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Rapid HPLC Method for Determination of Parachloroaniline in Chlorhexidine Antiseptic Agent in Mouthrinses, Ophthalmic and Skin Solution

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Abstract

We described a simple and rapid method to quantify simultaneously chlorhexidine (CHD) and its major metabolite, para Chloroaniline (pCA) by HPLC with UV detection without the additional need of mobile-phase amine modifiers or ion-pairing reagents, with good resolution between pCA and CHD, symmetry peak of the compound and short run time. HPLC-UV analyses were performed using a Dionex® Summit liquid chromatograph (Dionex Corp, Sunnyvale, CA, USA). Chromatographic separations were carried out on a Luna® 150 mm×3 mm i.d. column packed with 3 μm CN (cyano) particles (Phenomenex®), guarded by an on-line filter. Mobile phase consist of methanol:water with sodium chloride with 0.02% of formic acid (55:45). Wavelengths for pCA and for CHD are 238 and 255 nm respectively. Influence of methanol and of sodium chloride content in the eluant has been studied. Linearity of CHD is very good, from 0.5 up to 21.2 μg/l while linearity of pCA is in the range of 0.05 to 10 μg/l with correlation coefficients above 0.999. Resolution between the components is above 4, asymmetry is about 1.3 and 1.7 for pCA and CHD respectively and the run time is less than 5 minutes. This method has been applied to CHD solution of different medical devices. No interference has been reported, and the analysis of direct injection of solution, without any treatment is achieved in less than five minutes.In conclusion, we present a validated method for dosage of CHD and its major impurity pCA, known to be carcinogen, available into medical products or medicinal device for *in-vitro* diagnostic.

Keywords: Chlorhexidine, Chloroaniline, HPLC

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 [1,2]. The presence of two symmetrically positioned basic chlorophenyl guanide 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 [3].

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 al-

cohol, 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. Hydrolysis yields p-chloroan-
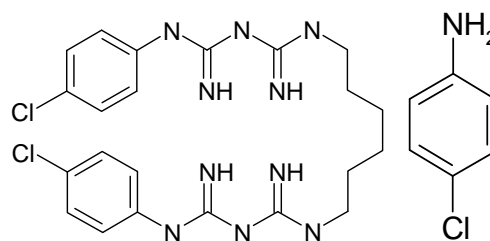

Figure 1. Chemical structure of CHD and pCA.

iline (pCA); the amount is insignificant at room temperature, but is increased by heating above 100°C, especially at alkaline pH [4]. CHD diacetate is soluble in alcohol, glycerol, propylene glycol, polyethylene glycols. According to the manufacturer, 1 part CHD is soluble in 15 parts of 96% ethanol.

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) [5].

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. The lethal dose 50 percent kill is 310 mg·kg^{-1} and 100 mg·kg^{-1} for rat and mouse respectively.

Liquid chromatography is the most widely used method for analysis of CHD [6]; UV detection around 250 nm is used for quantitative assays [7-9], while for the detection of impurities, mass spectrometry or photodiode array detectors are employed [10,11]. Other methods reported in the literature include fluorometry [12] and direct UV spectroscopy [13]; both of them have several disadvantages: lack of sensitivity, serious interference.

There are many reports about the determination of CHD in biological fluids using high performance liquid chromatography with a UV detector (HPLC-UV) [8,9, 14-18] and gas chromatography mass spectrometry (GC-MS) [19-23].

Column used are generally C18 [11,14,15,24], Amide [11] or phenyl [25]. Furthermore, reversed-phase ion-pair system is frequently used.

However, as pCA is the principal product degradation, and because of his toxicity and to be in line with actual recommendation for genotoxic impurities [26], it is important to quantify pCA in CHD solution. Therefore, we describe hereafter a new very sensible HPLC-UV method without the need of additional mobile-phase amine modifiers or ion-pairing reagents, with good resolution between pCA and CHD, symmetry peak of the compo-unds and short run time. To prove the reliability of this method, CHD and pCA were analyzed in medical products.

2. Materials and Methods

2.1. Chemicals and Reagents

CHD gluconate solution (20%, with a density of 1.06) was purchased from Sigma (St Quentin-Fallavier, France) and stored at 4°C in the dark. Methanol and formic acid was of analytical grade from Carlo Erba (Val de Reuil, France). Other chemicals used were of analytical grade.

