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Short communication

Development and validation of a liquid chromatographic method for the simultaneous determination of aniracetam and its related substances in the bulk drug and a tablet formulation

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ABSTRACT

Simultaneous determination of aniracetam and its related impurities (2-pyrrolidinone, p-anisic acid, 4-p-anisamidobutyric acid and (p-anisoyl)-4-methyl-2-pyrrolidinone) was accomplished in the bulk drug and in a tablet formulation using a high performance liquid chromatographic method with UV detection. Separation was achieved on a Hypersil BDS-CN column (150 mm × 4.0 mm, 5 μm) using a gradient elution program with solvent A composed of phosphate buffer (pH 4.0; 0.010 M) and solvent B of acetonitrile–phosphate buffer (pH 4.0; 0.010 M) (90:10, v/v). The flow rate of the mobile phase was 1.0 mL min^{-1} and the total elution time, including the column re-equilibration, was approximately 20 min. The UV detection wavelength was varied appropriately among 210, 250 and 280 nm. Injection volume was 20 μL and experiments were conducted at ambient temperature. The developed method was validated in terms of system suitability, selectivity, linearity, range, precision, accuracy, limits of detection and quantification for the impurities, short term and long term stability of the analytes in the prepared solutions and robustness, following the ICH guidelines. Therefore, the proposed method was suitable for the simultaneous determination of aniracetam and its studied related impurities.

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1. Introduction

Aniracetam [1-(4-methoxybenzoyl)-2-pyrrolidinone, ANIR, Fig. 1A] is a nootropic drug that belongs to the racetam chemical class and is used for improving cerebral insufficiency. It functions as a cognition enhancer and acts therapeutically in the treatment of emotional disturbances (anxiety, agitation and depressed mood), sleep disorders and behavior abnormalities (nocturnal delirium, wandering) that are associated with cerebral infarction and Alzheimer's and Parkinson's diseases [1,2]. It has been also reported to possess mechanisms for positively modulating cholinergic and glutaminergic nervous systems, as well as increasing synaptic efficacy and energy metabolism [2]. ANIR is sold as a dietary supplement in the United States while it is used as a prescription drug in Europe.

Several studies on aniracetam determination, its pharmacokinetics and metabolites in humans and rats have been reported using HPLC–UV and HPLC–MS/MS techniques [3–9]. However, there is no method in the literature, describing the determination of aniracetam and its related impurities in the bulk drug and pharmaceutical formulations.

The aim of this paper was the development and validation of a simple and reliable HPLC–UV method for the simultaneous determination of ANIR and four of its related impurities (2-pyrrolidinone, PD Fig. 1B; 4-methoxybenzoic acid or p-anisic acid, AA, Fig. 1C; 4-methoxybenzoylaminobutyric acid or 4-p-anisamidobutyric acid, ABA Fig. 1D; 1-(p-anisoyl)-4-methyl-2-pyrrolidinone, AMP Fig. 1E) in the bulk drug and in a tablet formulation.

The unique feature and the novelty of the proposed method is that it is the first time that these five compounds are determined simultaneously. In previous works [3,4] for the determination of ANIR and its three metabolites PB, AA and ABA in biological samples, two successive methods were proposed, one for ANIR, AA and ABA 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 (AA and ABA) and two hydrophobic components (ANIR and AMP). A Hypersil BDS CN column combined with a gradient elution program was chosen to compensate for the extreme compound polarities and their acidic–basic nature. The option of ion-pair chromatography was not considered in order

Abbreviations: ANIR, aniracetam; AA, anisic Acid; ABA, 4-p-anisamidobutyric acid; AMP, (p-anisoyl)-4-methyl-2-pyrrolidinone; PD, 2-pyrrolidinone; API, active pharmaceutical ingredient; ICH, International Conference of Harmonization; LLOQ, lower limit of quantification; RT, room temperature; MeOH, methanol; t_R, retention time; Rt_R, relative retention time; R_s, resolution; T_f, tailing factor; R^2, coefficient of determination.

