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Development and validation of a simple, fast, isocratic stability indicating RP-HPLC-UV method for the determination of chlorhexidine and its impurity para-chloroaniline in bulk and finished product

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Abstract:- A simple, isocraticRP HPLC-UV method was developed for the simultaneous determination of chlorhexidine (CHD) and p-Chloroaniline (pCA) inchlorhexidine mouth rinses. An excellent separation obtainedbyC18 column (200mm × 4.6 mm, 3µm). Mobile phasewas acetate buffer:methanol in a 45:55 ratio,flowrate was 1.0 ml/min. Both ingredient and an impuritywere detected at 254 nm,injection volume was 20µl and the analysis temperature was room temperature.Resolution4.7,retention times was3.1min and 5.7 min for pCAand CHD respectively. The proposed method was testedforsystem suitability, linearity, range, precision, accuracy, specificity, robustness, detection and quantification limits. The linearity range was40-160µg/ml forCHD and0.3-1.2 µg/ml forpCA.The correlation coefficient of the regression line was 1.000 for both components. Method robustness was tested under nine different conditions using sampleswith a known content. For CHD, the mean of the nine assays was 99.95% andthe RSD was 0.16%. ForpCA, the mean of the nine assays was 99.98% and the RSD was 0.24%. The results show that this is a simple method that can be applied to the analysis of Chlorhexidineproductswith satisfactory degrees of accuracy and precision. Due to the selected optimized conditions, this method can be used with the minimum requirements of an isocratic HPLC system.

Key words: *HPLC-UV; Isocratic; Method validation;Chlorhexidine; p-Chloroaniline*

1. INTRODUCTION

Chlorhexidine [CHD; 1,1'-hexamethylenebis [5-(4-chlorophenyl) biguanide]] has a wide spectrum of bactericidal and antiviral activity and is a common ingredient in various formulations ranging from skin disinfectants in healthcare products to antiplaque agents in dentistry **[1-5]**. The presence of two symmetrically positioned basic chlorophenylguanide groups attached to a lipophilic hexamethylene chain (Figure 1) aid in rapid absorption through the outer bacterial cell wall, causing irreversible bacterial membrane injury, cytoplasmic leakage, and enzyme inhibition. **[6].**This molecule exists as various forms of salts: diacetate, dihydrochloride, or digluconate, mainly differing by their solubilizing abilities in aqueous or oily media. CHD digluconate (or gluconate), as most soluble in water or alcohol, is the most used form in topical dermatology or cosmetic preparations. Aqueous solutions of CHD are most stable within the pH range of 5-8. Above pH 8.0 CHD base is precipitated and in more acid conditions there is gradual deterioration of activity because the compound is less stable. **[7,8]**. Chlorhexidine is a chemical antiseptic. It is effective on both gram-positive and gram-negative bacteria. It has both bactericidal and bacteriostatic mechanisms of action; the mechanism of action being membrane disruption, not ATPase inactivation as previously thought.It is also useful against fungi and enveloped viruses, though this has not been extensively investigated. Chlorhexidine is harmful in high concentrations, but is used safely in low concentrations in many products, such as mouthwash and contact lens solutions. By ionization it produces positive ions**[9].**Hydrolysis yields p-chloroaniline (pCA); the amount is insignificant at room temperature, but is increased by heating above 100°C, especially at alkaline pH.This cationic molecule (positively charged species) is thus generally compatible with other cationic materials, although compatibility will depend on the nature and relative concentration of the second cationic species. It is, however, possible for a reaction to occur between CHD and the counter-ion (anion) of a cationic molecule which is negatively charged, resulting in the formation of a less soluble CHD salt, which then may precipitate. CHD is incompatible with inorganic anions in all but extremely dilute solutions. CHD is also incompatible with organic anions, such as soaps, sodium lauryl sulphate, sodium carboxymethyl cellulose, alginates, and many pharmaceutical dyes. In certain instances, there will be no visible signs of incompatibility, but the antimicrobial activity may be significantly reduced because of the CHD being incorporated into micelles (ionic clusters). pCA is very toxic if inhaled, swallowed or absorbed through the skin.It may act as a human carcinogen. It is readily absorbed through the skin and it may act as a sensitizer **[10]**. However, as pCA is the principal product of degradation of CHD and toxic with actual recommend maximum limit of genotoxic impurity**[11]**, it is important to quantify pCA in CHD solution.CHD and pCAwasdetermined using several methodologies such as high

