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Short Communication

High-performance liquid chromatographic analysis of chlorhexidine and *p*-chloroaniline using a specialty column and a photodiode-array detector

Walter K. Gavlick

Calgon Vestal Laboratories, P.O. Box 147, St. Louis, MO 63166-0147 (USA)

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ABSTRACT

A high-performance liquid chromatographic method has been developed for the separation of chlorhexidine and its known degradation product, *p*-chloroaniline. These amine-containing compounds can be separated without the addition of ion-pairing reagents and/or amine modifiers if the proper specialty column is selected. A photodiode-array detector was used to acquire spectral data and demonstrate the importance of the mobile phase pH when optimizing the response of *p*-chloroaniline.

INTRODUCTION

Chlorhexidine (CH) exhibits a high level of antibacterial activity and is often used as a skin disinfectant in various surgical handscrubs, patient preoperative skin preparation products, healthcare personnel handwashing products, and wound cleansing products [1]. *p*-Chloroaniline (PCA) is a known degradation product which must be assayed in any skin care product which contains chlorhexidine. An assay which can quantitate both compounds is essential for product stability studies.

Since CH and PCA each contain amine functionalities, previous high-performance liquid chromatography (HPLC) analyses have used ion-pairing reagents and/or amine modifiers to minimize peak tailing and enact a separation with conventional reversed-phase columns [2–9]. Recent advances in column technology have resulted in commercially available columns which minimize residual silanol interactions and are well suited for the analysis of basic type compounds. In this work, this type of column was used to separate CH and PCA without the addition of these types of mobile phase modifiers, and a photodiode-array detector was used to optimize the mobile phase pH in order to best detect PCA.

Correspondence to: Dr. W. K. Gavlick, Calgon Vestal Laboratories, P.O. Box 147, St. Louis, MO 63166-0147, USA.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Varian (Walnut Creek, CA, USA) 9095 autosampler with a 50-μl injection loop, a Varian 9010 gradient pump, and a Varian 9065 photodiode-array detector. The data were collected and analyzed with a Varian Star workstation.

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The HPLC columns used in this study were a Supelco (Bellefonte, PA, USA) Suplex pkb-100 (25 cm × 4.6 mm I.D., 5-μm packing) with a Suplex pkb-100 guard cartridge (2 cm × 4.6 mm I.D.) and holder and a Polymer Labs. (Amherst, MA, USA) PLRP-S (25 cm × 4.6 mm I.D., 5-μm, 100-Å packing) with a PLRP-S guard cartridge (5.0 mm × 3.0 mm I.D.) and holder.

Reagents

The mobile phase was prepared with HPLC-grade reagents only. Fisher (Pittsburgh, PA, USA) Optima water, Fisher Optima acetonitrile, Fisher Scientific glacial acetic acid, Fisher Scientific trifluoroacetic acid (TFA), and Fisher Scientific sodium acetate trihydrate were used. PCA (Fisher Scientific, 98% purity) and CH (99.9+% purity, Aldrich, Milwaukee, WI, USA) were used to prepare standards. Chlorhexidine digluconate (CHG) (20%, w/v, Sigma, St. Louis, MO, USA) was used in the preparation of the spiked-sample recovery studies. The CH-containing handwash product was prepared by the Calgon Vestal Laboratories Skin Care Product Development group (St. Louis, MO, USA).

Mobile phase preparation

Reservoir A contained HPLC-grade acetonitrile which had been degassed by vacuum filtration through a 0.45-μm nylon filter (Gelman Sciences, Ann Arbor, MI, USA). Reservoir B contained various acetate and TFA buffers. The acetate buffers were prepared by dissolving HPLC-grade sodium acetate trihydrate in HPLC-grade water to produce a 50 m*M* acetate solution. The pH was then adjusted to a final value through the addition of glacial acetic acid, and the solution was vacuum-filtered through a 0.45-μm nylon filter. The TFA buffer was prepared by adding TFA to HPLC-grade water to produce a 0.1% (v/v) TFA solution. When the TFA buffer was used, 0.1% TFA was also added to the acetonitrile.

