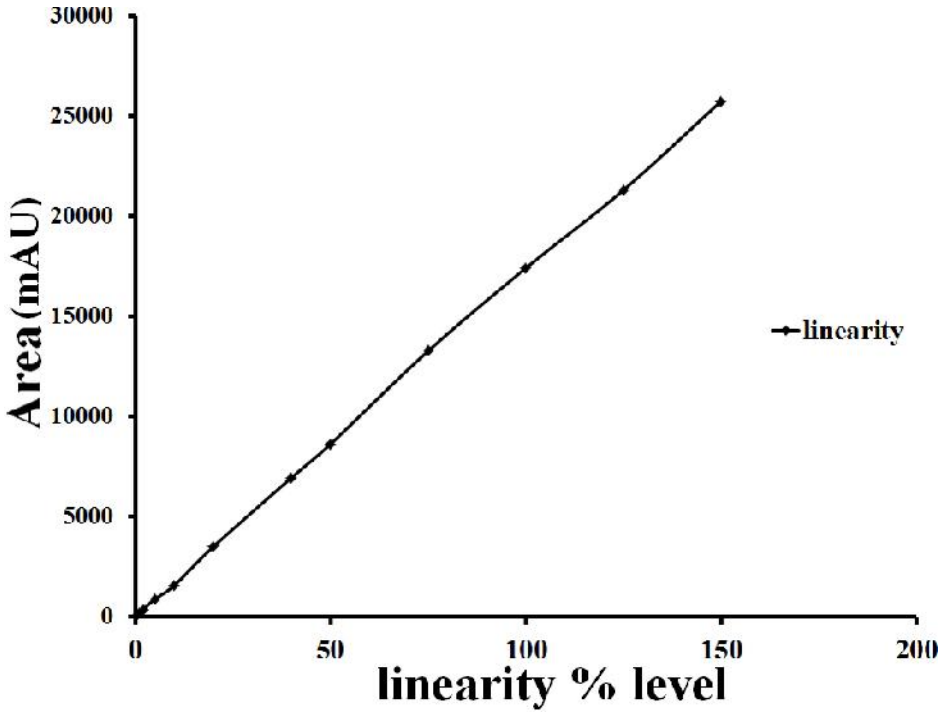

Figure 4
Plot of Area *verses* linearity level (0.5% to 150%) of AN.

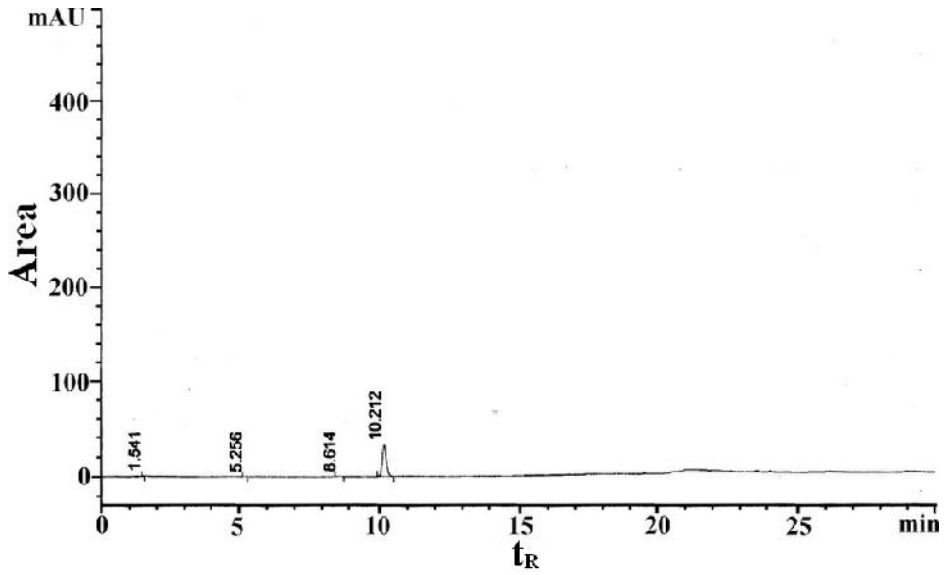

Figure 5
Chromatograph for method validation (Accuracy, Precision and Recovery) of AN and MBA,NAG,PD.

