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Development and Validation of RP-HPLC Stability-Indicating Methods for the Determination of Butamirate Citrate and Sodium Cromoglycate

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ABSTRACT

*Two novel high performance liquid chromatographic (HPLC) methods are presented for the determination of butamirate citrate (**BC**) and sodium cromoglycate (**NaCr**) in the presence of or with their degradation products. The first method was based on RP-HPLC separation of BC from its degradation product using an Agilent Zorbax ODS (C18) column with a mobile phase consisting of methanol: acetonitrile: water (100: 75: 25, v/v/v) adjusted with triethylamine to pH 9.8±0.1 with UV detection at 225 nm and using clobutinol hydrochloride (**CH**) as internal standard. The second method was based on HPLC separation of NaCr from its degradation product using an Agilent Zorbax ODS (C18) column with a mobile phase consisting of methanol: water (75: 25, v/v) using metronidazole (**MT**) as internal standard. Quantification was achieved with UV detection at 254 nm. The developed methods were applied for the determination of BC or NaCr in their pharmaceutical formulations. Both methods proved stability indicating power but in the second one, the degradation product could be evaluated along with the intact **NaCr**. The developed methods were validated as per ICH guidelines and were also; suitable for assay determination of the cited drugs in pharmaceutical dosage forms.*

Keywords: butamirate citrate, sodium cromoglycate, HPLC, degradation product.

INTRODUCTION

Butamirate citrate (**BC**), 2-(2-diethylaminoethoxy) ethyl-2-phenylbutyrate dihydrogen citrate, is widely used as a central cough suppressant for non productive cough [1, 2]. Literature survey reveals that the number of the analytical methods referring to the drug is rather limited. The drug has been determined by derivative spectrophotometric method in the presence of sodium benzoate [3]. Malliou et al. described derivative spectrophotometric and HPLC methods for the determination of the drug in cough syrups [4]. Enantioseparation of ***BC*** was investigated by electrophoresis [5].

Sodium cromoglycate, disodium 4,4'-dioxo-5,5'-(2-hydroxytrimethylenedioxy) di(4H-chromene-2-carboxylate), is believed to act primarily by preventing release of mediators of inflammation from sensitized mast cells through stabilization of mast cell membranes. It is used in the prophylaxis of many types of asthma [1, 2]. The official methods for the determination of ***NaCr*** are non aqueous titration using standard perchloric acid as titrant [6] and spectrophotometry in pure form and different pharmaceutical formulations using standard solutions [7] and A(1%, 1 cm) as the reference standard [8].

The drug has been also determined by spectrophotometric [9, 10], TLC-densitometric methods [11, 12] and also; electrochemical methods [13, 14]. Several HPLC methods were described for the determination of ***NaCr*** using UV detection [15-18], fluorescence detection [19] or tandem mass spectrometric detection [20-22]. Simultaneous determination of ***NaCr*** and salbutamol sulphate using HPLC was also adopted [23].

Literature survey revealed also published RP-HPLC assays for ***NaCr*** in presence of its related substances; one HPLC method was described for the analysis of ***NaCr*** in the presence of 3 related substances which are: 1-(2,6-dihydroxyphenyl)-ethanone (***A***), 1-{2-[3-(2-Acetyl-3-hydroxy-phenoxy)-2-hydroxy-propoxy]-6-hydroxy-phenyl}-ethanone (***B***), and diethyl cromolyn (***C***). Compounds were eluted on a C18 column using cetyl trimethylammonium bromide: acetonitrile: water (1g: 1l: 1l, w/v/v) as the mobile phase for drug assay, and (0.376 g: 920 ml: 1080 ml, w/v/v) as that for related compounds [24]. Another HPLC assay method was developed for the determination of ***NaCr*** in presence of 2 related substances: ***B*** and ***C***. Using a Nova-Pak C8 column and a mobile phase of methanol: tetrabutyl ammonium dihydrogen phosphate buffer (45:55, v/v), ***NaCr*** and ***B*** eluted at 6.2 and 47.5 minutes, respectively, with an obvious large retention time difference between the drug and its impurities. Furthermore, the authors mentioned that on using the same mobile phase on C18 column, ***B*** and ***C*** did not elute from the column up to more than 90 minutes [25]. For this reason, the same authors tried to shorten the chromatographic run time by developing another method consisting of Zorbax C8 column and a mobile phase consisting of myristyl trimethylammonium bromide buffer pH 6.5: methanol (45:55, v/v). Using these conditions, ***NaCr*** and ***B*** eluted at 9.5 and 5 minutes, respectively [26]. The most common stationary phases used are octadecyldimethyl (C18) phases with silica as the solid support [27-30].Thus, it was deemed useful to develop a new HPLC method with a simpler mobile phase composition and reasonable retention times for the drug and its degradation product.

EXPERIMENTAL SECTION

2.1. Instrumentation

A chromatographic system consisting of Agilent 1100 series; interface was equipped with an Agilent isocratic pump G1310A, Agilent UV-visible detector G1314A, an Agilent manual injector G1328B equipped with (20 µl) injector loop, separation and quantitation were made on Agilent Zorbax ODS column (C18) 5µm, 4.6 x 250 mm. Agilent syringe, LC 100 µl (CA, U.S.A.).

Infrared spectra were recorded on Bruker FT-IR spectrophotometer Vector 22 (Billerica MA, USA) as potassium bromide discs.

The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on Varian Mercury spectrophotometer (IL, USA) at 300 MHz.

Mass spectra were performed on Schimadzu GCMS-QP1000EX mass spectrophotometer (Kyoto, Japan).

2.2. Materials and reagents

BC was kindly donated by Novartis Pharma Company; Cairo, Egypt. Purity was 99.84±0.415 (n=6)according to the comparison method. ***NaCr*** was kindly donated by Amoun Pharmaceutical Company; Al-Obour City, Egypt. Purity was 99.79±0.722 (n=6) according to the comparison method.

