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Validated Stability Indicating HPLC Method for Determination of the Polyanionic Cromolyn Sodiumi in Presence of its Alkaline Degradate

El-Kosasy AM, Omar AA, Magdy N* and El Zahar NM

Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Ain Shams University, Organization of African Unity Street, Abassia, Cairo (11566), Egypt

***Corresponding author:** Magdy N, Faculty of Pharmacy, Organization of African Unity Street, Abassia, Cairo (11566), Egypt, Tel: +20 1223767106; E-mail: drnancymagdy@hotmail.com

Abstract

A simple precise accurate reversed phase HPLC method was presented for the quantitative determination of Cromolyn sodium in pure form, in combined pharmaceutical formulations and in presence of its alkaline degradate. The compounds were eluted isocratically on a C18 column and Cromolyn sodium was quantitatively determined at 235 nm. The mobile phase composed of acetonitrile: water (70: 30, v/v) at flow rate of 1.0 mL/ min. The total run time is 9 min. The standard curve of Cromolyn sodium was over the range of 1 to 100 µg/mL with a limit of quantitation (LOQ) of 30 ng/mL. The validity of the proposed method was assessed using the standard addition technique. The obtained results were statistically compared with those obtained by the official method, showing no significant difference with respect to accuracy and precision at p=0.05.

Keywords: *Cromolyn sodium; HPLC; Cromolyn sodium alkaline degradate*

Introduction

Cromolyn sodium (CS) (5,5′-(2-hydroxypropane-1,3-diyl)bis(oxy)bis(4-oxo-4H-chromene-2-carboxylic acid) Figure 1 is a mast cell stabilizer preventing the release of inflammatory chemicals as histamine. It blocks early and late asthmatic responses induced by allergen inhalation and exercise and also blocks the increase in bronchial hyper reactivity induced by chronic allergen exposure [1,2].

Determination of CS in pharmaceutical formulations and biological fluids has been reported by several methods including spectroscopy [3,4], radioimmunoassay [5], Thin layer chromatography[6,7], HPLC using UV [8-11], fluorescence [12] and tandem mass [13-15] detectors, capillary electrophoresis [16] and electrochemical methods [17-19].

The literature survey revealed that no methods have been reported for the analysis of CS in presence of both Oxymetazoline HCl (OXY) and CS alkaline degradate. The aim of the present work is to develop new, simple, accurate and precise method validated according to ICH guidelines.

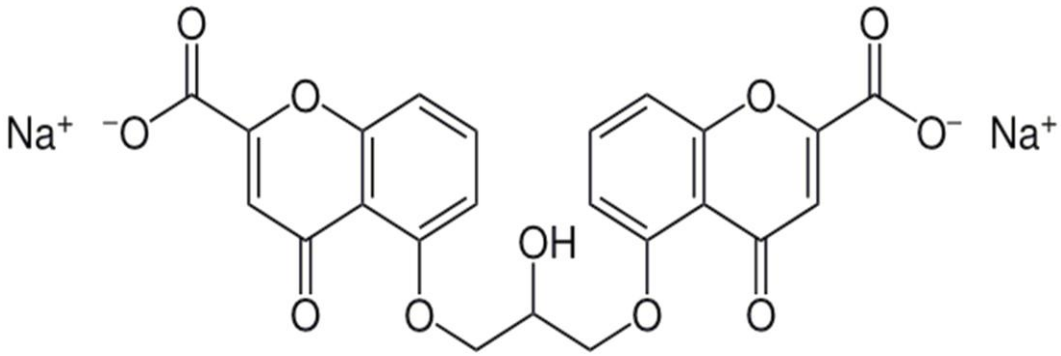


Figure 1: Structure of Cromolyn Sodium.

Experimental

Instrumentation

• Waters Alliance e2695 HPLC binary pump (Waters Technology, Milford, MA) equipped with an in-line vacuum degassing auto-sampler with capacity for 120 vials with programmable temperature control, heated column compartment and photodiode array detector (model 2998). All components of the HPLC system were controllable through the Empower 3 chromatography data software.

• Zorbax eclipse HC-C18 column (250 mm x 4.6 mm x 5 µm).

• 0.2 µm disposable membrane filters (Millipore corp., Milford, MA, USA).

• Jenway pH meter 3310 pH /mV/℃.

• Sonicator (Crest ultrasonics, scotch road. Mercer, country airport, Trenton, NJ 08628).

Chemicals and Reagents

• CS and OXY were kindly supplied by Sigma Pharmaceutical Industries (Egypt) and certified to contain 99.61% and 99.80; respectively.

• Nazocrom® nasal spray, labeled to contain 20 mg of CS and 0.25 mg of OXY per 1 mL (Batch number: 23888), was manufactured by Sigma Pharmaceutical Industries (Egypt).

• Epicrom® eye drop labeled to contain 40 mg of CS per 1 mL (Batch number: 1203205), was manufactured by Egyptian International Pharmaceutical Industries Company, (EIPICO), Cairo, Egypt.

• All reagents and solvents were of HPLC grade: acetonitrile (Sigma Gmbh, Germany), phosphoric acid (Reidel-deHaën, Germany), water (Sigma Gmbh, Germany), methanol (Sigma Gmbh, Germany).

Standard solutions

A stock solution of CS) (1.00 mg mL-1 was prepared by dissolving 0.10 gm of CS in water, transferred into 100-mL volumetric flask and completed to the mark with water and also used as the working solution.

A stock solution of OXY (1.00 mg mL-1) was prepared by dissolving 0.10 gm of OXY in water transferred into 100-mL volumetric flask and completed to the mark with water and also used as the working solution.

A stock solution of CS alkaline degradate (500.00 µg mL^{-1}) was prepared by dissolving 0.05 gm of CS alkaline degradate in 0.1 N NaOH, transferred into 100-mL volumetric flask and completed to the mark with 0.1 N NaOH and also used as the working solution and also used as the working solution.

