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RESEARCH ARTICLE

Validation of a stability-indicating HPLC method with diode array detection for the determination of beclomethasone dipropionate in aqueous suspension for nebulizer

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ABSTRACT

The aim of this study was to develop and validate a stability-indicating high performance liquid chromatographic method for the analysis of beclomethasone dipropionate in aqueous suspensions for nebulizer. This drug was submitted to accelerated degradation studies under acidic, alkaline and oxidation conditions, exposure to light and moisture and thermal stability. The separation of BD and its degradation products was achieved on Eclipse XDB Phenyl column (150 nn x 4.6 mm, i. d. 5 µm particle size) with an isocratic mobile phase containing methanol and water (80:20, v/v). The flow rate was 1.0 mL min^{-1} and detection wavelength was set at 240 nm, at 30 ºC. Different degradation products were formed in the stress conditions and the peak purity indexes of BD obtained by diode array detection were > 0.999, confirming the specificity of this method. The calibration curve was linear over the selected concentration range of 30.36 – 91.07 µg mL^{-1} with a correlation coefficient 0.99989. Recovery was within acceptable statistical limits and the limit of detection was 0.062 µg mL^{-1}. The intra-day repeatability and intermediate precision (RSD) amongst six sample preparations was 1.60 % and 1.43 %, respectively. In conclusion, the proposed method was found to be rapid and stability-indicating with adequate specificity, precision, accuracy and robustness and hence be suitable for monitoring the degradation process of BD during stability studies.

Keywords*:* beclomethasone dipropionate, diode array detection, method development, stability-indicating HPLC, stress degradation studies.

1. INTRODUCTION

Beclomethasone diproprionate (BD), 9-chloro-11-hydroxy-16-methyl-pregna-1,4-diene-3,20-dione (Fig. 1), is a glucocorticoid used as first-line, anti-inflammatory therapy to treatment of all asthmatic patients [1, 2]. BD is a highly hydrophobic steroidal drug which is formulated as an aqueous suspension for nebulizer therapy [3]. The inhalation of high doses of BD, which is often used in initial treatment, can be found to induce systemic side effects such as adrenocortical suppression, skin changes (thinning, bruising) and cataract formation [4].

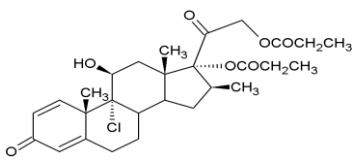
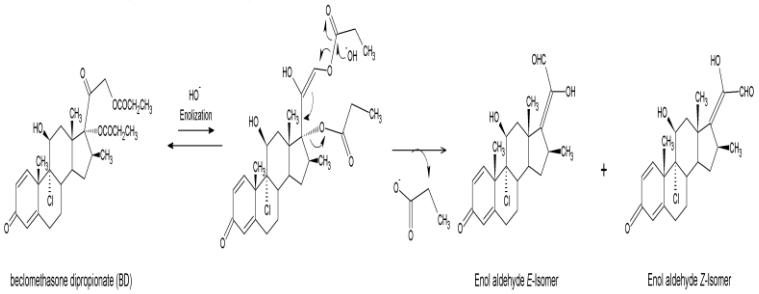


Fig. 1. Chemical structure of beclomethasone dipropionate (BD).

The beclomethasone dipropionate in the formulated drug products can form enol aldehyde via β-elimination of water from the D-rings containing 1,3-dihydroxyacetone side chain and this process is catalyzed by alkaline condition (Scheme 1).



Scheme 1. Formation of beclomethasone enol aldehyde from beclomethasone dipropionate via the revised Mattox rearrangement under alkaline conditions. Adapted from [5]

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The enol aldehydes are one type of the key degradants, which can further degrade into a number of secondary degradants dependent upon the formula and/or storage condition [5].

In pharmaceutical preparations and biological matrices, multiple analytical procedures have been reported for the analysis of BD using HPLC, capillary electrophoresis, liquid chromatography mass spectrometry and spectrophotometry [6 – 13]. However, no study describes a stability-indicating HPLC method for the determination of BD in aqueous suspension for nebulizer.

In recent times, there is an increased tendency towards the development and validation of stability-indicating assays, using the stress testing on the drug under a variety of conditions, including hydrolysis (at various pH), oxidation, photolysis and thermal degradation [14, 15]. Thus, the aim of this work was to develop and validate a stability-indicating reversed-phase HPLC method for beclomethasone diproprionate in aqueous suspension for nebulizer.