2.3. Pharmaceutical preparations

Sinecod® syrup (batch no. Y0012, containing 1.5 mg/ml of **BC**) of Novartis Pharma Company; Cairo, Egypt under license from Novartis Pharma AG., Basle, Switzerland was purchased from the market.

Nasotal® drops (batch number 2205, containing 2g **NaCr** per 100ml) of Amoun Pharmaceutical Company; Al-Obour City, Egypt was purchased from the market.

2.4. Chromatographic conditions

The mobile phase (A) used in the assay of **BC** was prepared by mixing methanol: acetonitrile: water (100:75:25, v/v/v) adjusted with triethylamine to pH 9.8 ±0.1. Quantification was carried out with a flow rate 1 ml/min, at ambient temperature and UV detection at 225 nm.

The mobile phase (B) used in the assay of **NaCr** was prepared by mixing methanol: water (75:25, v/v). Quantification was carried out with a flow rate 1.2 ml/min, at ambient temperature and UV detection at 254 nm.

2.5. Preparation of 2-phenylbutyric acid (PBA)

BC (500 mg) was refluxed with 0.1M sodium hydroxide (50 ml) at 100^{o}C for 10 hr. The reaction was followed till complete disappearance of **BC** with thin layer chromatography using chloroform: methanol (9:1, v/v) as the developing system. Subsequently, the solution was acidified using 1M hydrochloric acid and extracted with chloroform (4 x 10 ml). The aqueous layer was discarded. The combined chloroform extracts were dried over anhydrous sodium sulphate and evaporated to dryness to yield white precipitate of 2-phenylbutyric acid (**PBA**) (m.p.= 46 ^{o}C (reported 39-42 [31]). The stock solution of **PBA** was prepared by dissolving in the mobile phase (A) to prepare solution (1mg ml^{-1}).

2.6. Preparation of 1-{2-[3-(2-Acetyl-3-hydroxy-phenoxy)-2-hydroxy propoxy]-6-hydroxy-phenyl}-ethanone (Deg1)(previously mentioned B)

NaCr (500 mg) was refluxed with 10% aqueous potassium hydroxide (10 ml) for 2 hr. Subsequently, the solution was acidified with glacial acetic acid until complete precipitation of the degradation product (m.p. = 165^{o}C (as reported [32]). The precipitate was filtered and dried under vacuum. The stock solution of **Deg1** was prepared by dissolving in the mobile phase (B) to prepare solution (0.6mg ml^{-1}).

2.7. Preparation of 4-(2-{3-[2-(3-Carboxy-3-oxo-propionyl)-3-hydroxy-phenoxy]-2-hydroxy-propoxy}-6-hydroxy-phenyl)-2,4-dioxo-butyric acid tetrasodium salt (Deg2)

NaCr (500 mg) was heated with 10% alcoholic sodium hydroxide (10 ml) at 70^{o}C for 1 hr. Subsequently, the solution was carefully treated with glacial acetic acid to pH = 7.5. An orange yellow solid was precipitated, filtered and dried. The product (**Deg2**) was purified by column

chromatography using silica gel as the stationary phase and gradient elution of chloroform: methanol as the mobile phase.

2.8. Standard solutions and calibration graphs

Stock solutions of **BC** and **CH** were prepared by dissolving each compound in the mobile phase (A) to obtain concentrations of 2 mg ml^{-1} and 0.15 mg ml^{-1}, respectively. Standard solutions of **BC** were prepared by dilution of accurately measured aliquots of **BC** stock solution with the mobile phase (A), after the addition of **CH** stock solution (1ml), to reach concentration range of 100-1200 μg ml^{-1}.

Stock solutions of **NaCr** and **MT** were prepared by dissolving each compound in the mobile phase (B) to obtain concentrations of 2mg ml^{-1} and 0.8mg ml^{-1}, respectively. Standard solutions of **NaCr** and **Deg1** were prepared by dilution of accurately measured aliquots of **NaCr** and **Deg1** stock solutions, respectively, with the mobile phase (A), after the addition of **MT** stock solution (1ml), to reach concentration range of 100-600 μg ml^{-1} for **NaCr** and 30-180 μg ml^{-1} for **Deg1**.

Triplicate 20 μl injections were made for each concentration and chromatographed under the specified conditions described previously. The ratios (R) of the recorded AUPs of each compound to that of the corresponding internal standard were plotted versus the corresponding concentrations.

2.9. Sample preparation

2.9.1. For Sinecod syrup

A volume of syrup equivalent to 30 mg **BC** was introduced into a 50 ml volumetric flask and diluted to volume with the mobile phase (A) to reach concentration of 0.6 mg ml^{-1}. Aliquots equivalent to **BC** (1.44- 4.8 mg) were introduced into a series of 10 ml volumetric flasks. The general procedure for HPLC method of **BC** described under calibration was followed.

2.9.2. For Nasotal drops

A volume of nasal drops equivalent to 100 mg **NaCr** was introduced into a 50 ml volumetric flask and diluted to volume with the mobile phase (B) to obtain **NaCr** solution (2 mg ml^{-1}). Aliquots equivalent to **NaCr** (1.2- 3.6 mg) were introduced into a series of 10 ml volumetric flasks. The general procedure for HPLC method of **NaCr** described under calibration was followed.

2.10. Validation of the methods

Methods validation was carried out in accordance to ICH guidelines [33].

2.10.1. Optimization of the chromatographic conditions

The system suitability tests were used to verify that the chromatographic systems used are adequate for the analysis. Tests include number of theoretical plates, resolution, relative retention and capacity factor; all were calculated for each chromatographic system.

2.10.2. Linearity and range

The methods were validated as described under standard solutions and calibration graphs. The calibration range was established within the protocol range necessary, according to each drug concentration present in the pharmaceutical product.

