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Problems affecting the liquid chromatographic quantitation of chlorhexidine digluconate in ophthalmic solutions*

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Abstract: A liquid chromatographic (LC) assay was recommended by the Food and Drug Administration of the United States for the uptake and release study of chlorhexidine digluconate (CHDG) in ophthalmic solutions by contact lenses. The results from this and other reversed-phase LC assays of CHDG were inaccurate when the working standard and sample solution matrices were different. The error was caused by binding of the analyte onto the container surface and LC column packings. The loss of chlorhexidine due to binding was dependent upon, and very sensitive to, the counter ions in the sample solutions. Relative to water solutions of CHDG, solutions containing chloride, thiosulphate and edetate reduced the loss of the analyte, while solutions containing borate enhanced the loss. To assay CHDG reliably with reversed-phase LC, the media of the working standard and the sample solutions should be closely matched.

Keywords: *Chlorhexidine digluconate; LC; irreversible adsorption; dissociation equilibrium.*

Introduction

Due to its low toxicity and broad antimicrobial activity, chlorhexidine digluconate (CHDG) is widely used as an antiseptic agent in mouth rinses [1, 2] and as a preservative in ophthalmic solutions [3]. The CHDG concentrations in ophthalmic solutions are usually below 0.01% [4, 5]. Thus reliable and sensitive methods are needed for their quantitation. Methods based on colorimetry [6, 7], gas chromatography [8, 9] and liquid chromatography (LC) [10–14] have been reported. A sensitive stability-indicating LC assay was reported by Stevens *et al.* [15] and was recommended by the United States Food and Drug Administration (FDA) for monitoring the uptake and release of CHDG by contact lenses from ophthalmic solutions [16]. However, the accuracy of this assay was very sensitive to medium differences between the working standard and the sample (ophthalmic) solutions. Because of the importance of this LC assay, an understanding of the cause(s) and alternatives to overcome this unusual problem of CHDG quantitation by reversed-phase LC are needed. This paper addresses this issue and alternatives to overcome the problem.

Experimental

Materials and reagents

CHDG (20%) in water, was received from Sola/Barnes-Hind. SOFTMATE CONSEPT-2 (an isotonic solution containing 0.5% $Na_2S_2O_3$ and borate) and SOFTMATE Preservative Free Saline (an isotonic saline solution buffered with borate) were commercially available from Sola/Barnes-Hind. Other chemical reagents and HPLC solvents were analytical or HPLC grade.

Standard solutions

The 20% CHDG concentrate was diluted 1 to 200 with distilled water to form the stock solution (0.1% CHDG). Working standard solutions of CHDG were prepared by appropriate dilution of the stock with water, CONSEPT-2, or the Saline to 0.0005, 0.001, 0.002 and 0.003%.

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Liquid chromatography (LC)

System. An IBM LC/9533 liquid chromatograph was equipped with an IBM LC/9505 autosampler, a 5 μm C_6-Phenomenex spherisorb 250 × 4.6 mm i.d. stainless column, a Bio-Rad 1305 UV monitor, and a Dynamic Solution Maxima Data Station.

Method A. UV detection was monitored at 230 nm. The mobile phase, adapted from Stevens *et al.* [15], was prepared by adding 1.86 l acetonitrile to 1.0 l aqueous solution, pH 2.4, that contained 2.2 g sodium heptanesulphonate and 5.9 ml 85% phosphoric acid. The flow rate was 1.8 ml min^{-1}. Quantitation was performed with peak area comparison by the external standard method.

Method B. UV detection was monitored at 260 nm. The mobile phase consisted of 55 parts of acetonitrile and 45 parts of a pH 5.0, 0.05 M phosphate buffer. The flow rate was 1.0 ml min^{-1}. An aliquot of the internal standard solution (1 ng biphenyl/ml methanol) was added to an aliquot of each test solution just prior to chromatography. Quantitation was achieved by the internal standard method, using peak areas.