Standard and sample preparation

PCA standards were prepared by dissolving an appropriate amount of PCA in 1.0 ml of acetonitrile and then diluting to a final weight with buffer. The CH standards were prepared by adding approximately 1.5 ml of buffer to an appropriate amount of CH followed by three drops of glacial acetic acid. The resultant solution was then briefly shaken to dissolve the CH before the sample was diluted to its final weight by the addition of buffer.

Placebo handwash product samples were spiked to a 1% CHG concentration and then diluted with buffer for analysis. Placebo handwash product samples were also spiked to a low-ppm PCA concentration and then diluted with buffer for analysis. All handwash product samples were syringe-filtered with a 0.45-μm Gelman Acrodisc LC13 PVDF filter after dilution before being analyzed by HPLC.

RESULTS AND DISCUSSION

Effect of pH on the CH/PCA separation using the PLRP-S column

The PLRP-S column consists of cross-linked polystyrene. The column was chosen for the analysis of amine-containing compounds such as CH and PCA because the absence of silanol groups would prevent the residual silanol interactions which are known to occur when silica-based columns are used to chromatograph amine-containing compounds [10]. The mobile phase buffer pH was changed in a series of analyses in order to determine the effect of pH on the separation of CH and PCA. The chromatograms in Fig. 1 demonstrate the improved separation and peak shape of each compound that was found as the mobile phase pH was lowered from 5.8 to 2. It was also noted that the elution order of the two compounds changed under the pH 2 conditions since PCA (pK_a 4.15 [11]) is ionized at this pH.

Effect of pH on the CH and PCA UV spectra

Just as the mobile phase pH had a dramatic effect on peak shape and resolution, it also affects the UV spectrum of the compounds. The photodiode-array detector was used to optimize mobile phase pH for detection of the compounds in terms of the best absorbance wavelengths. The UV spectrum of CH is similar at all of the different pH mobile phases that were examined. The UV spectrum of PCA at pH 2, however, is noticeably different from that at 5.8, and Fig. 2 demonstrates the pH effect on the spectra. It is important that the analysis is optimized with respect to PCA detection since it must be quantitated in the low ppm range. The low-pH mobile phase results in poor PCA absorbance at the