2.10.3. Specificity and accuracy

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences including degradation products and related substances. Specificity was checked

by analyzing **BC** in presence of **PBA** in laboratory prepared binary mixtures and also **NaCr** and **Deg1**. Complete peak separation was achieved and laboratory prepared mixtures were used to determine the cited drugs in presence of/and their degradation products. The concentrations of the samples used to determine the accuracy were 240-1200 μg ml^{-1} for **BC** in presence of 50-100 μg ml^{-1} for **BPA** where it was 360-600 μg ml^{-1} for **NaCr** and 48-138 μg ml^{-1} for **Deg1**. Moreover, the standard addition technique was applied to assess the methods accuracy.

2.10.4. Precision

The %RSD of assay of **BC**, **NaCr** and **Deg1** were calculated using 6 injections. The intra-day and inter-day variability was determined by repeated injections of three different concentrations in triplicate for 3 days.

2.10.5. LOD and LOQ

LOD and LOQ were determined on the basis of standard deviation (σ) of the response and slope (S) of the calibration curve using 3.3 σ/S and 10 σ/S, respectively. (Values are the averages of 6 experiments).

2.10.6. Robustness

The robustness of the methods was checked by varying the pH of the mobile phase by ± 0.1% and its organic strength by ±0.2%.

RESULTS AND DISCUSSION

3.1. Identification of the degradation products

3.1.1. Identification of PBA

BC and oxeladin citrate (**OC**) are ester type drugs susceptible to hydrolysis. As reviewed in literature, possible ester hydrolysis of **OC** was achieved by refluxing the drug in alkaline aqueous solution for 3 hr [34]. Following the same published procedure, **BC** was boiled with 0.1M sodium hydroxide Fig. (1). Reaction was followed using thin layer chromatography till complete disappearance of **BC** spot. Complete degradation reaction required reflux for 10 hr. The assignment of the degradation product as 2-phenylbutyric acid (**PBA**) was based on spectral analyses. The mass spectrum of **PBA** showed a peak at m/z 164 representing the molecular ion peak Fig. (2). The IR spectrum of **PBA** lacked the characteristic ester C=O stretching band of **BC** at 1733.2 cm^{-1}. The ^{1}H-NMR spectrum of **PBA** was characterized by the presence of the aromatic protons at δ 7.31 ppm, protons of the ethyl chain at δ 0.93, 1.83 and 2.12 ppm, and benzylic proton at δ 3.48 ppm. Moreover, the spectrum lacked the characteristic signals of 2-(diethylamino) ethoxyethyl chain protons Fig. (3).

3.1.2. Identification of Deg1

According to Spath and Gruber method, khellin, which is a naturally occurring chromone compound, can be hydrolyzed in 1% aqueous potassium hydroxide to yield khellinone [35-37]. Consequently, a successful attempt has been carried out for the preparation of ***Deg1*** from ***NaCr*** through the application of the aforementioned Spath and Gruber method based on the similarity of the chromone nucleus in both compounds (***NaCr*** and ***khellin***) Fig. (4). The formation of ***Deg1*** could be confirmed by the UV spectrum of its alkaline solution which revealed disappearance of the characteristic absorption band of ***NaCr*** at 326 nm and appearance of other two absorption bands at 282 and 330 nm (as reported [9]). Fig. (5)

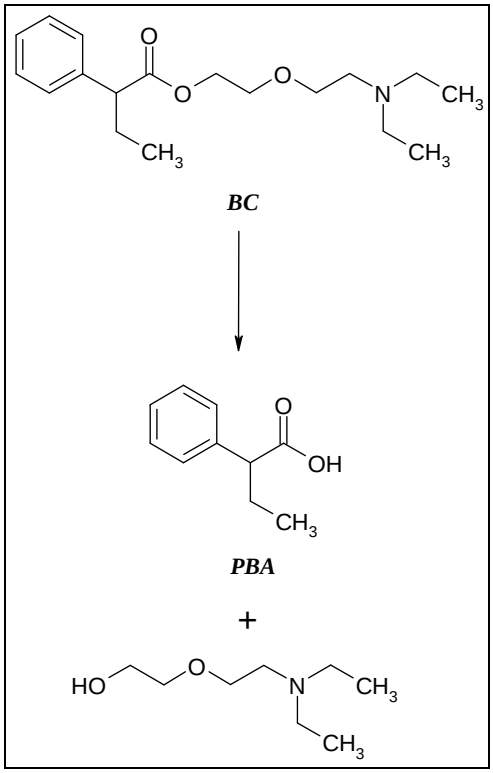

Figure (1): *BC* degradation scheme.

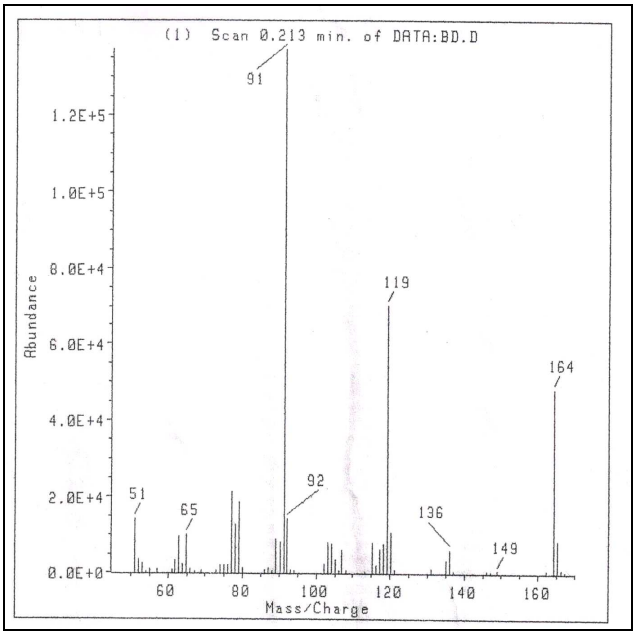

Figure (2): Mass spectrum of 2-phenylbutyric acid (*PBA*).