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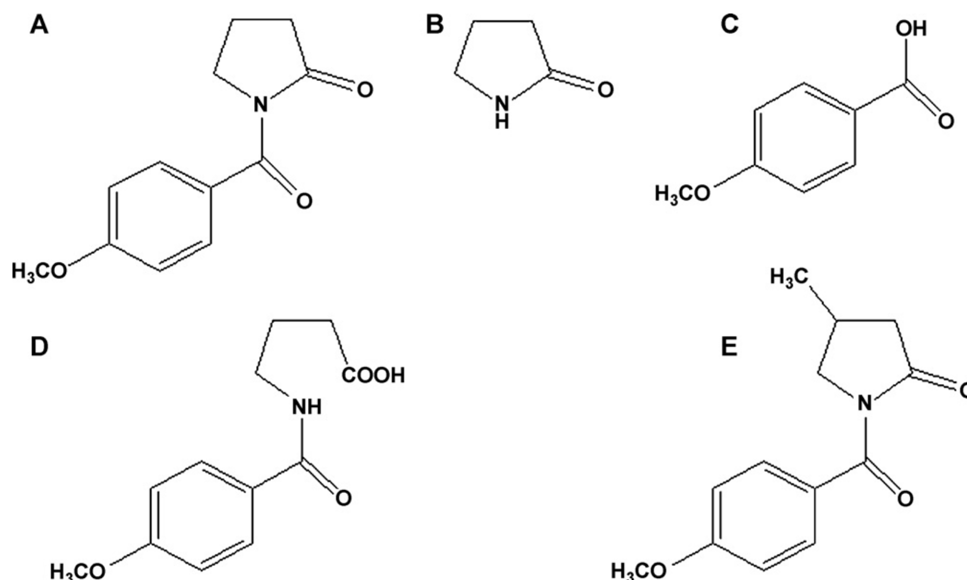

Fig. 1. The chemical structures of ANIR, A; PD, B; AA, C; ABA, D; and AMP, E.

to avoid the well-known drawbacks of this technique which are the long equilibration times, the substantial UV absorbance of the ion-pair reagents that precludes use with low-UV detection, the difficulty of the ion-pair reagents to be washed from the column and the almost impossible use of gradient elution.

The developed method was validated for the analysis of the bulk drug and a tablet formulation. The method was intended to be simple, sensitive, accurate, precise and robust for all studied compounds. The increased sensitivity for the four 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).

2. Materials and methods

2.1. Instrumentation and software

The liquid chromatographic system consisted of a Waters 600E gradient pump, a 600 controller, and a 486E Waters UV detector (Waters, Milford, MA, USA). The above system was controlled by the software package Millennium 2010. A Hettich centrifuge Rotofix 32 (Tuttlingen, Germany) was utilized to centrifuge the tablet samples. The maximum wavelength for each substance was verified using a Hitachi U-2000 spectrophotometer. The pH meter was a Jenway 3310 (Essex, USA). Finally, a Milli-Q reversed osmosis purification system (Millipore, Bedford, USA) was used for the preparation of HPLC grade water.

2.2. Chemicals and reagents

Pure ANIR and its four impurities [PD ($d = 1.103\,g\,mL^{-1}$), AA, ABA and AMP] along with bulk drug, Memodrin tablets 750 mg and the complete series of the excipients of this formulation were kindly donated by the pharmaceutical company Lavipharm (Athens, Greece). Methanol and acetonitrile were of HPLC grade (Merck, Darmstadt, Germany). Potassium dihydrogen phosphate was of analytical grade and obtained from Merck (Darmstadt, Germany). Hydrochloric acid (37%, w/w) was purchased from Panreac Quimica S.A. (Barcelona, Spain).

2.3. Chromatographic conditions

The separation was carried out on a Hypersil (Thermo Scientific, London, UK) BDS CN column (150 mm × 4.0 mm, 5 μm). Solvent A was composed of a phosphate buffer (pH 4.0; 0.010 M), and solvent B of acetonitrile–phosphate buffer (pH 4.0; 0.010 M) (90:10, v/v). The gradient program was varied linearly, as follows: 0 min, 0% B; 10 min, 100% B; 11 min, 100% B; 11.1 min, 0% B. The column was then re-equilibrated for 9 min with mobile phase A. The flow rate was set at $1.0\,mL\,min^{-1}$. Injection volume was 20 μL. The UV detector wavelength was varied as follows: 0 min, 210 nm; 3.2 min, 250 nm; 6.2 min 280 nm; 11.1 min, 210 nm. The total elution time, including the column re-equilibration, was approximately 20 min. Experiments were performed at ambient temperature.

2.4. Preparation of solutions

2.4.1. Stock and calibration standard solutions of aniracetam

Stock standard solution of $10{,}000\,\mu g\,mL^{-1}$ of ANIR was prepared by accurately weighing approximately 500 mg of ANIR into a 50 mL volumetric flask and making up to volume with ACN. The stock standard solution was diluted with mobile phase A to obtain five calibration standards of 75, 120, 150, 180 and $225\,\mu g\,mL^{-1}$ of aniracetam at 50–150% of the method nominal concentration ($150\,\mu g\,mL^{-1}$).

