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Original Article

HPLC Method for Determination of Chlorhexidine in Pharmaceutical Formulations alone and in presence of Hexamidine and p-chlorocresol, and in Spiked Human Saliva

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ABSTRACT

In the current study, a simple, reliable, and quantitative HPLC analytical method was designed to determine Hexamidine Di isethionate (HEX), Chlorhexidine Digluconate (CHX), and p-chlorocresol (CSOL) in various dosage forms including mouthwash and intimate douche in addition to chlorhexidine determination in spiked human saliva. HEX, CHX, and CSOL were determined in colored aqueous formulations without any sample pre-treatment or extraction steps. The proposed method showed linearity over a concentration range of 0.10 to 25.00 µg/mL of pure HEX, 2.00 to 30.00 µg/mL of pure CHX, and 0.10 to 30.00 µg/mL of pure CSOL and a detection limit of 0.02 µg/mL, 0.47 µg/mL & 0.03 µg/mL for HEX, CHX, and CSOL; respectively. The recoveries for Cyteal® were 100.43 %±1.70, 99.06 %±0.69 & 98.74 %±1.06 for HEX, CHX, and CSOL; respectively, whereas, for Hexitol® recovery was 100.79 %±1.57 for CHX. Furthermore, the proposed method has been employed to detect CHX in spiked human saliva with a recovery of 101.69%±1.38.

Keywords: *HPLC; Hexamidine; Chlorhexidine; p-chlorocresol; COVID-19.*

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1. INTRODUCTION

Chlorhexidine digluconate **(CHX)**; 1,1′-Hexamethylenebis[5-(4-chlorophenyl) biguanide] di-D-gluconate, Hexamidine **(HEX)** [1,6-di(4-amidinophenoxy)-n-hexane] and p-Chlorocresol **(CSOL)** [4-Chloro-3-methylphenol] with the structures shown in **Fig. 1,** are included in many products such as disinfectants, washes, cleansing lotions and creams that are applied to the skin, soft tissues, and wounds. In dental treatments, chlorhexidine 0.2% oral solution has played a role in oral cavity disinfection which is recommended in COVID-19 preventive strategies **[1–6]**. The mouth cavity can act as a base for transmission of COVID-19 viral infection during the dental care procedures in which the dentist and the patients are close to each other. Furthermore, it has the potential to minimize the likelihood of caries formation due to the antiseptic properties of CHX against a diverse range of gram-positive and gram-negative bacteria **[7]**.

There are several methods of analysis for the determination of HEX, CHX and CSOL reported in the literature including spectroscopic methods **[8–13]**, HPLC methods **[14–23]** electrochemical

methods **[24–29]**, and capillary electrophoresis **[30]**.

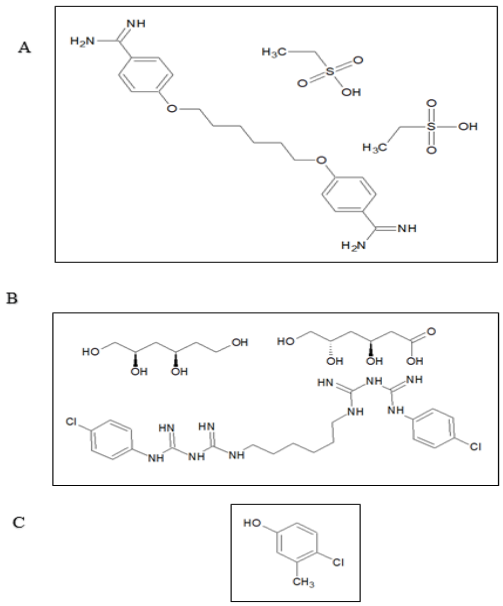

Fig. 1. The chemical structure of a) HEX b) CHX c) CSOL. (The structures were drawn by a Chem-Sketch program).

Our work aims to develop a rapid, simple, validated, and quantitative HPLC method that would be suitable for selective determination of a ternary mixture of HEX, CHX, and CSOL in their pure forms and in various dosage forms including mouthwash and intimate douche in addition to chlorhexidine determination in spiked human saliva. Also, our goal is to develop a method that overcomes many obstacles like the long duration of the method **[14]** or that the method was only involved in screening **[15]** and achieves the assay without pre-treatment steps or extraction of the sample. Several HPLC methods were reported for the determination of a single drug **[17, 18]**, binary Mixture **[14]**, or ternary mixture **[15]** of investigated drugs, and the comparison was shown in **Table 1**. Our study showed optimum separation and quantitation of a ternary mixture of the investigated drugs at a short run time with an optimum LOD and LOQ. Also, optimum system suitability parameters were obtained. Because of its quick run time, rapid analysis, and ability to analyze a large number of samples, this approach is recommended for routine analysis of the drugs specified. The method was defined following ICH guidelines **[31]**. The study of the effect of pH and interferents was performed.