Procedure

Preparation of CS alkaline degradate [20]

(1-{2-[3-(2-Acetyl-3-hydroxy-phenoxy)-2-hydroxypropoxy]-6hydroxyphenyl}-ethanone): 0.50 g of CS was refluxed with 10% aqueous potassium hydroxide (10.00 mL) for 2 hrs. Subsequently, the solution was acidified with glacial acetic acid until complete precipitation of the degradation product. The precipitate was filtered, washed and dried.

Chromatographic conditions: The mobile phase used was acetonitrile: water (70:30, v/v). All analyses were performed under isocratic conditions with a flow rate of 1.0 mL min-1 and at room temperature. Diode array detector was adjusted at 235.0 nm for quantitative determination of CS. All solvents were filtered through 0.2 µm membrane filter immediately before use and degassed in an ultrasonic bath. Zorbax eclipse HC-C18 column (250 mm x 4.6 mm x 5 µm) was equilibrated with mobile phase. Sample solutions were diluted with mobile phase and filtered through 0.45 µm syringe filter.

Method validation

Linearity: Different aliquots of CS standard stock solutions were transferred, separately, into 10 mL volumetric flasks, diluted to volume with mobile phase to obtain concentrations of (1.00- 100.00 µg mL-1) of CS.

A 50.00 µL volume of each solution was injected, in triplicates, the drug was separated using the above mentioned chromatographic conditions and the average peak areas were calculated. The calibration curve, representing the relationship between average peak areas and their corresponding concentrations was plotted and the regression equation for the investigated drug was computed.

Accuracy: The previously mentioned procedure under linearity was repeated for determination of different concentrations of pure samples of CS. The concentrations were calculated from the corresponding regression equation and the recovery percentages were then calculated.

Precision: The intraday and interday variations were evaluated by applying the previously mentioned procedure, for analysis of 10.00, 30.00 and 60.00 µg mL-1 of CS (n=9) on the same day and on three successive days, respectively. The concentrations were calculated from the corresponding regression equation, the recovery percentages and standard deviations were then calculated.

Specificity: Laboratory prepared mixtures were prepared as follows: Aliquots of (0.01-0.90) mL of CS working standard solution (1.00 mg mL-1) were transferred separately into a series of 10 mL volumetric flasks and to the previous solutions accurately measured volumes (0.01-0.80 mL) of OXY (1.00 mg mL-1) and (0.02-1.60 mL) of CS alkaline degradate (500.00 µg mL-1) were added. The volume was completed to mark with the mobile phase.

Using the proposed method, the samples were analyzed for CS contents. The concentrations were calculated from the corresponding regression equation; the mean percentage recoveries and standard deviations were then calculated.

LOD and LOQ: LOD and LOQ were calculated using the corresponding calibration curve. According to the ICH guidelines for determination of LOD and LOQ, the estimation was based on the standard deviation of response.

LOD=3.3 x σ / S

LOQ=10 x σ / S

Where, σ is the standard deviation of response and S is the slope of the calibration curve. Here, the standard deviation of the y-intercept of the regression line can be used as the standard deviation of response.

Robustness: The robustness was carried out by changing the ratio of the system acetonitrile: water from (70:30) to (80:20) and reinjection of 10.00 µg mL^{-1} sample again. Standard deviation was calculated.

Application of the proposed procedure for the determination of CS in its pharmaceutical formulations: Half mL and 1.00 mL were accurately transferred from Epicrom® eye drop and Nazocrom® nasal spray; respectively, to a 100 mL volumetric flask and diluted to the mark with distilled water to get 200.00 µg mL^{-1} of CS.

Five milliliters of the prepared solutions were transferred into 25 mL volumetric flask. The volume was completed with the mobile phase to obtain solution with a final concentration of 40.00 µg mL^{-1} CS. The procedure described under linearity was applied to the prepared samples.

Standard addition technique was applied to assess the validity of the proposed method by analyzing the pharmaceutical formulations spiked with different concentrations of pure standard drug.

Concentrations were calculated using the corresponding regression equation; the mean recovery percentages and standards deviations were then calculated.

Results and Discussion

A reversed phase HPLC method was suggested for the quantitative determination of CS in pure form, in its pharmaceutical formulations and in presence of its alkaline degradate.

The specified chromatographic conditions were adjusted; a 50.00 µl volume was injected into HPLC. Several mobile phases were tried to obtain satisfactory results, symmetric peak shape and short run time.

The best results were obtained upon using mobile phase acetonitrile: water (70:30, v/v) with a flow rate 1 mL min^{-1} at wavelength 235.0 nm.This wavelength shows maximum sensitivity for the studied drug. The average retention time ± SD for 3 replicate injections were found to be 1.99 ± 0.2 min (Figures 2 and 3).

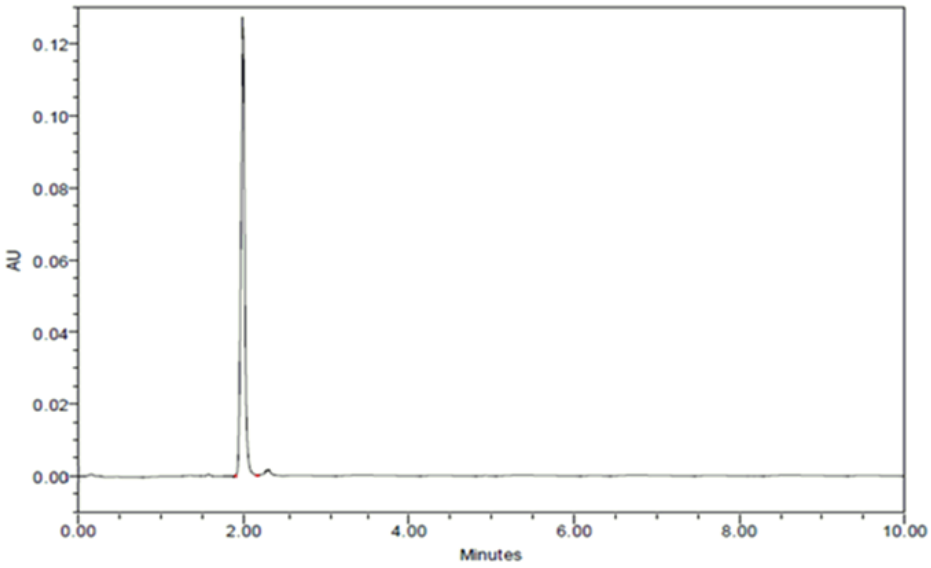


Figure 2: HPLC Chromatogram of Cromolyn sodium 10.00 µg mL-1 at λ235 nm..