performance liquid chromatography[12-27],gas chromatography-mass (GC-MS)**[28-34]**,fluorometry **[35],** UV spectroscopy **[36]** and time-of-flight secondary ion mass spectrometry **[37].**In the present work the objective is to design and validate a simple fast and isocratic stability indicating RP-HPLC-UV procedure for the assay of chlorhexidine in presences of its degradation product.

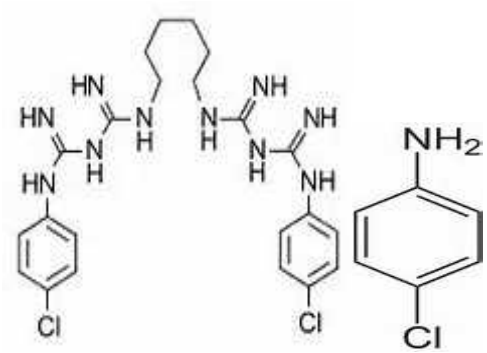

Figure 1Figure 2
Chlorhexidine Gluconatep-Chloroaniline

2. Experimental

2.1. Chemicals

Working standards of CHD and pCA, finished productsand excipientswere supplied from Yamani Medical Products. Methanol and Acetic acid were HPLCgrade(Scharlau Spain). HPLC gradewater was used.

2.2. Instrumentation

TheHPLC-UV system consisted of analytical apparatus(Analytical Technologies Limited Corporation, Mumbai,India) with a P2230 pumpSr No P2304051, UV2230 UV-Vis detectorSr No U2304633. This system was connected to a computer loaded with A2000-Solutions software. A C18column (200mmx4.6mm, I.D. 3 µm) was used.

2.3. Methods

2.3.1. Standard stock solution

To prepare stock solutions, 0.1000 g of CHDand 0.0075 g of pCAwere weighed accurately and transferred quantitatively to the same 100-ml volumetric flask. The flask was half-filled with the mobile phase and sonicated for 10 minutes, cooled to room temperature, then the volume was completed to the mark with the same solvent.

2.3.2. Standard solution

Subsequent dilutions were made from the stock solution with the mobile phase to make solutions with100µg/ml ofCHD and 0.3µg/ml of pCA. The resulting solution was filtered through a 0.45µm membrane nylon filter.

2.3.3. Assay preparation

2 ml of the mouthwash was diluted to 25 ml with the mobile phase to produce a solution containing 0.01% w/v of chlorhexidine gluconatesonicated for 10 minutes,theresulting solution was filtered through a 0.45µm membrane nylon filter.The recovered concentration wascalculated by comparing the analyte response of the samplewith that ofthe standard.

2.4.Optimized chromatographic conditions

The mobile phase was composed of acetate buffer and methanol (45:55), usingisocratic elution with a flow rate of1.0ml/min. The injection volume was 20µl, using a C18 (200mmx4.6mm, 3µm). The eluents were monitored at 245 nm.

II. RESULTS AND DISCUSSION

3.1. Validation of the developed and optimized method

The validation of the developed method was done according to ICH guidelines:system suitability, linearity, specificity, accuracy,interday precision, intraday precision and robustness.

3.1.1. System suitability

The system suitability test is an integral part of the analytical method. For this, amixed standard solution (target concentration) was injectedsix times. Parameters such as RSD% for the peak area, retention time, resolution and theoretical plates of the peaks were calculated. The resultsfor CHD and pCAare shown in Table 1 and Table 2, respectively.

