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Short communication

Rapid analytical procedure for neomycin determination in ointments by CE with direct UV detection

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ABSTRACT

The purpose of this study was the development of an analytical methodology for the determination of neomycin in a complex pharmaceutical preparation. The simplified methodology consisted of a primary liquid–liquid extraction, employing a mixture of chloroform and water (1.25:1, v/v) and subsequent analysis by CE applying a capillary zone electrophoresis method with a 30 cm (effective length), 50 μm (internal diameter) polyacrylamide-coated silica capillary. The background electrolyte consisted of 35 mM phosphate and 15 mM acetate buffer set at pH 4.7, under normal polarity mode and direct UV detection at 200 nm. The separation of the target analyte from the complex matrix was accomplished in less than 3 min.

The analytical method was successfully validated in order to verify its proper selectivity, linearity, accuracy and precision for the goal intended and its further implementation for the quantification of the active compound in the pharmaceutical speciality for quality control.

1. Introduction

Neomycin is an aminoglycoside antibiotic that is produced naturally by the actinomycete bacterium *Streptomyces fradiae* via the fermentation process [1]. Similar to other aminoglycosides, neomycin displays excellent activity against Gram negative bacteria, and partial activity against Gram positive bacteria.

As a therapeutic agent, neomycin is mainly used in its sulfate form, which consists of a mixture of three aminoglycosides: the major component, neomycin B (framycetin), which possesses the greatest therapeutic activity, and two secondary components, neomycin C and neomycin A (hydrolytic degradation product of neomycin B and C). Neomycin A possesses only 10% of the antibiotic activity of the major components. The molecular structures of these compounds are displayed in Fig. 1.

This API (active pharmaceutical ingredient) plays a topical antibacterial role in creams and eye drops, and is also widely included in ointments used as anti-hemorrhoidal treatment.

When preparing cream and ointment specialities, it is frequently co-formulated in combination with other ingredients either antibiotics or anti-inflammatory agents (bacitracin, dexamethasone, fluocinolone, fluorometholone, flurandrenolide, gramicidin, hydrocortisone, methylprednisolone, polymyxin, etc.) [2].

The analysis of aminoglycosides involves an analytical challenge in many respects. This class of antibiotics lacks a good UV-absorbing chromophore group, is highly polar and is a basic compound.

The official method in the European and United States Pharmacopoeia for the assay of neomycin in pharmaceuticals either for raw material and for formulations, food and tissues is a microbiological one [3]. This method contains many drawbacks as it is time-consuming, with low detectability and low precision.

Many procedures based on HPLC as a separation technique have employed several different strategies to overcome the lack of chromophore. Suitable derivatisating reagents that have been used prior to the analysis of neomycin with this separation technique include 1-fluoro-2,4-dinitrobenzene [4], benzoyl chloride [5] and 2-naphthalenesulfonyl chloride [6] for UV detection, and 9-fluorenylmethyl chloroformate [7], which is used in fluorescence detection.

Automatic derivatisation methods have also been reported either pre-column [8–9] or post-column [10].

Although a high sensitivity can be obtained using these methods, derivatisation is a time-consuming step which requires a good control of the reaction conditions to obtain accurate results due to the associated limitations of varying derivatisation efficiencies and reagent instabilities that compromise method ruggedness [11,12]. Compounds such as neomycin also have up to six derivatisation positions.

Other detection systems have been coupled to HPLC separations to overcome the lack of chromophore such as mass spectrometry [13], pulsed electrochemical detection [14-16] and refractometric