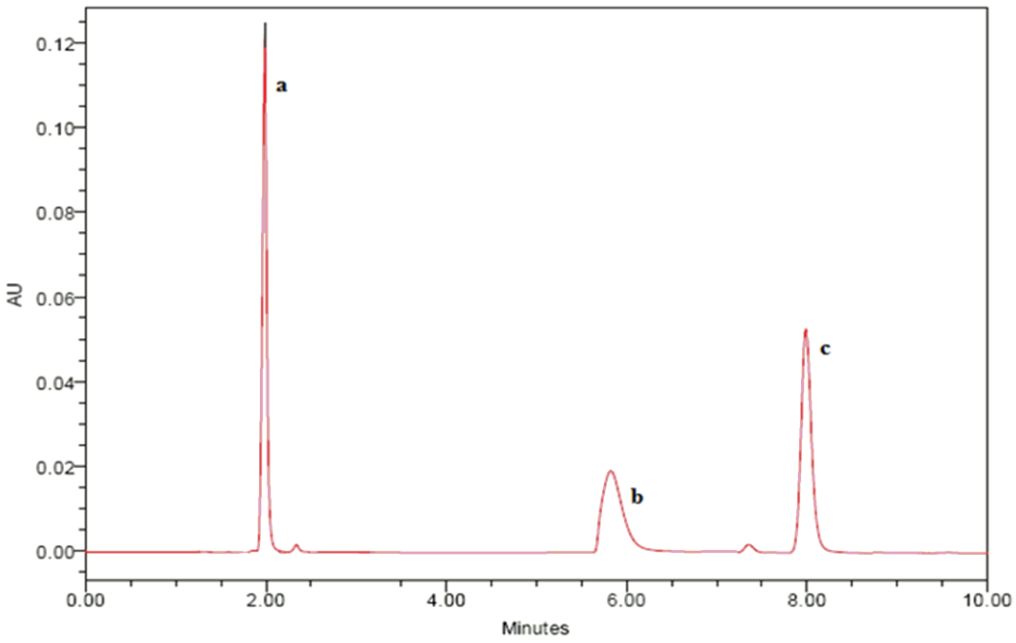
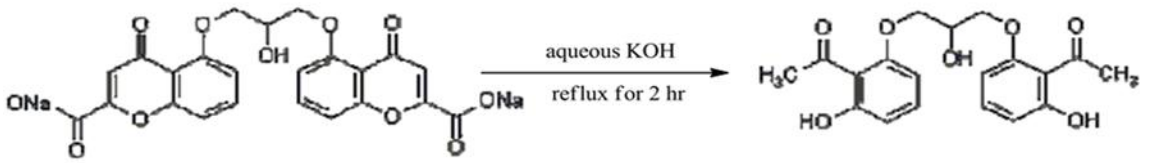


Figure 3: HPLC Chromatogram showing simultaneous separation of Cromolyn sodium 10.00 µg mL-1 (a), Oxymetazoline HCl 40.00 µg mL-1 (b) and Cromolyn sodium alkaline degradate 50.00 µg mL-1 (c) at λ235 nm

Identification of CS alkaline degradate.

A successful attempt has been carried out for the preparation of CS alkaline degradate through the application of the Spath and Gruber method based on the similarity of the chromone nucleus in both compounds (CS and khellin). khellin, which is a naturally occurring chromone compound, can be hydrolyzed in 1% aqueous potassium hydroxide to yield khellinone [21-23]. The formation of CS alkaline degradate (which is one of the related substances of CS in the European Pharmacopeia) [24] could be confirmed by the UV spectrum of its alkaline solution which revealed disappearance of the characteristic absorption band of CS at 326.0 nm and appearance of other two absorption bands at 282.0 and 330.0 nm [20,25] as shown in Figure 4.



Cromolyn sodium **Cromolyn sodium alkaline degradate**

Method validation

The proposed method was applied, the calibration curve was constructed and the regression equation was computed. A linear correlation was obtained between the average peak areas and the corresponding concentrations of the pure drug in the range of 1.00–100.00 µg mL-1.

The corresponding regression equation was computed and found to be:

A= 41662C + 7306.6 (r=0.9999)

Where, A is the peak area, C is the concentration (µg mL-1) and r is the correlation coefficient

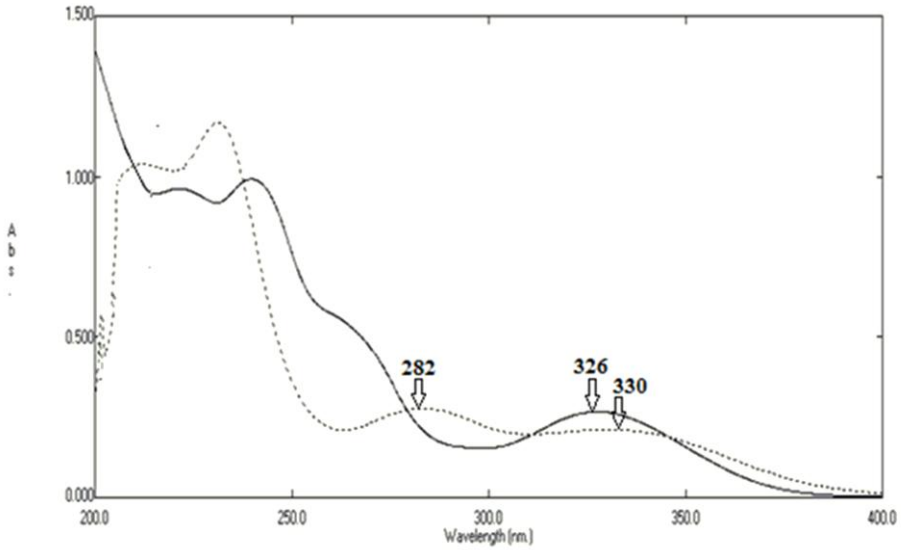


Figure 4: Zero order absorption spectra of 20.00 µg mL-1 Cromolyn sodium (____) and 20.00 µg mL-1 Cromolyn sodium alkaline degradate (……).