Table .1System suitability parameters for CHD

	Area	Retention time	Resolution	Theoretical plate	Asymmetry factor
STD 1	5659567	5.18	4.71	13420	1.13
STD 2	5651240	5.18	4.71	13619	1.14
STD 3	5656282	5.17	4.69	13580	1.13
STD 4	5650005	5.17	4.69	13576	1.13
STD 5	5660625	5.17	4.68	13576	1.12
STD 6	5648577	5.16	4.65	13516	1.14
Average	5654383	5.171666667	4.68833333	13547.83333	1.131666667
STDEV	5141.937	0.007527727	0.02228602	70.76840161	0.007527727
RSD	**0.090937**	**0.145557071**	**0.47535058**	**0.522359553**	**0.665189384**

Table. 2System suitability parameters for pCA

	Area	Retention time	Resolution	Theoretical plate	Asymmetry factor
STD 1	46534	3.77	4.71	15760	1.18
STD 2	46519	3.78	4.71	15801	1.09
STD 3	46500	3.78	4.69	15994	1.1
STD 4	45611	3.78	4.69	16001	1.14
STD 5	46566	3.78	4.68	16008	1.12
STD 6	45688	3.78	4.65	15994	1.11
Average	46236.33	3.778333333	4.68833333	15926.33333	1.123333333
STDEV	455.723	0.004082483	0.02228602	113.8220834	0.032659863
RSD	**0.985638**	**0.108049834**	**0.47535058**	**0.71467852**	**2.907406223**

Note: acceptance criteria for impurity (±15%).

3.1.2.Selectivity

The mixed standard solution was injected and its chromatogram was recorded (Fig. 3).The sample and placebo solutions were prepared by taking the weight of placebo equivalent to its weight in the test preparation. Based on the chromatograms of the sample (Fig. 4) and placebo (Fig. 5), the placebo solutions showed no peaks at the retention time of the CHD and pCA peaks. This indicates that the excipients used in the formulation did not interfere in the estimation of the active ingredients in the product. The system suitability parameters in the sample chromatogram(Fig. 4) was almost equalto that of the standard chromatogram(Fig. 3) indicating that theexcipientsin the sample did not affect separation.

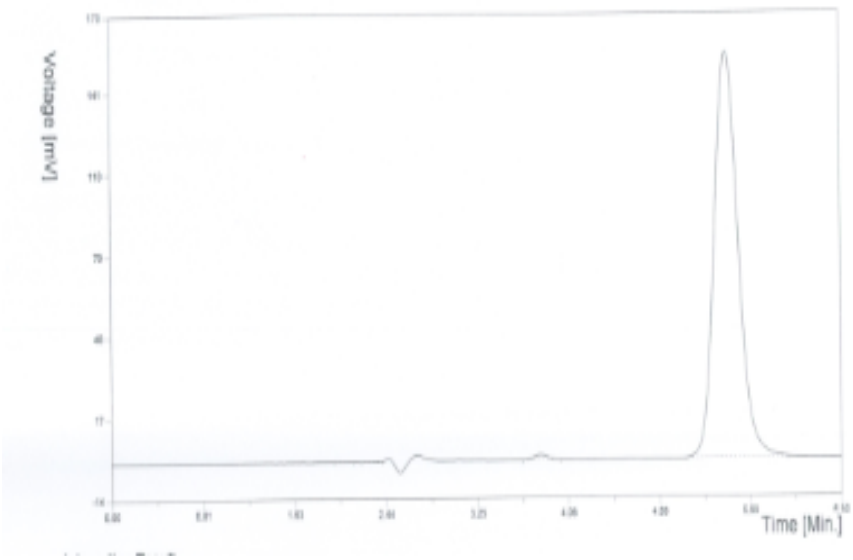

Fig. 3: Chromatogram of the mixed standard solution under the optimized conditions