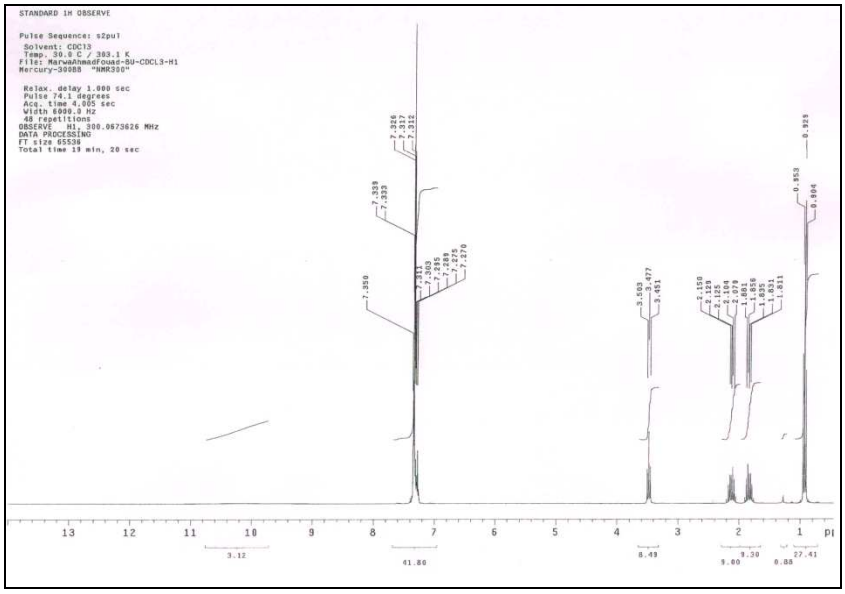

Figure (3): 1H-NMR spectrum of 2-phenylbutyric acid (*PBA*).

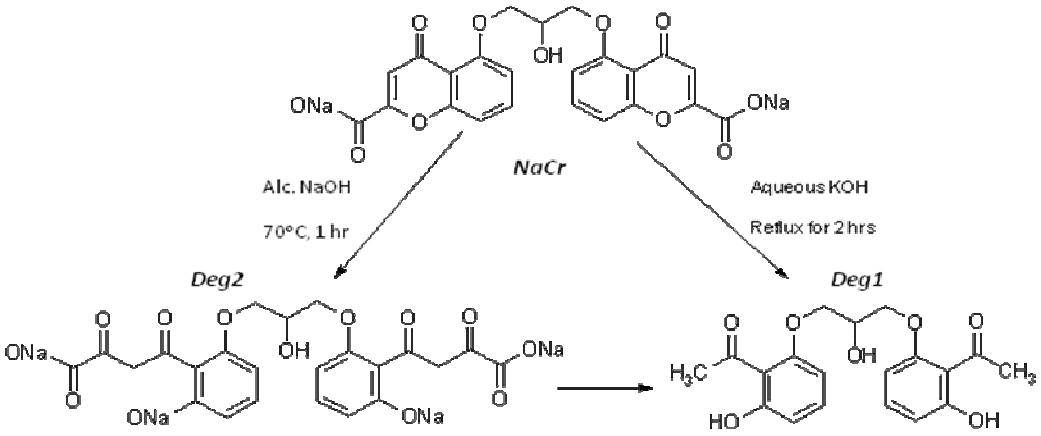

Figure (4): *NaCr* degradation scheme.

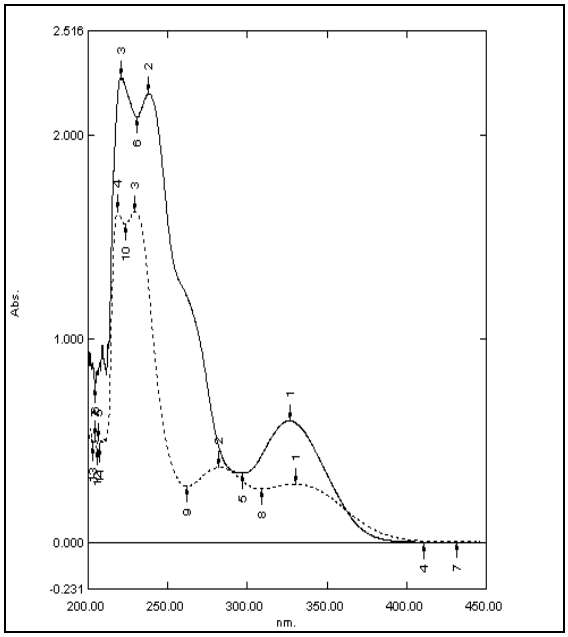

Figure (5): UV absorption spectra of *NaCr* ____ and *Deg1* …… in 0.1M sodium hydroxide.

3.1.3. Identification of Deg2

A thorough look in literature revealed the presence of a general method for carrying out partial degradation of the chromone ring using alcoholic and not aqueous alkaline solution [38-40]. Based on this knowledge, this method was successfully applied to ***NaCr*** in order to prepare ***Deg2*** Fig. (4).Careful acidification of the solution has to be carried out as ***Deg2*** is unstable in acidic medium leading to ring closure to NaCr again [9]. UV spectrum of ***Deg2*** in alkaline pH shows hypsochromic shift to 310 nm (***NaCr*** has an absorption maximum at 326 nm) with an increase in absorption intensity, while in neutral pH, the strong band at 310 nm disappears and is replaced by two weaker bands at 267 and 330 nm (as reported [9]) Fig. (6).IR spectra of ***NaCr*** and ***Deg2*** were not able to differentiate between the two compounds because the OH stretching and CO stretching bands appear nearly at the same frequency (***NaCr*** 3394, 1638 cm^{-1}) (***Deg2*** 3397, 1633 cm^{-1}). Mass spectrum of ***Deg2*** showed a peak at m/z 592 corresponding to molecular ion peak, Fig. (7).

