Accepted Manuscript

Title: Development and Validation of Impurity-Profiling UPLC Method for the Determination of Sodium Cromoglicate and Tetryzoline Hydrochloride: Application on Rabbit Aqueous Humor

Author: Hayam M. Lotfy Sarah S. Saleh Nagiba Y. Hassan Hesham Salem

PII: S1570-0232(15)30240-3
DOI: http://dx.doi.org/doi:10.1016/j.jchromb.2015.10.020
Reference: CHROMB 19673

To appear in: *Journal of Chromatography B*

Received date: 10-8-2015
Revised date: 10-10-2015
Accepted date: 15-10-2015

Please cite this article as: Hayam M.Lotfy, Sarah S.Saleh, Nagiba Y.Hassan, Hesham Salem, Development and Validation of Impurity-Profiling UPLC Method for the Determination of Sodium Cromoglicate and Tetryzoline Hydrochloride: Application on Rabbit Aqueous Humor, Journal of Chromatography B http://dx.doi.org/10.1016/j.jchromb.2015.10.020

Development and Validation of Impurity-Profiling UPLC Method for the Determination of Sodium Cromoglicate and Tetryzoline Hydrochloride: Application on Rabbit Aqueous Humor

Hayam M. Lotfy[a,b], Sarah S. Saleh[c*], Nagiba Y. Hassan[a], Hesham Salem[d]

[a]Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr-El Aini, 11562 Cairo, Egypt

[b]Pharmaceutical Chemistry department, Faculty of Pharmaceutical sciences & Pharmaceutical industries, Future University in Egypt (FUE), 12311 Cairo, Egypt

[c]Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 11787 6th October, Egypt

[d]Analytical Chemistry Department, Faculty of Pharmacy, Deraya University, Minia, Egypt

*Corresponding author, e-mail:drsarahsalah@gmail.com; Mobile: +201006095963

HIGHLIGHTS

- UPLC-UV as impurity profiling method with development of forced degradation study
- Sodium cromoglicate &tetryzoline hydrochloride with possible degradation impurities
- Degradation products were identified by IR and Mass spectra
- Method is validated for specificity, linearity, accuracy, precision and robustness
- Method was successfully applied to rabbit aqueous humor and dosage form

Abstract

Sodium cromoglicate (SCG), antihistaminic agent, and tetryzoline hydrochloride (TZH), a sympathomimetic agent, are formulated together as an ophthalmic preparation. An ultra-performance liquid chromatographic method with UV detection (UPLC‑UV) was developed and validated for the quantitative determination of SCG and TZH in rabbit aqueous humor. Due to the instability of both SCG and TZH under alkaline conditions, the UPLC method was applied for their determination in the presence of their possible degradation impurities. The separation was performed using C_{18} column (1.7 µm particle size) and isocratic elution system with methanol: 1 % *o*-phosphoric acid (65: 35, v/v).The optimum flow rate was 0.5 mL/min and the detection was done at 230 nm. The suggested method was validated in compliance with the ICH guidelines and was successfully applied for determination of sodium cromoglicate (SCG) and tetryzoline HCl (TZH) as prepared synthetically in laboratory mixtures, and in the presence of their alkali-induced degradation impurities. The suggested method was effectively applied the determination of spiked rabbit aqueous humor samples as well as commercial pharmaceutical formulation.

Keywords: Sodium cromoglicate; Tetryzoline HCl; UPLC; aqueous humor; degradation.

1. Introduction

Sodium cromoglicate (*SCG)* is a mast cell stabilizer that inhibits the release of histamine and other inflammatory mediators from sensitized mast cells, and it is used as an anti-histaminic agent for topical application. A survey of the literature revealed the reported methods for the determination of SCG such as UV spectrophotometry [1-3], HPLC [4], LC-MS [5], TLC [3, 6] and capillary electrophoresis [7]. Tetryzoline HCl (TZH) is a sympathomimetic agent with marked alpha adrenergic activity exhibiting a vasoconstrictor effect, and it is used as conjunctive and nasal decongestant. Different analytical techniques were reported for the determination of TZH in pharmaceutical preparations and biological fluids such as: colorimetric determination [8], spectrophotometry [9-13], HPTLC [14], HPLC [15-20], gas chromatography [21] and electrochemical method [22]. Both components are official [23] and they are formulated together as an ophthalmic solution for the treatment of allergic conjunctivitis and vernal keratoconjunctivitis [24].

Alkali-induced degradation was reported for both sodium cromoglicate (SCG) [25-27] and tetryzoline HCl (TZH) [16-19]. The chemical structure for both components and the pathways of degradation are shown in Figures 1 and 2. Due to their instability under alkaline conditions, the development of simple, sensitive and specific method for their determination in the presence of their possible degradation impurities was essential.

The International Conference on Harmonization of technical requirements for the registration of pharmaceuticals for human use (ICH) guideline on impurities in new drug substances [28], addressed the impurities in new drug products as degradation products of the drug substance or reaction products of the drug substance with an excipient and/or immediate container closure system (collectively referred to as “degradation products” in this guideline). [28, 29].

The drug impurities should preferably be identified, qualified and/or quantified either they reached the identification threshold (concentration) limits or not [28]. For this purpose, many pharmaceutical laboratory studies develop a drug impurity profile, which detect the identified and unidentified impurities present in a new drug substance. Moreover, impurities are often only present in minute amounts and have similar chemical structure to the parent drug. Thus sensitive and selective analytical methods are mandatory, as for chromatographic drug impurity profiles.

As reported in literature, only expensive, hyphenated methods, such as LC-MS and GC-MS, were reported for the determination of each component separately in biological samples. Spectrophotometric methods were reported for the determination of both components in pharmaceutical preparation [30]. Meanwhile, there was no method reported for the simultaneous determination of both components, in presence of their degradation products. So, the aim of this work is to develop and validate simple, sensitive and inexpensive method for the determination of SCG and TZH in presence of their possible degradation impurities, in pharmaceutical preparation and biological fluid, namely rabbit aqueous humor.

2. Materials and methods

2.1. Chemicals

Pure samples. Sodium cromoglicate (SCG) reference standard was kindly supplied by Sigma Pharmaceutical Industries Limited, Al-Monofeya, Egypt, while TZH reference standard was supplied by Sigma–Aldrich, USA, with purity of 100.80 ±0.742 and 100.06±0.592, respectively. The purity testing was done according to BP official methods [23].