2.4.2. Stock and calibration standard solutions of impurities

Stock standard solutions of $1000\,\mu g\,mL^{-1}$ of each impurity (PD, AA, ABA and AMP) were prepared in the following way: diluting 9.1 μL ($d = 1.103\,g\,mL^{-1}$) of the pure solution of PD into a 10 mL volumetric flask, weighing 10 mg of AA into a 10 mL volumetric flask or 5 mg for each of ABA and AMP into 5 mL volumetric flasks. All solutions were diluted to volume with ACN. For the calibration curves of (i) PD and AMP, five calibration standards containing $150\,\mu g\,mL^{-1}$ of aniracetam were prepared at LLOQ, 0.075, 0.113, 0.150 and $0.225\,\mu g\,mL^{-1}$ and (ii) AA and ABA, six calibration standards containing $150\,\mu g\,mL^{-1}$ of aniracetam were prepared at LLOQ, 0.037, 0.075, 0.113, 0.150 and $0.225\,\mu g\,mL^{-1}$ by diluting the stock standard solutions of each impurity with mobile phase A.

2.4.3. Preparation of test solution

Ten tablets (750 mg/tablet) of Memodrin were carefully pulverized with mortar and pestle, and 116.4 mg of this powder, which approximates to 100 mg of ANIR, were accurately weighed, transferred into a 10 mL volumetric flask and diluted to volume with ACN in order to prepare an ANIR solution of 10,000 $\mu g\,mL^{-1}$. This solution was used for the standard addition method.

For the analysis of a commercial formulation, twenty tablets of Memodrin (750 mg/tablet) were processed using the same preparation procedure as that of the test solution, described above.

2.5. Method validation

Validation of the developed method for the determination of aniracetam and the four impurities was performed according to the ICH guidelines [10,11] with standards, bulk drug and Memodrin tablets. Thus, system suitability along with method selectivity, specificity, linearity, range, precision (repeatability and intermediate precision), accuracy, limits of detection and quantification for the impurities, short term and long term stability of the analytes in the prepared solutions and robustness were demonstrated.

2.5.1. System suitability

The system suitability solution (diluted in mobile phase A and contained each compound at a concentration level of 1 $\mu g\,mL^{-1}$) was prepared as a mixed standard solution of ANIR and the four impurities.

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 samples and samples spiked with the API (active pharmaceutical ingredient) in the presence of all impurities in the mobile phase, the bulk drug, and the formulation. In this work, due to the lack of an appropriate placebo solution, the verification of selectivity in the tablet was assessed using a complete series of the excipients. Solutions of each excipient were prepared in ACN, filtered, diluted in mobile phase and injected in the HPLC.

2.5.3. Linearity and range

Standard calibration curves were prepared as discussed in Section 2.4.1 for aniracetam and Section 2.4.2 for the impurities. The data of peak area of each analyte versus the corresponding concentration were treated by linear least square regression analysis. At least, six complete calibration curves were constructed in six consecutive days for each compound.

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 ANOVA for six consecutive days using the daily calibration curves. Five concentration levels were used for the first two days performing determinations in triplicate. For the remaining four days three concentration levels (low, medium and 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 analyte 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. The intra-assay accuracy was determined 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 days as a whole, over the theoretical concentration.

2.5.5. Recovery

Recovery studies of ANIR and its four impurities were performed both in bulk drug and in the tablet formulation using the standard addition method. In particular, for the estimation of the recovery of ANIR in Memodrin tablets, a series of four solutions was prepared. The first solution was a tablet test solution prepared in such a way to contain a nominal concentration of 75 $\mu g\,mL^{-1}$ of ANIR. The other three solutions were prepared by spiking the tablet test solution with appropriate aliquots of the stock solution of ANIR so that these solutions contained increased amounts of ANIR by 50 $\mu g\,mL^{-1}$. For the recovery studies on the impurities, a similar procedure was followed. The only difference was that the concentration of ANIR in this series of solutions was the method nominal concentration of the tablet test solution (150 $\mu g\,mL^{-1}$). In this solution, four standard additions of all impurities were performed (75, 150, 188 and 225 $\mu g\,mL^{-1}$).

2.5.6. LOD and LLOQ

The evaluation of the detectability of the method was approached in the following way: A rough estimation of the probable LOD and LLOQ values of each component was accomplished by constructing individual calibration curves close to the LOD and LLOQ concentrations of each substance. Then, a better evaluation was attempted by preparing five independent spiked standards of all analytes at appropriate low concentration levels, and the signal to noise ratio (S/N) was used to establish LOD at S/N 3:1, and the LLOQ at S/N 10:1. Finally, the LOD and the LLOQ values were verified by preparing five spiked standards at these levels for every compound in mobile phase A and measured them. The precision of these standards at the LLOQ level was also calculated by the relative standard deviation (% RSD) of the mean values of the back-calculated concentrations.