Seven pharmaceutical specialities: Prexidine 0.12%® mouthrinses (Expanscience, France), Septisol® eye drops (Merck, France), Correctol 0.1%® eye drops (Alcon, France), Sophtal® eye drops (Alcon, France), Septeal® for topical application (Pierre Fabre Dermatologie, France), Chlorhexidine Aqueuse Gilbert à 0.05%® for topical application (Gilbert, France) and Dacrine® eye drops (McNeil, France) were purchased in pharmacy. CHD is used as active substance except in Septisol®, Correctol 0.1%® and Sophtal® where it is used as excipient.

2.2. Apparatus and Chromatographic Conditions

HPLC-UV analyses were performed using a Dionex® Summit liquid chromatograph (Dionex Corp, Sunnyvale, CA, USA), equipped with a vacuum solvent degassing unit, a binary high pressure gradient pump P680, an ASI100 automated sample injector, an UVD340U variable wavelength UV–VIS detector.

Chromatographic separations were carried out on a Luna® 150 mm × 3 mm i.d. column packed with 3 μm CN (cyano) particles (Phenomenex®), guarded by an on-line filter. Data were collected and analyzed using the Chromeleon® software v6.50SP4 from Dionex®.

Since it is known that amines tend to form stable salts with acetic acid, ammonium acetate and phosphate salts, the mobile phases for chlorhexidine quantitative assay consisting of methanol:water (55:45, v/v) with sodium chloride with 0.02% of formic acid (pH = 3) and pumped at a flow rate of 0.5 ml/min.

Wavelengths for pCA and for CHD are 238 and 255 nm respectively.

2.3. Sample Preparation

Pharmaceutical specialities were directly injected (20 μl) into the chromatograph system (without any extraction) after or not dilution.

2.4. Mobile Phase

Published HPLC assays also suffer from quantitation

problems caused by irreversible adsorption of CHD onto the silica-based reversed phase (RP)-HPLC columns [27, 28]. These problems can be overcome by adding NaCl to the mobile phase [28] and explain the presence of this compound in our mobile phase. The effect of NaCl can be explained by the two simultaneously occurring processes: the salting out effect, and the electrostatic interactions between polar molecules and salt ions in sample solution [29].

Chlorhexidine is a dicationic compound with Ka1 = 6.3×10^{-3} and Ka2 = 5.0×10^{-11} and is almost completely ionized (1+) at pH = 3, pH of our mobile phase.

Because chlorhexidine and some of its degradation products are strongly basic, they are intensely retained on most silica-based reversed phase columns [30]; the difficulty was overcome by using a cyano column and an acid mobile phase (pH = 3), without the need to use ion-pairing reagents in the mobile phase. It is known that chlorhexidine is quite stable at this low pH (some HPLC methods use pH = 2.5) [9].

2.5. Statistics

Results are presented as the mean ± SD and results were analyzed using Excel® 10, WinStat® v2003.1 and Chromeleon®. A p value < 0.05 was considered as significant.

2.6. Validation Criteria

2.6.1 Linearity
Working standards were prepared by dilution in water and taking account of density for CHD gluconate. Height reference samples were used for calibration curves of CHD (0.53 to 21.20 mg/l) and pCA (0.01 to 10.0 ng/ml). Each determination was done in triplicate. The calibration factors were calculated according to least-squares linear regression .

2.6.2 Precision and Accuracy
Precision was determined for both inter- and intra-day variability. These measurements were made by HPLC analysis of CHD (5.3 and 12.72 mg/l) and pCA (0.50, and 2.0 mg/l), on three consecutive days (inter-day variation or reproducibility) or during the same day for intra-day variation determination (repeatability).

2.6.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)
Standard curves were prepared for the two analytes and the following equation were used to calculate the LOD and the LOQ for each compound [31]:

$$LOD = 3.3\sigma / S; LOQ = 10\sigma / S$$

where σ is the standard deviation of the response (estimated from the standard deviation of y-intercepts of regression lines) and S is the slope of the standard curve.

At the LOQ, bias and precision should not exceed 12% [32].

3. Results

To optimise the mobile phase, the influence of concentration of methanol and sodium chloride was first studied.

4. Discussion

Based on the results presented in **Figures 2** and **3** and in **Table 1**, the concentration of methanol in the mobile phase was chosen as 55% in order to have a short run time (less than 5 minutes) and a good resolution (about 4.1) (**Figure 4**) between the two components.

There is few influence of sodium chloride concentration on retention time and peak asymmetry of pCA and CHD. It is noted that asymmetry for CHD (1.7) and for pCA (1.3) is very good with this cyano column from Phenomenex® (**Table 2**). However, height of CHD decrease, when concentration of sodium chloride increase, while pCA height peak increase too. A concentration of 12‰ of sodium chloride was chosen, the goal is to avoid an important decrease of CHD and to increase the sensibility of pCA detection, especially because of its toxicity.