2.EXPERIMENTAL

2.1. Materials

Beclomethasone diproprionate (BD, 99.5 % purity) used as analytical standard was purchased from Sigma Chemical (St. Louis, Missouri, USA). HPLC grade methanol (J. T. Baker, Mexico City, Mexico) and purified water obtained by reverse osmosis (Millipore) were used throughout the study. The aqueous suspension for nebulizer (Clenil® A) labeled to contain 400 μg mL^{-1} of BD and the excipients: sodium phosphate, potassium phosphate, cetostearyl alcohol, sodium chloride, polisorbate 20, sodium chloride, sorbitan monolaurate and deionized water. All chemicals used were of pharmaceutical or special analytical grade.

2.2. Instrumentation and chromatographic conditions

The Agilent 1200 HPLC system consisting of a diode array detector (DAD) (set at 240 nm) was used for the quantification of beclomethasone dipropionate (BD) in suspensions in the presence of its degradation products. The analytical column employed was RP-18 (Eclipse XDB Phenyl) (250 mm x 4.6 mm, i.d. 5 μm particle size). The mobile phase consisted of methanol-water (80:20, v/v) and the injection volume was 30 μL. All separations were performed isocratically at a flow rate of 1.0 mL min^{-1}, the column temperature was thermostated at 30 ºC, and the run time was 15 min.

2.3. Forced degradation studies

All stress degradation experiments of BD were performed in accordance with the ICH guidelines and Brazilian Legislation in order to demonstrate the stability-indicating feature of the assay. Beclomethasone diproprionate (standard stock solution and sample preparation) at concentration of 400 μg mL^{-1} was submitted to acid, alkaline and oxidation conditions. The experiments were carried out as follows: solutions of BD were prepared in HCl or NaOH or hydrogen peroxide (H_2O_2) solutions, respectively. These samples were protected from light and stored in an incubator at room temperature. Aliquots were taken at appropriate time intervals depending on decomposition rate (until 240 hours or above 10 % of degradation of BD) and analyzed immediately for the determination of BD and degradation products.

In order to check the degradation of samples against moisture, light and temperature, the samples were prepared as described above, without addition of any degradation solution, and stored at room temperature or UV-light or in an incubator (80 ºC), respectively.

The placebos were prepared like the samples, with the degradation solutions and stored in the same conditions.

2.4. Standard stock solution and sample preparation

An amount of 1.2 mg of BD was accurately weighed and dissolved in absolute methanol to prepare a stock solution at a concentration of 400 μg mL^{-1}. This solution was diluted appropriately with mobile phase to obtain working standards (at five different levels which were 50%, 75%, 100%, 125% and 150% of the test concentration) (in the concentration range of 30.36 – 91.07 μg mL^{-1}).

The suspension vial was stirred on a magnetic stir plate during 2 min to assure the homogeneity at the time to take the aliquot. An aliquot of 1 mL of the suspension was quantitatively transferred to a 10 mL volumetric flask containing 5 mL of mobile phase, and stirred in an ultrasonic bath for 45 min. The volume was completed with the mobile phase to obtain the final concentration of 40 μg mL^{-1}, and the resulted solution was filtered through a 0.45 μm nylon membrane before use.

2.4.1. Stability of the sample solution

The stability of sample solution was tested after 72 hours, using to analysis the first sample of repeatability test. The solution was injected two times and the degradation percentage was calculated in relation of initial concentration.

2.5. Method validation

The analytical method was validated according to Brazilian Legislation (RE nº 899/03) and ICH [15, 16]. The parameters evaluated were selectivity/specificity (with forced degradation), linearity, precision (repeatability and intermediate precision), accuracy, limit of quantitation and robustness.

2.5.1. Selectivity/Specificity

Specificity and selectivity, according to Brazilian Legislation [15], is the ability of the method to measure accurately and specifically the analyte of interest in the presence of other components such as impurities, degradation products and compounds of matrix. To examine the selectivity of the proposed method, placebo (sample without drug) was injected three times and

samples submitted to accelerated degradation studies were injected two times to determine possible interferences in the drug peak region.