2.5.7. Stability

The stability of the studied compounds was estimated based on the comparison of the peak areas of the analytes at t_0 h over those after t_h h. First, short term stability of aniracetam for 48 h was examined in stock solutions prepared in MeOH or ACN at 5 °C and measured in standard solutions of 10 $\mu g\,mL^{-1}$ in mobile phase A, at 5 °C. Second, the stability of aniracetam (150 $\mu g\,mL^{-1}$) was additionally studied in mobile phase A at RT and 5 °C. Third, the stability of PD, AA, ABA, and AMP was tested in mobile phase A at RT by preparing four independent standard solutions, each solution containing 1 $\mu g\,mL^{-1}$ of the corresponding impurity. In the case of AA, it was necessary to test its stability at 5 °C, as well. Finally, the long term stability of the stock solution of ANIR and all impurities in ACN at −20 °C was examined in a 2-month period.

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 quantitation of the analytes. Three major parameters were individually altered; the flow rate, the pH of the buffer and the percentage of the organic solvent in the mobile phase B. The evaluation of the robustness of the proposed method was approached by recording the retention time, resolution and peak areas of the analytes. The relative retention time of each impurity was calculated dividing their t_R by the t_R of ANIR.

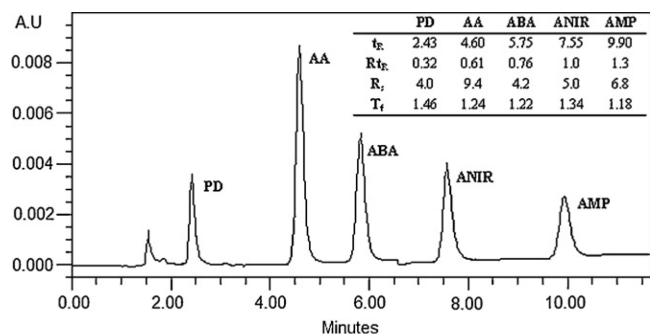

Fig. 2. A typical chromatogram of ANIR and its four impurities (PD, AA, ABA, and AMP) obtained after method development and optimization, using a Hypersil BDS-CN column (150 mm × 4.0 mm, 5 μm) along with the chromatographic characteristics of the separation. The gradient elution program was varied linearly, as follows: 0 min, 0% B; 10 min, 100% B; 11 min, 100% B; 11.1 min, 0% B; detection wavelength: 0 min, 210 nm; 3.2 min, 250 nm; 6.2 min, 280 nm; 11.1 min, 210 nm; total analysis time: approximately 20 min; injection volume: 20 μL; ambient temperature.

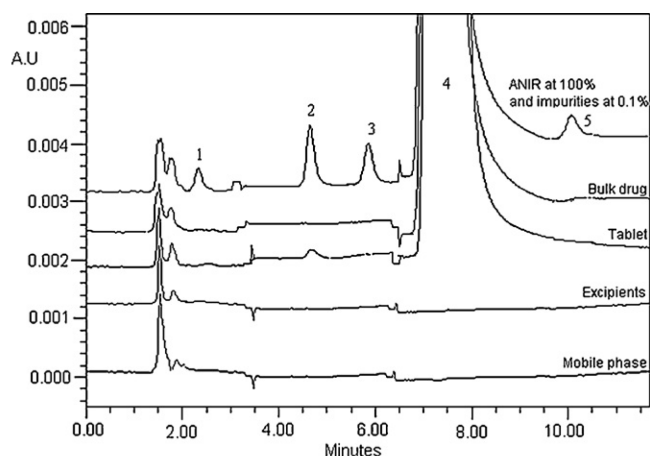

Fig. 3. An overlay of representative chromatograms of mobile phase (blank), excipients of the tablet formulation, the tablet and the bulk drug at the method nominal concentration of ANIR (150 μg mL^{-1}) and a spiked standard solution in mobile phase containing ANIR at 100% of the method nominal concentration (150 μg mL^{-1}) and all impurities at 0.1% of ANIR concentration (0.150 μg mL^{-1}). The peaks 1, 2, 3, 4 and 5 correspond to the compounds PD, AA, ABA, ANIR and AMP, respectively.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The presence of the polar compound PD among the other constituents, that necessitated an almost aqueous mobile phase, led to the selection of a BDS CN column for the development of the proposed method. The highly polar nature of PD combined with the acidic nature of AA and ABA and the relatively non-polar behavior of ANIR and AMP demanded extensive optimization of the pH of the mobile phase and the need of a gradient elution. The selection of the pH of the mobile phase was very critical. For pH <4, the peaks of the acidic substances AA and ABA would move to the right, closer to the aniracetam peak. For pH >4, these two substances would coelute and move to the left of the chromatogram. Finally, a linear gradient program with an initial mobile phase consisted of 0.010 M KH_2PO_4, pH 4.0 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 215 nm, while AA, ABA, ANIR and AMP had their λ_{max} at 250, 252, 285 and 280 nm, respectively. Thus, the detector was set at 210 nm 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 250 nm for maximum sensitivity of AA and ABA, while for the last pair of compounds, the 280 nm was selected in order to favor the sensitivity of the impurity AMP over that of ANIR. Finally, the wavelength was set at the initial value of 210 nm in order prepare the system for the next injection. The optimal wavelength scheme along with the other chromatographic conditions is described in details 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

