Validated RP-HPLC and TLC-Densitometric Methods for Analysis of Ternary Mixture of Cetylpyridinium Chloride, Chlorocresol and Lidocaine in Oral Antiseptic Formulation

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Abstract

This work was concerned with development, optimization, application and validation of reversed phase high performance liquid chromatography (RP-HPLC) and thin layer chromatography (TLC)-densitometric methods for analysis of cetylpyridinium chloride, chlorocresol and lidocaine in Canyon® gel. The first developed RP-HPLC method depended on chromatographic separation on a ZORBAX Eclipse Plus C8 column, with elution with a mobile phase consisting of 0.05% phosphoric acid solution : acetonitrile : methanol (15 : 24 : 61, by volume), pumping the mobile phase at a flow rate of 1.00 mL min⁻¹, with ultraviolet detection at 220 nm. While in the subsequently developed method, the TLC-densitometric method, complete separation of the studied mixture was achieved using methanol : acetone : acetic acid (7 : 3 : 0.2, by volume) as a mobile phase, aluminum plates pre-coated with silica gel 60 F₂₅₄ as a stationary phase and 215 nm as the scanning wavelength. Factors affecting the developed methods were studied and optimized; moreover, methods had been validated as per the International Conference of Harmonization guideline and the results indicated that the suggested methods were reproducible, reliable and applicable for rapid routine analysis. Statistical comparison of the two developed methods with the reported HPLC ones using F- and Student’s t tests showed no significant difference.

Introduction

Cetylpyridinium chloride (CE) is chemically designated as 1-hexadecylpyridinium chloride (1, 2) (Figure 1A). It is an antiseptic detergent (1), a cationic quaternary ammonium compound used in some types of mouthwashes, toothpastes, lozenges, throat, breath and nasal sprays. It is effective in preventing dental plaque and reducing gingivitis (3, 4). Chlorocresol (CH) is chemically designated as 4-chloro-3-methylphenol (1, 2) (Figure 1B). It is an antimicrobial preservative. Lidocaine (LI) is chemically designated as 2-(diethylamino)-N-[2,6-dimethylphenyl]acetamide, (1, 2) (Figure 1C). LI is a common local anesthetic and anti-arrhythmic drug (5). It is used topically to relieve itching, burning and pain from skin inflammations (6) and as a local anesthetic for minor surgery (5–8). It is characterized by rapid onset of action and intermediate duration of efficacy (9–11). A combination of CE, CH and LI works to relieve pain caused by teething, mouth ulcers or denture irritation. Antiseptics help to prevent the irritated areas from getting infected leading to fast healing. CE, CH and LI were analyzed separately by titrimetric methods (1), while only LI was determined in United States Pharmacopoeia (2) by the HPLC method. The literature survey revealed several analytical methods for the determination of CE either alone or in combination with other drugs such as spectroscopic (12–15), chromatographic
was a deuterium lamp, slit dimensions were adjusted to 6 × 0.45 mm and scanning speed was 20 mm s⁻¹.

Materials and reagents

Pure standards
CE, CH and LI pure standards were kindly supplied by Bioregional international group for phoenic for advanced products, Massken Sheraton, Cairo, with claimed purities of 98.53, 97.94 and 99.96%, respectively, according to the manufacturer’s certificate.

Pharmaceutical formulations
Canyon® Gel: Batch No. 100004 manufactured by Bioregional international group for phoenic for advanced products and labeled to contain 0.2, 1 and 10 mg of CE, CH and LI, respectively, per gram.

Chemicals and solvent
All chemicals used throughout this work were of analytical grade and were used without further purification: deionized water (SEDCO Pharmaceutical Co., Cairo, Egypt); methanol, acetone and phosphoric acid (El-Nasr Pharmaceutical Chemicals Co., Abu Zabaal, Cairo, Egypt); acetic acid, methanol and acetonitrile HPLC grade (Chromosolve®, Sigma-Aldrich, Chemie GmbH, Germany—supplied by the Egyptian International Center for Import, Cairo, Egypt).