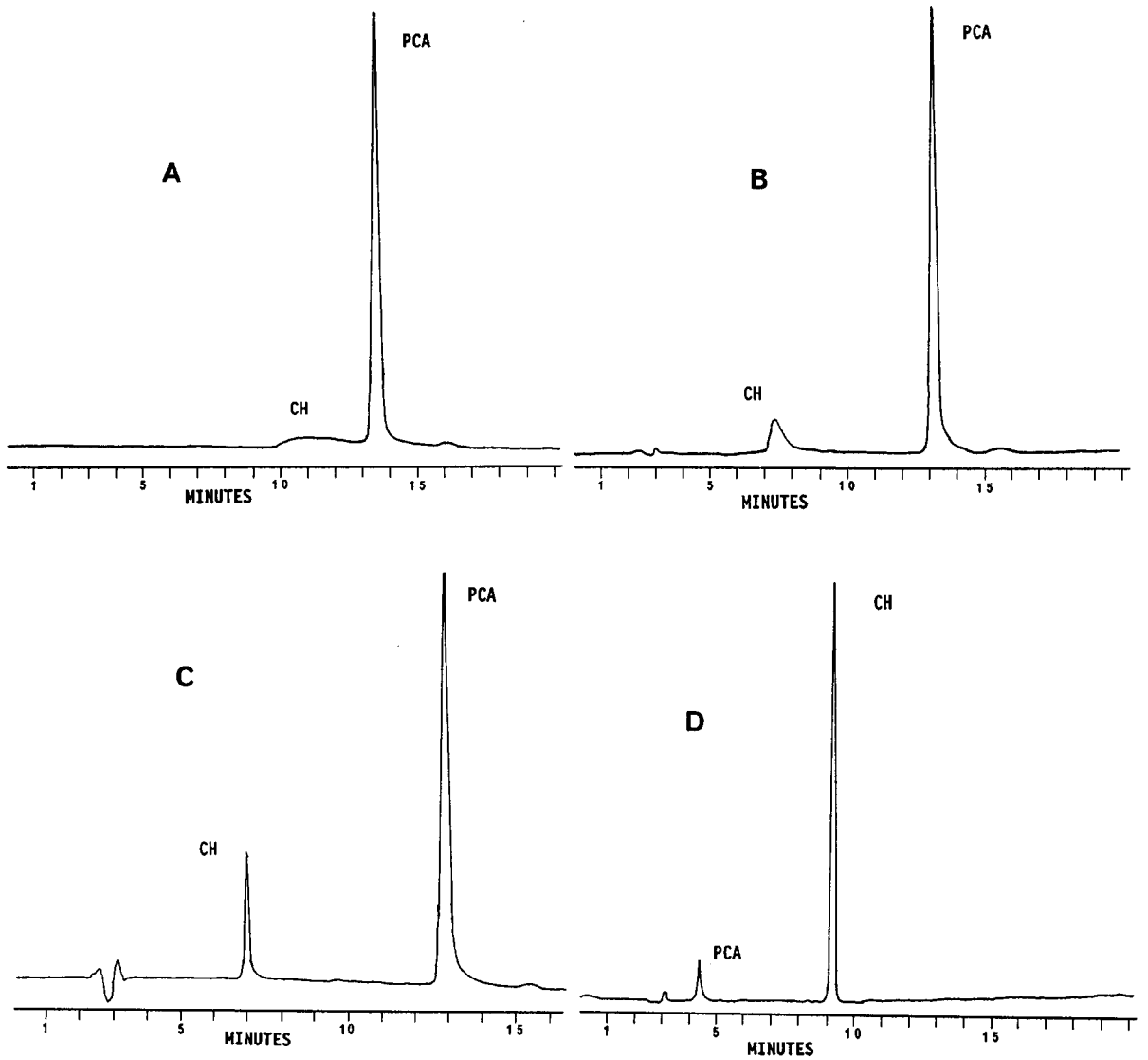

Fig. 1. Effect of the mobile phase pH on the CH/PCA separation. Column, PLRP-S, 25 cm × 4.6 mm I.D.; flow-rate, 1.0 ml/min; gradient elution, from 30 to 80% A in 15 min, hold at 80% A for 5 min; detection, 244 nm. (A) Mobile phase component A = acetonitrile, B = 50 m*M* acetate buffer, pH 5.8. (B) Mobile phase component A = acetonitrile, B = 50 m*M* acetate buffer, pH 4.8. (C) Mobile phase component A = acetonitrile, B = 50 m*M* acetate buffer, pH 3.8. (D) Mobile phase component A = 0.1% TFA in acetonitrile, B = 0.1% TFA buffer, pH 2.

higher UV wavelengths so the higher-pH mobile phase is needed to maximize the sensitivity of the assay for low levels of PCA.

The PLRP-S column produces the best chromatography when the pH 2 buffer is used in the mobile phase, but this does not produce the optimum UV spectrum for PCA. If the pH is raised to improve the spectral characteristics of PCA, then the chromatography suffers. In order to optimize both the chromatographic and spectral aspects of the analysis, another column which is often used for the analysis of basic compounds was investigated.