2. Experimental

2.1. Instrumentation

An in-line vacuum degassing system, an autosampler, programmable temperature control, a heated column chamber, and a photodiode array detector (2998 PDA Detector) are all included in the HPLC waters Alliance e2695 Separating Module. To gather data and control all the equipment components, the empower-3 chromatography data software was employed. X-SELECT® RP C18 column (250 mm x 4.6 mm i.d. x 5 µm particle size) was used for separation. 0.22 µm disposable membrane filters (Millipore corp., USA). Sonicator Crest Ultrasonics (USA). Analytical balance SBA 3 (Scaltec)(Germany). A Jenway pH glass electrode type 3510 (Essex, UK) was utilized for the adjustment of pH.

2.2. Materials

2.2.1. Chemicals and reagents

O-phosphoric acid and potassium dihydrogen phosphate were purchased from El Nasr Pharmaceutical Co. (Egypt). 10 mM of potassium dihydrogen phosphate solution was prepared and adjusted to pH 3 with O-phosphoric acid. Methanol, acetonitrile, and water of HPLC grade (Fisher Scientific, UK). All the other chemicals were of analytical grade and didn't require any additional processing. Cyteal® intimate wash (batch number 190359), described to include 0.5 mL chlorhexidine di-gluconate of a solution of 20% (w/v), 0.1 g% Hexamidine di-isethionate, and 0.3 g% p-chlorocresol, and Hexitol® mouthwash (batch number 2030405), described to include 125 mg% chlorhexidine hydrochloride, were purchased from the local market, Cairo, Egypt.

2.2.2. Pure standards

Chlorhexidine Di-gluconate of purity 99.92%±0.04 was kindly supplied by NODCAR (Egypt). Hexamidine di-isethionate and p-chlorocresol both with a certified purity of 99.00%±0.04 were kindly purchased from Alibaba (China).

2.2.3. Acidified phosphate Solutions (Mixture A)

10 mM of potassium dihydrogen phosphate solution was prepared by dissolving 0.68 g potassium dihydrogen phosphate in water, magnetic stirred till dissolution, and sonicated for 10 min till complete dissolution. Afterward, it was completed to the mark with water in a 500-mL volumetric flask. The solution was adjusted to pH 3 with O-phosphoric acid using a pH meter.

Table 1. Comparison of the results obtained using the proposed HPLC method versus the reported methods **[14,15,17,18]** for HEX, CHX, and CSOL determination

Parameter	Proposed method	Reported method[14]	Reported method [15]	Reported method [17]	Reported method [18]
Type of column	X-SELECT® RP C18 column (250mm x 4.6mm i.d. x 5 µm	µBondapakX18 (300 mm X 4 mm, 10 pm)	A 125 mm x 4.6 mm I.D. stainless-steel column slurry-packed with 10µm µBondapak C18	(Luna) C18 (250 mm × 4.6 mm, 5µm	a C8 column, ZORBAX Eclipse Plus, (25 cm × 4.6 mm i.d., 5 µm particle size)
Flow rate	1.00 mL/min	2.00 ml/min	2.50 mL/min	1.50 mL/min	1.00 mL/min
Detector	UV detection at 265, 260 & 230 nm for HXD, CHX & CSOL; respectively	UV detection at 264 nm	UV detection at 264 nm	UV detection at 240 nm	UV detection at 220 nm
Retention time (minutes)	2.50 (HXD) 5.90 (CHX) 9.30 (CSOL)	13.50 (HEX) 22.50 (CHX)	11.50 (CSOL) 13.30 (HXD) 16.40 (CHX)	8.80 (CSOL)	2.70 (CSOL)
Applications	Intimate douche, mouthwash & saliva	Creams	Creams	Cream	Canyon® Gel
Measured drugs under investigation	HEX, CHX, & CSOL	HEX, CHX	HEX, CHX, & CSOL	CSOL	CSOL
Concentration range (µg/ml)	0.10 to 25.00 (HEX), 2.00 to 30.00 (CHX), & 0.10 to 30.00 (CSOL)	---		64.00-96.00 (CSOL)	0.50–30.00 (CSOL)
LOD (µg/ml)	HEX (0.02) CHX (0.47) CSOL (0.03)	----	This method was only implemented for the screening of several preservatives but not for quantitation.	----	0.314
LOQ (µg/ml)	HEX (0.07) CHX (1.41) CSOL (0.08)	----		----	0.950
Number of theoretical plates (N)	12925.19	---		50388.00	7,642
Tailing factor	1.08 (CSOL)	---		1.16 (CSOL)	----

2.2.4. Preparation of Mixture B

Methanol was mixed with Mixture A and the final solution was adjusted to pH 3 with O-phosphoric acid using a pH meter in a ratio of 1:1 to prepare Mixture B.