The accuracy of the method was assessed by the determination of different concentrations of the studied drug in its bulk powder. The mean recovery percentage was found to be 100.17 ± 1.s04 for CS. Results were shown in Table 1.

Taken µg mL^{-1}	**Found* µg mL^{-1}**	**Recovery %**
1.00	0.99	99.00
5.00	5.06	101.20
10.00	9.87	98.70
20.00	19.80	99.00
30.00	30.21	100.70
40.00	40.28	100.70
50.00	50.69	101.38
60.00	60.00	100.00
80.00	79.71	99.64
100.00	101.35	101.35
	Mean ± SD	**100.17 ± 1.04**

*Average of three determinations

Table 1: Accuracy results for the determination of CS in the bulk powder by the proposed method.

The intraday and the interday precision were evaluated and the intraday standard deviation value was found to be 0.25 while that of the interday was 1.02.In order to assess the specificity of the proposed method, several synthetic mixtures, within the linearity ranges with different concentration ratios of CS, OXY and CS alkaline degradate were analysed.

The percentage recoveries obtained for each mixture are given in Table 2. It is obvious that the proposed method could be applied to resolve CS in presence of OXY and CS alkaline degradate with good selectivity.

Ternary mixtures ratios	**CS**		
CS:OXY:CS alkaline degradate	**Taken µg mL^{-1}**	**Found* µg mL^{-1}**	**Recovery %**
1 : 4 : 5	10.00	10.14	101.40
1 : 1 : 3	20.00	20.10	100.50
3 : 7 : 4	30.00	29.78	99.27
2 : 3 : 2	40.00	39.75	99.38
1 : 1 : 1	50.00	49.62	99.24
3 : 2 : 1	60.00	60.13	100.22
7 : 3 : 2	70.00	69.92	99.89
4 : 1 : 4	80.00	80.15	100.19
9 : 1 : 1	90.00	89.41	99.34
60 : 1 : 1	60.00	59.24	98.73
80 : 1 : 1	80.00	80.44	100.55
Mean ± SD	**99.88 ± 0.77**		

Table 2: Specificity results for the determination of CS in the laboratory prepared CS, OXY and CS alkaline degradate mixtures by the proposed method.

Parameter	**CS**
Accuracy (mean ± SD)	100.17 ± 1.04
Precision (SD)	
Repeatability[a]	0.25
Intermediate precision[b]	1.02
Specificity	99.88 ± 0.77
Range (µg mL^{-1})	1.00-100.00
Linearity	
Intercept	7306.6
slope	41662
Correlation coefficient	0.9999
LOD (µg mL^{-1})	0.07
LOQ (µg mL^{-1})	0.03
Robustness	1.34

[a]The intraday and [b]the interday standard deviations of samples of concentration 10.00, 30.00 & 60.00 µg mL^{-1} performed as triplicates.

Table 3: Results of assay validation parameters of the proposed method for the analysis of CS in pure form.

System suitability

System suitability testing was done in order to demonstrate that the instrumental system is performing properly. Calculations revealed good results as shown in Table 4.

Parameter	Value	Reference value
Capacity factor (K')	3.98	K' > 2
Tailing factor (T)	1.06	T ≤ 2
Number of theoretical plates (N)	1584	N > 2000
Height equivalent to theoretical plates (HETP)	0.16	The smaller the value, the higher the column efficiency
Selectivity*(α)	3.42	α > 1
Resolution**(R_S)	9.65	R_S > 2

*Selectivity was calculated according to the capacity factors of two successive peaks.

** Resolution was calculated according to the retention times of the drugs in two successive peaks.

Table 4: System suitability parameters for the analysis of CS using the proposed HPLC method.

Statistical analysis of the results

The proposed method was successfully used for the determination of the studied drug in its bulk powder and in its pharmaceutical formulations with good accuracy and precision.

The validity of the proposed method was checked by applying the standard addition technique. Results obtained are given in Table 5 which indicates that different additives with the studied drug did not interfere.

	HPLC method			**Standard addition technique**		
	Taken	**Found**	**Recovery***	**Added**	**Found**	**Recovery***
	µg mL^{-1}	**µg mL^{-1}**	**%**	**µg mL^{-1}**	**µg mL^{-1}**	**%**
Nazocrom® nasal spray labeled to contain 20 mg of CS per 1 mL	40	41.96	104.9	30	30.04	100.13
	40	42.1	105.25	40	39.77	99.43
	40	41.9	104.75	50	49.57	99.14
	Mean ± SD		104.97 ± 0.26	Mean ± SD		99.57 ± 0.51
Epicrom® eye drop labeled to contain 40 mg of CS per 1 mL	40	41.62	104.05	30	30.37	101.23
	40	41.55	103.88	40	39.82	99.55
	40	41.58	103.95	50	50.74	101.48
	Mean ± SD		103.96 ± 0.09	Mean ± SD		100.75 ± 1.05

*Average of three determinations

Table 5: Application of Standard addition technique on the pharmaceutical formulations of CS by the proposed method.

Statistical comparison showed that there is no significant difference between the results obtained from the proposed method and those obtained from the official method [26]. The proposed method was found to be accurate and precise since the t and F values are less than the tabulated ones as shown in Table 6.

	Average recovery of CS	
Parameter	**HPLC method**	**Official method[a]**
Mean	100.17	100.34
SD	1.04	0.67
Variance	1.08	0.45
n	10	6
t	0.37 (2.14)*	--
F	2.39 (4.77)*	--

*The values between parentheses are the corresponding theoretical values for **t** and **F** at the 95% confidence level.