Fig. 4: Chromatogram of a sample solution under the optimized conditions

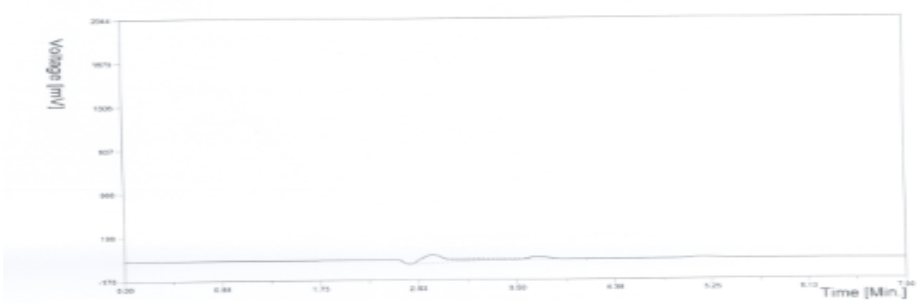

Fig. 5: Chromatogram of a placebo solution under the optimized conditions

3.1.3. Linearity

Seven concentrations of mixed standard of CHD and pCA ranging from 40% to 160% of the target analyte concentrationswere prepared in mobile phase solution;20, 40.60, 80, 100,120, 140 and 160µg/ml,and 0.3,0.45, 0.6,0.75,0.9,1.05 and 1.2 µg/ml, respectivly. Eachstandard mixture was injected in triplicate and the mean value of the peak area was used for the calibration curve. The calibration graphwas obtained using XL-STAT 2016. Thelinear regression plotsforCHD (Fig. 6) and pCA(Fig. 7) show that the regression equationswere**Area=-12044.1666666688+56570.3083333333*µg/ml**and**Area=81.8214285714494+61736.4285714285*µg/ml**, respectively. The regression coefficient values (R^2) were found to be 1.000 for both analytes,indicating an excellent degree of linearity.