2.2.5. Standard Solutions

2.2.5.1. HEX stock solution (100 µg/mL)

A stock solution (100 µg/mL) was prepared by dissolving 0.01 gm of HEX in 80 mL of Mixture B, sonicated for 10 min, and completed to the mark with Mixture B in a 100 mL volumetric flask followed by another sonication for 5 min.

2.2.5.2. CHX stock solution (100 µg/mL)

A stock solution (100 µg/mL) was prepared by dissolving 0.01 gm of CHX in 80 ml of Mixture B, sonicated for 10 min, and completed to the mark with Mixture B in 100 mL volumetric flask followed by another sonication for 5 min.

2.2.5.3. CSOL stock solution (100 µg/mL)

A stock solution (100 µg/mL) was prepared by dissolving 0.01 g of CSOL in 80 ml of Mixture B, sonicated for 10 min, and completed to the mark with Mixture B in a 100 mL volumetric flask followed by another sonication for 5 min.

2.3. Procedures

2.3.1. Chromatographic conditions

A gradient elution technique as shown in **Table 2** was applied with a flow rate of 1.00 mL/min at room temperature. The photodiode array detector was adjusted at 265, 260, and 230 nm for HEX, CHX, and CSOL; respectively in a timed-wavelength protocol provided by the apparatus software. All solvents were filtered through 0.22 µm membrane filters followed by degassing under ultrasonication in an ultrasonic bath. X-SELECT® RP C18 column (250 mm x 4.6 mm i.d. x 5 µm particle size) was conditioned with the mobile phase for 10 min or more till stabilization of pressure. Additionally, the sample solutions were filtered through 0.22 µm syringe filters.

Table 2. The developed gradient elution technique:

Time (minutes)	Flow rate (mL/min)	Methanol	Acetonitrile	Mixture A
0.00	1.00	55.00 %	5.00 %	40.00 %
3.00	1.00	55.00 %	5.00 %	40.00 %
3.50	1.00	5.00 %	50.00 %	45.00 %
10.00	1.00	5.00 %	50.00 %	45.00 %
11.00	1.00	55.00 %	5.00 %	40.00 %

2.3.2. Method Validation

The proposed method was validated according to ICH guidelines **[31]**.

2.3.2.1. Selectivity

Separation selectivity of the proposed method, which means the ability of specific separation of the investigated drugs in presence of other materials, was assessed by the absence of any interfering peaks at the defined retention times of the drugs under investigation. “A mixture of HEX, CHX, and CSOL (10.00 µg/mL each); respectively, was prepared and injected in triplicates under the selected chromatographic conditions.

2.3.2.2. Linearity and range

A series of dilutions ranging from 0.10 to 25.00 µg/mL of pure HEX, 2.00 to 30.00 µg/mL of pure CHX, and 0.10 to 30.00 µg/mL of pure CSOL were prepared separately from their stock standard solutions (100 µg/mL) by transferring appropriately measured volumes (0.01-2.50 mL) of HEX standard stock solutions, (0.20-3.00 mL) CHX standard stock solutions and (0.01-3.00 mL) of CSOL standard stock solutions into three separate sets of 10 mL volumetric flasks. A combination of methanol and acidified phosphate solution was used to fill the volume to the mark. A volume of 20 µL of each solution was injected

in triplicates. The chromatographic conditions stated above were applied and the mean peak areas were calculated for HEX, CHX, and CSOL; respectively. For each drug, a calibration curve was constructed representing the relationship between the concentration of each dilution versus the corresponding peak area, and regression equations were assessed.

2.3.2.3. LOD and LOQ

Calculations of LOD & LOQ were performed through the following equations:

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

Where σ is the standard deviation of intercept and S is the slope of the calibration curves of drugs under investigation.

2.3.2.4. Accuracy

Five different concentrations of pure samples of HEX (5.00, 10.00, 15.00, 20.00, 25.00 µg/mL), CHX (5.00, 8.00, 10.00, 15.00, 25.00 µg/mL), & CSOL (5.00, 10.00, 15.00, 20.00, 25.00 µg/mL) were measured three times, the concentrations were assessed from the corresponding regression equation, and the percentage recoveries related to each one were calculated to estimate accuracy.

2.3.2.5. Precision

2.3.2.5.1. Repeatability (intraday precision)

The recoveries of three different concentrations of pure samples of HEX (5.00, 10.00, 20.00 µg/mL), CHX (5.00, 10.00, 15.00 µg/mL), and CSOL (5.00, 10.00, 20.00 µg/mL), were determined three times on the same day. The concentrations were assessed from the corresponding regression equation, the percentage recoveries related to each one, and standard deviations were calculated.