2.5.2. Linearity

The linearity is the ability of an analytical procedure to demonstrate that obtained test results are directly proportional to the concentration of analyte in the sample, within a given range. The linearity of the method was studied by injecting BD at five different concentration levels, from 50% to 150%. Each concentration was prepared in triplicate and analyzed three times. The mean peak areas versus concentration data was treated by least-squares linear regression and analysis. The relative standard deviation (RSD) for the slope and Y-intercept of the calibration curve was calculated. In linearity studies, the acceptance criterion is a correlation coefficient of at least 0.998 and RSD between replicates of injection less or equal to 2.0 %.

2.5.3. Precision

The precision of the method is the parameter that expresses the closeness of agreement (or degree of scatter) among a series of measurements obtained from multiple analysis of the same sample.

The intra-day repeatability was determined by replicate analysis of six samples. The intermediate precision of the method was validated using six separate samples prepared by a second analyst and in another day of the repeatability assay. Every sample was injected once. The results of intermediate precision and repeatability were compared each other.

2.5.4. Accuracy (Recovery Method)

The accuracy of an analytical method is the exactness or the closeness of agreement between the true value and the value found by the method under study.

To demonstrate the accuracy of the proposed method, recovery studies were carried out by the standard addition technique in which quality control samples were prepared by spiking methanol with known amounts of the BD to obtain three different concentration levels (80, 100 and 120 %) and in parallel with the linearity assay. Each level of the drug was determined in triplicate (n = 3) by the HPLC assay. The amount of BD recovered was calculated in relation to the added amount (recovery percent).

2.5.5. LOQ

The quantitation limit (LOQ) is defined as the lowest amount of analyte in a sample which can be determined with precision and accuracy under the stated operational conditions. The signal-to-noise of 10:1 was considered. The solution was injected five times and the LOQ were determined using Eq. (1):

$$LOQ = 10\sigma/S \qquad \text{Eq. (1)}$$

where σ is the standard deviation of the Y-intercept and S is the slope of the standard curve.

2.5.6. Robustness

The robustness of an analytical method is the measure of its capacity to resist small and deliberate variations of analytical parameters and indicates its reliability during normal use. The robustness was evaluated by the effect of some operational parameters in the obtained results. These results were compared with the results obtained from the first injection of repeatability test. Three factors were selected from the analytical procedure: flow rate (0.9 and 1.1 mL min^{-1}), mobile phase ratio (v/v) methanol:water (82:18 and 78:22) and column temperature (28 ºC and 32 ºC).

3. RESULTS AND DISCUSSION

3.1. Stress degradation of beclomethasone diproprionate

Under stress conditions the BD peak reduced over time (Table 1) with appearance of different unknown degradation products peaks.

Percentage of degradation of BD (in relation to initial time)

Stress condition	Time				
	1 h	24 h	96 h	168 h	240 h
Acidic	-	14.46	-	-	-
Alkaline	17.72	-	-	-	-
Oxidation	-	0.92	0.46	0.41	0.06
Moisture	-	0.62	0.01	0.38	1.05
Light	-	1.49	23.37	-	-
Temperature	-	10.39	-	-	-

Table 1. Effect of stress conditions on degradation of BD.

The results showed that this drug degrades when submitted to the stress conditions tested at the following time: 1 hour to alkaline condition (Fig. 2), 24 hours to acidic condition and thermal stress (Fig. 3 and 4) and 96 hours to light (Fig. 5). In oxidative condition and moisture, the drug was found to be stable for 240 hours, and these conditions did not promote the formation of degradation products (Fig. 6 and 7). There was no degradation of placebo in any of the conditions tested (data not showed).

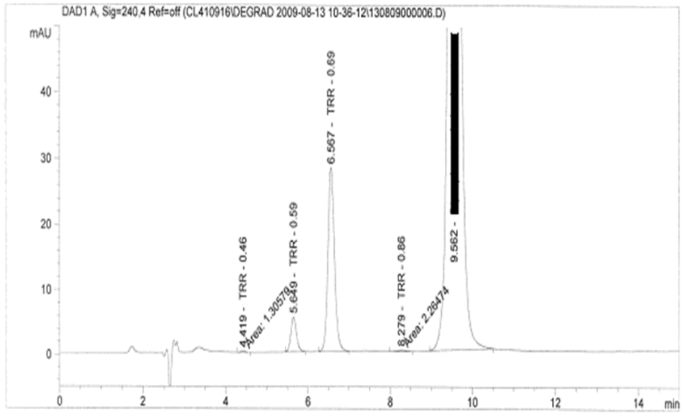

Fig. 2. HPLC chromatograms of BD solution degraded in NaOH at room temperature for 1 h.