Market sample. Croma® eye drops, labeled to contain 40 mg of sodium cromoglicate, 0.5 mg of TZH and 0.1 mg of benzalkonium chloride per 1 mL. The sample was manufactured by Jamjoompharma, Kingdom of Saudi Arabia, and purchased from the local market.

Solvents. Methanol (HPLC grade) was supplied from (LabScan Limited–Dublin, Ireland); ortho phosphoric acid (85%) was supplied from (Adwic-El Nasr pharmaceutical Chemicals Co., Egypt) and distilled water was used. Phosphate buffer solution (prepared according to B.P. using 0.2 M potassium dihydrogen phosphate).

Biological samples. Fresh aqueous humor was extracted from albino rabbits.

2.2. Instrumentation

Waters Acquity UPLC system (Waters, Manchester, UK) equipped with binary solvent manager, thermostat column compartment, autosampler, tunable ultra-violet (TUV) detector and the data

was processed using Masslynx software. The column used was Kinetex C_{18} (2.1 mm ID x 50 mm L, porosity 135 A°, particle size 1.7 µm (Phenomenex, USA).

Shimadzu 435 spectrometer using KBr discs (for IR spectra) at Faculty of Pharmacy, Cairo University).

Shimadzu QP-2010 spectrometer (for GC Mass) and Hewlett Packard 5988 spectrometer (for Mass spectra) at the Microanalytical center, Cairo University, Egypt.

2.3. Standard solutions

Stock solutions were prepared in the optimized mobile phase of concentration 1 mg/ml for *SCG* and *TZH*; while working solutions were freshly prepared by dilution from the stock solutions with the same solvent to obtain a concentration of 100 µg/mL of *SCG* and 10 µg/mL of *TZH*.

2.4. Procedure

2.4.1. Chromatographic conditions

The mobile phase consisted of methanol: 1 % o-phosphoric acid in the ratio of (65: 35, v/v). The mobile phases were filtered using 0.45 µm Millipore membrane filter (Billerica, MA). The injection volumes were 2 µL and the flow rate was 0.5 mL/min kept at 40 ˚C on UPLC column (2.1 mm ID x 50 mm L, porosity 135 A°, particle size 1.7 µm). UV detection was done at 230 nm.

2.4.2. System suitability

According to USP [31], system suitability parameters are checked for evaluating the performance of the analytical instrument and the analytical method before it is used routinely for sample analysis. The mentioned injection volumes of the working solutions were injected and the chromatographic conditions were applied. The software gathers data about separated peaks (as peak area and height …etc) to statistically calculate those parameters which are: Theoretical plate number or column efficiency (N), capacity factor (**k')**, separation actor (**α)**, resolution (Rs) and tailing factor (T). The values calculated by the software are then compared to reference values given by USP guidelines [31] to detect whether the results are accepted or not.

2.4.3. Linearity

Different aliquot volumes were separately transferred from each working solutions into 10 mL volumetric flasks and diluted to volume with the mobile phase to form working solutions with concentrations of: (2.5 – 100 µg/mL) of *SCG*, and (0.5-10 µg/mL) of *TZH*. The chromatographic conditions were applied and the chromatograms were recorded. The calibration curve of each drug was constructed by plotting the relative peak area [the peak area found to that of an external standard of the same drug] against the corresponding concentration, from which the regression equations were calculated. The external standard was chosen to be 5 µg/mL of SCG and 1 µg/mLof *TZH*. The calibration curves were constructed using the average of three experiments.

2.4.4. Preparation of alkali-induced degradation products of SCG

A weight of (500 mg) of standard SCG was refluxed with 10% aqueous potassium hydroxide (10 ml) for 2 hr. Then the solution was acidified with glacial acetic acid until complete precipitation of the degradation product. The precipitate was filtered and dried (*Deg 1*). Anther weight of (500 mg) was heated with 10% alcoholic sodium hydroxide (10 ml) at 70 ^{o}C for 1 hr. Then the solution was carefully treated with glacial acetic acid to pH = 7.5. An orange yellow solid was precipitated, filtered and dried (*Deg 2*) [27, 32]. Both structures were confirmed using Mass spectrometry. The stock solutions of both degradation products were prepared using the mobile phase to prepare solutions of concentration (100 µg/ml). Different concentrations of the products were prepared by dilution from the stock solution using the same mobile phase to obtain concentrations of (10, 50, 90 µg/ml).

2.4.5. Preparation of alkali-induced degradation products of TZH

A weight of 50 mg of standard TZH was transferred into 100 mL volumetric flask and dissolved in 20 mL of distilled water. Five milliliters of 10 M NaOH were added and the solution was placed in an oven at 50 ^{o}C for 24 hr. The pH was then adjusted to 7 with 10 M HCl and the volume was completed to the mark with the mobile phase [18]. The structure of the induced degradation product (*Deg 3*) was elucidated using the IR spectrometry. Different concentrations of the products were prepared by dilution from the stock solution using the same mobile phase to obtain concentrations of (1, 5, 9 µg/ml).

2.4.6. Application to pharmaceutical preparation

One milliliter of the eye drops were transferred into 100 mL volumetric flask and the volume was completed with mobile phase to obtain concentrations of 400 µg/mL of SCG and 5 µg/mL of TZH. An appropriate dilution was made with the mobile phase to prepare the working solution to obtain the concentrations of 80 µg/mL of SCG and 1 µg/mL of TZH. The chromatographic conditions were applied for the prepared solution and the concentration of each component was calculated using the corresponding regression equation. When carrying out the standard addition technique, different known concentrations of pure standard of each component were added to the pharmaceutical dosage form before proceeding in the previously mentioned procedure.

2.4.7. Preparation of spiked rabbit aqueous humor samples

Five albino rabbits were used to obtain a fresh aqueous humor. Two drops of 0.4% solution of benoxinate HCl (Local anesthetic) were instilled into rabbit's eye. Samples of aqueous humor were immediately removed from the anterior chamber of each eye using a 26- gauge needle attached to 1 ml tuberculin syringe. The procedure was repeated 2 times a day for about 3-5 days till the wanted volume was collected. The samples were stored frozen until the experiment was carried out [33]. After removal of aqueous humor samples at each time interval, the ocular surface was irrigated with isotonic phosphate buffered saline and dried with soft tissue. Aliquots of 500 µl and 1 ml of the working solutions of standard SCG and TZH solutions were separately introduced to 2 volumetric flasks (5-ml) and the volume was completed with the collected aqueous humor, and then transferred into test tube and vortex for 60 seconds. One milliliter aliquot of the spiked aqueous humor samples was pipetted into a Wassermann tube and then one milliliter aliquot of methanol was added for deproteinization. The samples were vortex-mixed vigorously for 5 min and centrifuged at 4000 rpm for 15 min. The supernatants were extracted using a syringe needle and the solution was filtered through a 0.45 µm Millipore filter membrane. Then 2 µL was injected into the UPLC system.