[a]Direct spectrophotometric determination of Cromolyn sodium using pH 7.4 sodium phosphate buffer at the maximum absorbance 326.0 nm

Table 6: Statistical comparison between the results obtained by applying the proposed method and the official method[(14)] for the analysis of CS in pure form.

Conclusion

The proposed method offers distinct advantage in simplicity and sensitivity and could be easily used in quality control laboratory for the analysis of CS in its bulk powder and in its pharmaceutical formulations.

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Instrument

Analysis was performed on a chromatographic system Jasco LC-Net II/ADC (Japan) equipped with UV detector (UV-2070 plus), isocratic pump (PU-2080 plus) and 4-line degasser (DG-2080-54). A chromatographic separation was achieved by Inertsil C-18, 250 x 4.6mm, 5µ analytical column. Data acquisition was made with ChromNAV software.

Mass Spectrometer for structure elucidation of the degradation product

This is done by MS/MS detection in positive ion mode using a MDS Sciex (Foster City, CA, USA) API-3200 MS/MS triple quadrupole mass spectrometer, equipped with a Turbo ion spray interface at 350°C. The common parameters, nebulizer gas (GS1), heater gas (GS2) and collision activated dissociation gas (CAD), were set at 30, 40, and 5 psi, respectively. The compound parameters, declustering potential (DP), collision energy (CE) and collision exit potential (CXP) were set at 56, 31, and 12 V respectively. The analytical data were processed using Analyst software (version 1.4.2).

Procedure

Preparation of the basic degradation products of the studied drugs

BENZ and AML were subjected to basic degradation. 100 mg of each powder was weighed, transferred separately to two conical flasks. Then, 0.1 N NaOH was added separately on the two volumetric flasks. Then the flasks were refluxed for 0.5 hour for basic degradation. After the reflux, the conical flasks were neutralized against 0.1 N HCl, and then completed to 100 mL by distilled water. The complete degradation was confirmed using HPLC and mass spectrometry.

Chromatographic conditions

Separation of the two analytes along with the degradation products and the IS was accomplished using Inertsil C18 column (250 x 4.6 mm, 5 µ column) as a stationary phase. The mobile phase used was acetonitrile-potassium hydrogen phosphate buffer pH=7 adjusted by 0.1 N NaOH (40/60 v/v) was used as a mobile phase. The flow rate was 1 mL/min and the detection wavelength was 242 nm.

Preparation of standard stock and working solutions

Primary stock solutions of BENZ, AML, and MOX internal standard (IS) (all at 1.0 mg/mL) were separately prepared by dissolving 100 mg of each standard powder in the least amount of methanol and completed to the volume by distilled water, where BENZT was prepared by degradation [as explained earlier] . Primary stock solutions were diluted with the mobile phase for the RP-HPLC to prepare standard working solutions of BENZ, BENZT, AML, and MOX (100 µg/mL). All solutions were stored at 4◦C, and equilibrated to room temperature before use.

Linearity and Calibration standards of the pure bulk powder

Accurately measured aliquots of BENZ, BENZT, and AML were transferred from their working standard solution (0.1 mg/mL) into three series of 10-ml volumetric flasks and complete to volume with the mobile phase (acetonitrile-potassium hydrogen phosphate buffer (40/60 v/v) pH=7 adjusted by 0.1 N NaOH). The calibration samples consist of six concentrations of BENZ (0.5 – 100 µg/mL), BENZT (0.5-100), and AML (5–100 µg/mL). The samples were injected separately along with

MOX (IS) into the Inertsil C18 column under a flow rate of 1 mL/min. The relative peak area of each analyte was recorded against its concentration, the linearity curves were constructed and the regression equations computed.

Validation

Accuracy: The accuracy of the results was checked by applying the proposed methods for determination of three replicates of different concentrations of the analytes. The concentrations were obtained from the corresponding regression equations, from which the percentage recoveries suggested good accuracy of the proposed methods.

Precision

Repeatability: Three concentrations of the analytes were analyzed three times intra-daily using the proposed methods under the same experimental conditions. The relative standard deviations were calculated.

Reproducibility (Intermediate precision): The previous procedures were repeated inter-daily on three different days for the analysis of the three chosen concentrations. The relative standard deviations were calculated.

Laboratory prepared mixtures (Selectivity): Solutions containing different ratios of the analytes were prepared by transferring accurately measured aliquots from their standard working solutions into a series of 10-ml volumetric flasks and the volume was completed to the mark with mobile phase. The final concentration ranges were 5 - 20 µg/ml for BENZ, 10 - 25 µg/ml for BENZT, and 5-25 for AML. The chromatograms of these different laboratory prepared mixtures were recorded and the procedure under linearity was then followed. Concentrations of the analytes in the prepared samples were calculated from the corresponding computed regression equations.

Application to pharmaceutical formulation

To determine the content of BENZ and AML in commercial capsuless (Loadless®) (each capsule labeled to contain 20 mg BENZ and 5 mg AML), 20 capsules were weighed and finely powdered. A portion of powder equivalent to one capsule was weighed accurately and transferred to a 100-ml beaker. 50 ml of methanol was added, stirred using a magnetic stirrer for 15 min and filtered through 0.5µm Whatman filter paper into a 100-ml volumetric flask. The residue was washed three times each with 10 ml of methanol and the solution was completed to the mark with the same solvent. From the above prepared solution, further dilutions were prepared in the obtained linearity ranges using mobile phase. The general procedure described under linearity was followed to determine the concentration of both drugs in the prepared dosage form solution. The analysis was done in triplicates. Concentrations of BENZ and AML in the prepared samples were calculated from the corresponding computed regression equations.

Application to spiked human plasma sample

Aliquot volumes from the standard working solutions of BENZ, BENZT, and AML were added on 500 µL human plasma, then 30 µL IS was added. Then, 3 mL of acetonitrile was added, the samples were mixed on a vortex for 1 min, followed by centrifugation for 10 min at 10,000 rpm. The organic phase solution was transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen gas at 40 ºC. The residue was reconstituted with 100 µL mobile phase, and 20 µL was injected. Then, the same procedure was repeated but spiking was done after the extraction step to calculate the recovery %.