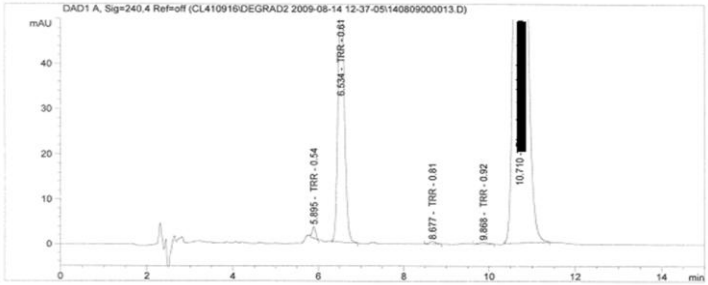

Fig. 3. HPLC chromatograms of BD solution degraded in HCl at room temperature for 24 h.

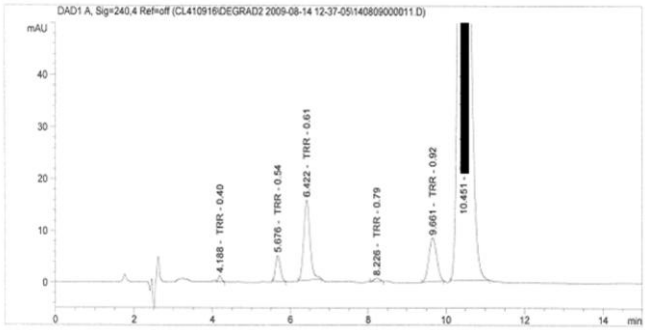

Fig. 4. HPLC chromatograms of BD solution degraded in temperature at 80 ºC for 24 h.

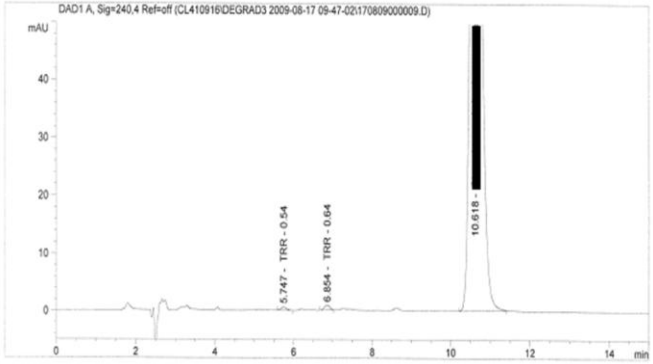

Fig. 5. HPLC chromatograms of BD solution degraded in light for 96 h.

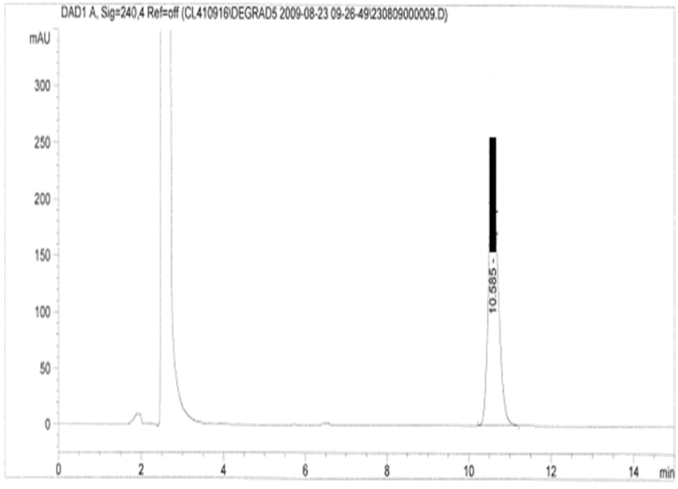

Fig. 6. HPLC chromatograms of BD solution degraded in H_2O_2 at room temperature for 240 h