2.3.2.5.2 Intermediate precision (interday precision)

The recoveries of three different concentrations of pure samples of HEX (5.00, 10.00, 20.00 µg/mL), CHX (5.00, 10.00, 15.00 µg/mL), and CSOL (5.00, 10.00, 20.00 µg/mL) were determined three times on three consecutive days. The concentrations were assessed from the corresponding regression equation, the percentage recoveries related to each one, and standard deviations were calculated.

2.3.2.6. Robustness

The robustness of a method is determined by modifying the analysis of experimental conditions. It reflects the method's capacity to stay unaffected by minor experimental differences. Two different gradient systems as shown in **Table 3 (A & B)** and measurements at two different pH (2.8 and 3.2) values were applied to assess robustness with the other parameters remaining fixed. The concentration of 10 µg/mL of each drug was prepared and measured under the same chromatographic conditions as other parameters.

Table 3. Two different gradient systems (A & B)

(A)

Time (minutes)	Flow rate (mL/min)	Methanol	Acetonitrile	Mixture A
0.00	1.00	45.00 %	15.00 %	40.00 %
3.00	1.00	45.00 %	15.00 %	40.00 %
3.50	1.00	5.00 %	50.00 %	45.00 %
10.00	1.00	5.00 %	50.00 %	45.00 %
11.00	1.00	45.00 %	15.00 %	40.00 %

(B)

Time (minutes)	Flow rate (mL/min)	Methanol	Acetonitrile	Mixture A
0.00	1.00	45.00 %	15.00 %	40.00 %
4.00	1.00	45.00 %	15.00 %	40.00 %
4.50	1.00	5.00 %	50.00 %	45.00 %
10.00	1.00	5.00 %	50.00 %	45.00 %
11.00	1.00	45.00 %	15.00 %	40.00 %

2.3.3. Application to pharmaceutical formulations

A volume of 125.00 µL of Cyteal® is labeled to contain (2.50, 12.50, & 7.50 µg/mL) of HEX, CHX & CSOL; respectively and another volume of 80 µL of Hexitol® is supposed to contain (10.00 µg/mL) of CHX were separately transferred into two 50 mL volumetric flasks, completed to the mark with Mixture A and sonicated for 10 min till complete mixing. The resulting solutions were filtered through 0.22 µm syringe filters. The filtered solutions were injected in triplicates and analyzed under the selected chromatographic conditions. Concentrations were assessed from the calibration curves regression equation. Mean recoveries and standard deviation were then assessed.

2.3.4. Application to Spiked Human Saliva

An accurately measured volume of 0.50 mL of pure CHX stock solution was mixed with 0.50 mL of human saliva from a healthy volunteer in a 25 mL volumetric flask, completed to the mark with Mixture A, and sonicated for 10 min till complete mixing. The resulting solution was filtered through a 0.22 µm syringe filter followed by measuring in triplicates under the selected chromatographic conditions. A blank was prepared by the same procedure without the addition of the drug. Concentrations were assessed from the calibration curve regression equation. Mean recoveries and standard deviation were then assessed.

3. Results and Discussion

Several pharmaceutical formulations like Cyteal® vaginal douche & Solo fresh® antiseptic solution contain the 3 drugs under investigation. That is why our study focused on the development of HPLC quantitative analytical technique to assess this ternary mixture in a single run without any interference. It is important to develop a reliable, quantitative, and simple analytical technique to facilitate the determination of the investigated drugs with a reasonable run time and optimum system suitability parameters. During method development, many parameters were optimized to get the best measurement conditions.

3.1. Optimization of the experimental conditions

Several mobile phase systems were tried in the method development and the most convenient one was selected that gave peaks of suitable sensitivity and selectivity showing high resolution, optimum baseline separation, and sharp peaks. In addition, different gradient eluting modes were also tried. The flow rate was changed during the measurements from low to high value (0.50-2.00 mL/min). The isocratic system was tried where different solvents such as (methanol: water and acetonitrile: water…etc) and different ratios were tried. Several binary and ternary mixtures of solvents were also tried. The best conditions for efficient separation with high resolution (R_s > 1.5) and sharpest peaks were found to be presented by the ternary mixture (methanol: acetonitrile: mixture A) under the selected gradient conditions at a flow rate of 1.00 mL/min. Many solvents and solvent ratios were tried with different flow rates in different modes

of elution. They gave broad peaks, no optimum baseline separation, or inefficient separation.

It is important to note that two drugs under investigation are compounds with basic functionalities. To separate them using reverse-phase HPLC, one of the eluting solvents was adjusted to be acidic (pH= 3) to facilitate their chromatographic separation.