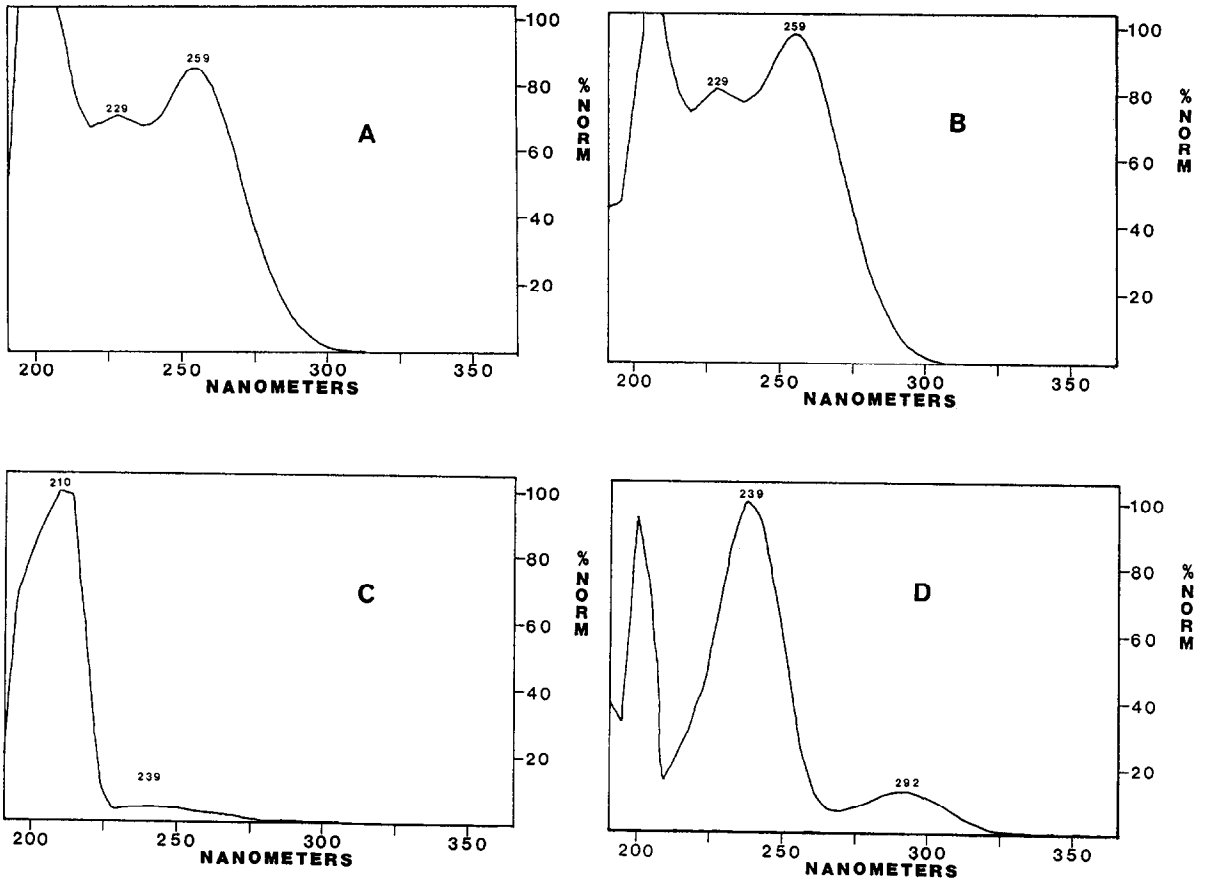

Fig. 2. Effect of the mobile phase pH on the CH and PCA photodiode array-generated UV spectra. (A) CH in pH 2, 0.1% TFA-containing mobile phase. (B) CH in pH 5.8, 50 m*M* acetate buffer-containing mobile phase. (C) PCA in pH 2, 0.1% TFA-containing mobile phase. (D) PCA in pH 5.8, 50 m*M* acetate buffer-containing mobile phase.

CH/PCA separation using the Suplex pkb-100 column

The Suplex pkb-100 is a silica based column which is used for the analysis of amine-containing (*i.e.* basic) compounds. The proprietary chemistry of the column allows for the analysis of such compounds without the addition of ion-pairing reagents and/or amine modifiers. A pH 5.8, 50 m*M* acetate buffer was used in the mobile phase to enact baseline resolution of CH and PCA with minimal tailing of the two peaks (Fig. 3). The pH 4.8 and 3.8 mobile phases also enacted a baseline separation of CH and PCA with minimal tailing of the two peaks, but were not considered to be the optimum mobile phase conditions since the spiked sample recovery studies of the handwash product yielded inconsistent results at these pH values relative to those obtained at pH 5.8. The pH 2 mobile phase separation was similar to that found with the PLRP-S column. The high mobile phase pH allows for optimum PCA sensitivity at a high (239 nm) UV wavelength. CH and PCA can thus be analyzed by HPLC without the addition of ion-pairing agents and/or amine modifiers to the mobile phase if the Suplex pkb-100 column is used. The column also allows for the optimum mobile phase pH to be used.