3.2.1. System suitability

The system suitability test is very important because it ensures the validity of the analytical procedure. In the present work, a mixed standard, consisted of ANIR and the four 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 ANIR (t_R) and the area of the peaks along with their asymmetry factor (T_f, the USP tailing factor), the relative retention times (Rt_R), and the resolution (R_s) between adjacent peaks, were the critical parameters examined every day. During the validation of the method the critical parameters of R_s and T_f met the acceptance criteria ($R_s \geq 1.5$, $T_f \leq 2.0$) and assured for a good separation between the five analytes and their reliable quantification [10–12].

3.2.2. Selectivity

Under the optimized chromatographic conditions in all studies, the obtained resolution between the adjacent peaks of ANIR and the four impurities in the way they eluted (PD, AA, ABA, ANIR and AMP), was satisfactory (R_s > 3.2). The critical resolution between PD and the 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, Rt_R, R_s and T_f obtained with the system suitability solution, are shown in Fig. 2.

In addition, selectivity in the Memodrin tablet 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 1 presents the analytical parameters of typical standard calibration curves of ANIR and its four related impurities in mobile phase A. All calibration curves for ANIR presented coefficient of determination $R^2 \geq 0.9999$, while for the impurities R^2 was greater than 0.995, as required [11,12]. A lack-of-fit test was performed for all calibration curves and the calculated *P*-values of the representative curves, included in Table 1, were the following at 95% confidence level: $P_{PD} = 0.193$, $P_{AA} = 0.280$, $P_{ABA} = 0.360$, $P_{ANIR} = 0.793$ and $P_{AMP} = 0.215$.

3.2.4. Precision and accuracy

The repeatability and intermediate precision were expressed as the % relative standard deviation (% RSD) of each analyte concentration using one-way ANOVA and the results are presented in Table 2A. The repeatability (s_r) of the method at each concentration level was calculated by the square root of the MS_{within} (within mean square), while intermediate precision (s_R) was calculated based on the following equation:

$$s_R = \sqrt{s_r^2 + s_L^2}$$

Table 1
Linearity parameters of ANIR and its related impurities. Limits of detection and quantification of the impurities along with % RSD at the LLOQ values.

Substance	Range (μg mL^{-1})	Slope (b_1) (×10^2) (mean ± SD) (×10^2)	Intercept (b_0) (×10^2) (mean ± SD) (×10^2)	Coefficient of determination (R^2)	LOD[a] (μg mL^{-1})	LLOQ[a] (μg mL^{-1})	(%)[a] RSD_{LLOQ}
PD[b]	0.049–0.225	301.5 ± 8.2	−0.87 ± 1.1	0.998	0.0163	0.049	7.9
AA[c]	0.015–0.225	846.6 ± 4.3	−0.029 ± 0.53	0.9998	0.005	0.015	1.0
ABA[c]	0.025–0.225	585.2 ± 7.1	−0.37 ± 0.89	0.9994	0.0083	0.025	2.9
ANIR[d]	75–225	445.6 ± 2.4	746 ± 385	0.9999	0.015	0.045	5.3
AMP[b]	0.068–0.225	410.3 ± 8.5	−0.020 ± 0.12	0.999	0.0226	0.068	1.5

[a] The LOD and LLOQ values were calculated based on the S/N, using five independent measurements.
[b] Five concentration levels for the first two days, three (0.075, 0.150, 0.225 μg mL^{-1}) for the next four days and 2–5 independent replicates at each level.
[c] Six concentration levels for the first two days, three (0.075, 0.150, 0.225 μg mL^{-1}) for the next four days and 2–5 independent replicates at each level.
[d] Five concentration levels for the first two days, three (75, 150, 225 μg mL^{-1}) for the next four days and 2–4 independent replicates at each level.