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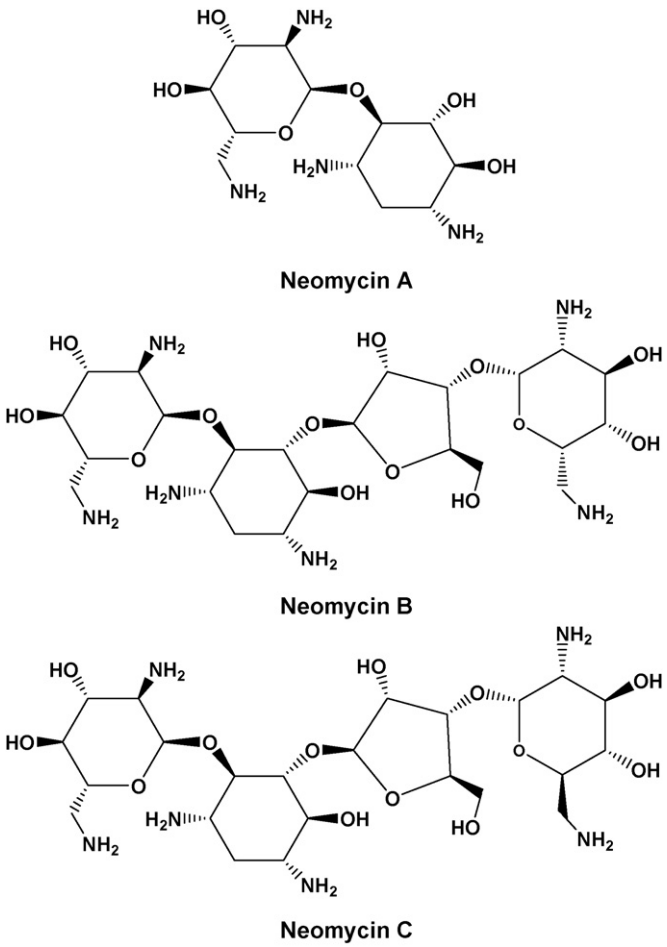

Fig. 1. Chemical structures of neomycin.

detection [17] and indirect fluorescence detection based on a ligand displacement reaction [18], evaporative light scattering detection [19,20] and integrated pulsed amperometric detection [21].

TLC has not really been employed for the determination of this API. One method that allows the simultaneous determination of neomycin, polimixin, bactracin methyl and propyl hydroxybenzoates in ophthalmic ointment by TLC has been published. However, the method validation pointed out the semi-quantitative aspect of this technique [22].

In relation to CE, no direct UV detection at low wavelengths has been reported to date. Test mixtures of aminoglycoside antibiotics, including neomycin, have been separated employing micellar electrokinetic capillary chromatography (MEKC) with indirect UV detection [23] and capillary zone electrophoresis (CZE) with electrochemical detection based on copper electrodes [24]. A CZE with indirect UV detection for the simultaneous determination of neomycin and polymixin B sulfates [25] in eye–ear drops as a pharmaceutical form as well as a CE system with potential gradient detection (PGD) for measuring native neomycin components have been developed [26].

Summarising, to the best of our knowledge, to date, no analytical procedure for the determination of neomycin sulfate in ointments that does not require either sophisticated and expensive instrumentation or time-consuming methodology for sample preparation has been described.

The present work has addressed the development of an analytical procedure which allows the determination of the total content of neomycin sulfate in ointments as a pharmaceutical form. As the purpose of this methodology is its implementation in pharmaceutical quality control laboratories, the methodology was restricted to two different requirements: first, the employment of an analytical tool available in a quality control laboratory and second, reliable results in a reasonable time frame.

This goal involves an analytical challenge in many respects. Apart from the low UV-absorptivity, the target analyte is co-formulated in a complex pharmaceutical preparation with several API's which can interfere with its determination. In addition, the nature of the matrix in which is delivered in a lipophilic matrix.

2. Experimental

2.1. Chemicals

Standards of neomycin sulfate, hydrocortisone acetate, benzocaine and tanic acid, neomycin 30 g ointment from Cinfa Laboratories and excipients (methylparaben, butylparaben, cholesterol, Multiwax W445 and vaseline) were kindly provided by CINFA S.A. (Pamplona, Spain).

Chloroform was purchased from Sigma Aldrich (Steinheim, Germany), NaOH (99%) from Panreac (Barcelona, Spain), H_3PO_4 (85%) from Merck (Darmstadt, Germany) and acetic acid (99%) from Panreac (Barcelona, Spain). Water was purified with a Milli-Q plus system from Millipore (Bedford, MA, USA).

2.2. CE analysis

All the experiments were carried out in a Beckman's P/ACE 5500 Capillary Electrophoresis System (Fullerton, CA, USA) with a UV-absorbance detector. The injection was by pressure at 0.5 psi for 20 s. The polyacrylamide-coated silica capillary tubing coated with linear polyacrylamide was from Beckman Coulter (Fullerton, CA, USA). It was properly cut to 37 cm of total length (30 cm of effective length) and 50 μm ID capillary. The background electrolyte consisted of 35 mM phosphate (ortho-phosphoric acid) and 15 mM acetate (acetic acid) buffer set at pH 4.7 with NaOH 4.0 M. The running potential was 20 kV in normal polarity mode (injection in the positive end and detection at the negative end) and temperature was maintained at 25 °C during the analysis. The current observed under these conditions was around 37 μA. Detection was performed at 200 nm.