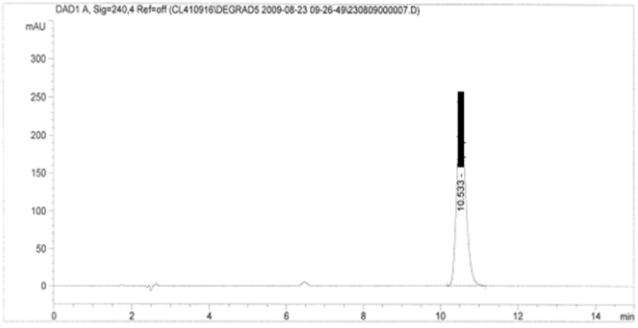

Fig. 7. HPLC chromatograms of BD solution degraded in moisture for 240 h.

The Table 2 presents the peak purity and retention time (t_R) of BD under all forced degradation conditions. It was observed that all the peak purity was greater than 980 and this confirm the absence of other co-eluting substances.

Stress condition	Time (hours)	t_R	Peak purity
Alkaline	1	9.539	999.314
Acidic	24	10.710	999.975
Temperature	24	10.451	999.983
Light	96	10.618	999.987
Moisture	240	10.533	999.978
Oxidation	240	10.585	999.984

Table 2. Peak purity and retention time for BD under stress degradation studies.

3.2. Stability of the sample solution

According to the results obtained, it can be noticed that solutions were stable for 72 h, as during this time the results does not decrease below the minimum percentage (98 %).

3.3. Validation of stability-indicating HPLC method of BD

3.3.1. Selectivity/Specificity

The mean retention time (t_R) of BD was found to be 10 min. The drug peak did not overlap with peaks of placebo, diluents (Fig. 8) or even any degradation products, as indicated by peak purity. The peak obtained was sharp with clear baseline separation. The method is highly specific.

	BD
Linearity range (µg mL^{-1})	30.36 – 91.07
Slope	50.40104
Y-intercept	-10.17651
Correlation coefficient (*r*)	0.99989
RSD of slope (%)	0.06052
RSD of intercept (%)	2.64399

[a]Mean of three injections.

Table 3. Characteristics of BD calibration plots[a].

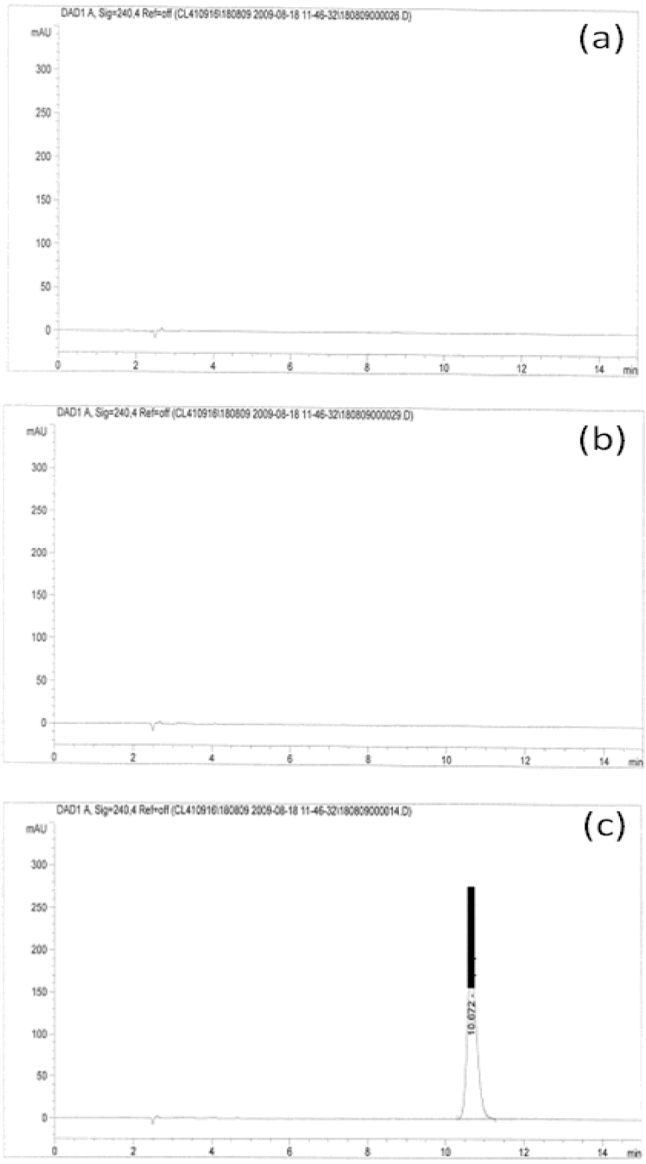

Fig. 8. HPLC chromatograms of (a) placebo; (b) diluents; (c) 60 µg mL^{-1} BD in mobile phase.