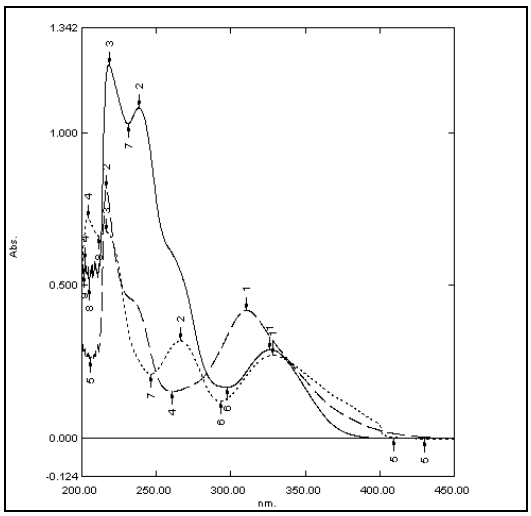

Figure (6): UV absorption spectra of *NaCr* in 0.1M sodium hydroxide _____ , *Deg2* in 0.1M sodium hydroxide --------- and *Deg2* in methanol …….. .

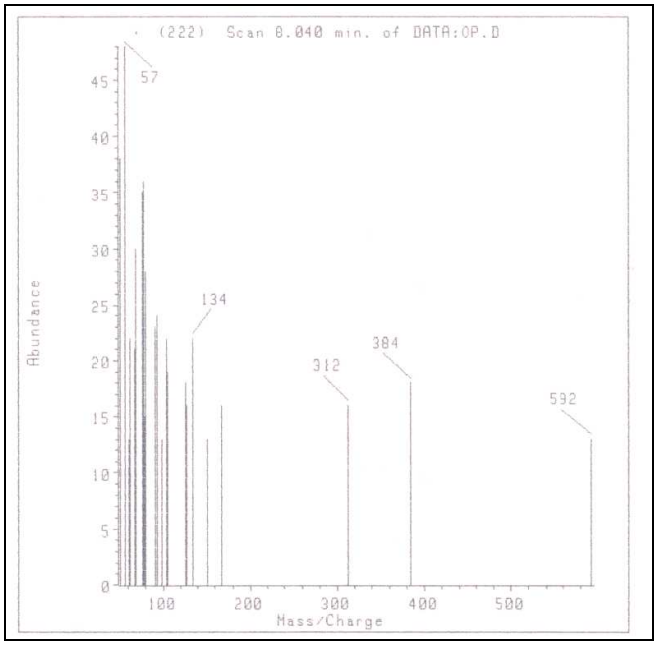

Figure (7): Mass spectrum of *Deg2*

3.2. Method development and optimization

3.2.1. For BC and PBA

One method for the determination of ***BC*** by HPLC was described. A mobile phase, containing tetraethylammonium hydrogen sulfate, methanol and acetonitrile adjusted to pH 3.5, was used to separate ***BC*** (t_R= 8.26 min) in cough preparation, "Sinecod" syrup, using Lichrosorb RP-18 column and UV detection at 258 nm [4]. Another HPLC method was described in literature to separate oxeladin citrate; which possess high structure similarity with the drug under investigation, from its alkaline degradation product. This method simply uses a mobile phase consisting of acetonitrile: 0.1% phosphoric acid (60:40, v/v) and a Nucleosil C18 column at 220 nm UV detection [34]. This mobile phase was prepared, overall pH was measured and it was found to be 2.38. Thus, it was concluded from both methods that acidic pH was necessary for the drug elution from the RP-stationary phase and to separate it from its alkaline degradation product. Many trials have been performed with different solvents in various proportions at acidic pH-values to reach the optimum stationary/mobile phase matching. Acidity was adjusted using either phosphoric acid, glacial acetic acid or ammonium acetate/acetic acid buffer. The chosen mobile phase was water: methanol (25:75, v/v) adjusted to pH= 4.58 using phosphoric acid Fig. (8). Nevertheless, all these attempts failed to resolve ***BC*** from its syrup components, thus, it was decided to determine the drug in cough preparation using syrup extract, prepared by extracting the butamirate base from other excipients after alkalinisation of the syrup. Injecting this extract produced blank chromatogram from which we could conclude that the peak eluted by injecting ***BC*** was actually the peak of the drug counter ion, citric acid (***CA***). This finding was supported by injecting citric acid solution which eluted at the same retention time (t_R= 2.554 min) Fig. (9).

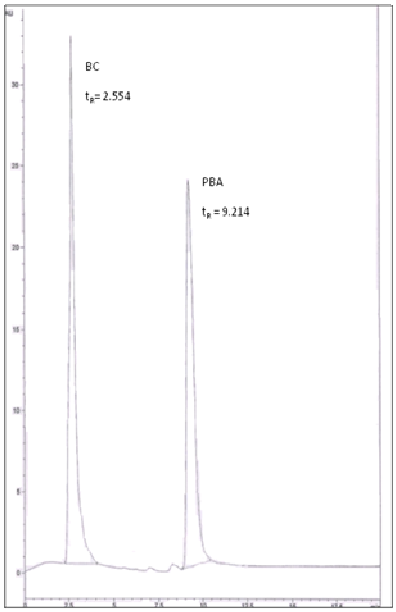

Figure (8): Retention time of butamirate citrate (*BC*), 2-phenylbutyric acid (*PBA*) using water: methanol (25: 75, v/v) adjusted with phosphoric acid to pH 4.58 at a flow rate of 2 ml/min and UV detection at 225 nm.

Several mobile phase compositions with different alkaline pH-values were employed. A satisfactory separation was obtained with a mobile phase composed of methanol: acetonitrile: water (100:75:25, v/v/v) adjusted with triethylamine to pH 9.8 ±0.1 as these excipients (saccharin sodium and vanillin) showed good separation of butamirate base and ***PBA***. The internal standard, ***CH,*** was chosen as it eluted at reasonable retention time and showed good separation from mixture components Fig. (10). Injection of "Sinecod" syrup in mobile phase revealed separation of syrup excipients from ***BC*** but it showed complete overlap with the

degradation product; which prevents its determination in the pharmaceutical formulation Fig. (11). Due to the low UV sensitivity of ***BC***, a full study was performed to choose optimum wavelength of detection and flow rate based on capacity factor and peak area. This study revealed that the best capacity factor and peak area were obtained using UV detection at 225 nm and a flow rate of 1 ml/min (Table 1).