In-between runs, the capillary was just rinsed with water (2 min at 10 psi) and then with BGE (background electrolyte) (2 min at 10 psi) prior to each injection. The BGE was replenished every eight runs in order to avoid the variability due to buffer deterioration.

2.3. Standard solutions and sample preparation

Neomycin sulfate stock solution was prepared with 400.0 mg of neomycin sulfate standard dissolved with milli-Q water in a 100 mL volumetric flask.

Neomycin sulfate standard solution was prepared as follows: 2.5 mL of chloroform and 1.5 mL of milli-Q water were dispensed into a glass tube, and 0.5 mL of neomycin sulfate stock solution was added. The mixture was then agitated during 1 min with a vortex prior to its centrifugation at 3000 rpm during 3 min. The upper layer was filtered with 0.45 μm nylon filters prior to the injection.

For quantification, 400.0 mg of the ointment were directly weighed in a glass tube. A volume of 2.5 mL of chloroform was added and this mixture was shaken manually until total dispersion before adding 2.0 mL of milli-Q water. The final mixture was then agitated during 1 min with a vortex, prior to its centrifugation at 3000 rpm for 3 min. The upper layer was filtered with 0.45 μm nylon filters prior to the injection.

In all cases, the theoretical concentration of neomycin sulfate was 1.0 mg/mL.

Table 1
Preparation of solutions assayed for the linearity test of the validation. Relative standard deviation achieved within each concentration level assayed..

Concentration level	Volume aliqouted from stock (mL)	Volume of water (mL)	Neomycin concentration (mg/mL)	Volume of chloroform (mL)	RSD of response factor (%)
75%	0.375	1.625	0.750	2.50	1.9
90%	0.450	1.550	0.900	2.50	2.1
100%	0.500	1.500	1.000	2.50	2.2
110%	0.550	1.450	1.100	2.50	1.8
125%	0.625	1.375	1.125	2.50	2.3

2.4. Validation

The linearity was tested by preparing three replicates of standard solutions at five concentration levels, from 75% to 125% of the target analyte concentration. In this case, neomycin raw material (base) concentrations ranged from 0.75 mg/mL to 1.25 mg/mL. The preparation of these solutions was exactly the same as described in Section 2.3, only varying the volumes of neomycin stock solution and water added. Table 1 shows the individual preparation for each concentration level assayed.

To determine accuracy, the analytical procedure was applied to synthetic mixtures of the ointment components to which known quantities of drug substance to be analysed had been added. Solutions were prepared as indicated in Table 1 except for the addition of 400.0 mg of the matrix. This matrix contained all the excipients (methylparaben, butylparaben, cholesterol, Multiwax W445 and vaseline) and the rest of the API's present in the formulation (hydrocortisone acetate, benzocaine and tanic acid). These solutions were equally prepared and tested in triplicate at three levels (75, 100 and 125%) and in parallel with the linearity assay.

Intra-assay precision data were obtained by repeatedly analysing, in one laboratory on one day, ten samples and ten standards, each of which was independently prepared according to the procedure of the method. Data for intermediate precision were obtained by repeating the intra-assay experiment on a different day with freshly prepared solutions.

3. Results and discussion

3.1. Exploration of possibilities with CE

HPLC was the first choice for a method to be employed in pharmaceutical quality control, but all attempts to develop a simple method for neomycin with this technique, either with direct or indirect detection proved to be unsuccessful. The compound with six primary amine groups stuck to the column providing very broad peaks even with last generation columns, while the addition of triethylamine precluded detection at 200 nm.

According to this laboratory experience the problem possessed characteristics that lead to CE as the technique of choice. The first step was method development for standards. As the method to be developed was aimed at the determination of the total amount of neomycin, the reported methods based on this technique, where neomycin components are separated, were discarded. Consequently, the only method left out of this criterion was that reported by Srisom et al. [25].