Table 2
Precision and accuracy data of the proposed method.[a]

	Repeatability (% RSD) Theoretical concentrations (μg mL^{-1})			Intermediate precision (% RSD) Theoretical concentrations (μg mL^{-1})		
A						
API	75	150	225	75	150	225
ANIR	1.0	1.0	1.3	1.1	1.1	1.3
	0.075	0.150	0.225	0.075	0.150	0.225
Impurity						
PD	5.1	3.7	3.4	5.8	5.7	3.4
AA	2.4	1.8	1.8	2.4	1.8	1.8
ABA	2.7	0.87	2.2	2.7	1.3	2.2
AMP	2.6	2.7	1.5	2.6	2.7	1.5

	Intra-day accuracy (%) Theoretical concentrations (μg mL^{-1})			Inter-day accuracy (%) Theoretical concentrations (μg mL^{-1})		
B						
API	75	150	225	75	150	225
ANIR	98.4–100.6	99.4–101.3	99.5–100.2	99.3	100.6	99.8
	0.075	0.150	0.225	0.75	0.150	0.225
Impurity						
PD	94.3–106.6	92.2–102.8	98.8–102.1	100.3	99.2	99.9
AA	98.1–100.5	99.5–102.1	99.1–100.3	99.1	100.8	99.7
ABA	97.3–100.2	100.5–102.4	98.9–99.7	98.9	101.5	99.4
AMP	97.8–102.0	97.7–102.0	98.3–100.8	99.9	99.4	100.1

[a] For aniracetam: at each concentration level three independent replicates for two days and two independent replicates for four days were performed, while for the impurities: 2–5 independent replicates for six days were measured.

Table 3
Recovery of aniracetam and impurities.

	Added concentration (μg mL^{-1})[a]		
	50	100	150
API			
ANIR	97.6 ± 3.8	101.8 ± 1.2	99.2 ± 2.3

	Added concentration (μg mL^{-1})[a]			
	0.075	0.150	0.188	0.225
Impurities				
PD	97.33 ± 0.85	97.9 ± 1.2	96.42 ± 0.48	97.5 ± 2.2
AA	104.9 ± 1.7	101.80 ± 0.46	102.2 ± 1.1	101.9 ± 2.9
ABA	99.0 ± 1.8	102.82 ± 0.90	103.4 ± 2.9	105.9 ± 2.6
AMP	105.93 ± 0.92	102.1 ± 1.2	101.50 ± 0.10	100.1 ± 1.1

[a] The initial solution (before addition) in the case of ANIR, contained the nominal concentration of 75 μg mL^{-1} of ANIR and in the case of impurities 150 μg mL^{-1} of ANIR.