Results and Discussion

This manuscript describes the use of a RP-HPLC method to quantify BENZ, its active metabolite BENZT, and AML in the pharmaceutical dosage form. Also the paper describes the application of the proposed method to determine the three analytes in spiked human plasma without interference form endogenous plasma constituents. All the determinations were done in a short run time and with high sensitivity.

Method development

Different organic modifiers proportions along with different buffers with different pH were tried. Our goal is to obtain optimum resolution, symmetric peak shape, reasonable run time, and best sensitivity. Methanol and acetonitrile were tried, methanol caused longer run time where the run time extended to more than 20 min. Phosphate buffer with different pH's (3, 5, 7) were tried. The optimum resolution and peak shape obtained with acetonitrile-potassium hydrogen phosphate buffer pH=7 adjusted by 0.1 N NaOH (40/60 v/v) as a mobile phase. The flow rate for better resolution and rapid separation was adjusted to 1mL/min. Also, two types of stationary phases C8 and C18 were investigated for the optimum resolution of the analytes' peaks, however the more hydrophobic Inertsil C18 was found to be more superior in separating analytes' peaks in a reasonable run time. Full separation of the three peaks of BENZ, BENZT, and AML was obtained, where the retention time of the analytes was 6.3, 2.4, and 7.8 min., respectively, where the retention time for MOX (IS) was 5.3 min. as shown in Figure 2. System suitability parameters are shown in Table 1.

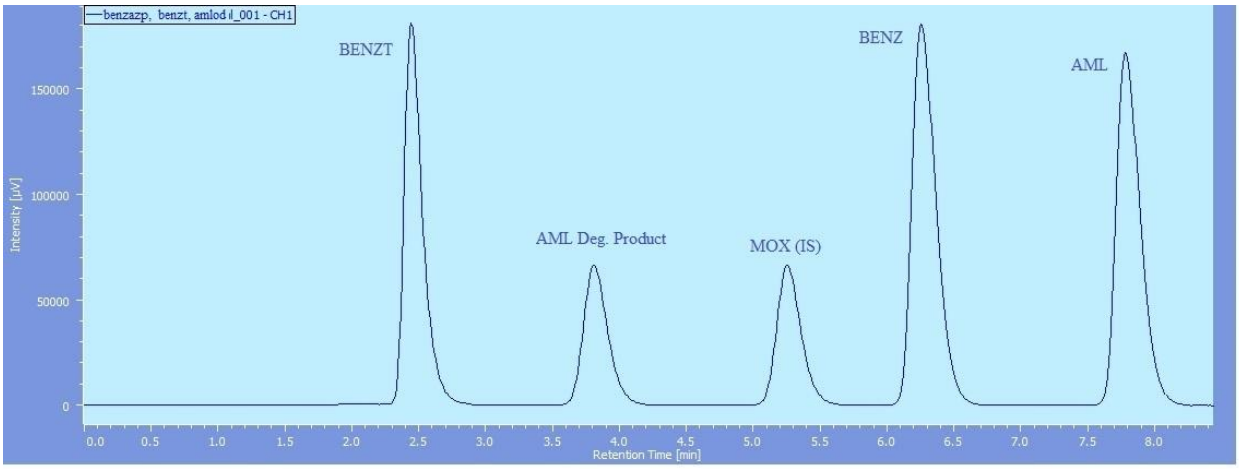

Figure 2: HPLC chromatogram showing the complete resolution of BENZ, BENZT, and AML (80 ug/mL each) with MOX (IS) and AML deg. Product.

Parameter	Benazepril	Benzeprilat	Amlodipine
Retention time	6.3	2.4	7.8
Resolution*	3.5	4.2	———
Tailing factor	1.2	1.2	1.3
Column Capacity	4.73	1.18	6.09
Column effeciency (No. of theoritical plates)	6879	1955	6348
HETP	0.004	0.013	0.004

* Resolution is calculated relative to the next peak.

Table 1: System suitability parameters of the proposed RP-HPLC method.

Stability of BENZ and AML

BENZ with a molecular weight (424.5) is an ester pro-drug which is hydrolyzed in vivo by esterase enzyme to the pharmacologically active biacid BENZT with a molecular weight (396). So to obtain this active form, Hydrolysis of BENZ was tried by exposing to 0.1 N NaOH and refluxing for 0.5 hour for basic degradation. Also complete degradation of AML was done by the same method. AML has a molecular waight of (408.8) where its basic degradation has a molecular weight (366). The complete degradation was confirmed by HPLC (with the disappearance of BENZ and AML peaks at retention times 6.3, and 7.8 min, respectively. and appearance of the BENZT and basic degradation product of AML peaks at retention times 2.4, and 3.8 min., respectively. (Figure 3a and 3b), and mass spectrometry (with the appearance of m/z for BENZT and degradation product of AML at 397 and 367.2, respectively, and disappearance of m/z for BENZ and AML at 425.2 and 409.2, respectively) as shown in (Figure 4a and 4b).