Based on the instrumental conditions of this method, a fused silica with a surfactant for reversing the EOF under reverse polarity mode was chosen as starting condition. The specific experimental conditions were a 37 cm (total length) $\times$75 μm ID fused silica capillary under reverse polarity with a BGE phosphate (ortho-phosphoric acid) buffer at pH 6.0, including CTAB (hexadecyltrimethyl-ammonium bromide) to create a cationic surface on the capillary surface and, consequently, reverse the direction of EOF.

Srisom's method employed *N*-(4-hydroxy-phenyl) acetamide as a chromophoric ion for the background, to perform indirect UV detection. However, as is well known, the incorrect choice of a chromophoric ion suitable for indirect detection leads to peak tailing or fronting of the peaks if the chromophoric ion and the analytes do not have similar electrophoretic mobilities. Therefore, we decided not to add any chromophoric ion in the buffer and performed the detection of the API in the direct mode.

The outcome of this trial revealed that although neomycin possesses a low UV-absorptivity at low UV wavelengths, it can still be measured with direct UV detection at 200 nm with the assayed concentration (2 mg/mL). The neomycin standard under these conditions migrated as two differentiated species, as its displacement against the EOF provides enough resolution. That would be interesting for a better knowledge of the API, but it was not desirable for quantification in pharmaceutical quality control, as only the total amount in the formula is registered and therefore that is the value to be submitted to regulatory agencies.

Attending to this first result, it was necessary to work out the separation from a different perspective. CZE of positively charged analytes in uncoated fused silica capillaries might be problematic due to the interaction with the capillary wall, causing peak fronting and tailing. In order to avoid the interactions between neomycin and the silanol groups of the silica surface, a separation using a neutral coated capillary was attempted.

For these experiments, a coated silica capillary with linear polyacrylamide was used. In this case, neomycin migrates solely because of its electrophoretical mobility (μ_{eo}) compared to the previous separation where mobility due to electroosmotic flow (μ_{EOF}) took part. This fact provides a high selectivity. A capillary with the same length but smaller internal diameter was employed, the cationic surfactant (CTAB) was not introduced in the running buffer and two different pH were assayed for the BGE. For this condition normal polarity was mandatory.

The electrophoretic profiles obtained revealed an asymmetry for pH 3.0 and a split of the standard into two differentiated peaks at pH 7.0. In accordance with the results reported by Yuan et al. [26], the first peak may correspond to neomycin C and the second one to neomycin B. These two components are diasteroisomers, therefore their p*K*a may be different and consequently, a differentiation in migration times may be expected.

Although equal ionic strength is not maintained if salt composition changes, similarity was attempted by replacing 30% of phosphoric acid with acetic acid (p*K*a 4.8), which allows the buffering of the uncovered pH range (pH 4.0 and pH 6.2).

In order to obtain an adequate peak shape without separating neomycin components, a study with a narrower pH range (from 4.1 to 5.1) was performed. Fig. 2 shows the resulting effect of the pH of the running buffer over the migration time and shape of neomycin peak. As can be seen, the lower the pH, the faster the neomycin migration, as the grade of ionisation for this compound is higher. Tailing was observed with the lower pH values, while a total separation for this peak was obtained with the higher pH values. Finally, a balanced situation was found at pH 4.7, where the tailing was minimised and no split of the peak took place.

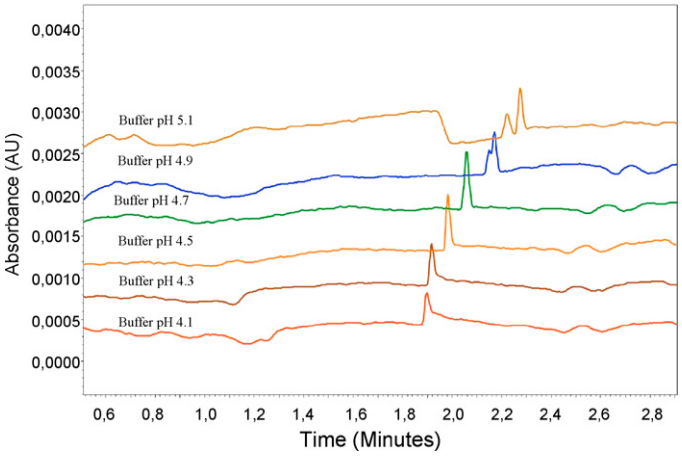

Fig. 2. Effect of pH value in buffer on electrophoretic times and shape of neomycin peak. Conditions: silica capillary 37 cm, 50 μm. BGE: 35 mM phosphate and 15 mM acetate, +20 kV at 25 °C. UV detection 200 nm.