Solutions
Stock standard solutions of CE, CH and LI (S = 1 mg mL⁻¹): 100 mg of CE, CH and LI were accurately and separately weighed into three separate 100-mL volumetric flasks and the volume was then completed to the mark with methanol.

Working standard solutions of CE, CH and LI (W = 0.1 mg mL⁻¹): They were prepared by placing 10 mL of their respective stock solutions (S = 1 mg mL⁻¹) into three separate 100-mL volumetric flasks and completing the volume using methanol.

Pharmaceutical dosage form solution (D = 0.02, 0.1 and 1 mg mL⁻¹ for CE, CH and LI, respectively): 10 g of Canyon®gel was emulsified into and triturated well in a mortar. A sample containing CE, CH and LI equivalent to 0.2, 1 and 10 mg, respectively, was transferred into a 10-mL volumetric flask. About 7 mL of methanol was added and the flask was sonicated for 15 min. The solution was filtered and the volume was completed to the mark with methanol to obtain a stock sample solution (D) containing 0.02, 0.1 and 1 mg mL⁻¹ of CE, CH and LI, respectively.

Procedure

Chromatographic conditions
RP-HPLC method
Chromatographic separation was performed on a C₈ column (Agilent Technologies, ZORBAX Eclipse Plus) (25 cm × 4.6 mm i.d., 5 µm particle size) using a mobile phase of 0.05% phosphoric acid solution : acetonitrile : methanol (15 : 24 : 61, by volume) delivered at a flow rate of 1 mL min⁻¹; samples were injected in volumes of 20 µL at ambient temperature and monitored at 220 nm using a DAD, and then peak areas were recorded.

TLC-densitometric method
Samples were applied on aluminum plates precoated with silica gel 60 F₂₅₄ (20 × 10 cm) as bands of 6 mm width with a 100-µL sample syringe using an autosampler. The space between bands was 8.9 mm and a constant application rate of 0.1 µL/s was used. The scanning...
speed was 20 mm/s and the slit dimension was 6.0 × 0.3 µm. The mobile phase consisted of methanol: acetone: acetic acid (7:3:0.2, by volume). A glass chamber saturated with the mobile phase was used for linear ascending development. Development of the plates was allowed till the mobile phase migrated a distance of 8 cm. Following the development, the plates were air-dried. Densitometric scanning was performed using a CAMAG TLC Scanner in the reflectance-absorbance mode at 215 nm and operated by WINCATS software using a deuterium lamp as a radiation source and then the peak areas were recorded.

**Linearity and construction of calibration curves**

External standards were used to construct calibration curves for the two proposed methods because their use allows analysis of a series of samples using a single calibration curve, which is an important advantage when we have many samples to be analyzed (53).

**RP-HPLC method**

Calibration graphs for CE, CH and LI were constructed by recording and storing the peak areas of different concentrations of each in the ranges of 1–30, 0.5–30 and 1–50 µg mL\(^{-1}\), respectively, prepared by suitable dilutions of their respective working standard solutions (W = 0.1 mg mL\(^{-1}\)) then separately injecting a volume of 20 µL of each solution in triplicate. The procedure under chromatographic conditions was then followed. Calibration graphs were constructed by plotting peak area ratios (using a concentration of 10 µg mL\(^{-1}\) of each drug as an external standard to its respective calibration curve) against the corresponding concentration, and regression equations were then computed.

**TLC-densitometric method**

Accurate volumes of 0.1–5, 0.2–3 and 0.4–6 mL were transferred from CE, CH and LI stock standard solutions (S = 1 mg mL\(^{-1}\)), respectively, into three separate sets of 10 mL volumetric flasks; the volumes were completed to the mark with methanol. A volume of 10 µL from each flask was spotted in triplicate on TLC plates. The procedure under chromatographic conditions was performed. The peak area ratios (using 1 µg band\(^{-1}\) each of CE, CH and LI as external standards) were plotted against their respective concentrations to obtain the calibration graphs.