3. Results and discussion

The aim of this work was to develop and validate simple, accurate, specific and precise UPLC method for the determination of SCG and TZH in the presence of their possible alkali-induced degradation impurities and in biological samples.

3.1. Identification of the degradation products

IR spectra were not suitable to differentiate between SCG and the two possible alkali-induced degradation products because the OH stretches and CO stretching bands appear nearly at the same frequencies. So, mass spectrometry was performed for the identification of the two degradation products. *Deg1* is one of the related substances of SCG listed in the European Pharmacopeia [34]. According to the reported degradation pathway [27, 32], *Deg 1* showed a peak at m/z 342 corresponding to the dehydrated molecular ion peak ($-H_2O$) as shown in Figure 3. While *Deg 2* showed a peak at m/z 593 corresponding to the molecular ion peak (+1) as shown in Figure 4.

The alkali-induced degradation of TZH was tested by TLC till complete degradation product was obtained. The structure of the degradation product (*Deg 3*) was elucidated by IR spectroscopy as shown in Figure 5, where stretching bands appeared at 3459 cm^{-1} (N−H group) and at 1637 cm^{-1} (C=O group). Those bands are characteristic to the reported structure of the degradation product only [16] and absent in the intact drug structure.

3.2. Optimization of the method

To optimize this method, it was necessary to test the effect of different variables. Mobile phases of similar – yet slightly modified – compositions have been tested. Optimum resolution was achieved upon using a mobile phase composed of methanol: 1 % o-phosphoric acid in the ratio of (35: 65, v/v). Different stationary phases were also tested, where a compromise was achieved using C_{18} column (2.1 mm ID x 50 mm L, particle size 1.7 µm). Good resolution and linearity were obtained at a flow rate of 0.5 ml/min, where short run time was achieved with moderate backpressure. The detection was carried out at 230 nm to overcome the problem of the interference caused by the added preservative (benzalkonium chloride). The column temperature

was kept at 40 ˚C to obtain optimum separation. Complete resolution was achieved for the peaks of SCG and TZH together with the separated peaks of their degradation products were obtained as shown in Figure 6. The retention times were found to be 4.26 and 5.96 for SCG and TZH respectively; while for *Deg 1*, *Deg 2* and *Deg 3*, the retention times were found to be 1.76, 3.45 and 2.70, respectively.

3.3. Method validation for standard solutions

The UPLC method was validated according to ICH guidelines for standard solutions [35] as follows:

3.3.1. System suitability

System suitability was checked by calculating different parameters including column efficiency (N), capacity factor (**k')**, separation actor (**α)**, resolution (Rs) and tailing factor (T). The obtained values in Table 1 were found to be in the acceptable ranges when compared to USP reference values [31].

3.3.2. Range and linearity

The concentration ranges were selected on the basis of the anticipated drugs concentration during the assay of the pharmaceutical formulation. The linearity of the proposed methods was evaluated by processing the different calibration curves (of 8 points) on three different days. The external standard compensated instrumental errors. However in this study, there was no need for an internal standard as the sample preparation steps were so simple and didn't affect the sample matrix due to low protein content in aqueous humor. Calibration curves were constructed in the range of (2.5 – 100 µg/ml) for SCG and (0.5 – 10 µg/ml) for TZH. The corresponding assay parameters and validation sheet for the proposed methods were listed in Table 2.

3.3.3. Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte in standard calibrators of the curve and

quality control samples at multiple levels. To study the accuracy of the proposed method, procedure under study of linearity, were carried at a minimum of five determinations per concentration and a minimum of three concentrations in the expected range of concentrations within linearity. Accuracy are recommended to be low, mid and high QC levels to cover the whole analytical range for each drug. The tested concentrations for SCG were: (5, 50 and 85μg/ml) and for TZH were: (1, 5 and 8.5 μg/ml). The accuracy was expressed in Table 2. Accuracy was also tested via the interference of excipients in the pharmaceutical formulations by applying standard addition to the pharmaceutical formulation. Good accuracy was proved and the results were shown Table 3.

3.3.4. Specificity

Specificity is the ability to unequivocally assess the analyte in the presence of components that may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. Specificity of this method was assessed by comparing the retention time and area of the peaks of standard sodium cromoglicate and tetryzoline hydrochloride to that of the samples peaks where good correlation was obtained. By injecting the individual identification solutions of degradation products of each drug, the peaks of SCG and TZH were completely resolved in presence of their degradation impurities.

3.3.5. Precision

Precision was studied with respect to both repeatability and intermediate precision through the analysis of three different concentrations of each component at the 80, 100 and 120% levels of label claim, by three replicate analyses on a single day and on three consecutive days, respectively. The results expressed as relative standard deviation (RSD) were illustrated in Table 2.

3.3.6. Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated for each component using the standard deviations of the blank and the slope of the calibration lines, as shown in Table 2.

3.3.7. Robustness

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications, as shown in Table 1. In addition, the analysis of samples was done under a variety of experimental conditions, such as small changes in proportions of the organic part of the developing system (methanol) in the ratio of (61, 63, 67 v/v) and by varying the column temperature (38, 36, 42 °C). A slight change in the retention time and peak parameters were observed, however the peak areas were conserved. The effect of robustness was shown in Table 2.

3.4. Method validation for spiked biological samples

The proposed method was validated according to FDA Bioanalytical validation guidelines [36].

3.4.1. Selectivity

The selectivity was studied by analyzing different spiked humor samples. Placebo and blank samples were tested under the same chromatographic conditions. Placebo samples resembled the matrix of the pharmaceutical preparation, prepared by adding all the excipients; while the blank consisted of the extracted supernatant of deprotenized blank aqueous humor samples. There were no peaks observed when the placebo and blank were injected where no interferences occurred, hence the method is considered to be specific. System suitability parameters were checked for both peaks including resolution, tailing factor and number of theoretical plates. The results were shown in Table 4.