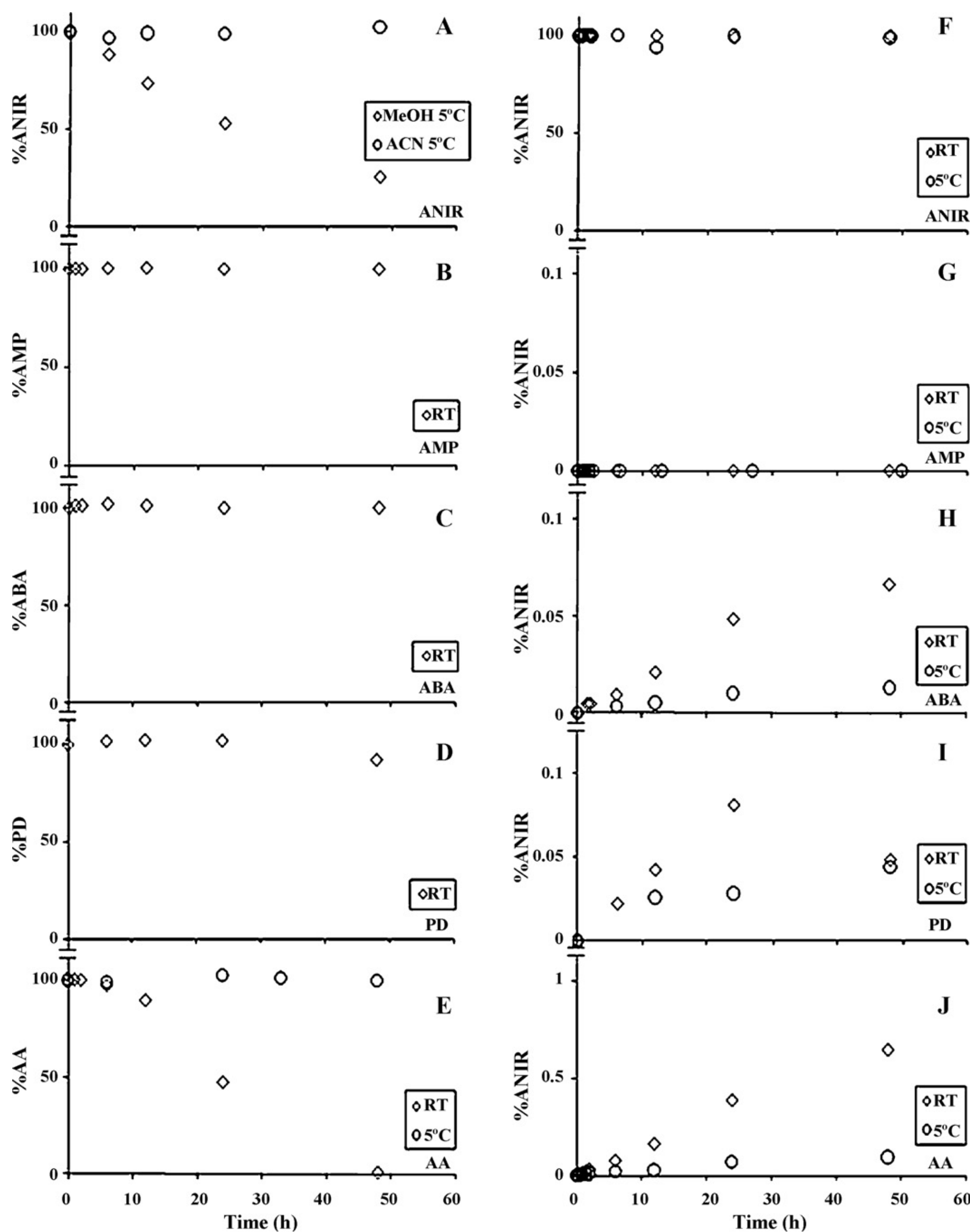

Fig. 4. Stability studies of ANIR and its related substances (PD, AA, ABA, and AMP). Short term stability of ANIR in stock solutions of MeOH and ACN (10 μg mL^{-1}), A. Short term stability of impurities (AMP, ABA, PD and AA 1 μg mL^{-1}), B–E, respectively. Short term stability of ANIR standard solution in mobile phase A (150 μg mL^{-1}), F, along with the rate of impurities production in this solution (AMP, ABA, PD and AA) G–J, respectively.

where $s_L^2 = (\mathrm{MS_{between}} - \mathrm{MS_{within}})/m$, $\mathrm{MS_{between}}$ is the between mean square and m is the 'normalized' number of replicates used to obtain the mean value per day. Due to the different number of replicates used per day, the m value is given by the equation:

$$m = \frac{n - \Sigma n_j^2/n}{k-1}$$

where n is the total number of observations, n_j is the number of observations on the j day and k is the number of days [13].

The repeatability and the intermediate precision for the API found to be less than 1.3%, while the acceptance criteria were less than 2.0% and 3.0% [11], respectively. In addition, the repeatability for all impurities was less than 5.1% and the intermediate precision was less than 5.8%, while the acceptance limits were below 15% [11]. Thus, it was concluded that the method was considered precise for the determination of all compounds.

The intra- and inter-assay accuracy was examined for all substances according to Section 2.5.4 and the results were summarized in Table 2B. For ANIR the intra-assay accuracy ranged from 98.4 to 101.3%, and the inter-assay accuracy ranged from 99.3 to 100.6% while the acceptance range was 98–102% [11]. In addition, the intra-assay accuracy for the impurities ranged from 92.2 to 106.6% and the inter-assay accuracy ranged from 98.9 to 101.5%, while the acceptance range was 75–125% [11].

3.2.5. Recovery

Recovery studies were performed by the standard addition method in the bulk drug and in the tablet formulation. The slopes

Table 4
Robustness of the proposed method.

Method parameter	Relative retention time					Resolution					% Recovery				
	PD	AA	ABA	ANIR	AMP	PD	AA	ABA	ANIR	AMP	PD	AA	ABA	ANIR	AMP
Nominal conditions	0.32	0.61	0.76	1.0	1.3	3.2	6.1	4.2	3.9	6.3	97.5	101.9	105.9	99.2	100.1
Flow rate (mL min^{-1})															
0.9	0.34	0.63	0.81	1.0	1.4	2.1	5.7	3.1	2.6	5.0	107.6	94.0	99.8	96.4	104.5
1.1	0.33	0.63	0.80	1.0	1.4	1.8	6.0	3.0	2.5	5.0	108.8	94.3	100.9	98.9	92.5
pH															
3.9	0.34	0.63	0.80	1.0	1.4	2.2	5.2	3.4	2.9	5.1	105.7	95.3	104.8	97.8	104.6
4.1	0.34	0.59	0.78	1.0	1.4	2.0	5.9	3.1	2.6	5.1	100.3	94.0	102.5	99.0	107.0
Buffer:ACN (v/v)															
85:15	0.33	0.60	0.78	1.0	1.5	2.1	5.5	3.3	2.8	5.6	97.1	94.2	104.6	96.8	98.4
95:5	0.34	0.62	0.80	1.0	1.4	2.0	5.5	3.4	2.9	5.1	103.1	94.3	102.2	97.4	94.2