**Application to pharmaceutical formulations**

**RP-HPLC method**

Four sample solutions, D\(_1\), D\(_2\), D\(_3\) and D\(_4\), were prepared from Canyon® gel stock sample solution (D) by accurately transferring 1 mL, 0.2 mL, 0.02 and 0.5, respectively, into four separate 10-mL volumetric flasks and the volume was completed to the mark with methanol. Twenty microliters of the last prepared solutions were injected in triplicate, for each, following the procedure described for the chromatographic conditions. Concentrations of CE in D\(_1\) and D\(_4\), CH in D\(_2\) and D\(_4\), and LI in D\(_1\) and D\(_3\) sample solutions were then calculated from the respective constructed regression equations. A standard addition technique was carried out to prove the accuracy of the suggested method, and it was performed by spiking the preanalyzed CE, CH and LI samples in D\(_1\), D\(_2\) and D\(_3\), respectively (2 µg mL\(^{-1}\) for each) with an extra 50, 100, 150 and 200% of standard CE, CH and LI.

**TLC-densitometric method**

A sample of 5 µL of Canyon® gel solution (D) containing 0.02, 0.1 and 1 mg mL\(^{-1}\) of CE, CH and LI, respectively, was spotted on TLC plates in triplicate following the procedure described for the chromatographic conditions to obtain concentrations of 0.1, 0.5 and 5 µg band\(^{-1}\), respectively, and the chromatographic method was then continued as before. The peak area ratio of each spot was determined and the concentrations were calculated from the respective previously computed regression equations. The standard addition technique was carried out to ensure accuracy of the suggested method, and it was performed by spiking the preanalyzed CE, CH and LI samples (0.1, 0.5 and 5 µg band\(^{-1}\), respectively) with an extra standard concentrations of CE, CH and LI, respectively.

**Results**

Although different methods were reported for determining each of CE, CH and LI either alone or with other drugs, none of these methods determined CE, CH and LI in their ternary mixture. The main task of this work was to develop sensitive, selective and accurate RP-HPLC and TLC chromatographic methods for the determination of CE, CH and LI in their ternary mixture and in pharmaceutical formulations, with satisfactory precision for good analytical practice.

The proposed methods were applied for the determination of CE, CH and LI in their pharmaceutical formulation; firstly, for the RP-HPLC method, calibration curves were obtained relating the peak area ratios with the corresponding concentrations in the ranges of 1–30, 0.5–30 and 1–50 µg mL\(^{-1}\) for CE, CH and LI, respectively (using 10 µg mL\(^{-1}\) of each drug as a respective external standard). The concentrations of CE, CH and LI were calculated from the corresponding regression equations. Regression equation parameters are given in Table I. Secondly, for TLC-densitometric method, calibration curves were obtained relating the peak area ratios with the corresponding concentrations in the ranges of 0.1–5, 0.2–3 and 0.4–6 µg band\(^{-1}\) for CE, CH and LI, respectively (using a concentration of 1 µg band\(^{-1}\) of CE, CH and LI as an external standard). The concentrations of CE, CH and LI were calculated from the corresponding regression equations. Regression equation parameters are given in Table I.

To evaluate the applicability of the proposed methods, the methods were applied to Canyon® gel. Three peaks were detected at \(t_R = 3.7 ± 0.2\), 2.7 ± 0.1 and 2.1 ± 0.1 min for the RP-HPLC method and at \(R_I = 0.12 ± 0.01\), 0.81 ± 0.02 and 0.48 ± 0.01 for the TLC-densitometric method for CE, CH and LI, respectively, indicating no interference from the excipients that routinely occur in gel pharmaceutical formulations. The mean percentage recovery of the drug content was found to be acceptable (Table II).

The suggested methods were compared favorably with the reported HPLC methods (51, 29) as shown from the values of the calculated Student’s t and F values, confirming that there was no significant difference within a probability of 95% between the proposed methods and the reported ones (Table III).

**Discussion**

**Development and optimization of methods**

**Optimization of the RP-HPLC method**

**Optimization of the mobile phase.** Several mobile phases were tried: water : methanol, water : acetonitrile with different ratios and at different flow rates. Water was replaced with phosphoric acid solution at different strengths and with different ratios. Finally, a mobile phase of 0.05% phosphoric acid solution : acetonitrile : methanol (15 : 24 : 61, by volume), a stationary phase of ZORBAX Eclipse Plus C\(_8\) column, a flow rate of 1.00 mL min\(^{-1}\) and UV detection at 220 nm resulted in a stable baseline, adequate separation and sharp peaks in a suitable analysis.
time, where the $t_R$ values for CE, CH and LI were $3.7 \pm 0.2$, $2.7 \pm 0.1$ and $2.1 \pm 0.1$ min, respectively (Figures 2 and 3).