3.4.2. Accuracy

Accuracy was expressed as percentage recovery of the target value and assessed by means of validation standards in aqueous humor matrix at three concentration levels (5, 50 and 85 µg/ml for SCG and 1, 5 and 8.5 µg/ml for TZH). Samples were analyzed in triplicate for seven days for aqueous humor. The results were shown in Table 4.

3.4.3. Precision

Precision was estimated by measuring repeatability and intermediate precision of the same concentration levels mentioned in *section 3.3.5* in aqueous humor samples. Variance of repeatability and intermediate precision were computed from estimated concentrations and precision was expressed by relative standard deviation (RSD) at each level as shown in Table 5.

3.4.4. Stability

3.4.4.1. Short-Term Matrix Stability

The same concentration levels of each f SCH and TZH mentioned in *section 3.3.5* in aqueous humor samples were frozen for 24 hours and thawed unassisted at room temperature. The freeze–thaw cycle was repeated two more times, then analyzed on the third cycle and the concentrations were calculated. The same three aliquots of each component were thawed at room temperature and kept at this temperature for 24 hours and then the concentrations were calculated. The analytes concentrations were compared to the calculated concentrations at the beginning of the experiment, and the samples were found to be stable through the three freeze–thaw cycles and stable through 24 hours at room temperature.

3.4.4.2. Long-Term Temperature Stability

The previous three aliquots of each component were stored under the same conditions as the study samples. The concentrations of all the stability samples were compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing. The samples were found to be stable through 90 days.

3.5. Application to pharmaceutical preparation

The proposed method was valid and applicable for the analysis of the pharmaceutical preparation (Croma®) and the separated peaks are shown in Figure 7a. The validity of the proposed methods was further assessed by applying the standard addition technique, which showed accurate results. The results confirm the suitability of the proposed method for the routine determination of the two components in their combined formulation with no interference of the added excipients. The results were shown in Table 3.

3.6. Application to Biological samples

The biological samples were prepared in order to reach the low concentration in the calibration range were the concentration levels of SCG and TZH in aqueous humor samples. There was no need for further extraction procedures due to low protein content in aqueous humor samples, therefore no interference was observed during the blank analysis, as shown in Figure 7b.The proposed method was applied successfully for the determination of both components in the biological fluid as shown in Figure 7c. The results demonstrated in Table 4 demonstrated the overall recoveries of SCG and TZH in aqueous humor. This method could be used as an alternative method to the hyphenated GC-MS and LC-MS methods to determine the SCG and TZH in different biological samples.

4. Statistical analysis

Table 6 showed statistical comparison of the results obtained by the proposed method and official methods [2]. The calculated t and F values were less than the theoretical ones indicating that there was no significant difference between the proposed and the official methods with respect to accuracy and precision.

6. Conclusion

An ultra-performance liquid chromatographic method with UV detection (UPLC‑UV) was developed and validated as an impurity-profiling and stability-indicating method for the quantitative determination of SCG and TZH in presence of their possible degradation products. The method was also applied for the quantitative determination of SCG and TZH in pharmaceutical preparation and spiked samples of rabbit aqueous humor. As a final conclusion, the results obtained by the proposed method were reliable, accurate and precise. Hence it can be employed for routine quality control analysis. Nevertheless, our proposed UPLC method is a simple useful tool for further investigations of the pharmacokinetics of sodium cromglicate and tetryzoline HCl.

7. References

[1] E.-Z.A. Hassib ST, Fouad MA, Drug Test. Anal., 3 (2011) 306-318.

[2] D.W.W. J. Tillman, Analyst, 69 (1971) 689-698 .

[3] S.S. Saleh, H.M. Lotfy, N.Y. Hassan, S.M. Elgizawy, Saudi Pharm. J., 21 (2013) 411–421.

[4] M.S. Ali, S. Rafiuddin, D.A. Al-Jawi, Y. Al-Hetari, M.U.H. Ghori, A.R. Khatri, J. Sep. Sci., 31 (2008) 1645-1650.

[5] M.L. Ozoux, J. Girault, J.M. Malgouyat, O. Pasquier, J. Chromatogr., B: Biomed. Sci. Appl., 765 (2001) 179–185.

[6] R.D. Kocić-Pesić V, Pećanac D, Zivanović L, Farmaco, 12 (1992) 1563-1567.

[7] S.H. Anne Helle, Leena Peltonen, Jouni Hirvonen, Susanne K. Wiedmer, J. Chromatogr. A, 1178 (2008) 248–255.

[8] M.S. Rizk, E.Y.Z. Frag, G.G. Mohamed, A.A. Tamam, Int. J. Res. Pharm. Chem., 3 (2013) 168-183.

[9] T.G. Altuntas, F. Korkmaz, D. Nebioglu, Pharmazie, 55 (2000) 49-52.

[10] E. Hassan, I. Hewala, A. Wahbi, Y. Hassan, Farmaco, 48 (1993) 1137-1151.

[11] H.M. Lotfy, S.S. Saleh, N.Y. Hassan, H. Salem, Spectrochim. Acta, Part A, 126 (2014) 112–121.

[12] S.S. Saleh, H.M. Lotfy, N.Y. Hassan, H. Salem, Spectrochim. Acta, Part A, 132 (2014) 239–248.

[13] H. Salem, H.M. Lotfy, N.Y. Hassan, M.B. El-Zeiny, S.S. Saleh, Spectrochim. Acta, Part A, 135 (2015) 1002-1010.

[14] A. Bekele, A. Hymete, A.A. Bekhit, Thai J. Pharm. Sci., 37 (2013) 134-145.

[15] F. Al-Rimawi, W. Zareer, S. Rabie, M. Quod, J. Pharm. Anal., 2 (2012) 67-70.

[16] J. Bauer, S. Krogh, J. Pharm. Sci., 72 (1983) 1347–1349.

[17] J.A. De Schutter, W. Van Den Bossche, P. De Moerloose, J. Chromatogr., 391 (1987) 303-308.

[18] G. Andermann, A. Richard, J. Chromatogr., 298 (1984) 189-192.

[19] A. Nicolas, M. Mirjolet, J.M. Ziegler, Talanta, 31 (1984) 229-231.