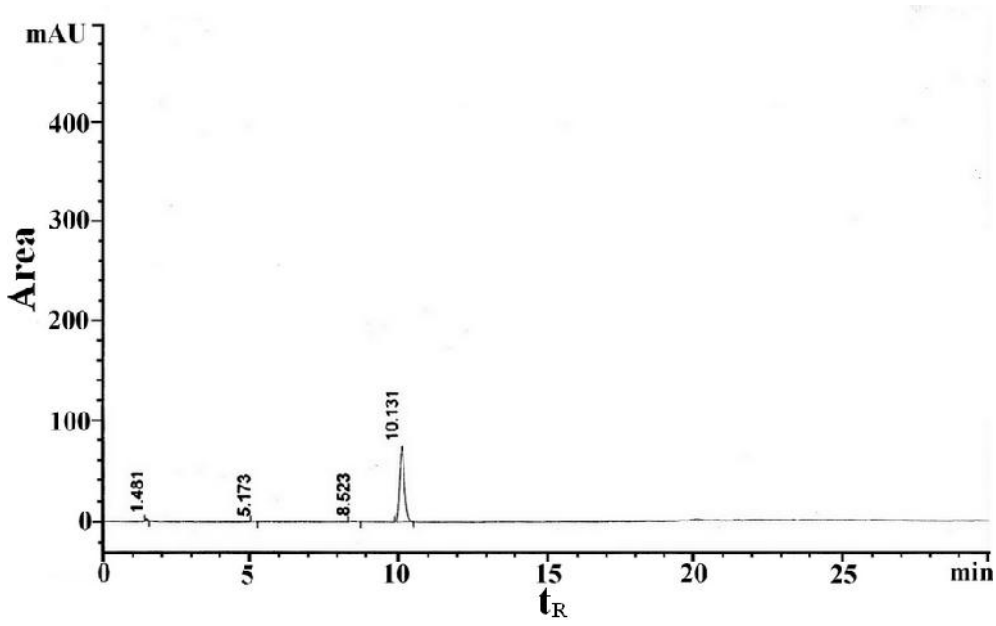

Figure 6
Chromatograph of Aniracetam andrelated impurities for precision and accuracy on LOQ level of AN and MBA,NAG,PD.

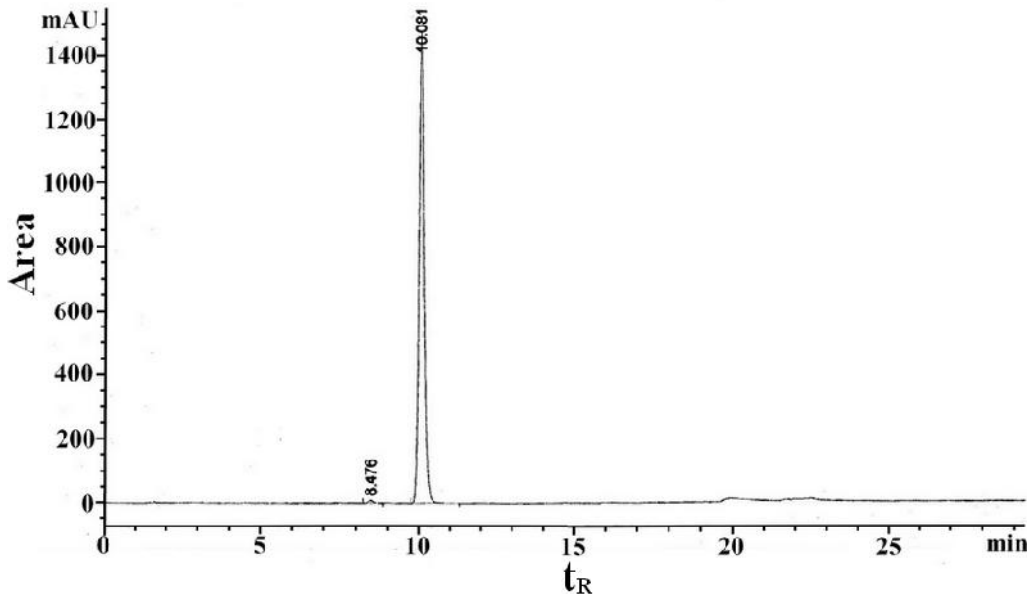

Figure 7
Chromatograph for mixture of AN (99.5%) and MBA (0.5%) .

3.2.9 Analysis of a commercial formulation
The validated method was used in the analysis of a commercial product containing 750mg of Aniracetam tablet representative chromatogram is shown in Fig.7.AN99.49% content was found to be of the nominal value while impurities PD,NAG and unknown impurity below the LOD values, which were much lower than the acceptance level (100% of AN).Only the impurity MBA was found to be0.50 % of (AN content 0.5mg/mL) nevertheless it was lower than the acceptance limit.
An online (IPC) synthesis for drug and drug intermediate Check drug and impurities to Aniracetam(AN), 4-Methoxy benzoic acid(MBA),N-anisoylGABA(NAG), 2-pyrrolidinone(PD),online % of analysis for (IPC) monitoring in reaction and final drug analysis.

4. CONCLUSION
Reversed-phase high performance liquid chromatography method for the simultaneous determination of AN and its three related impurities PD,MBA,NAG and unknown in the bulk drug, synthesis drug and drug intermediate, and a tablet formulation was fully validated and proved to be reliable, sensitive, accurate, precise and robust. It is the first time that such method appears in the literature and a can be useful for routine analysis and quality control of AN in relevant forms.

ACKNOWLEDGEMENTS
Authors thank Advance Analytical Research &Training Institute, Ahmedabad, India for providing instrumental facilities.

Supplementary data
All experimental data in detail are embedded in supplementary material.

Abbreviations:
AN: Aniracetam;
MBA: 4-Methoxy benzoic acid;
NAG: N-anisoyl GABA;
PD: 2-pyrrolidinone;
API: Active pharmaceutical ingredient;
ICH: International conference of Harmonization;
LLOQ: Lover limit of Quantification;
RT: Room temperature;
t_R: Retention time;
Rt_R: Relative time;
R_S: Resolution;
T_f: Tailing factor;
R^2: Coefficient of determination

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