Selection of stationary phase. ZORBAX Eclipse plus C18 and C8 columns were tried; the C8 column provided higher resolution and more symmetric peaks.

Selection of scanning wavelength. Different wavelengths were tried; however, 220 nm provided the best results with respect to peak sensitivity.

Optimization of column temperature. The column compartment was adjusted to different temperatures (20, 25 and 30°C). Note that

Table I. Regression Parameters of the Proposed HPLC and TLC-Densitometric Methods for the Determination of CE, CH and LI

| Parameters       | CE    |     | CH    |     | LI    |     |
|------------------|-------|-----|-------|-----|-------|-----|
|                  | HPLC  | TLC | HPLC  | TLC | HPLC  | TLC |
| Linearity        |       |     |       |     |       |     |
| Range            | 1–30 µg mL$^{-1}$ | 0.1–5 µg band$^{-1}$ | 0.5–30 µg mL$^{-1}$ | 0.2–3 µg band$^{-1}$ | 1–50 µg mL$^{-1}$ | 0.4–6 µg band$^{-1}$ |
| Slope            | 0.077 | 0.554 | 0.080 | 0.401 | 0.066 | 0.459 |
| Intercept        | 0.228 | 0.432 | 0.207 | 0.590 | 0.349 | 0.536 |
| $r$              | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 |
| Accuracy (mean ± %RSD) | 100.58 ± 0.502 | 100.43 ± 0.554 | 100.05 ± 0.555 | 100.25 ± 1.194 | 99.99 ± 0.988 | 99.97 ± 1.324 |
| Precision        |       |     |       |     |       |     |
| Repeatability    | 0.675 | 1.386 | 0.834 | 0.964 | 0.417 | 1.316 |
| Intermediate     | 1.642 | 1.864 | 1.752 | 1.694 | 1.054 | 1.829 |
| LOD              | 0.327 | 0.029 | 0.314 | 0.064 | 0.330 | 0.125 |
| LOQ              | 0.990 | 0.090 | 0.950 | 0.193 | 1.000 | 0.380 |

Table II. Determination of CE, CH and LI in Pharmaceutical Formulation by the Proposed HPLC and TLC-Densitometric Methods and Application of a Standard Addition Technique

| Pharmaceutical formulations | Taken (µg mL$^{-1}$) | % Recovery ± RSD | Standard addition technique | Pure added concentrations | % Recovery |
|-----------------------------|----------------------|------------------|-----------------------------|--------------------------|-----------|
| Canyon Oral gel B.N. 100004 each gram labeled to contain 0.2, 1 and 10 mg of CE, CH and LI, respectively | | | | | |
| CE HPLC | 2.00 µg mL$^{-1}$ | 103.92 ± 0.175 | 1.00 µg mL$^{-1}$ | 99.76 |
| TLC | 0.10 µg band$^{-1}$ | 103.94 ± 0.178 | 0.20 µg band$^{-1}$ | 100.97 |
| CH HPLC | 2.00 µg mL$^{-1}$ | 101.04 ± 0.139 | 1.00 µg mL$^{-1}$ | 100.07 |
| TLC | 0.50 µg band$^{-1}$ | 101.09 ± 0.228 | 0.20 µg band$^{-1}$ | 100.95 |
| LI HPLC | 2.00 µg mL$^{-1}$ | 97.93 ± 0.175 | 1.00 µg mL$^{-1}$ | 97.75 |
| TLC | 5.00 µg band$^{-1}$ | 97.97 ± 0.224 | 0.20 µg band$^{-1}$ | 98.04 |

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the column temperature neither affected the peak sharpness nor the chromatographic separation.

Optimization of flow rate. Different flow rates were tried and the best flow rate was 1 mL min⁻¹.