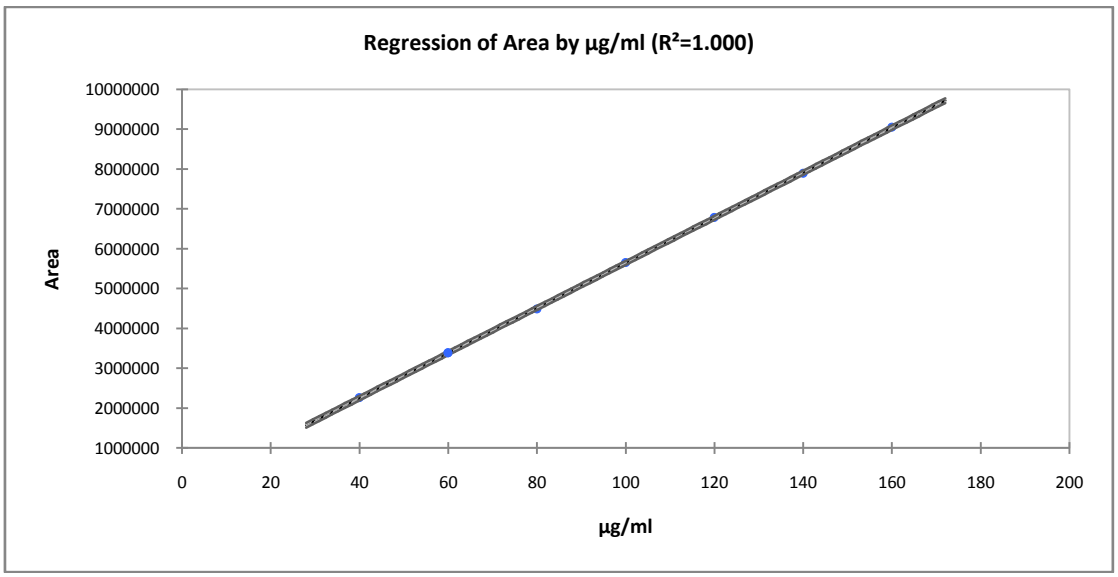

Fig. 6: XL- STAT 2016 plot of (µg/ml) Vs (peak area) - CHD

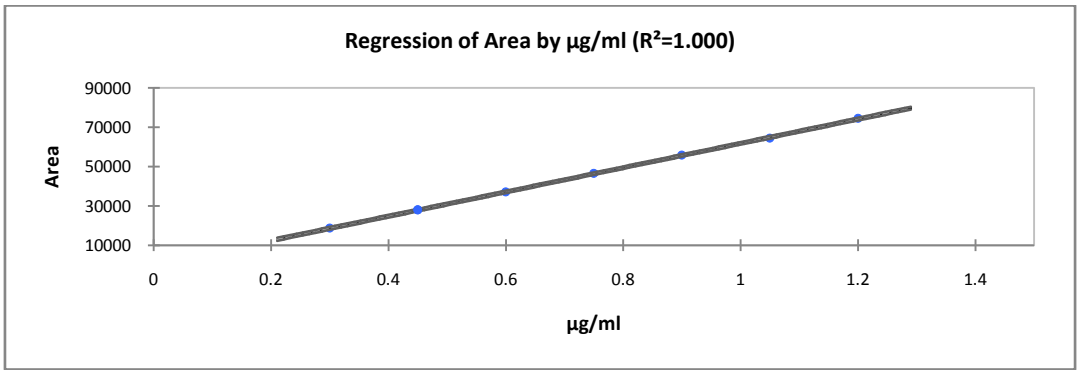

Fig. 7: XL- STAT 2016 plot of (µg/ml) Vs (peak area) - pCA

3.1.4. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated from linearity data according to ICH [22]:LOD = 3.3* (SD/S) and LOQ = 10 * (SD/S). TheLOD was found to be 0.97626µg/mland 0.01151 µg/ml for CHD and pCA, respectively, while the LOQ valueswere 3.2541 µg/mland 0.038389 µg/ml, respectively.

3.1.5. Accuracy

Seven 100- ml volumetric flasks were labeled, and the placebo equivalent to a tablet's weight was transferred to a different flask. The volume of the mixed standard stock solution required to produce 40%, 60%, 80%, 100%, 120%, 140%and 160% of thetarget concentration of both CHD and pCA was added to the flasks. The flaskswere half-filled with the mobile phase, sonicated for 10 minutes, cooled to room temperature, then completed to the mark with the same solvent. Subsequent dilutions were made with the mobile phase in the same manner as the standard preparation. The assay was performed on the seven solutions.The recovery percentagefor CHD and pCAwasfound to bewithin the acceptance criteria, i.e. the mean, standard deviations and relative standard deviationsof the recovery percentage of the seven different concentrations (Table 3).

Table 3- Accuracy of the method for CHD and pCA

Amount added%	CHD		pCA	
	Recovery	Recovery%	Recovery	Recovery%
40	40.00906	100.0227	40.23675	100.5919
60	59.91577	99.85962	60.41281	100.688
80	79.28594	99.10742	80.10367	100.1296
100	99.96527	99.96527	100.4801	100.4801
120	120.042	100.035	120.5589	100.4657
140	139.5668	99.69056	139.4567	99.61193
160	160.0863	100.0539	160.9888	100.618
Av		99.81921		100.36932
STDEV		0.3389236		0.3798261
RSD		**0.3395374**		**0.3784285**

3.1.6. Precision

3.1.6.1.Interday precision

Three 25- ml volumetric flasks were labeled, and the placebo equivalent to target concentration was transferredto each flask. The volume of the standard stock solution required to produce 80%, 100% and 120% of the product content of both CHD and pCA was added. The flaskswere half-filled with the mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. The assay of CHD and pCA in these solutionswas performedfive times in one day; each solution was injected three timesfor each assay. The means, standard deviations and relative standard deviations of the assays were calculated;the method'sinterday precision was foundto be within the permissible limits. The results are shown in Table4.