Results and Discussion

LC Method A was reported by Stevens *et al.* [15] and was recommended by the FDA to monitor the uptake and release of CHDG by contact lenses from ophthalmic solutions [16]. When tested on CHDG standard solutions prepared with water, the method gave excellent precision (within 1%), sensitivity (LOD = 0.1 ng CHDG), and detection linearity (r = 0.9997) within the testing concentration range 0.0005–0.0030%. The use of these standard solutions to assay CHDG concentrations of solutions prepared with CONSEPT-2, an ophthalmic solution medium, gave results that were about 10% higher than actual. A similar anomaly with other commercial ophthalmic solutions was observed by Stevens *et al.* The cause of this anomaly was explained as the interaction of sodium ions in the ophthalmic solutions with the association–dissociation mechanism of CHDG and heptanesulphonate (an ion-pair agent) in the mobile phase [15]. In an attempt to overcome this quantitation problem, Method B was developed. In this method, the ion-pair agent was eliminated from the mobile phase, an internal standard (biphenyl) was added and the pH of the mobile phase was adjusted from 2.4 to 5 to improve the reproducibility and ruggedness of the assay. Although the mode of separation was ion-pair for Method A and was reversed-phase for Method B, the separation of CHDG from its hydrolytic products by both methods was similar (Fig. 1). When tested with three sets of CHDG standard solutions prepared with water (W), CONSEPT-2 (C), and SOFTMATE Preservative Free Saline (S), Method B yielded two working curves of different slopes and intercepts (Fig. 2). The detection responses [R = CHDG peak area/biphenyl peak area] of the S solutions were twice those of the other two solutions for the entire CHDG concentration range. At <0.003% CHDG, the R values of the C solutions were 10% higher than those of the W solutions, a phenomenon similar to the quantitation problem observed in Method A. The problem could not have been caused by the ion-pair agent since none was used in Method B. It persisted even when the C6 column was replaced with an ODS column and the LC system was pre-conditioned with large injections of CHDG solutions. The addition of sodium chloride or the 1:1 addition of S to the W solution increased R relative to that of the W solution. However, the addition of S to the C solution had much smaller effect on R (Fig. 3). Therefore, the quantitation anomaly caused by sample solution matrix differences cannot be overcome simply with the addition of sodium chloride, as suggested by Stevens *et al.* [15].

Conceivably, different amounts of CHDG in different solutions might have adsorbed onto sample container surfaces to result in non-equivalent detection responses. However, the UV results in Table 1 revealed that the loss of CHDG was significant only for the 0.001% CHDG in W solution in autosampler vials (Type I glass, borosilicate). Other solutions in both volumetric flasks (Pyrex) and autosampler vials did not show an appreciable loss of CHDG. The loss of the preservative from the 0.001% CHDG in W solution in autosampler vials was 9.5%, much less than the 50% decrease in LC detection response seen relative to the S solutions. Therefore, the major loss of CHDG for the W and C solutions probably occurred during LC.