Linearity of CHD is very good, from 0.5 up to 21.2 µg/l (**Table 3**). This range is in the same order of that describes by Hebert *et al*. [7], but with a run time of 22 minutes. Some authors [9,11,14,30] present better sensibility for CHD (LOD is 0.05 mg/l) quantitation but they don't quantify pCA. But for Usui [33] which works with LC/MS, it was difficult to estimate the reliable values at the concentration range below 0.1 mg/l. Only Below and Kramer [16] present a dosage method for both pCA and CHD, with a run time of 20 minutes.

Linearity of pCA is very good (range from 0.05 to 10 mg/l), for a product known to be difficult to be analysed [9,30] and with LOD and LOQ very low.

This method has been applied to a CHD solution included into pharmaceutical specialities (**Table 4**) (**Figure 5**). In conclusion, we present a validated method for dosage of CHD and its major impurity pCA. No interference has been reported, and the analysis of direct injection of solution, without any treatment except an eventually dilution is achieved in less than five minutes. In all cases, pCA was less than LOD, and CHD is in the range generally accepted in pharmaceutical products (95% -
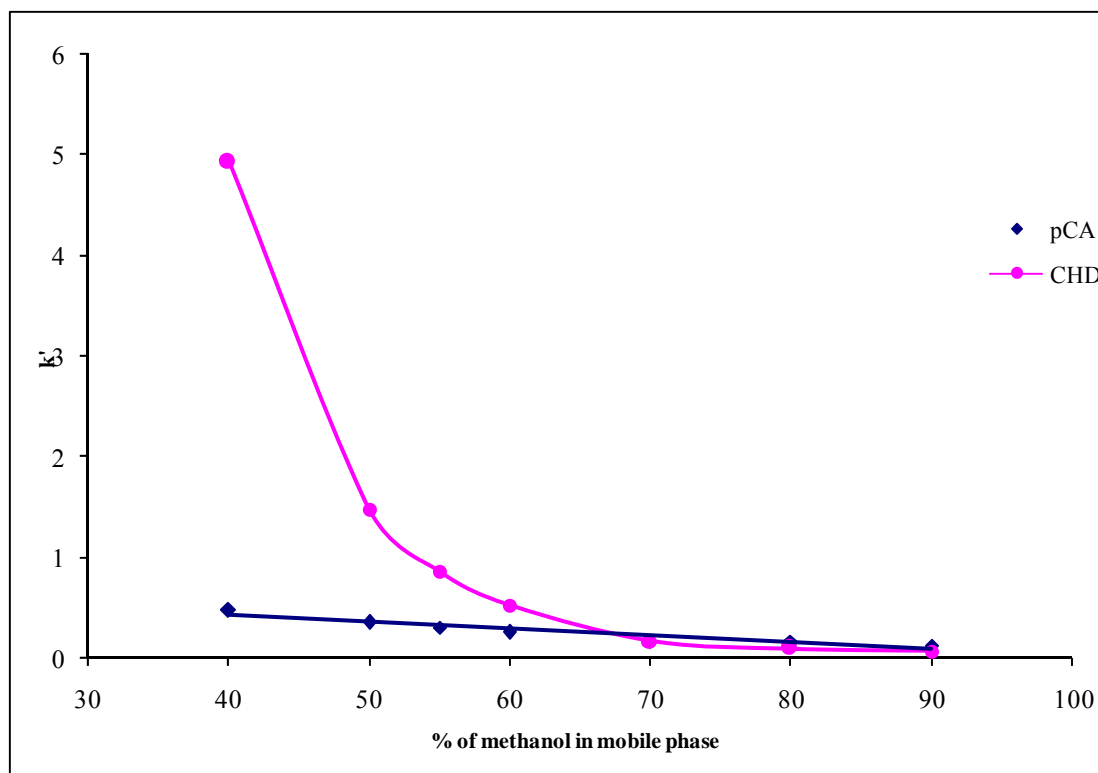
Figure 2. Influence of the methanol content in the eluant (methanol: 8‰ of sodium solution with 0.02% of formic acid (pH = 3) on the capacity factor of CHD and pCA (Luna CN, 150 × 3.0 mm, 3 μm, 0.5 ml/min); k' (capacity factor) for ◆ pPC and ● CHD.
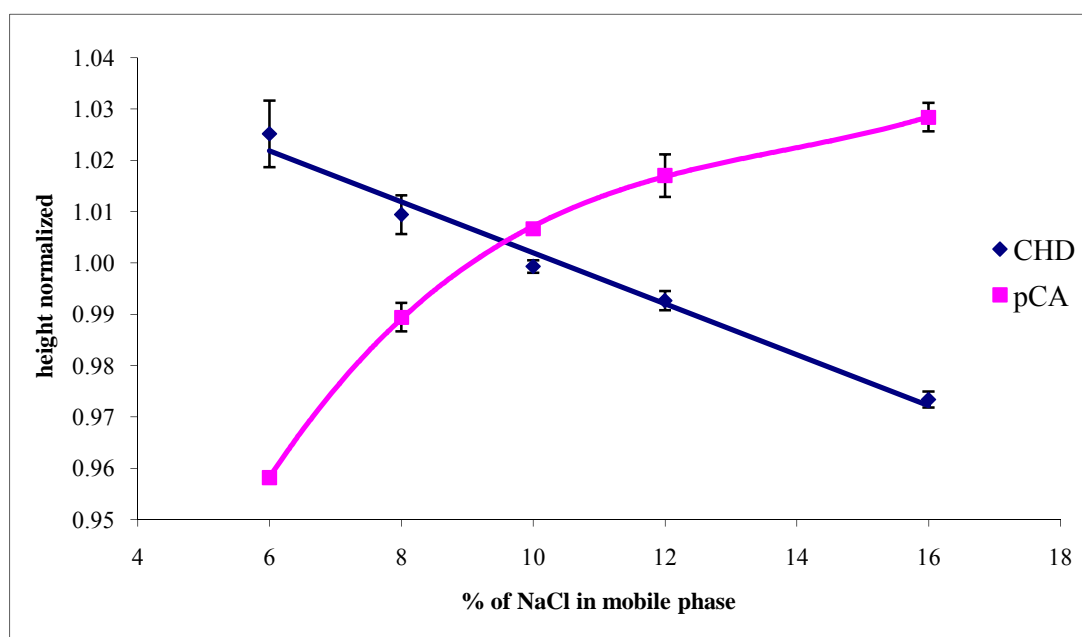