Table 4-Interday precision of the method for CHD and pCA

	80 %		100 %		120 %	
	CHD	pCA	CHD	pCA	CHD	pCA
1st trial	99.10742	100.1296	99.96527	100.4801	100.035	100.4657
2nd trial	98.99985	100.1629	100.0016	99.99784	100.0008	100.5486

3rd trial	99.21989	100.1846	99.78585	100.0137	99.61658	100.4723
4th trial	99.07551	100.19	99.96394	100.0807	100.006	100.4843
5th trial	99.09971	100.2476	99.98999	100.013	99.79884	100.0199
Avg.	99.10048	100.1829	99.94132	100.1171	99.89142	100.3982
STDEV	0.07915	0.043258	0.088395	0.205474	0.180131	0.213999
RSD	**0.079869**	**0.043179**	**0.088447**	**0.205234**	**0.180327**	**0.21315**

3.1.6.2. Intradayprecision

Three 25 ml volumetric flasks were labeled, and aplacebo equivalent to target concentration was transferred to each flask. The volume of the standard stock solution required to produce 80%, 100% and 120% of the product content of both CHD and pCA was added. The flasks were half-filled with the mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Theassay was performed on these solutions three times on three different days. The solutions were injected three times for each assay. The means, standard deviations and relative standard deviations of the assays were calculated; the method's intraday precision was foundto be with the permissible limits. The results are shown in Table 5.

Table 5- intraday precisionof the method for CHD and pCA

	80%		**100%**		**120%**	
	CHD	**pCA**	**CHD**	**pCA**	**CHD**	**pCA**
Day 1	99.102559	100.1375	99.960367	100.4881	100.03007	100.4737
Day 2	98.753796	100.6066	99.765273	99.85019	99.21924	100.1698
Day 3	100.28873	100.249	99.475039	100.4019	100.56168	99.90645
Avg.	99.381696	100.331	99.73356	100.2467	99.890462	100.1833
STDEV	0.8046402	0.245054	0.2442133	0.346107	0.9492505	0.283862
RSD	0.8096463	0.244245	0.2448657	0.345256	0.9502914	0.283343

3.1.7Robustness

The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. An assay was performed with the following variations:optimum conditions, 5ºChigher or lower, 5% more or less organic solvent in the mobile phase, 5% increase or decrease in the flow rate of the mobile phase, and detection 3nm above or below the detection wavelength. The results were collected and subjected to statistical treatments.The means, standard deviations and relative standard deviations for the assay under all studied conditions are shown in Table6.

Table. 6- Robustness of the methodof the method for CHD and pCA

No	**Condition**	**CHD**	**pCA**
1	Optimized conditions	99.96036703	100.4801419
2	less 5 degree Celsius	99.91153242	99.97778052
3	More 5 degree Celsius	99.98848653	99.65306379
4	5% less flow rate	100.2310422	100.065966
5	5% more flow rate	99.9106038	99.96361541
6	5% less Organic solvent	99.66974347	99.68066608
7	5% more Organic solvent	100.1049996	99.9772377
8	3nm less	99.98613261	100.0817496
9	3nm more	99.78474648	99.98299257
	Avg	99.94973935	99.98480151
	STDEV	0.163854928	0.240950102
	RSD %	0.163937324	0.240986728

III. CONCLUSIONS

In this study, a specific and reliable HPLC-UV procedure was developedto assessCHD and pCA in their mouth rinse pharmaceutical formulations. The most important feature in the proposed method is its simplicity, as this method can be used with the minimum requirements of an isocratic HPLC system (one pump), UV detection at the same wavelength (the most common detector and no gradient program required). The method was optimized to be used at ambient temperature (no column oven required). Moreover, the method is economically (flow rate 1.0ml/min for 6 min per injection), the buffer solution is easy to prepare (Acetatebuffer solution without pH adjustment), and the method passed all tests of robustness. To the best of our knowledge, no simpler method has been reported for an assay ofthis drug and its degradation product.

Themethod is applicable evenwith conventional HPLC systems,and there for, it can be used forquality control purposes.

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