The run time of each injection was 16 min showing reasonable and short time intervals allowing rapid analysis. The photodiode array detector was adjusted at 265, 260, and 230 nm for HEX, CHX, and CSOL; respectively in a timed-wavelength protocol provided by the apparatus software. Other wavelengths were tried but gave lower sensitivity. All solvents were filtered through 0.22 µm membrane filters followed by degassing in an ultrasonic bath. X-SELECT® RP C18 column (250 mm x 4.6 mm i.d. x 5 µm particle size) was conditioned with the mobile phase for 10 min or more till stabilization of pressure. Additionally, the sample solutions were filtered through 0.22 µm syringe filters. The mean retention time ± SD for three replicates were 2.50±0.20 min, 5.90±0.20 min & 9.30±0.20 min for HEX, CHX & CSOL; respectively.

3.2. Method Validation

3.2.1. Selectivity

Ternary mixtures of HEX, CHX, and CSOL were simultaneously separated and determined using the proposed HPLC method. In **Fig. 2,** the chromatogram showed simultaneous separation of HEX, CHX & CSOL (10.00 µg/mL each) at a timed wavelength of 265, 260 & 230 nm; respectively. Sharp peaks were revealed with optimum baseline separation and optimum resolution.

3.2.2. Linearity and range

Linear relationships between the peak areas and the corresponding concentrations over the ranges of 0.10 to 25.00 µg/mL of pure HEX, 2.00 to 30.00 µg/mL of pure CHX, and 0.10 to 30.00 µg/mL of pure CSOL were constructed (**Fig. 3, 4 & 5)** and the regression equations were calculated.

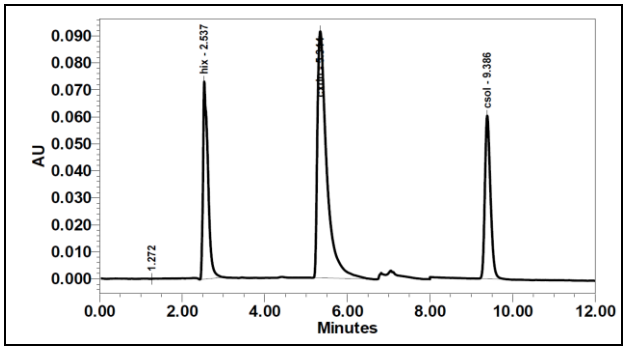

Fig. 2. HPLC chromatogram of the pure drugs showing simultaneous separation of HEX, CHX & CSOL (10.00 µg/mL each) at a timed wavelength of 265, 260 & 230 nm; respectively.

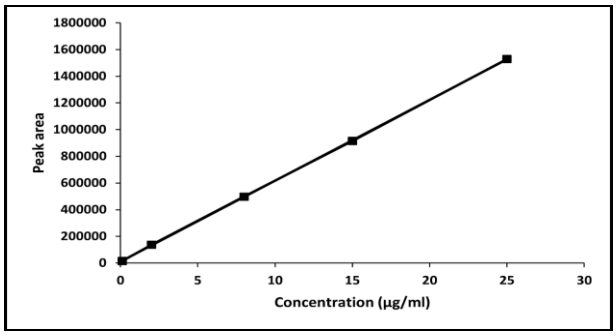

Fig. 3. Calibration curve for determination of HEX in pure form at λ_{max}= 265 nm using the proposed method.

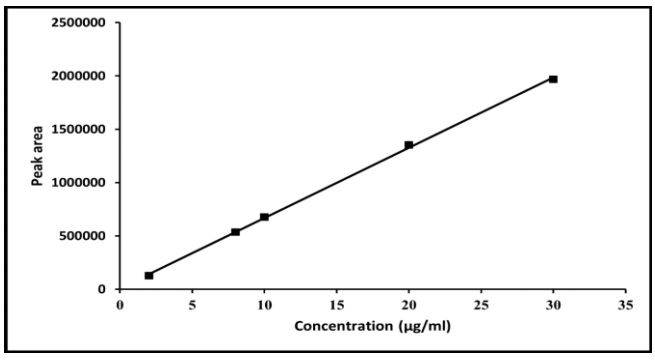

Fig. 4. Calibration curve for determination of CHX in pure form at λ_{max}= 260 nm using the proposed method.

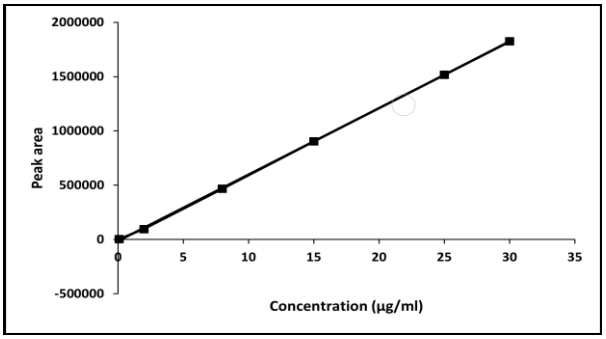

Fig. 5. Calibration curve for determination of CSOL in the pure form at λ_{max}= 230 nm using the proposed method.