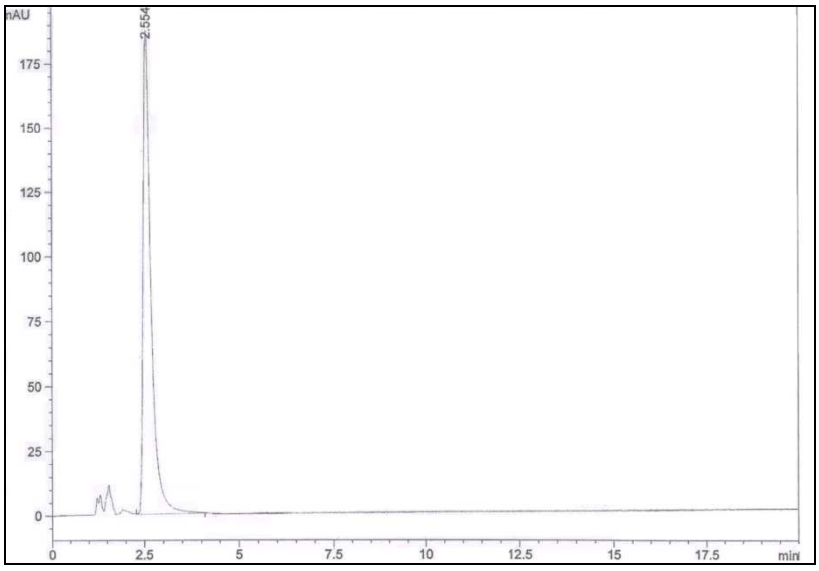

Figure (9): Retention time of citric acid using water: methanol (25:75, v/v) adjusted with phosphoric acid to pH 4.58 at a flow rate of 2 ml/min and UV detection at 225 nm.

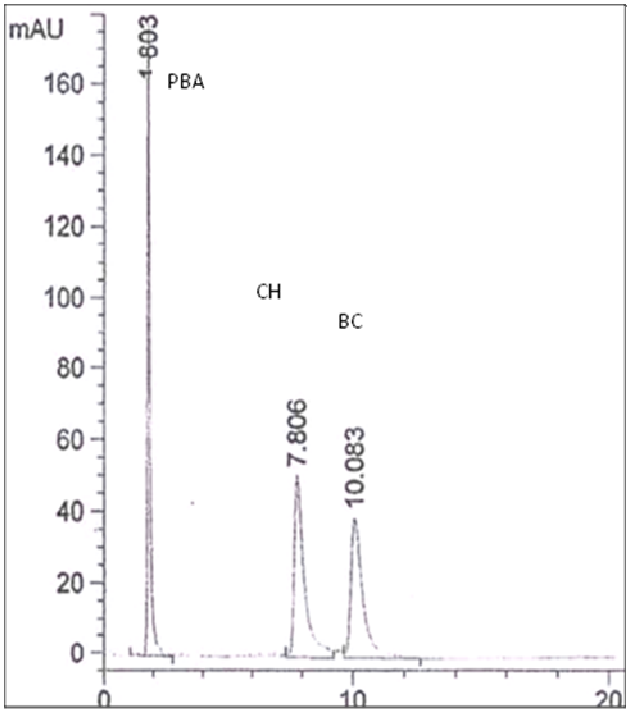

Figure (10): The chromatogram of *BC, CH* and *PBA* using methanol: acetonitrile: water (100:75 25, v/v/v) adjusted with triethylamine to pH 9.8 ± 0.1 at a flow rate of 1 ml/min and UV detection at 225 nm.

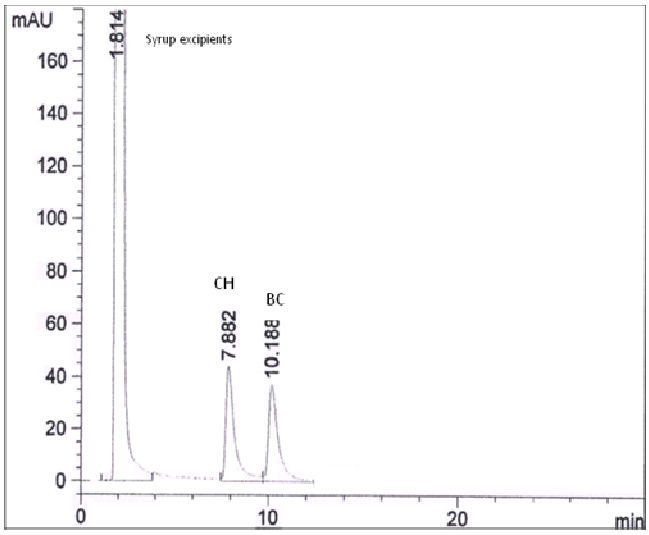

Figure (11): Retention time of syrup excipients, clobutinol hydrochloride (*CH*) and butamirate citrate (*BC*) using methanol: acetonitrile: water (100:75:25, v/v/v) adjusted with triethylamine to pH 9.8 ±0.1 at a flow rate of 1 ml/min and UV detection at 225 nm.

Table(1): Effect of different wavelengths and flow rates on capacity factor and peak area ratio of BC and its internal standard(CH)

flow rate	0.8 ml/min			1 ml/min			1.6 ml/min		
Drug	**BC**	**CH**		**BC**	**CH**		**BC**	**CH**	
Retention time	**13 min**	**9.9 min**	**Peak Area Ratio**	**10 min**	**7.8 min**	**Peak Area Ratio**	**6.3 min**	**4.9 min**	**Peak Area Ratio**
225 nm	5546	1675	3.311	4470	1323	3.379	2764	817	3.383
230 nm	3029	824	3.676	2326	629	3.698	1434	402	3.567
235 nm				1103	118	9.311	637	74	8.597
240 nm				494	18	27.319	300	12	24.544
[a]t_a, min	0.92			0.7			0.5		
[b]T_R of PBA, min	2.223			1.786			1.142		
[c] (K')	1.416			1.551			1.284		

[a]Void time; [b]Retention time; [c]Capacity factor

3.2.2. For NaCr and Deg1

The developed HPLC has been applied for the determination of ***NaCr***, ***Deg1*** and ***Deg2***. To optimize the HPLC assay parameters, the mobile phase composition was studied. Due to their difference in polarity, a satisfactory separation between ***NaCr*** and ***Deg1*** was obtained with a mobile phase consisting of methanol: water (75:25, v/v) Fig. (12).