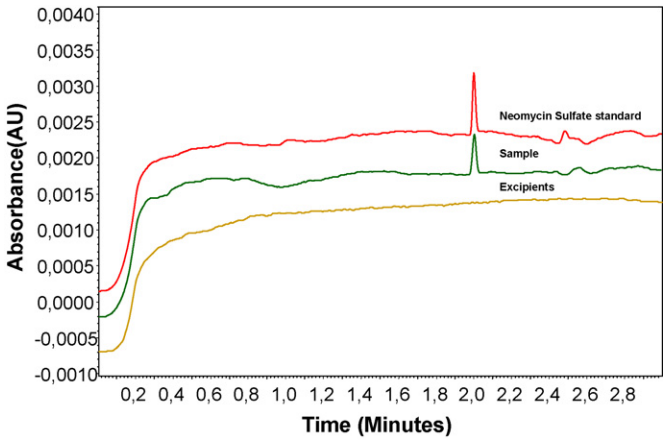

Fig. 3. Electropherograms of neomycin sulfate standard, excipients, and ointment sample under the CE method developed. Silica capillary 37 cm, 50 μm. BGE: 35 mM phosphate and 15 mM acetate buffered at pH 4.7, +20 kV at 25 °C. UV detection 200 nm.

3.2. Sample preparation optimisation

Once the analytical tool for measuring neomycin was established the next obstacle to overcome was sample preparation. The sensitivity achieved with this method is in the mg/mL range. The pharmaceutical speciality presents a content of 150 mg of the target compound per 30 g of ointment. Therefore, an amount of approximately 0.4 g should be diluted in 1 mL of water to reach the standard concentration assayed (2 mg/mL), which was obviously not viable.

Furthermore, neomycin is a highly polar analyte in a mainly high lipophilic matrix. Taking into consideration the enormous amount of water necessary to dissolve neomycin and the length of this process (stirring the solution overnight), this procedure calls, in any case, for further concentration steps. Because of the aforementioned reasons, a liquid–liquid extraction approach was considered for sample preparation.

Lipids are usually extracted from tissue matrices with chloroform–methanol (2:1, v/v) by a procedure described originally by Folch et al., in which the crude extract is washed with one-fourth its volume of saline solution (0.9 M NaCl), and lipids remain in the lower chloroform layer, while non-lipid impurities are washed out with the upper aqueous layer [27].

Based on this methodology, its adaptation was examined to remove the lipophilic substances in one organic phase and to dissolve neomycin in the smallest volume possible of the aqueous phase.

First experiments were run with a neomycin sulfate standard stock solution. Neomycin standard solution was prepared as follows: 2.0 mL of chloroform and 1.5 mL of milli-Q water were dispensed in a glass tube, and 0.5 mL of neomycin stock solution was added. After vortexing and centrifugating the upper layer was analysed. Recoveries around 100% were obtained.

A mixture of the excipients (methylparaben, butylparaben, cholesterol, Multiwax W445 and vaseline) and all the API's except for neomycin (hydrocortisone acetate, benzocaine and tanic acid) was prepared in order to verify the effect of this matrix on the solubility process. Sample was prepared as follows: 2.0 mL of chloroform and 2.0 mL of 2 M NaCl solution were dispensed in a glass tube. After vortexing and centrifugating the upper layer was analysed.

Lower recoveries were obtained when excipients were present in the liquid–liquid extraction system. This failure was solved by increasing the chloroform volume from 2.0 mL to 2.5 mL. Finally, 2 M NaCl solution was replaced with water, finding no influence on the analyte recovery. With this operation the ionic strength in the sample decreased and the stacking effect will be more effective.

Several compounds were tested as internal standards without satisfactory results, because they either increased the run time unnecessarily or gave incomplete recoveries. Even with arginine as one of the best options, method precision was found to be poorer during pre-validation tests as variability coming from the two analytes was additive.

Finally, in order to maintain the sensitivity achieved the time injection was increased from 10 s to 20 s to counteract the effect of injecting 1 mg/mL concentration solutions instead of 2 mg/mL.