3.2.3. LOD and LOQ

The results, as indicated in **Table 4,** showed adequate sensitivity to the proposed HPLC methods with a low detection limit and adequate LOQ. For HEX, CHX & CSOL, LOD values were found to be 0.02, 0.47 & 0.03 µg/mL; respectively and LOQ were 0.07, 1.41 & 0.08 µg/mL.

Table 4. Assay validation findings of the proposed HPLC method for determination of HEX, CHX & CSOL

Parameter		HEX	CHX	CSOL
Slope		60567.00	65,872.55	61297.00
Intercept (mV)		12135.00	9,812.8828	-15433.00
Correlation coefficient (r)		1.00	0.9997	0.99995
Concentration range (µg/mL)		0.10-25.00	2.00-30.00	0.10-30.00
Accuracy[a] ± S.D.		99.11 ± 0.84	99.34 ± 1.34	99.39 ± 0.44
Repeatability[b] (RSD%)		0.30	0.56	0.30
Intermediate precision[c] (RSD%)		0.32	0.56	0.32
LOD (µg/mL)		0.02	0.47	0.03
LOQ (µg/mL)		0.07	1.41	0.08
Robustness	**Gradient system[d] (RSD%)**	0.21	0.33	0.06
	pH[d] (RSD%)	0.14	0.33	0.04

[a] Mean of five determinations.

[b] Repeatability: (n=9), mean of 3 concentrations repeated 3 times within the same day (the intraday precision).

[c] Intermediate precision: (n=9), mean of 3 concentrations repeated 3 times over 3 consecutive days (the interday precision).

[d] RSD% (n=6).

3.2.4. Accuracy

As shown in **Table 4**, Five concentrations of each drug under investigation were measured in triplicates. The average recoveries were presented for HEX, CHX & CSOL, respectively as follow: 99.11 % ± 0.84, 99.34 % ± 1.34, 99.39 % ± 0.44.

3.2.5. Precision

As shown in **Table 4**, Three concentrations of each drug under investigation were measured in triplicates within the same day (intraday precision) and on three consecutive days (interday precision). RSD% for HEX, CHX & CSOL, of intraday precision were 0.30, 0.56, 0.30; respectively and of interday precision were 0.32, 0.56, 0.32, respectively suggesting excellent precision.

3.2.6. Robustness

The robustness of the method was assessed where two different pH values (2.8 & 3.2) were applied. RSD% of the method was 0.14, 0.33, and 0.04 for HEX, CHX, and CSOL; respectively. On the other hand, upon applying two gradient eluting systems, the RSD% of the method was 0.21, 0.33, and 0.06 for HEX, CHX, and CSOL; respectively. Low values of RSD% show good robustness of the proposed HPLC method.

3.2.7. System suitability

Optimum results for system suitability parameters are shown in **Table 5**. They showed that the instrumental system is in good working status, peaks exhibit good resolution and selectivity, as well as theoretical plates, are of high value.

Table 5. System suitability parameters of the proposed HPLC method for determination of HEX, CHX & CSOL in pure form

Parameter	**HEX**	**CHX**	**CSOL**	**Reference[35] value**
Capacity factor (K')	1.32	4.05	7.43	K' is > 2
Tailing factor (T)	1.24	2.26	1.08	T is ≤ 2
Selectivity (α)	---	2.79	1.79	(α > 1)
Number of theoretical plates (N)*	1774.78	2178.35	12925.19	N is > 2000
Resolution (R_s)**	---	8.28	9.28	R_s is > 2

* Measure column efficiency.

** resolution was assessed regarding the retention times of 2 successive peaks.

3.3. Application to pharmaceutical formulations

The peaks appeared at the selected retention time for each drug. They showed good resolution with minimal tailing, as shown in **Fig. 6 & 7**. For Cyteal® douche, the recoveries were 100.43 % ± 1.70, 99.06 % ± 0.69 & 98.74 % ± 1.06 for HEX, CHX, and CSOL; respectively, whereas, for Hexitol® mouth wash, recovery was 100.79 % ± 1.57 for CHX. These results are indicated in **Table 6.** The precision of the method was satisfactory as presented by the RSD% obtained from replicate analyses. The obtained results were found to be consistent with the drugs' claimed content. The analysis was not affected by the other constituents in the formulations. The solvent chosen for dilution was only mixture A without the addition of alcohol like methanol to avoid any precipitation of some ingredients present in the formulations like the surface-active agents and thus there was no need for sample purification or pre-treatment. As a result, the formulation containing the drugs under investigation and that colored one, like Hexitol® mouth wash, were easily analyzed without any sample pre-treatment.