This mobile phase could not separate ***NaCr*** and ***Deg2*** due to the great similarity in the structure and in the polarity of the two compounds. The partial overlap of the two eluents using same previously mentioned chromatographic conditions with 1 ml min^{-1} flow rate is shown in Fig. (13). Several attempts have been carried out to separate the peak of the open ring structure from that of ***NaCr***, these trials include changes in methanol: water proportions, addition of acetonitrile to the mobile phase with different ratios, and finally increasing the basicity of the mobile phase by adding different amount of triethylamine. All these attempts did not achieve the required separation of the two peaks. Removal of methanol from the mobile phase causes retention of ***Deg1*** on the column up to more than 30 minutes. ***MT*** was chosen as internal standard as it eluted at reasonable retention time and showed good separation from mixture components Fig. (12). Quantification was achieved with UV detection at 254 nm based on peak area. The method

showed complete separation between the drug, its degradation product, the internal standard and drops excipients (methylparaben sodium and propylparaben sodium) Fig. (14).

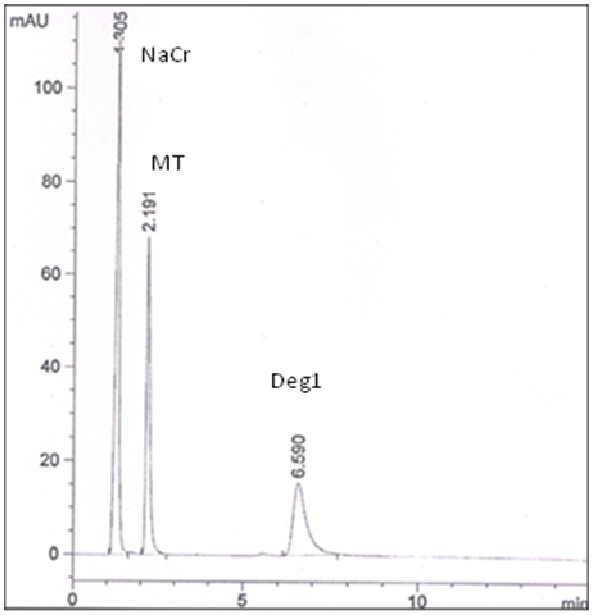

Figure (12): The chromatogram of *NaCr*, *Deg1* and *MT* using methanol: water (75:25, v/v) at a flow rate 1.2 ml/min and UV detection at 254 nm.

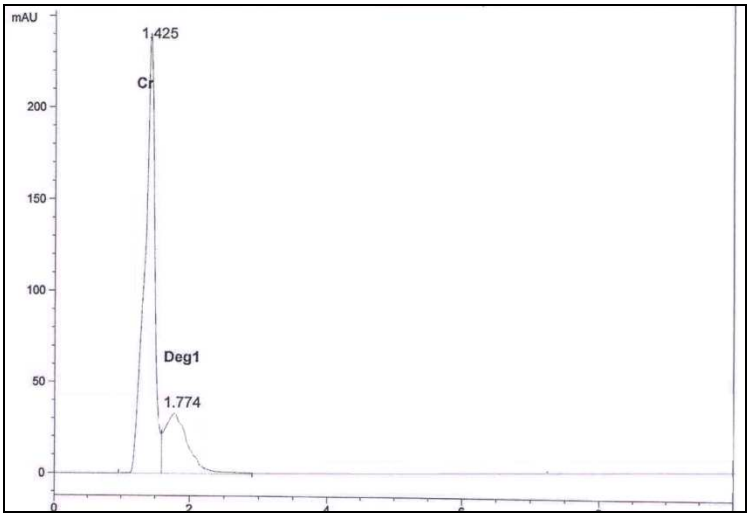

Figure (13): Retention time of sodium cromoglycate (*NaCr*), its degradation product (*Deg2*) using methanol: water (75:25, v/v) at a flow rate 1 ml/min at ambient temperature and UV detection at 254 nm.

3.3. Validation of the method

3.3.1. Optimization of the chromatographic conditions

The system suitability tests verify the resolution and reproducibility of the chromatographic system. The results obtained are presented (Table 2).

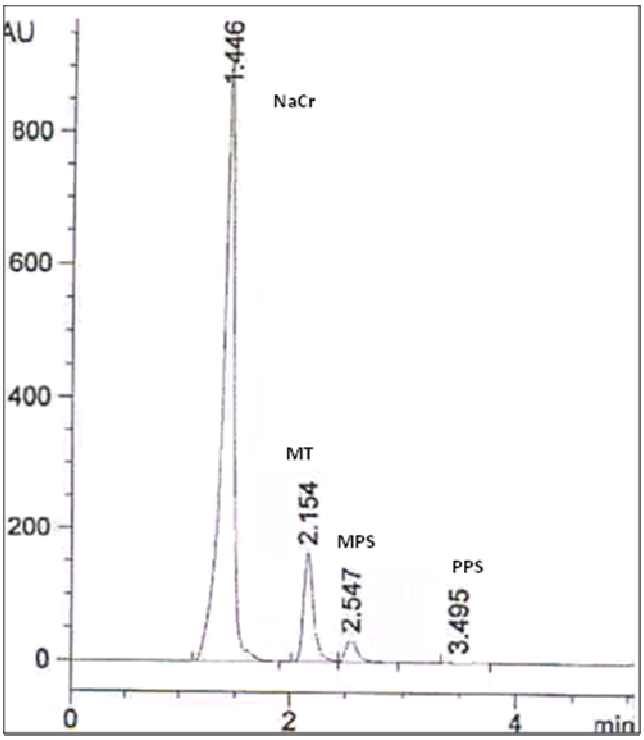

Figure (14): Retention time of sodium cromoglycate (*NaCr*), metronidazole (*MT*), methylparaben sodium (*MPS*) and propylparaben sodium (*PPS*) using methanol: water (75:25, v/v) at a flow rate 1.2 ml/min at ambient temperature and UV detection at 254 nm.