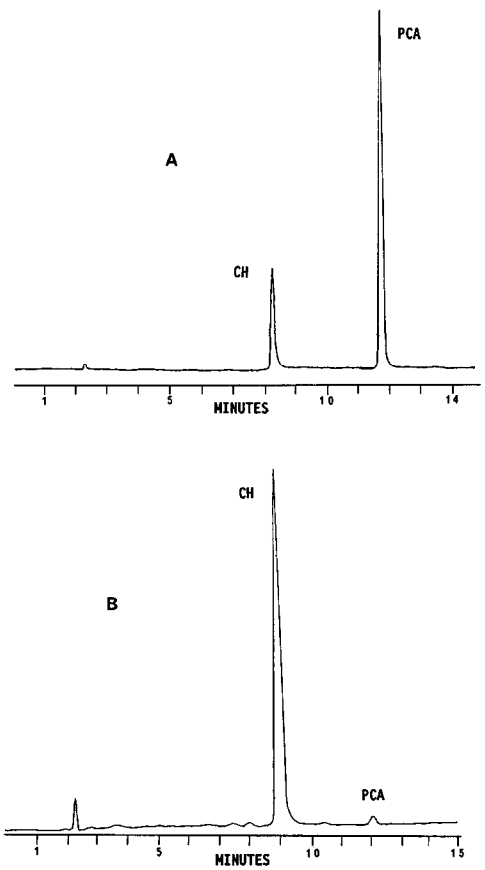

Fig. 3. Chromatograms of (A) CH and PCA standards and (B) CH and PCA separation in a dilute handwash product sample. Column, Suplex pkb-100, 25 cm × 4.6 mm I.D. ; flow-rate, 1.5 ml/min; gradient elution, from 20 to 55% A in 15 min; A: acetonitrile; B: 50 m*M* acetate buffer, pH 5.8; detection, 239 nm.

Calibration curve data

Calibration curve data were generated for CH and PCA standards. Two runs (analyses) of CH standards were performed but no detection limit was established since this is usually not an issue for CH analysis. The run 1 calibration curve consisted of standards ranging from 23.8 to 152 ppm CH, and the run 2 CH calibration curve consisted of standards ranging from 19.1 to 146 ppm CH with a linear response found over each standard concentration range.

Three PCA calibration curves were generated, and a limit of quantitation was established. The run 1 PCA calibration curve consisted of standards ranging from 1.58 to 177 ppm PCA, the run 2 standards ranged from 0.10 to 13 ppm PCA, and the run 3 standards ranged from 0.043 to 26.0 ppm PCA with a linear response found over each standard concentration range. The 0.043 ppm standard could be quantitated and lower amounts could be detected but not quantitated. Table I contains a summary of the CH and PCA calibration curve data.

Analysis of formulated handwash product

The HPLC assay was developed for CH and PCA analysis of a Calgon Vestal Laboratories (C.V.L) handwash product. In order to test the accuracy of the assay, spiked samples were prepared with respect to CHG and PCA. CHG is the CH salt form commonly used in this type of handwash product, and it was spiked into a placebo to yield an approximately 1% (w/w) sample. PCA was also spiked into a placebo to yield a low ppm range sample in an effort to duplicate a typical product sample. The spike recovery data generated from four separate spiked sample preparations for each compound were 103.5 ± 2.4% for CH and 96.2 ± 2.2% for PCA. The spiked sample recovery data indicate that the method provides accurate quantitation of both CH and PCA in the handwash product. Fig. 3 contains a chromatogram of a dilute handwash product sample. Photodiode array generated purity correlation data indicate that the CH and PCA peaks are homogeneous. These results are obtained by comparing spectra which are taken at different points across the chromatographic peak. When the spectra were compared, the best correlation value for the CH peak was 1.000000 and the worst was 0.999996. The best correlation value for the PCA peak was 0.999926 and the worst was 0.999643. The more similar the spectra, the closer the correlation value is to one and the greater the probability that the peak is a one component peak and no matrix components coelute.

In summary, an HPLC method has been developed in which a Suplex pkb-100 column is used to enact a separation of CH and PCA without the addition of ion-pairing reagents and/or amine modifiers to the mobile phase. The analysis is performed under pH conditions which optimize the PCA response. The method was demonstrated to be effective in the quantitation of CH and PCA in a handwash product.

TABLE I

CH AND PCA CALIBRATION CURVE DATA SUMMARY

Run	Number of standards	Correlation coefficient	Slope	*y*-Intercept
Chlorhexidine				
1	5	0.9997	6330	−31 594
2	6	0.9998	6258	−25 718
S.D.			51	4155
p-Chloroaniline				
1	5	1.000	11 916	3619
2	5	1.000	12 159	−582
3	6	1.000	11 948	−69
S.D.			132	2292

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