Figure 3. Influence of the sodium chloride content in the eluant (55:45 of methanol: sodium solution with 0.02% of formic acid (pH = 3) on the variation of height (± SD) normalized by mean of ◆ pPC and ● CHD (Luna CN, 150 × 3.0 mm, 3 μm, 0.5 ml/min).
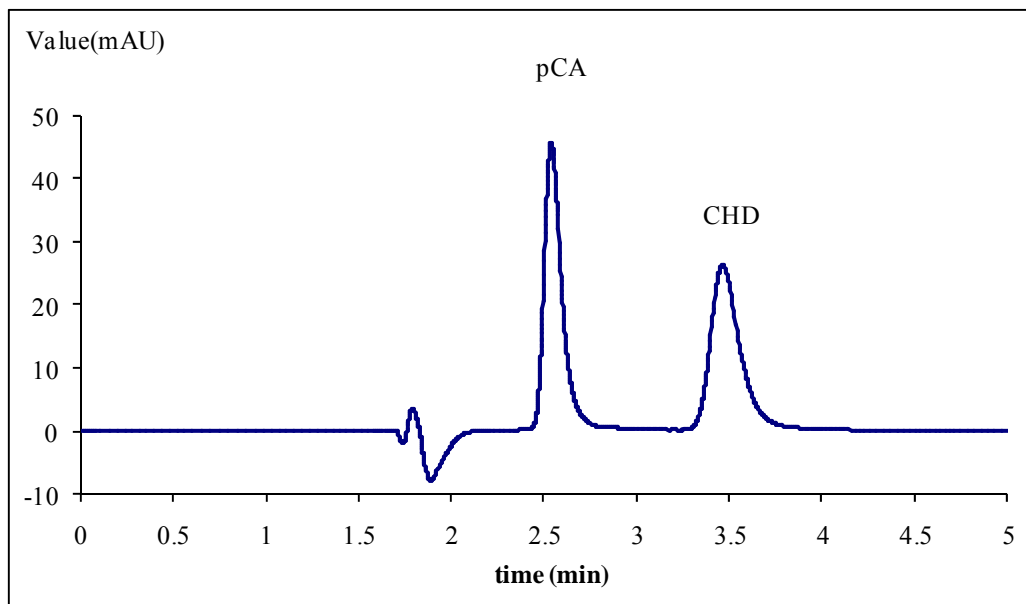
Figure 4. Typical chromatogram of a solution with pCA (1 mg/l) and CHD (5 mg/l).
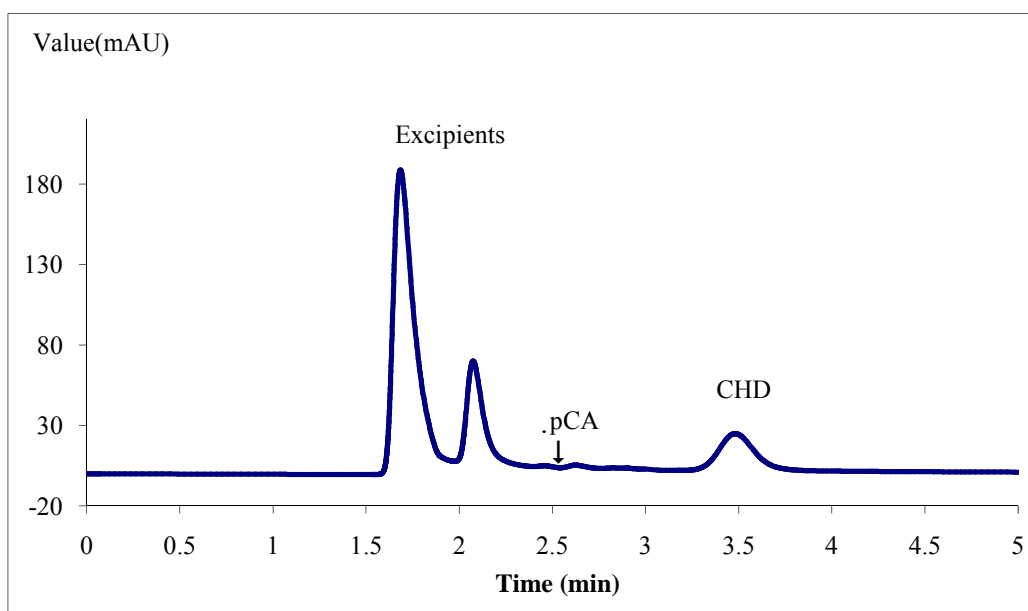
Figure 5. Chromatogram of sophtal solution, directly injected. The two first peaks are excipients. pCA is not detected.