Table(2): System suitability tests for the HPLC method proposed for the determination of BC and NaCr and their degradation products

Parameter	PBA	CH	BC	NaCr	MT	Deg 2
[a]N	1840	1586	2441	1062	2442	1676
[b]R	14.910	2.850		4.843	9.705	
[c]α	6.442	1.320		2.101	3.601	
[d]K'	1.576	10.151	13.404	1.610	3.382	12.180
[e]RSD,%	---	---	0.208	0.889	---	1.182

[a]*Number of theoretical plates*
[b]*Resolution*
[c]*Relative retention*
[d]*Capacity factor*
[e]*Relative standard deviation*

3.3.2. Linearity and range

The linearity of the calibration curves and adherence of the system to Beer's law were verified by the high correlation coefficient. The analytical data for the calibration curves including the mean recovery, SD, confidence limits of slopes, slope and intercept standard deviations (S_b, S_a) and standard errors of the estimations are summarized (Table 3).

3.3.3. Specificity and accuracy

The HPLC methods were found to be specific with complete separation of **BC** (t_R 10.08±0.1 min.) and complete separation of **NaCr** (t_R 1.305±0.1 min.) from its degradation products under the experimental conditions. The peaks obtained were sharp with clear baseline separation. Good resolution and absence of interference between the drugs determined are shown in Fig. (8,12). Repeatability and the application of the proposed methods to a pharmaceutical formulation were done using standard addition techniques. Satisfactory results were obtained indicating the specificity and accuracy of the proposed methods (Table 3).

3.3.4. Precision, LOD, LOQ

Results of %RSD and that of the intra and inter-day precision along with that of LOD and LOQ were satisfactory as presented in Table 3.

3.3.5. Statistical studies

Statistical comparison between the results of the proposed methods and those of the reference methods, obtained by using the student's t-test and F- ratio showed no significant differences. It can be concluded that the proposed analytical methods are sufficiently accurate and precise (Table 4).

Table(3): Results obtained by the HPLC methods for the determination of BC and NaCr and their degradation products

Parameter	*BC*			*NaCr*			*Deg 2*		
Retention time, min	10.083			1.35			6.61		
Wavelength of detection, nm	225			254			254		
Calibration range, μgmL^{-1}	100-1200			100-600			30-180		
Regression equation	y= 0.0074 x +0.058			y= 0.0346 x+0.2611			y = 0.0225 x- 0.0573		
Correlation coefficient (r)	0.9999			0.9997			0.9999		
$^{a}S_b$	3.53×10^{-5}			3.82×10^{-4}			9.59×10^{-5}		
$^{b}S_a$	0.025			0.149			0.011		
^{c}LOD	15.608			6.359			3.665		
^{d}LOQ	47.297			38.805			6.275		
Confidence limit of the slope	$0.0074 \pm 9.07 \times 10^{-5}$			$0.0346 \pm 1.06 \times 10^{-3}$			$0.0225 \pm 2.67 \times 10^{-4}$		
Confidence limit of the intercept	0.058±0.064			0.2611±0.414			-0.0573±0.030		
Standard error of the estimation	0.035			0.16			0.012		
Intra day									
Mean of concentrations (µg/ml) n=3	201.239	598.613	1200.769	185.84	367.704	544.976	55.101	67.405	135.979
^{e}RSD,%	0.441	0.149	0.888	0.319	0.17	0.123	0.264	0.472	0.306
Inter day									
Mean of concentrations (µg/ml) n=3	201.785	601.636	1200.777	186.172	364.161	537.125	54.94	66.837	133.247
RSD,%	0.345	0.068	0.087	0.249	0.05	0.251	0.747	0.654	0.223
Results									
1) Drug in laboratory made mixture,%	100.07 ± 0.208			100.31 ± 0.892			99.77 ± 1.179		
2) Drug in dosage form, %	96.56 ± 0.705			108.05 ± 0.746					
3) Drug added,%	99.52± 0.655			100.63± 0.478					

[a]Standard deviation of the slope *[c]Limit of detection* *[e]Relative standard deviation*
[b]Standard deviation of the intercept *[d]Limit of quantification*

Table(4): Results Of tests of significance for the HPLC methods for the determination of BC and NaCr and their degradation products

Statistical Term	BC		NaCr	
	Reference method[b]	HPLC method	Reference method[c]	HPLC method
Mean	99.84	100.07	99.79	100.31
S.D.±	0.415	0.208	0.722	0.892
S.E.±	0.169	0.085	0.295	0.364
RSD, %	0.416	0.208	0.723	0.889
n	6	6	6	6
V	0.172	0.043	0.521	0.796
t (2.228)[a]	1.216		1.110	
F (5.050)[a]	4.000		1.528	

CONCLUSION

The proposed HPLC methods developed are precise, accurate, linear, robust and specific for analysis of the drugs in pure form and in their respective dosage form. The first proposed method has the advantage of novelty as there is no reported HPLC method for the determination of ***BC*** in presence of its alkaline degradation product using simple component mobile phase. The second

proposed method has the advantages of overcoming the high retention time previously reported for ***NaCr*** and its degradation product and using simpler and cheaper mobile phase composition than ion pairing which is still considered a complicated method. Moreover; the second proposed method is a stability indicating assay that can determine ***NaCr*** and ***Deg1*** simultaneously. Satisfactory results were obtained from validation of the methods, thus both methods can be used for routine analysis and quality control of the cited drugs in small laboratories.

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