Commercial ophthalmic solutions contain,

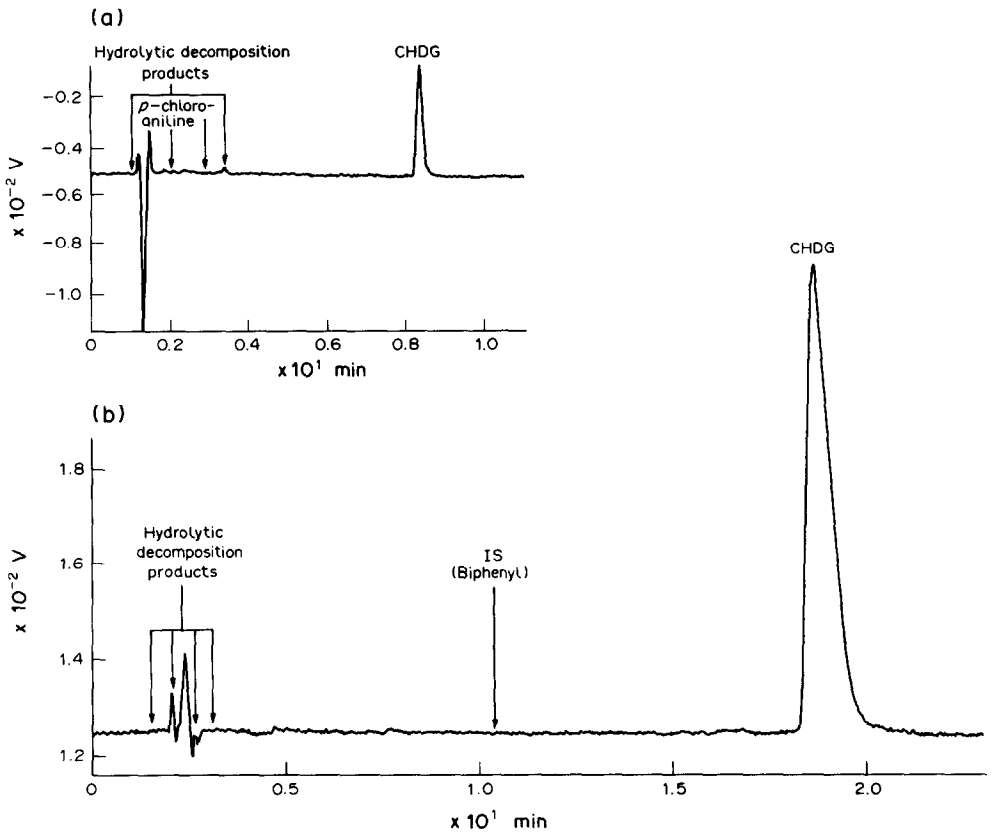

Figure 1
LC chromatograms of chlorhexidine digluconate (CHDG) in water, obtained with LC Method A (a) and LC Method B (b). Also shown on the chromatogram are *p*-chloraniline and other hydrolytic products. (See text for LC conditions.)

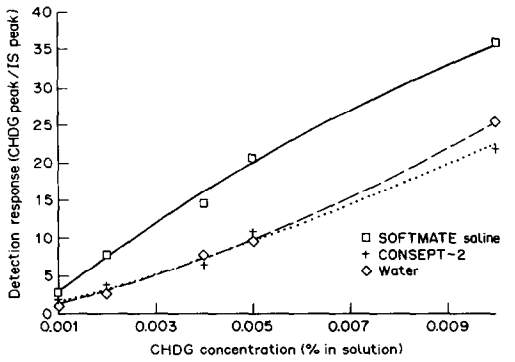

Figure 2
Working curves for the three sets of CHDG standard solutions prepared with water (W), CONSEPT-2 (C), or SOFTMATE Saline (S). Data were generated with LC Method B.

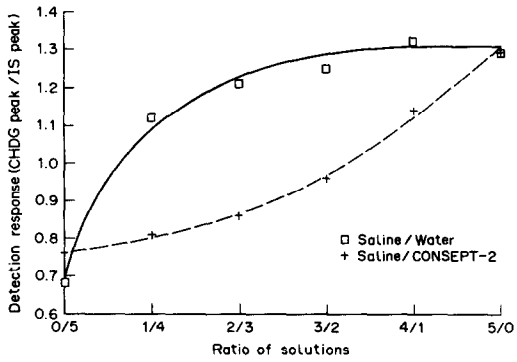

Figure 3
Effect of saline on the detection response of CHDG in water or CONSEPT-2. Data were generated with LC Method B.

apart from preservatives such as CHDG, sodium salts of chloride (0.7%), thiosulphate (0.5%), edetate (0.1%) and borate [15]. One or more of these salts may have interacted with CHDG and resulted in different losses of CHDG during LC. In order to identify the particular component(s) responsible for the loss, solutions of identical CHDG concentration were prepared with solutions of each salt. The detection response, R, of each solution was determined with Method B. The data in Table 2 indicated that the R values of

Table 1
Loss of CHDG to containers during storage (measured by UV*)

CHDG solution	Storage container	Relative UV absorbance† 0 h	2.5 h	7 h
0.001% in S	Volumetric flask	1.00	0.98	1.01
	Autosampler vial	0.99	1.00	1.03
0.001% in W	Volumetric flask	1.00	0.99	na
	Autosampler vial	0.92	0.87	0.94
0.005% in S	Volumetric flask	1.00	1.01	1.00
	Autosampler vial	1.01	1.00	0.99
0.005% in W	Volumetric flask	1.00	1.01	1.00
	Autosampler vial	1.00	0.99	0.99

Each CHDG stock solution was first prepared in individual glass containers. The UV spectrum (300–200 nm) of each stock solution was recorded. Portions of each stock solution were then transferred to volumetric flasks and to autosampler vials. At the indicated time after transfer, the UV spectrum of each solution was recorded. CHDG = chlorhexidine digluconate. S = SOFTMATE Saline. W = water. Volumetric flasks were made of Pyrex glass. Autosampler vials were made of borosilicate. na = not available.