[20] H. Salem, N.Y. Hassan, H.M. Lotfy, S.S. Saleh, J. Chromatogr. Sci., doi:10.1093/chromsci/bmu109 (2014).

[21] J. Peat, U. Garg, Methods Mol. Biol., 60 (2010) 501-508.

[22] N.Y. Hassan, H.M. Lotfy, S.S. Saleh, H. Salem, Anal. Bioanal. Electrochem., 7 (2015) 75-90.

[23] British Pharmacopoeia, Volume II, London, 2009.

[24] S.C. Sweetman, in, Pharmaceutical Press, London, 2005.

[25] R. Mansfield, J. Huang, S. Thatcher, R.B. Miller, C.W. Davis, J. Liq. Chromatogr. Relat. Technol., 22 (1999) 2187-2209.

[26] M. Barnes, R. Mansfield, S. Thatcher, J. Liq. Chromatogr. Relat. Technol., 25 (2002) 1721-1745.

[27] S.T. Hassib, A.A. El-Zaher, M.A. Fouad, J. Chem. Pharm. Res., 3 (2011) 243-258.

[28] International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) guideline Q3A(R2), Impurities in new drug substances, 2006.

[29] S. Ahuja, Impurities evaluation of pharmaceuticals, Marcel Dekker, New York, 1998.

[30] H.M. Lotfy, S.S. Saleh, N.Y. Hassan, H. Salem, Spectrochim. Acta, Part A, 151 (2015) 628-643.

[31] United States pharmacopeia, USA, 2004.

[32] J. Tillman, D.W. Whymark, Analyst, 96 (1971) 689-698

[33] M. Nebsen, G.M. Elsayed, M. Abdelkawy, S.Z. Elkhateeb, Anal. Bioanal. Electrochem., 5 (2013) 368 - 380.

[34] European pharmacopeia, London, UK, 2004.

[35] Q.B. International Conference on Harmonization (ICH), 62, 2005.

[36] Guidance for Industry, Bioanalytical Method Validation. Food and Drug Administration (FDA)-Center for Drug Evaluation and Research (CDER), 2001.

List of figures

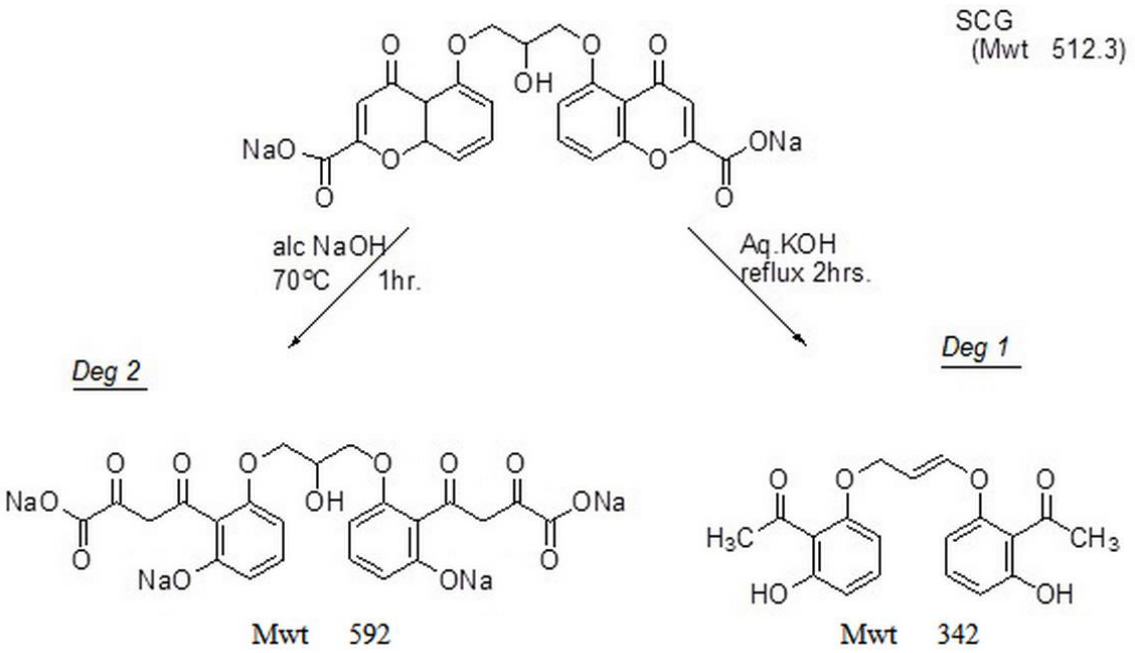

Fig.1. The chemical structure for SCG and the pathway of alkali-induced degradation.