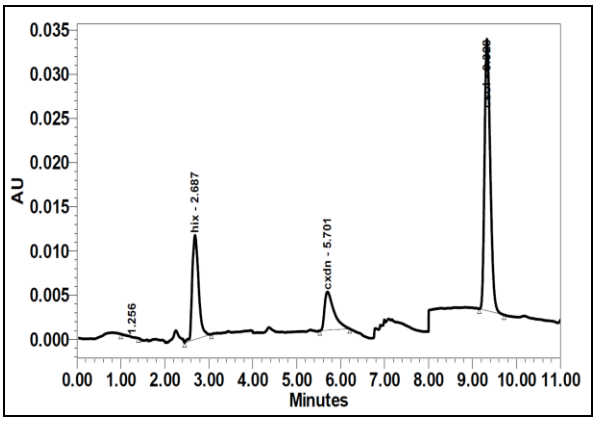

Fig. 6. HPLC chromatogram of Cyteal® douche showing simultaneous separation of 2.50 µg/mL HEX, 12.50 µg/mL CHX & 7.50 µg/mL CSOL at a timed wavelength of 265, 260 & 230 nm; respectively.

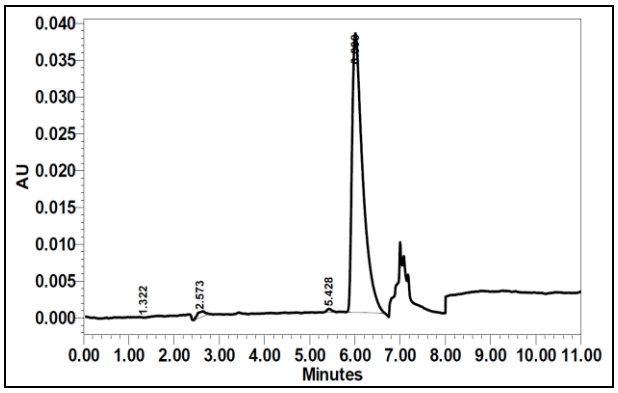

Fig. 7. HPLC chromatogram of Hexitol® solution showing a peak of 10.00 µg/ml of CHX at a timed wavelength of 265, 260 & 230 nm.

3.4. Application to Spiked Human Saliva

The proposed method has been employed to detect CHX in spiked human saliva. Upon comparing the chromatograms (**Fig. 8** and **9)**. A peak appeared at the R_t=5.79 min relating to CHX. As indicated in **Table 7**, the percentage recovery was 101.69 % ± 1.38. The precision of the method was satisfactory as indicated by the RSD% obtained from replicate analyses. Saliva components did not interfere with the drug analysis.

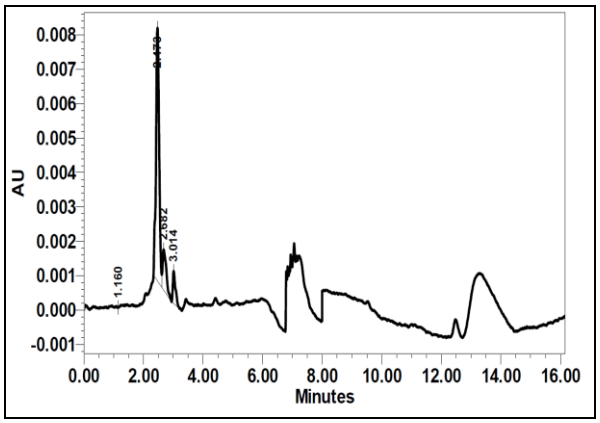

Fig. 8. HPLC chromatogram of blank saliva at a timed wavelength of 265, 260 & 230 nm.

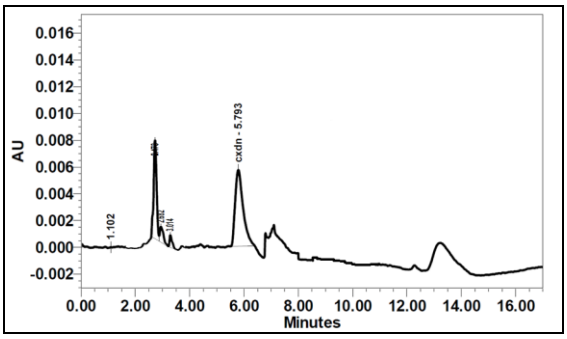

Fig. 9. HPLC chromatogram of saliva spiked with 2.00 µg/mL CHX at a timed wavelength of 265, 260 & 230 nm.