The nominal condition of the mobile phase were described in Section 2.3 (flow rate: 1 mL min^{-1}, pH (of the aqueous phase): 4.0 and buffer: ACN, in mobile phase B 90:10 (v/v)).

of the standard calibration curves and those of the standard addition method were statistically the same [13]. Recovery values of ANIR and its four impurities have been found in the following ranges: ANIR 97.6–102.1%, PD 96.4–98.0%, AA 101.5–105.0%, ABA 99.0–106.0% and AMP 99.5–105.0%. Representative recovery results in Memodrin tablets are included in Table 3.

3.2.6. LOD and LLOQ

As mentioned in Section 2.5.6 a reliable estimation of LOD and LLOQ values of the analytes was based on the signal to noise (S/N) ratios. The resulted values are included in Table 1 along with the % RSD at each LLOQ for an additional verification. It is obvious that the LLOQ values for all impurities were below 0.05% (<0.075 μg mL^{-1}) of the method nominal concentration of ANIR (150 μg mL^{-1}), needed according to the guidelines, considering that each impurity should be lower than 0.1% of the parent drug in the bulk drug and its formulation.

3.2.7. Stability

At the beginning of the method development it was observed that aniracetam stock solutions, prepared in methanol, seemed to be very unstable and additional peaks appeared in the chromatograms. Therefore, based on this observation, a stability study on ANIR stock solution in MeOH at 5 °C for 48 h followed which revealed an extensive and fast degradation of this drug (Fig. 4A). As a consequence, the stock solutions of ANIR were then prepared in ACN where they were stable, as shown in Fig. 4A. Moreover, the stock solutions of all substances were prepared in ACN and stored at −20 °C, where no degradation was observed in a period of 2 months (concentrations varied in the range of 98–102% of the initial measurement). Furthermore, a standard solution of ANIR (150 μg mL^{-1}) in mobile phase A was prepared and its stability was tested for 48 h at RT and 5 °C. Although no significant degradation of ANIR was observed (∼1%, Fig. 4F), impurities PD, AA and ABA were produced (Fig. 4I, J, H, respectively) at a very small rate, capable of elevating their concentration. In the case of AA, where the hydrolysis was faster, the concentration exceeded the acceptance limits at RT (Fig. 4J). Specifically, in a 12 h period, the formation of PD was 0.04%, AA was 0.16% and ABA was 0.02% of the API. Standard solutions of AMP in mobile phase A seemed to be very stable (Fig. 4G).

The stability of individual standard solutions of impurities in mobile phase A was also examined. This study showed that PD, ABA and AMP could be considered as stable at RT for 48 h (Fig. 4D, C, B, respectively). However, the standard solution of AA was unstable with a tremendous peak area reduction after a period of 48 h at RT, while it was stable at 5 °C (Fig. 4E). It should be noted here, that zero level concentrations of impurities in Fig. 4 meant unmeasurable quantities, much below the LOD levels.

3.2.8. Robustness

The robustness of the method was evaluated by analyzing standards and test solutions at the method nominal concentration of ANIR (150 μg mL^{-1}) in the presence of all impurities at 0.150 μg mL^{-1} (0.1% of the parent drug peak, Fig. 3). The parameters altered were, the flow rate (±10%), 0.9, 1.1 mL min^{-1} instead of 1.0 mL min^{-1}, pH (±0.1 pH unit), 3.9, 4.1 instead of 4.0 and the ACN content in mobile phase B 85, 95% instead of 90%. The results were summarized 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 ($R_s \geq 1.8$) met the acceptance limit ($R_s \geq 1.5$) for all compounds of interest.

3.2.9. Analysis of a commercial formulation

The validated method was used in the analysis of a commercial product, containing 750 mg of Aniracetam/tablet. Representative chromatogram is shown in Fig. 3. ANIR content was found to be 97% of the nominal value, while impurities PD, ABA and AMP were below the LOD values, which were much lower than the acceptance levels (0.1% of ANIR). Only the impurity AA was found to be 0.016% of ANIR content (≈0.024 μg mL^{-1}). Nevertheless, it was lower than the acceptance limit.

4. Concluding remarks

The liquid chromatographic method with gradient elution developed for the simultaneous determination of ANIR and its four related impurities PD, AA, ABA, and AMP in the bulk drug 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 can be useful for routine analysis and quality control of ANIR in the relevant forms.

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