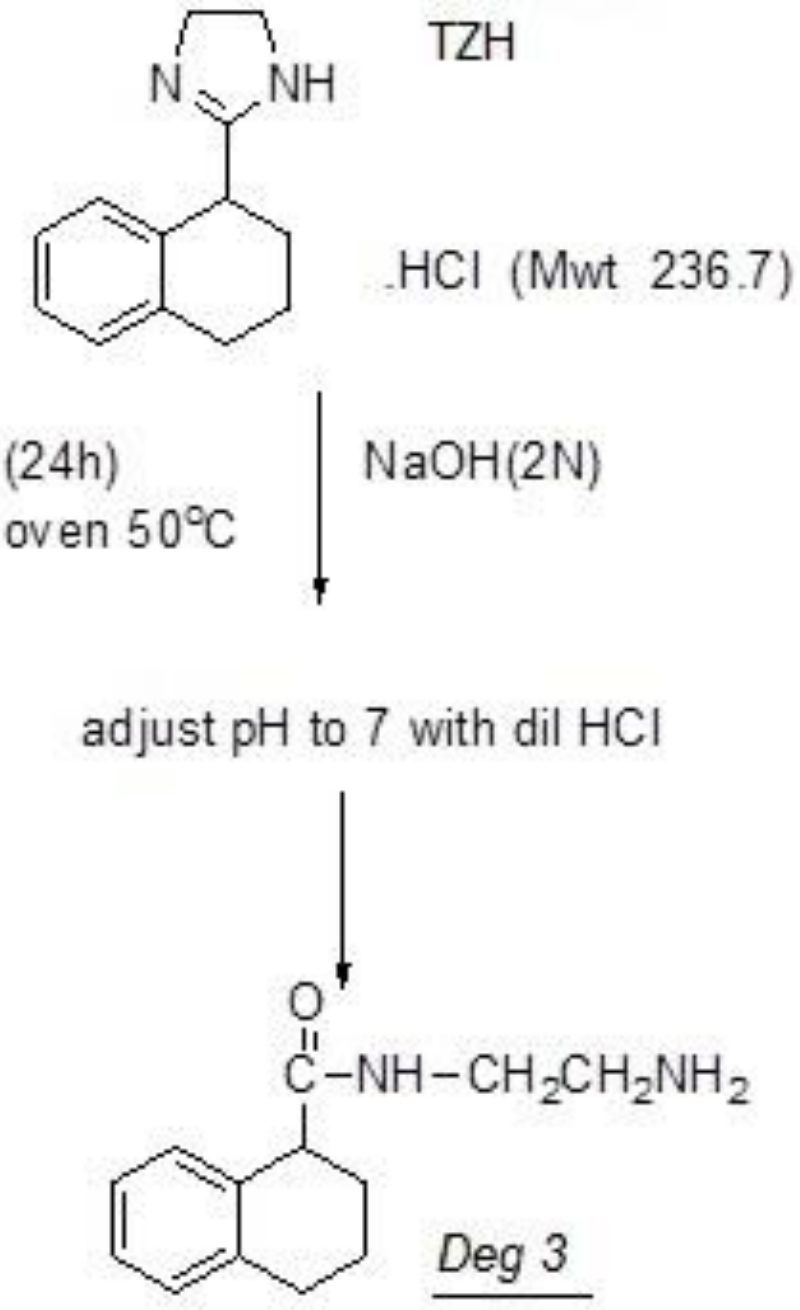

Fig.2. The chemical structure for TZH and the pathway of alkali-induced degradation.

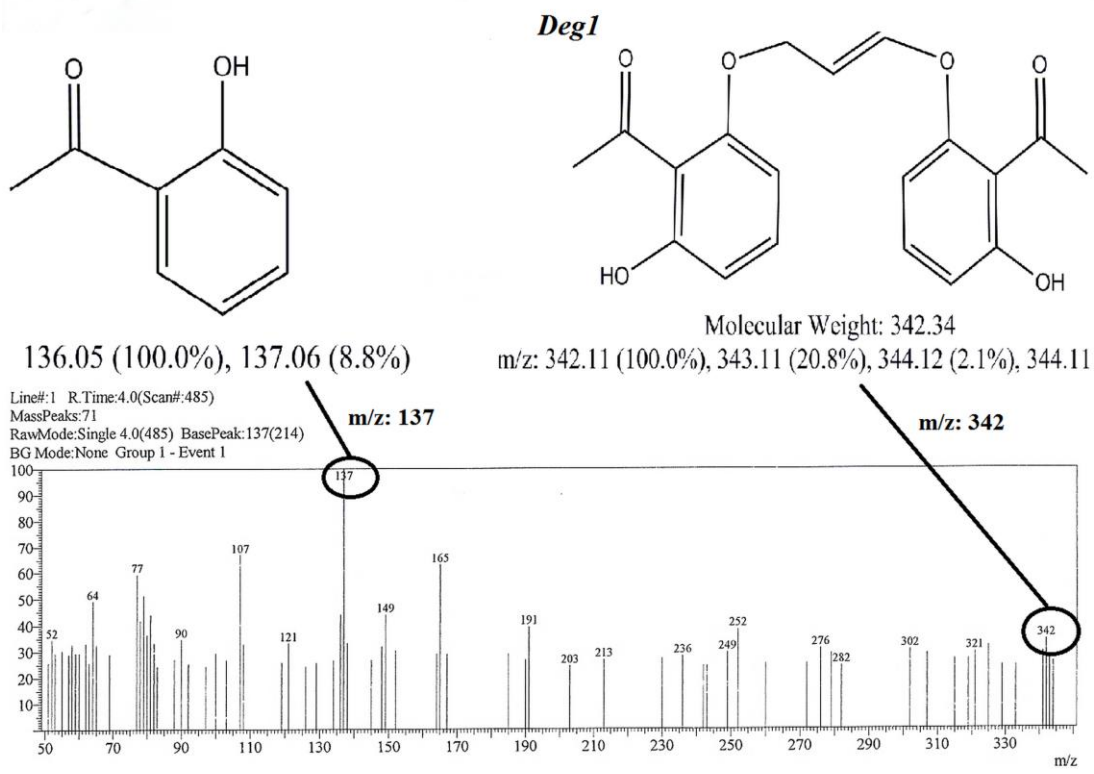

Fig.3. Mass spectrum identifying (*Deg 1*) showing a peak at m/z 342 corresponding to the dehydrated molecular ion peak and a base peak at m/z 137.

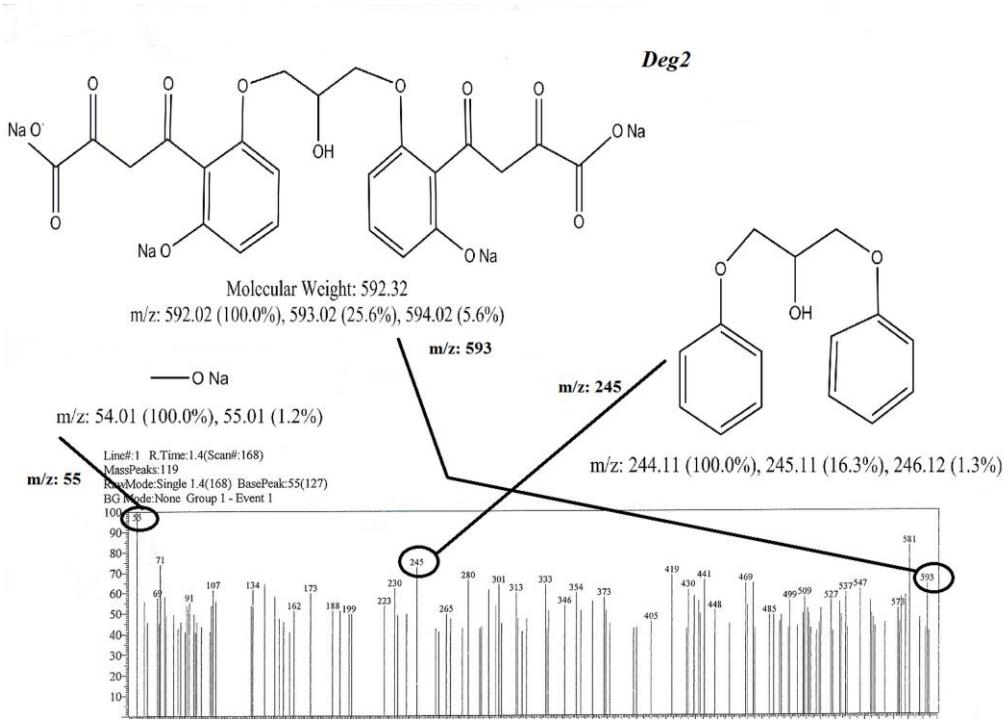

Fig.4. Mass spectrum identifying (*Deg 2*) showing a peak at m/z 593 corresponding to the molecular ion peak (+1) and base peaks at m/z 245 and 55.