*Absorbance was measured at 255 nm, the absorption maximum of CHDG.

†Absorbance of each solution was normalized by the absorbance of the respective stock solution.

Table 2
Effect of various salts on the LC detection response (R) of aqueous solutions of CHDG

CHDG solution medium	R (ratio of CHDG peak to IS peak) From 0.001% CHDG	From 0.005% CHDG
Distilled water	1.60	9.8
Sterile Saline	3.93	19.0
SOFTMATE Saline	2.84	20.6
0.5% $Na_2S_2O_3$	3.76	17.1
0.1% EDTA (acid form)	3.44	20.3
0.6% $Na_2B_4O_7$ (borate)	0*	4.7*
0.1% $Na_2B_4O_7$	1.33*	6.0*
0.01% $Na_2B_4O_7$	1.68	8.9*
0.1% Boric acid	3.19	18.6

The 0.001 and 0.005% CHDG were prepared by diluting a stock solution (0.1% CHDG in distilled water) appropriately with the various media. Sterile Saline (Abbott Laboratory) is a 0.9% NaCl in water solution. SOFTMATE Saline (Sola/Barnes-Hind) is an isotonic saline solution buffered with borate. Other media were prepared with reagent grade chemicals. LC was performed with Method B described in the text.

*Severe tailing was observed in these CHDG peaks.

saline, $Na_2S_2O_3$, EDTA and boric acid solutions were similar to one another, but were approximately twice those of the water solutions. The R values of the borate solutions were generally smaller than those of the water solutions. The decrease was proportional to the borate concentration. These data, consistent with those observed in Fig. 2, indicated that gluconate and borate anions interacted with chlorhexidine such that the LC detection response was suppressed. CHDG was known to have strong affinity for mouth tissues [1, 2, 17] and plastic surfaces [5] that are lipophilic and non-ionic. Similar affinity was likely to have occurred between CHDG and the LC stationary phases (C6 and ODS). The affinity would be strongest when CHDG existed as the undissociated ion-pair (CH--DG) and weakest when CHDG existed as the dissociated chlorhexidinium ion (CH^{++}). The strong affinity between CH--DG and the stationary phases could have resulted in loss of CHDG due to irreversible adsorption during LC to cause the decrease in LC detection response. As depicted schematically in Fig. 4, CH--DG was at equilibrium with CH^{++} and digluconate ions ($2G^-$) in aqueous solution. This equilibrium was disturbed by the presence of other counter anions. Chloride, thiosulphate, and EDTA enhanced the dissociation of CH--DG and thereby increased the CH^{++} for detection. Borate formed a complex with CH^{++} and was also adsorbed onto the column packings, leading to an even lower detection response. This

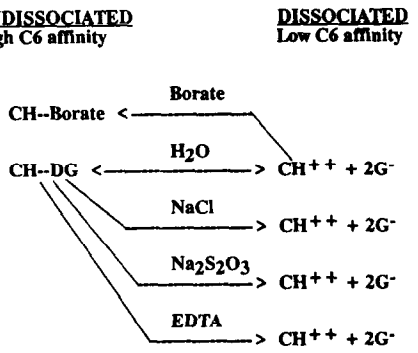

Figure 4
Proposed interaction of CHDG with the sample solution media and the LC stationary phase (C6 and ODS).

explanation is consistent with the 9.5% loss of CHDG to the autosampler vials that are made of borosilicate.

The reported strong affinity between CHDG and contact lens materials [13, 18, 19] that are organic polymers is probably due to the interaction between the neutral CH--DG and organic polymers. The type and concentration of anions in ophthalmic solutions could affect the CH--DG $\rightleftarrows$ CH^{++} + $2G^{-}$ equilibrium, which would have a profound effect on the sorption and elution of chlorhexidine from the contact lens. A better understanding of solution equilibria and the mechanism of surface interaction of CHDG would lead to a better and safer use of CHDG in ophthalmic solutions.

This paper presented a specific and sensitive reversed-phase LC assay for CHDG as an alternative to that of Stevens *et al.* [15]. However, due to the potential interaction of CHDG with the LC stationary phase, these LC quantitations may not be accurate unless the solution media of the working standard and the sample solutions are closely matched. In addition, due to sorption of CHDG to certain container surfaces, pre-conditioning of those containers with sample solutions is required if accurate quantitation of low CHDG concentrations (<0.01%) is desired.

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