3.5. Statistical analysis of the results

Statistical comparison was performed between the proposed method and those obtained from the reported ones **[32–34]**. The t and f values were calculated and found to be lower than the tabulated ones, indicating that there was no significant difference. The findings are provided in **Table 8**, and the proposed method was found to be accurate and precise

Table 6. Application of the proposed HPLC method by standard addition method for determination of HEX, CHX & CSOL in pharmaceutical formulations

	HEX			**CHX**			**CSOL**		
Cyteal® vaginal douche labelled to contain 0.1 g% HEX, 5 mL CHX of solution of 20% (w/v) & 0.3 g% CSOL**	**Taken (µg/mL)**	**Found (µg/mL)**	**Rec*%**	**Taken (µg/mL)**	**Found (µg/mL)**	**Rec*%**	**Taken (µg/mL)**	**Found (µg/mL)**	**Rec*%**
	2.50	2.55	101.97	12.50	12.30	98.40	7.50	7.36	98.16
	Pure added (µg/mL)	**Pure found (µg/mL)**	**Rec*%**	**Pure added (µg/mL)**	**Pure found (µg/mL)**	**Rec*%**	**Pure added (µg/mL)**	**Pure found (µg/mL)**	**Rec*%**
	1.50	1.48	98.65	10.00	9.88	98.85	5.00	287144.70	98.73
	2.50	2.55	102.05	12.50	12.31	98.50	10.00	592563.70	99.19
	3.50	3.52	100.57	15.00	14.98	99.84	15.00	904495.30	100.05
	Mean ± S.D.		100.43± 1.70	**Mean ± S.D.**		99.06 ± 0.69	**Mean ± S.D.**		98.74 ± 1.06

	CHX		
Hexitol® labeled to contain 125 mg% CHX.	**Taken (µg/mL)**	**Found (µg/mL)**	**Rec*%**
	10.00	10.20	102.00
	Pure added (µg/mL)	**Pure found (µg/mL)**	**Rec*%**
	5.00	4.95	98.99
	10.00	10.19	101.87
	15.00	15.23	101.51
	Mean ± S.D.		100.79 ± 1.57

*Mean of three determinations.

** Each 100 mL of Cyteal® vaginal douche contains 5 mL of 20% w/v chlorhexidine.

Table 7. Application of the proposed HPLC method for determination of CHX in spiked human saliva:

Added (µg/mL)	**Recovery ± SD*** **CHX**
2.00	101.69 ± 1.38

*Average of 3 determinations.

Table 8. Statistical comparison of the results obtained using the proposed HPLC method versus the reported methods for HEX, CHX, and CSOL determination

	HEX		**CHX**		**CSOL**	
	Proposed method	**Reported[32] method**	**Proposed method**	**Reported[33] method**	**Proposed method**	**Reported[34] method**
Mean	99.11	100.37	99.34	99.78	99.39	99.98
S.D.	0.84	1.59	1.34	1.26	0.44	0.95
Variance	0.71	2.55	1.8	1.59	0.19	0.91
n	5	5	5	5	5	5
Student's t-test	1.569 (2.306)		1.217 (2.306)		0.062 (2.447)	
F test	3.645 (6.388)		1.126 (6.388)		3.769 (6.388)	

(32) The chromatographic separation was carried out on a metallic microcolumn (2 x 64 mm) filled with a silasorb C1a adsorbent with a particle size of 5 µm. A mixture of 30 ml of acetonitrile and +70 mL of a 0.02 M solution of potassium hydrophosphate was used as an eluent at a rate of I00 µL/min.

(33) The chromatographic separation analysis was carried out on a column of Nucleosil 100-5 C18 (5 µm, 250 x 4,6 mm i.d.). Substances were eluted by a mobile phase consisting
of 40 mM triethylamine containing phosphate buffer (10 mM, pH 3.0) and acetonitrile (65:35, v/v) at a flow rate of 1 ml min^{-1}.

(34) The chromatographic separation was carried out on a Phenomenex Gemini column C18
(250 9 4.6 mm, 5 µm) by a Hitachi L-7200 autosampler.

Conclusion

In our work, a simple, reliable, and quantitative HPLC analytical method was developed for simultaneous separation and determination of a ternary mixture of HEX, CHX, and CSOL in their pure form and in various dosage forms including mouthwash and intimate douche in addition to chlorhexidine determination in spiked human saliva. The mentioned drugs were determined in colored aqueous formulation, like Hexitol® mouthwash, without any sample pre-treatment or extraction steps. The chromatograms showed high selectivity and sensitivity with a short run time of only 16 min. So, a lot of samples can be manipulated and measured in short time intervals which has significant importance in routine analysis in various quality control laboratories. Additionally, the proposed HPLC method requires no pre-treatment, and the sample could be manipulated without any interference from the matrix of saliva or the excipients in the dosage forms and other interfering species. The suggested method was validated following ICH guidelines showing linearity over a concentration range of 0.10 to 25.00 µg/mL of pure HEX, 2.00 to 30.00 µg/mL of pure CHX, and 0.10 to 30.00 µg/mL of pure CSOL with excellent detection limits.

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publish

All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Data analyzed during this study are all included in the main manuscript.

Competing interests

No competing interests were declared by the authors.

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