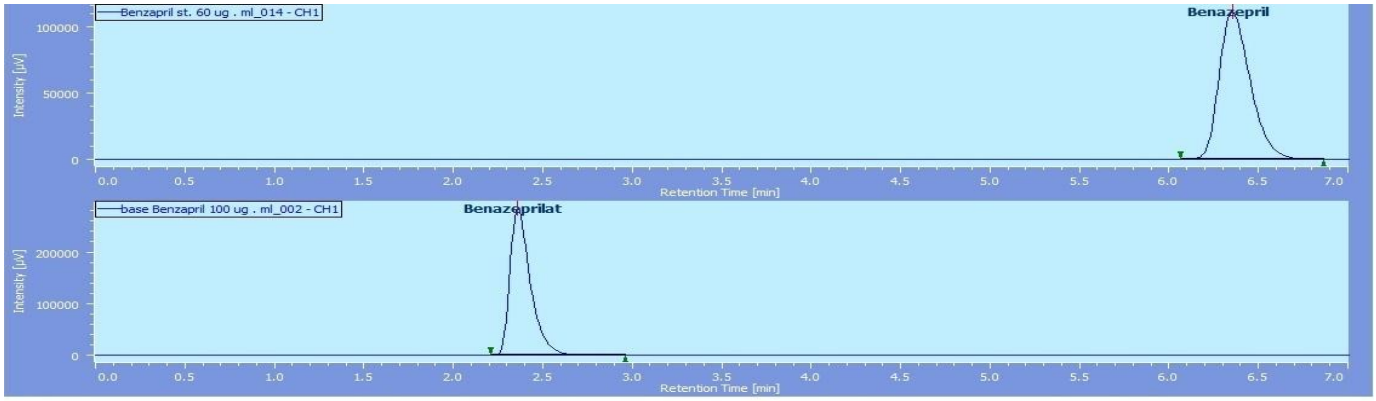

Figure 3a: HPLC chromatograms of BENZ (60 ug/mL) and BENZT (100ug/mL) confirming the complete degradation of BENZ by basic and acidic hydrolysis.

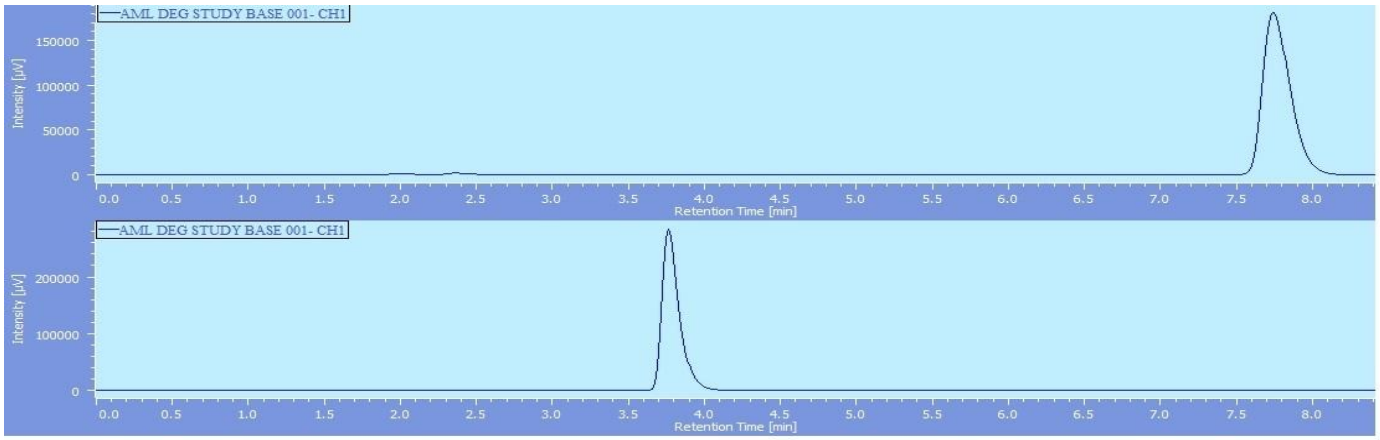

Figure 3b: HPLC chromatograms of AML (80 ug/mL) and AML basic degradation product, confirming the complete degradation of AML by basic hydrolysis.

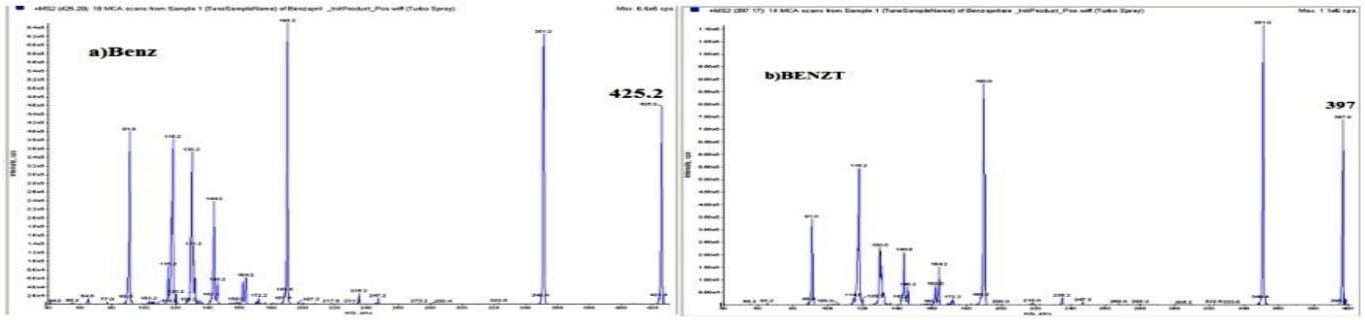

Figure 4a: MS spectra of a)BENZ and b)BENZT confirming the complete degradation by basic hydrolysis.

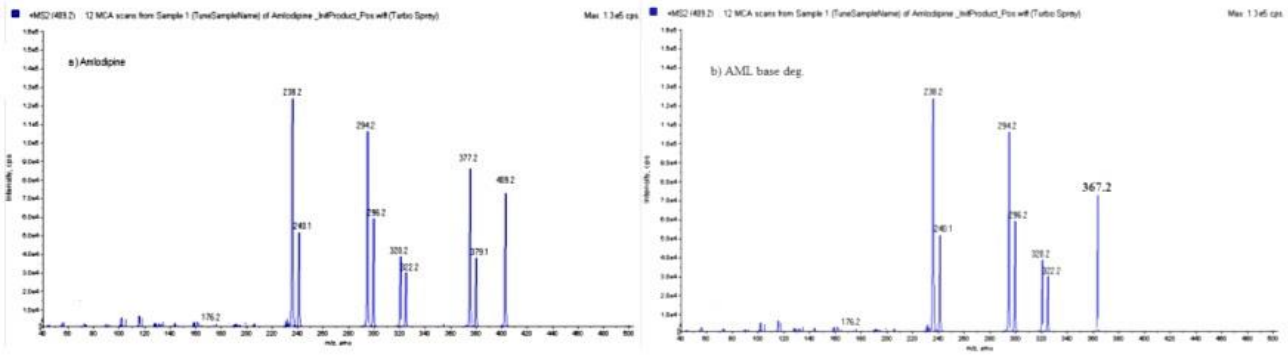

Figure 4b: MS spectra of a)AML and b) AML basic degaradtion product confirming the complete degradation by basic hydrolysis.