Table 1. Intra-day and inter-days precision and accuracy.

Substance	Theoretical concentrations	intra-day precision (n = 9)	inter-day precision (n = 3) (3 days)	RSD	Accuracy (%)
	(µg/l)	Concentrations found		(%)	
		(mean ± S.D.)			
pCA	0.50	0.511 ± 0.023		0.90	97.8
	2.0		2.022 ± 0.084	4.16	98.9
CHD	5.30		5.203 ± 0.152	2.92	101.9
	12.72	12.90 ± 0.14		0.56	98.6

Table 2. Influence of sodium chloride content in the eluant (55:45 of methanol:sodium chloride solution with 0.02% of formic acid (pH = 3)) on the variation (expressed as mean ± standard deviation for a triplicate injection) of retention time (TR) in minutes, height (H) in mAU and asymmetry (As) of pCA and CHD.

‰ NaCl	TR CHD		H CHD		As CHD		TR pCA		H pCA		As pCA	
	m	SD	m	SD	m	SD	m	SD	m	SD	m	SD
6	3.54	0.00	533.6	3.38	1.69	0.01	2.57	0.00	86.8	0.10	1.38	0.06
8	3.48	0.00	525.4	1.96	1.69	0.01	2.56	0.02	89.6	0.25	1.37	0.01
10	3.48	0.00	520.2	0.64	1.70	0.01	2.55	0.00	91.2	0.00	1.34	0.01
12	3.47	0.00	516.7	0.96	1.69	0.01	2.54	0.00	92.1	0.38	1.32	0.01
16	3.48	0.00	506.7	0.80	1.66	0.01	2.53	0.01	93.2	0.25	1.31	0.00

Table 3. Linearity, limits of detection and quatification of standard curvese.

Substance	Linear range (mg/l)	Calibration equation (y = area, x = mg/l)	Correlation coefficients (r)	LOD (mg/l)	LOQ (mg/l)
pCA	0.05 – 10	y = 5.5237 x – 0.1299	0.99996	0.0035	0.0105
CHD	0.53 – 21.2	y = 1.6084 x + 1.2123	0.99932	0.15	0.50

Table 4. CHD amounts in pharmaceutical specialities.

	CHD theoretical concentration (mg/l)	CHD observed concentration (mg/l)	Recovery (%)
Prexidine 0.12%	1200	1174	97.8
Septisol	30*	30.5	101.7
Correctol 0.1%	30*	31.3	104.3
Sophtal	30*	30	100.0
Septeal 0.5%	5000	4707	94.1
Dacrine	100	95.3	95.3
Chlorhexidine Gilbert 0.05%	500	517	103.4

* amounts of CHD generally used but not specified when CHD is used as excipients.

105%).

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