An acceptable separation and peak symmetry were obtained using the above optimum conditions where the \( t_R \) values were 3.7 ± 0.2, 2.7 ± 0.1 and 2.1 ± 0.1 min for CE, CH and LI, respectively (Figures 2 and 3).

Optimization of the TLC-densitometric method

Optimization of developing system. Different developing systems of different compositions were tried as methanol : chloroform, methanol : ethyl acetate and methanol : acetone in different ratios. Ammonia and acetic acid solutions were added separately to the last system in different ratios, whereby addition of acetic acid solution to make a system of methanol : acetone : acetic acid (7 : 3 : 0.2, by volume) removed tailing, improved spot shape and resulted in optimum separation with good peak symmetry (Figures 4 and 5).

Optimization of scanning wavelength. Different scanning wavelengths were tried. The best scanning wavelength was 215 nm, which showed a good signal-to-noise ratio for all components resulting in high sensitivity.

Optimization of slit dimensions of scanning light beam. Different band dimensions were tested. The optimum bandwidth chosen was

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**Table III. Statistical Comparison of the Proposed Methods and the Reported Ones for the Determination of CE, CH and LI in their Pharmaceutical Formulation**

| Items    | HPLC method | TLC-densitometry | Reported HPLC methods (51, 29) |
|----------|-------------|------------------|-------------------------------|
|          | CE          | CH               | LI   | CE          | CH               | LI   | CE          | CH               | LI   |
| Mean     | 103.92      | 101.04           | 97.93 | 103.94      | 101.09           | 97.97 | 103.87      | 100.90           | 98.06 |
| %RSD     | 0.175       | 0.139            | 0.175 | 0.178       | 0.228            | 0.224 | 0.164       | 0.159            | 0.170 |
| \( N \)  | 8           | 8                | 8    | 8           | 8                | 8    | 8           | 8                | 8    |
| Student’s \( t \) test (2.144)b | 0.469       | 1.811            | 1.539 | 0.759       | 1.900            | 0.950 | 8           | 8                | 8    |
| \( F \) value (3.787)b        | 1.135       | 1.311            | 1.058 | 1.181       | 2.073            | 1.735 | 8           | 8                | 8    |

*CE and LI: HPLC method: ZORBAX SB-C8, 5 µm, 250 x 4.6 mm column, gradient elution, 0.05 M phosphoric acid and acetonitrile, UV detection at 214 and 258 nm. CH: HPLC method: C8, 150 x 3.9 mm column, 1.00 mL min⁻¹ flow rate, 1.5% w/v aqueous ammonium acetate buffer–acetonitrile, 55 : 45 (v/v) of pH 3.8, UV detection at 240 nm.

bFigures between parenthesis represent the corresponding tabulated values of \( t \) and \( F \) at \( P = 0.05 \).
4 mm with an 8.9 mm inter-space between bands. Different slit dimensions were tried, where 4 × 0.45 mm proved to be the slit dimensions of choice that provided high sensitivity.

Acceptable chromatographic separation for the ternary mixture was achieved upon using the above optimum conditions. The respective compounds were well separated at reasonable retention times, where the $R_f$ values were $0.12 \pm 0.01$, $0.81 \pm 0.02$ and $0.48 \pm 0.01$ for CE, CH and LI, respectively (Figures 4 and 5).

**Validation of methods**

Methods validation was performed with respect to ICH guidelines (52).

**Linearity**

The Beer–Lambert law was obeyed in the concentration ranges of 1–30, 0.5–30 and 0.1–50 µg mL^{-1} for CE, CH and LI, respectively (for the RP-HPLC method) and in the range of 0.1–3, 0.2–3 and 0.4–6 µg band^{-1} for CE, CH and LI, respectively (for the TLC-densitometric method). Regression parameters like correlation coefficients, intercept and slope values were calculated and are presented in Table I.

**Accuracy**

Accuracy was calculated as percentage recovery of pure CE, CH and LI as presented in Table I. Accuracy was further evaluated by application of a standard addition technique by addition of known amounts of pure drugs, at different levels (50, 100, 150 and 200%), to known concentrations of Canyon® gel and then analyzing the prepared mixtures. Acceptable results are given in Table II.