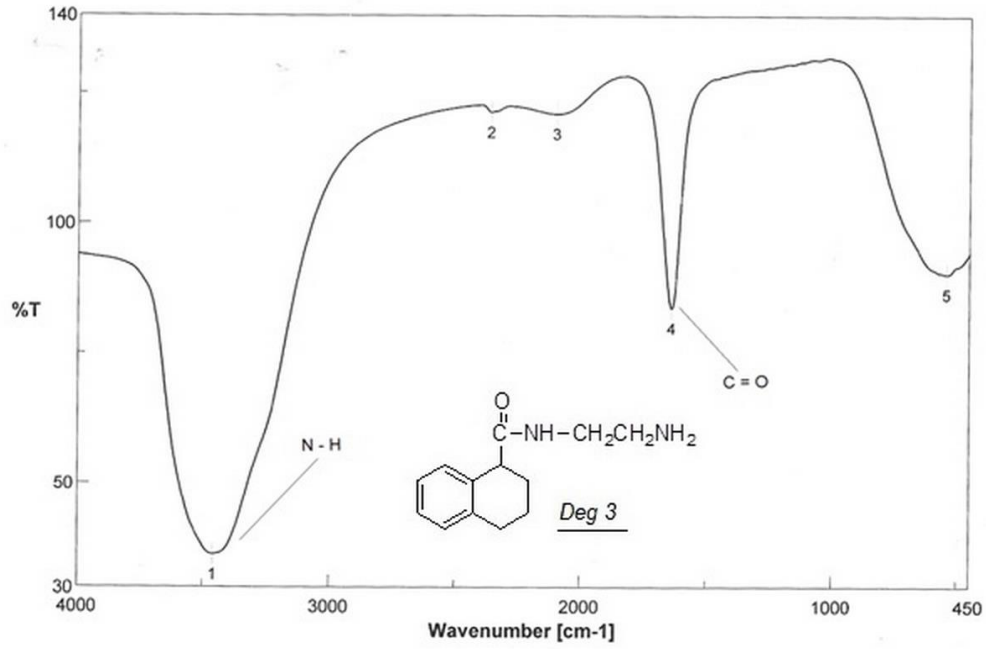

Fig.5. IR spectrum identifying (*Deg 3*) showing stretching bands at at 3459 cm^{-1} (N−H group) and at 1637 cm^{-1} (C=O group).

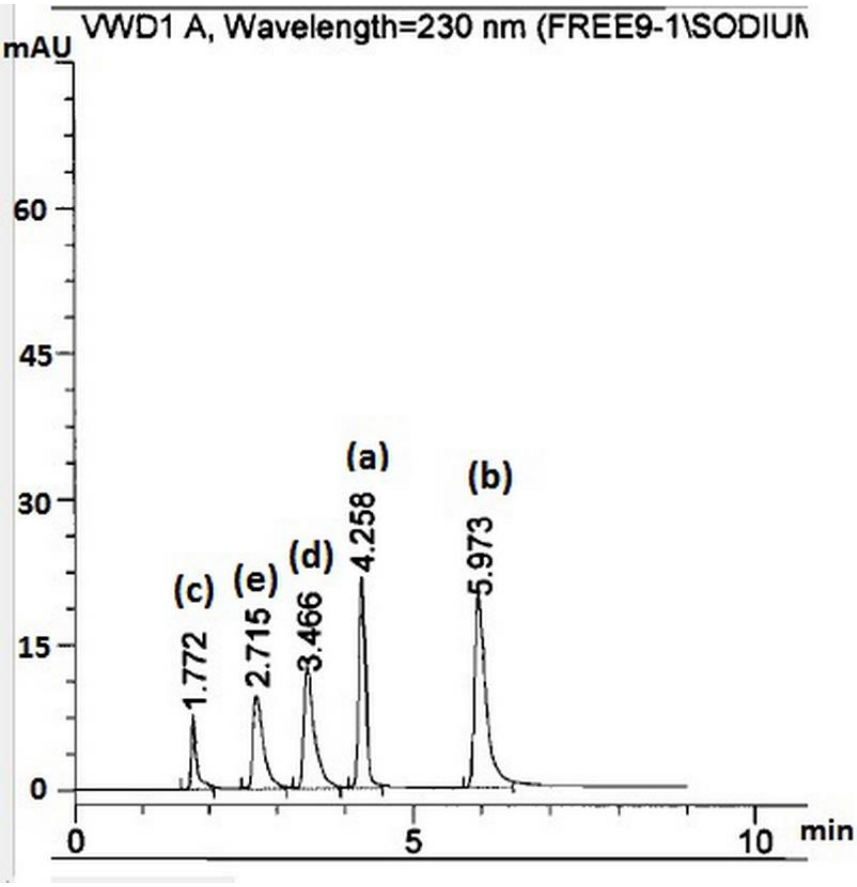

Fig.6. UPLC chromatogram showing resolution of five components: (a) SCG, (b) TZH, (c) *Deg 1*, (d) *Deg 2* and (e) *Deg 3*.