Using the conditions described above it was possible to separate neomycin without the interference of other API's and the excipients in less than 3 min. Fig. 3 shows the electropherograms obtained for the neomycin sulfate standard, an ointment sample and the matrix prepared under these conditions. Therefore, the selectivity of the CE method was demonstrated for the separation of API in this pharmaceutical speciality at 2.1 min migration time. The remaining components in the sample are rinsed out of the capillary once the analyte has appeared in each run, that is one of the advantages of CE for complex sample analysis.

3.3. Validation of the method developed

After the development and the optimisation of the electrophoresis method, experiments for evaluating its validity for neomycin determination in ointments were carried out. Validation was performed following ICH guidelines [28] with neomycin standard and the pharmaceutical speciality of anti-hemorrhoid ointment.

Validation parameters for linearity are shown in Table 2. This includes the experimental values plus the specifications for passing the test, established following generally accepted criteria [29]. These parameters showed a good linearity, with correlation coefficient >0.999 for neomycin. Moreover, no bias was found in the regression line, because the intercepts with their limits of confidence included the zero value. Both tests were also examined with the Student *t*-test (13 freedom degrees, $p < 0.05$), and the same conclusions were reached. Additionally, the RSD (relative standard deviation) values achieved within each concentration level assayed are shown in Table 1.

The recovery found was $99.93 \pm 0.81\%$, which does not statistically differ from 100% (Student *t*-test, $p < 0.05$) and passed the specifications established for the method (95–105%). The results obtained for each concentration level were 0.79, 1.60 and 0.45 for 75%, 100% and 125% concentration levels, respectively. Accuracy

Table 2
Main validation parameters for the linearity of the analytical method.

Variable		Specification	Results
Range (mg/mL)		–	0.76–1.26
Correlation coefficient (r)		>0.999	0.999
Linearity test	Response factor RSD (%)	<5%	3.2%
	Slope		1472.11
	Confidence interval	0 value not included	1446.00–1498.22
	Experimental "t"	"t"$_{exp}$ > "t"$_{tab}$	121.81 > 2.16
Proportionality test	Intercept		−20.88
	Confidence interval	0 value included	(−47.55)–5.79
	Experimental "t"	"t"$_{exp}$ < "t"$_{tab}$	1.69 < 2.16

Table 3
Main validation parameters for the precision of the analytical method.

Test	Variable	Specification	Results
Instrumental precision	RSD	<1.9%	1.7%
Standard precision method			
Intra-assay	RSD	<2.7%	1.4%
Intermediate	RSD	<5.5%	1.7%
Sample precision method			
Intra-assay	RSD	<2.7%	2.0%
Intermediate	RSD	<5.5%	2.5%

was calculated by evaluating the linear regression of the concentration obtained for the samples, interpolating the results in the regression line obtained for the standards *vs* their true assayed concentration. The parameters from this study were for the $b \pm t_{exp}\, b$ (0.987 ± 0.039) and $a \pm t_{exp} a$ (0.01 ± 0.04), which include values 1 and 0, respectively.

Table 3 presents the results obtained for the precision tests plus the specification established to consider the result as acceptable. The RSD value in the instrumental precision was 1.7%, fulfilling the fixed specification. The method precision for standards, the RSDs of the analytical response ranged from 1.4% to 2.4% on different days. When considering results from both days together, a 1.7% RSD value was obtained.

In relation to the method precision: sample RSDs for the data collected ranged from 2.0% to 3.1% for the intra-assay precision and 2.5% for the intermediate precision. The outcome of these experiments reveals the influence of sample preparation. With these values and the intervals of acceptance established for the release of batches (95–105%), two replicate of each sample ought to be measured for quantification.

4. Conclusions

An analytical approach has been developed for the rapid determination of neomycin in ointments. This approach has been optimised, saving time with respect to the methodology published to date, in terms of sample preparation employing a liquid–liquid extraction. It is simplified in terms of analytical method, applying a CZE method with direct UV detection that allows the separation of the target analyte from the complex matrix, in less than 3 min of total run. The employment of this separation technique offers the additional advantage of low costs attending to the currently instability of acetonitrile prices in market.

The method has been validated and has been shown to be reliable, linear, accurate and precise for concentration range assayed within this method. Therefore, it can be applied for the quantification of the active compound. Finally, the validity of the method has been proved by applying it to samples throughout long-term stability assays.

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