**Precision**

Precision was studied intra-day (repeatability) and inter-day (intermediate precision). Repeatability was evaluated by repeating the assay of three different concentrations three times in the same day, while intermediate precision was evaluated by assaying the same samples in triplicate on three successive days, using the developed chromatographic methods and calculating the percentage recoveries and percent relative standard deviation (%RSD) values. Results in Table I confirmed the satisfactory precision of the proposed methods.

**Limits of detection and quantitation**

Limits of detection and quantitation for CE, CH and LI are presented in Table I, which showed adequately small values, indicating the high sensitivity of the proposed methods.

**Selectivity**

The selectivity of the methods was confirmed by the good resolution of the proposed drugs as shown in the chromatograms (Figures 2 and 4). Also good percentage recoveries obtained upon applying the methods to Canyon® gel proved the high selectivity of the proposed methods and that there was no interference from excipients (Figures 3 and 5) (Table II).

**Robustness**

Robustness of the methods was evaluated by calculating the %RSD of peak areas for each studied parameter. It was established by making small deliberate changes in the chromatographic parameters, e.g., changing the flow rate by ±0.1 mL min^{-1}; changing the phosphoric acid, acetonitrile and methanol ratios in the mobile phase by ±2, ±1 and ±3%, respectively; changing the strength of phosphoric acid by ±0.001 (for the RP-HPLC method); changing the methanol and acetonitrile ratios of the developing system by ±1 and ±0.3%, respectively; changing the acetic acid percent by ±0.02 (for the TLC-densitometric method), and then calculating the resolution among the studied drugs. It was found that the changes in the studied parameters have no significant effect on the chromatographic resolution.

**System suitability testing parameters**

The system suitability test confirms that the analytical procedure is valid as well as ensures the resolution between different peaks of interest. System suitability testing was carried out during methods development and optimization according to ICH guidelines (52). Resolution (Rs) and selectivity ($\alpha$) factors were calculated and were found to be >2 and 1.5, respectively, for all drugs. In addition, the symmetry factors were calculated for the three drugs and nearly equaled 1. Other parameters such as capacity factor, number of theoretical plates and height equivalent to theoretical plates were calculated, and their values were within the acceptable limits (Table IV).
Table IV. System Suitability Testing Parameters of HPLC and TLC-Densitometric Methods for the Determination of CE, CH and LI

| Parameters                  | Obtained value |         |         |         |         |
|-----------------------------|----------------|---------|---------|---------|---------|
|                             | HPLC           | TLC     |         |         |         |
|                             | CH             | CE      | LI      | CH      | CE      |
| Symmetry factor             | 1.12           | 1.00    | 0.98    | 1       |         |
| Resolution (Rs)             | 2.21           | 3.96    | 3.63    | 2.41    |         |
| Capacity factor (k)         | 2.16           | 3.25    | -       | -       |         |
| Selectivity factor (α)      | 1.62           | 2.01    | 2.95    | 2.18    |         |
| Number of theoretical plates (N) | 4,427         | 7,642   | 13,496  | -       | -       |
| HETP                        | $5.65 \times 10^{-3}$ | $3.27 \times 10^{-3}$ | $1.85 \times 10^{-3}$ | -       | -       |

HETP = height equivalent to theoretical plates (cm/plate).

Conclusion

The developed chromatographic methods were the first developed methods for the determination of CE, CH, and LI in their ternary mixtures in pure form with successful application to pharmaceutical formulations. The proposed RP-HPLC method had some advantages over the reported HPLC-DAD method (51), in that it provided separation for CE, CH and LI in their ternary mixture within 4 min of using the simple isocratic elution protocol, while the proposed TLC-densitometric method had the high sensitivity required for the determination of CE, CH and LI even in the pharmaceutical formulation ratio of 1 : 5 : 50, with high precision and selectivity. Also the proposed TLC-densitometric method saved time so that several samples could be run simultaneously using a small quantity of the developing system. The aforementioned advantages made them preferable methods for quality control of the studied drugs. Moreover, all the obtained results confirmed the applicability, accuracy and precision of these methods.

Conflict of interest statement. None declared.

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