The cell wall of algae consists of chitin, lipids, polysaccharides and proteins. These macromolecules provide different functional groups, such as thiother, carboxyl, imidazole, hydroxyl, carbonyl, phosphate, phenolic, etc., which can form coordination complexes with heavy inorganic heavy metals [8,10].

The typical method of optimization involves varying one independent variable while maintaining all others at a fixed level which require to very long times. To overcome this difficulty, experimental factorial design and response methodology can be employed to optimize the biosorption of inorganic heavy metal. For better understanding of different stages of biosorption at varying heavy metal concentration, pH and sorbent dosages, RSM was used to optimize inorganic heavy metal uptake [11].

Response surface methodology (RSM) is an empirical statistical technique employed for multiple regression analysis of quantitative data obtained from statistically designed experiments by solving the multivariate equations simultaneously. The graphical representation of these equations are called as response surfaces, could be used to describe the individual and cumulative effect of the test variables on the response and to determine the mutual interaction between the test variables and their subsequent effect on the response [12-14].

In this study the effects of cooperating parameters on the lead (II) biosorption are described using response surface methodology (RSM). Four parameters namely, contact time, pH, temperature and biomass dose were taken as input variables in a Box-Behnken model and 27 experiments were performed to obtain the data. Subsequently, theoretical values were obtained for the best conditions for removal of lead [15,16].

Materials and Methods

Reagents

99.27% of levocetirizine working standard and 98.92% pseudoephedrine were obtained purchased from Sigma, UK. Combined tablet formulation Levocetirizine HCl 5 mg and Pseudoephedrine HCl 120 brand Verizet D, Manufactured by Cipla pharmaceuticals. Acetonitrile and water (HPLC grade) were obtained from Merck, Darmstadt, Germany. Analytical grade Phosphoric acid, potassium dihydrogen phosphate and ortho phosphate used were from Merck, Darmstadt, Germany. perrmanganate [37-41], ammonium metavanidate [42-44], chromium trioxide, and potassium dichromate[45,46]. None of these reagents have not been previously used for the spectrophotmetric analysis of neither acyclovir nor amantadine HCl. For these reasons, the present study was dedicated to investigate the application of these reagents in the spectrophotometric analysis of both drugs in their pharmaceutical dosage forms. of infection[4], as well as in the management of herpes zoster[1]. Amantadine has mild antiparkinsonism activity and also has been used in the management parkinsonism of mainly in early disease stage when symptoms are mild. Amantadine is usually given by mouth as the hydrochloride salt[1]. The

therapeutic importance of acyclovir and amantadine HCl has promoted the development of many analytical methods for their quantitative determination. These methods include high-performance liquid chromatography (HPLC)[5-18], micellar liquid chromatography[19], gas chromatography[20,21], capillary electrophoresis [22-23], radioimmunoassay [24], and potentiometry [25]. Most of these methods were devoted to biological fluids like plasma[5-7], serum[8], and/or urine[9]. Although some HPLC methods have been applied for the determination of acyclovir and amantadine HCl in their pharmaceutical dosage forms[15-18], however the procedures lack the sensitivity [15], besides being tedious and difficult to perform[16], or require selective and expensive detectors, which could not be available in many laboratories[17]. Spectrophotometric and fluorimetric analysis are considered more convenient alternative techniques because of their inherent simplicity and high sensitivity. Since acyclovir contains a weakly absorbing chromophore, and amantadine has no any light-absorbing chromophores in their molecules, few spectrophotometric methods[26-30] have been reported for their determination. These methods were laborious, time consuming, or/and require derivatization of the drug. Therefore, our laboratory involved to develop new simple spectrophotometric and fluorimetric methods that overcome these drawbacks. In our previous report [32].

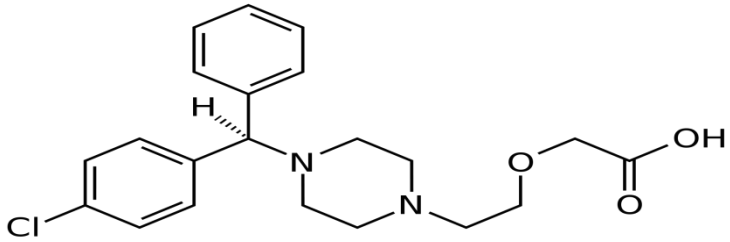

Figure 1: Levocetirizine Chemical structure.

the development of a simple fluorimetric method for determination of acyclovir and amantadine HCl was described. The present study was dedicated to the development of new simple spectrophotometric methods for determination of both drugs in their pharmaceutical dosage forms. Redox reactions have been used as the basis for the development of simple and sensitive spectrophotometric methods for the determination of many pharmaceutical compounds [33-46]. For these reasons, these reactions were attempted for use in the present study. This proposal was promoted by our previous report [32] that described the susceptibility of acyclovir and amantadine HCl for oxidation. In oxidimetric reactions, the most commonly used oxidizing agents are ceric ammonium sulphate [34-36], potassium permanganate [37-41], ammonium metavanidate [42-44], chromium trioxide, and potassium dichromate[45,46]. None of these reagents have not been previously used for the spectrophotmetric analysis of neither acyclovir nor amantadine HCl. For these reasons, the present study was dedicated to investigate the application of these reagents in the spectrophotometric analysis of both drugs in their pharmaceutical dosage forms.

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Table 1: HPLC linearity data for Levocetirizine.

Sno	Concentration (µG/Ml)	Peak Area
1	5	17233
2	10	34194
3	15	50814
4	20	66559
5	25	81069

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