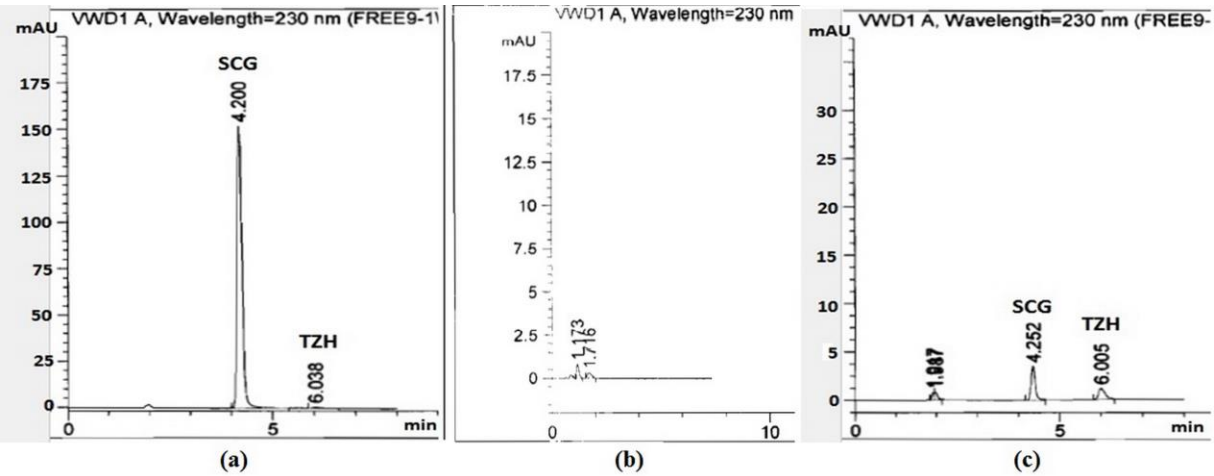

Fig.7. UPLC chromatogram showing separated peaks (a) in pharmaceutical preparation (80 ug/ml) SCG and (1 ug/ml) TZH; (b) in blank rabbit aqueous humor, (c) in spiked samples of rabbit aqueous humor (10 ug/ml) SCG and (1 ug/ml) TZH.

Table 1. Statistical Analysis of Parameters Required for System Suitability of the proposed UPLC method.

Parameter	*SCG*	*TZH*	*Deg1*	*Deg2*	*Deg3*	Reference USP value
t R (Retention time)	4.26	5.97	1.77	3.47	2.72	
N (Column efficiency)	9606	7084	2932	3138	4505	N > 2000
k' (Capacity factor)	3.27	4.98	0.97	2.46	1.71	1-10 acceptable
α (separation factor)	1.33	1.52		1.44	1.76	> 1
T (tailing factor)	1.01	1.21	1.09	1.14	1.10	$T \leq 2$ T=1 for symmetric peak
Rs (Experimental Resolution)	3.83	7.45		2.91	4.72	Rs > 2

Table 2. Assay parameters and validation sheet obtained by applying the proposed UPLC method for pure standard solutions.

Parameters	SCG	TZH
Calibration range [a] µg/mL	2.5 - 100	0.5 - 10
LOD [b]	0.831	0.152
LOQ [b]	2.505	0.462
Slope	0.1865	1.1345
Intercept	0.0114	- 0.1822
Correlation coefficient (r)	0.9999	0.9999
Mean [a]	99.93	100.02
RSD %	1.143	0.910
Accuracy [ac]	99.56 ± 0.87	100.55 ± 0.91
Repeatability [ad]	1.033	0.967
Inter-day precision [ad]	0.965	1.125
Robustness [ad]	0.732 [e] 1.145 [f]	0.643 [e] 0.995 [f]

[a] Average of three experiments

[b] LOD: limit of detection; LOQ: limit of quantitation.

[c] Mean ± standard deviation of 5 determinations of 3 concentrations of each drug.

[d] Relative standard deviation of 3 determinations of 3 concentrations of each drug.

[e] Robustness was checked by varying the methanol ratio (61, 63, 67 v/v).

[f] Robustness was checked by varying the column temperature (38, 36, 42 °C).

Table 3. Application of standard addition technique to the analysis of Croma® eye drops by the proposed UPLC method.

Croma®	Claimed µg/ml	Found µg/ml [a]	Recovery % [a]	Added µg/ml	Recovery % [b]
SCG	80	79.66	99.58 ± 1.00 [b]	5	100.22
				10	99.54
				20	100.98
					100.25 ± 0.72 [b]
TZH	1	1.00	100.21 ± 0.55 [b]	3	99.05
				6	100.87
				9	99.65
					99.86 ± 0.93 [b]

[a] Average of six experiments.

[b] Average of three experiments.

[c] Mean ± standard deviation.

Table 4. Determination of SCG and TZH in spiked rabbit aqueous humor samples in quality control samples using the proposed UPLC method.

Claimed concentrations in spiked samples (µg/ml) SCG : TZH	SCG		TZH	
	Found (µg/ml)	Recovery %	Found (µg/ml)	Recovery %
5 : 0	5.02	100.42		
10 : 0	9.98	99.83		
50 : 0	50.42	100.84		
0 : 0.5			0.50	100.80
0 : 1			1.00	100.42
0 : 5			5.02	100.45
Mean ± SD		100.36 ± 0.51		100.56 ± 0.21
10 : 1	9.68	96.78	0.98	97.66
5 : 0.5	4.88	97.69	0.49	98.59
Mean ± SD		97.24 ± 0.64		98.13 ± 0.66

Table 5. Representative data for repeatability and inter-day precision validation parameters for standard solutions, expressed as RSD%.

Concentration level [b]	Repeatability [a]		Inter-day precision [a]	
	SCG	***TZH***	***SCG***	***TZH***
80%	0.986	1.055	0.799	1.247
100%	0.885	1.129	0.963	0.996
120%	1.025	0.997	1.005	1.236
Average	0.965	1.060	0.922	1.160

[a] values expressed as RSD % of 3 determinations of each concentration level.

[b] concentration levels of label claim (80 µg/ml SCG and 1 µg/ml TZH).

Table 6. Statistical comparison between the results obtained by the proposed UPLC method and the official methods for the determination of SCG and TZH in pure powder form.

Items	SCG		TZH	
	UPLC	Official BP method [a]	UPLC	Official BP method [a]
Mean %	99.93	100.80	99.91	100.06
SD	1.14	0.74	0.90	0.59
Variance	1.2996	0.5476	0.8100	0.3481
n	8	5	8	5
Student's *t*-test (2.201) [b]	1.504		0.323	
***F* value (6.094) [b]**	2.361		2.287	

[a] BP method for SCG and TZH are potentiometric titration methods.

[b] Figures between parentheses are the corresponding tabulated *t* and *F* values of at P=0.05.