3.3.2. Linearity

The calibration curve of BD was constructed in mobile phase. Good linearity was found to be linear within the concentration range. The correlation coefficient (*r*) was found to be 0.99989, indicating a functional linear relationship between the concentration of analyte and area under the peak (Table 3).

3.3.3. Precision

The system repeatability and intermediate precision were calculated from six replicate injections of beclomethasone diproprionate at the analytical concentration of about 75 µg mL^{-1} quality control samples. RSD found for the intra-day repeatability was 1.60 %, while the R.S.D. found for the intermediate precision was 1.43 %, as shown in Table 4. These values indicate excellent precision, according the acceptance criterion adopted (RSD among 6 determinations ≤ 2.0 %)

3.3.4. *Accuracy (Recovery Method)*

Sample	Intra-day repeatability		Intermediate precision	
	BD (µg mL^{-1})	BD (mg dose^{-1})	BD (µg mL^{-1})	BD (mg dose^{-1})
1	76.78	0.492	76.21	0.496
2	74.02	0.497	73.46	0.498
3	74.93	0.494	74.81	0.496
4	74.10	0.495	76.20	0.495
5	73.86	0.494	74.38	0.494
6	73.54	0.493	75.27	0.497
Mean	74.54	0.494	75.06	0.496
RSD (%)	1.60	NA	1.43	NA

Table 4. Intra-day repeatability and intermediate precision of quality control samples of BD (n = 6).

Recovery data are presented in Table 5. The obtained values were within 80 – 120 %, satisfying the acceptance criteria reported by Brazilian Legislation (2003) [15].

Concentration added (µg mL^{-1})	Recovery (%)[a]	RSD (%)
48.57	99.31	1.05
60.72	98.77	0.75
72.86	99.51	0.43
Mean	99.20	0.74

[a]Mean of three determinations.

Table 5. Accuracy for BD.

3.3.5. *LOQ*

The limit of quantitation for BD was set at 0.062 µg mL^{-1}, indicating the excellent sensitivity of the analytical method.

3.3.6. *Robustness*

The robustness of the current method was evaluated by altering of conditions including changes in column temperature, organic-to-aqueous ratio, and mobile phase flow rate. The results obtained to each changed condition are shown in Table 6.

Drug content in the reference sample* - 0.4917 mg dose^{-1}				
Parameter	Modification	Mean (mg dose^{-1})	RSD	Difference in relation to reference (%)
Flow rate (mL min^{-1})	0.9	0.4924	0.03	0.14
	1.1	0.4934	0.09	0.35
Mobile phase ratio (v/v) methanol-water	82:18	0.4937	0.03	0.41
	78:22	0.4933	0.03	0.33
Temperature (ºC)	28	0.4934	0.01	0.34
	32	0.4932	0.00	0.30

*Reference: sample 1 of repeatability.

Table 6. Influence of changes in experimental parameters of chromatographic system.

The results showed that, under various deliberately altered HPLC conditions, the method demonstrated sufficient ruggedness, since relative standard deviation (RSD) between the determinations and the absolute difference in relation to reference is lowest 2.0 %. Therefore, this method is considered acceptable for the analysis of BD.

4. CONCLUSION

A simple and rapid stability-indicating RP-HPLC method was developed and validated for determination of beclomethasone diproprionate in presence of its degradation products in suspensions formed in different conditions. The degradation of BD was in following order: alkaline > acidic and heat > light exposure. At moisture and oxidation conditions, there was no significant degradation of BD over time. Since the forced degradation showed no interference with the beclomethasone diproprionate peak, the proposed stability-indicating method is specific, accurate, precise, and requires a simple sample preparation procedure encouraging application in stability studies and routine quality control analysis.

4. ACKNOWLEDGMENTS

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Conflict of Interest: None Declared

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