Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, Nos 10–12, pp. 1031–1036, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

High-performance liquid chromatographic analysis of chlorhexidine phosphanilate, a new antimicrobial agent*

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Abstract: Chlorhexidine phosphanilate (CHP) is analysed by two separate reversed-phase HPLC methods. CHP was found to be a non-stoichiometric compound with a phosphanilic acid to chlorhexidine ratio of 1.83. By careful choice of solvents, solution pH and HPLC columns, loss of sample due to incomplete dissolution and adsorption to surfaces is avoided. Both methods are shown to be stability-indicating and accurate.

Keywords: *Chlorhexidine phosphanilate; chlorhexidine; phosphanilic acid; HPLC; stoichiometry; stability; chlorhexidine acetate.*

Introduction

Chlorhexidine phosphanilate (CHP) is a salt of chlorhexidine and phosphanilic acid (Fig. 1). It is a non-stoichiometric compound with a chlorhexidine to phosphanilic acid ratio of 1.83 and is amorphous in nature. In solution it dissociates into chlorhexidine and phosphanilate ions. It has a remarkably broad spectrum of antibacterial activity encompassing the predominantly gram-positive spectrum of chlorhexidine and the broad gram-negative activity of phosphanilic acid [1]. Its lack of cross resistance with sulfonamide resistant strains [2] makes it especially attractive for burn wound treatment. The broad spectrum activity of this compound suggests potential applications for prevention and treatment of skin infections. The release of CHP from a cream vehicle and its skin permeation characteristics has been reported [3].

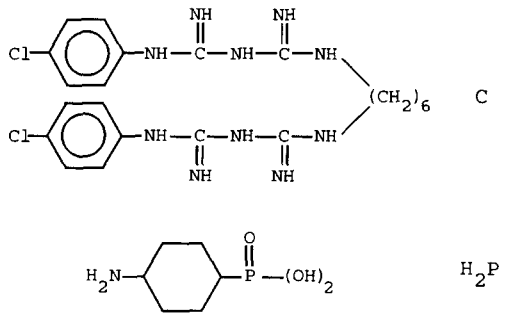

Figure 1
Chemical structures of chlorhexidine (C) and phosphanilic acid (H_2P).

CHP is practically insoluble in most common solvents with the exception of dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF). Its solubility in water is about 0.4 mg ml^{-1}. Chromatographically, it is observed as its two component ion peaks, chlorhexidine and phosphanilate, and not as a single CHP peak. Since both chlorhexidine and phosphanilate are therapeutically active and because CHP is a non-stoichiometric compound, it is important to monitor both chlorhexidine and phosphanilate during the development of the drug. Because of their difference in polarity and the ionic interactions of the chlorhexidine and phosphanilate ions, it is difficult to develop a single HPLC method for both ions.

A number of HPLC methods for chlorhexidine have been reported and a good bibliography of these and other analytical methods may be found in refs 4–6. Most of these HPLC methods use an octadecylsilane (C_{18}) column and a mobile phase of aqueous methanol or acetonitrile containing an ion-pairing agent. Only one HPLC method was reported in the literature for phosphanilic acid [7]. Direct application of any of these methods

* Presented at the "Third International Symposium on Pharmaceutical and Biomedical Analysis", April 1991, Boston, MA, USA.

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to CHP analysis is not possible because of the poor solubility of CHP in water and in most other solvents used in HPLC mobile phases. In addition, phosphanilic acid is also very sparingly soluble in water and it is insoluble or sparingly soluble in the usual organic solvents [8]. The problems associated with low solubilities are losses due to incomplete dissolution, precipitation and surface adsorption. These problems have been circumvented in the two individual HPLC assay methods reported in this paper. Practical details for easy application of these methods, along with method development and validation information, are presented.

Experimental

Instrumentation

A Waters Associate (Milford, MA, USA) chromatograph equipped with Model 6000A pump, WISP Model 710 injector, Model 440 absorbance detector (254 nm) was used. A Hewlett–Packard (Palo Alto, CA, USA) Model 1040A diode array spectrophotometric detector was used as needed to simultaneously monitor the chromatogram and absorption spectra of the component peaks. Data acquisition and integration of peaks were performed with a Hewlett–Packard Model 3352D Laboratory Automation System.

For chlorhexidine assay, a Waters μBondapak C_{18} column (30 cm × 3.9 mm i.d.) was used. A Waters μBondapak-NH_2 column was used in phosphanilic acid analysis. The eluent flow rate was 1.0 ml min^{-1} for the former analysis and 2.0 ml min^{-1} for the latter. In both cases, an injection volume of 10 μl was used.

Reagents and solvents

Chlorhexidine acetate (Sigma, St Louis, MO, USA) was used as the chlorhexidine standard. Its purity was assessed by non-aqueous titration with 0.1 M perchloric acid in glacial acetic acid and its water content by Karl Fischer titration. The purity of phosphanilic acid (Bristol–Myers Squibb Co., Syracuse, NY, USA) was determined by potentiometric titration with nitrite. CHP was manufactured under Bristol–Myers Squibb control. Ethyl benzoate was purchased from Aldrich (Milwaukee, WI, USA).

Tetrahydrofuran (THF), acetonitrile, 85% m/m phosphoric acid and ammonium dihydrogen phosphate were all of HPLC grade (Fisher Scientific, Pittsburgh, PA, USA). All other solvents and reagents were of ACS grade from Fisher Scientific. The water used was deionized and organic free quality from Milli Q System (Millipore, Bedford, MA, USA).

Mobile phase

For the chlorhexidine analysis, the mobile phase was 40:10:50 (v/v/v) mixture of methanol, THF and 0.1 M aqueous sodium sulphate, the pH of which was adjusted to 2.2 with sulphuric acid. The mobile phase for the phosphanilic acid analysis was a 10:90 mixture of acetonitrile and 0.012 M aqueous ammonium dihydrogen phosphate.

Standards preparation

The chlorhexidine standard for HPLC analysis was prepared to contain 0.04 mg ml^{-1} chlorhexidine acetate and 0.33 mg ml^{-1} ethyl benzoate (internal standard) using methanol as the solvent.

Separate stock solutions of phosphanilic acid (1 mg ml^{-1}) and salicylic acid (14 mg ml^{-1}) were prepared in 0.5 N sodium hydroxide solution to facilitate easy dissolution. To prepare the standard, phosphanilic and salicylic acid stock solutions, 4 and 5 ml respectively, were mixed with 9 ml of dilute phosphoric acid (4.34% w/v) and the mixture diluted to 100 ml with 45:55 (v/v) acetonitrile–water mixture. This sample preparation resulted in the diluent being approximately equivalent to the mobile phase so as to minimize baseline disturbances.

Sample preparation

The solution of CHP sample (2.5 mg ml^{-1}) was prepared in DMSO. For the phosphanilic acid assay, 4 ml of this sample solution was mixed with 5 ml of the salicylic acid stock solution (see standard preparation above) and 5 ml of dilute phosphoric acid (4.34% w/v) solution. The resultant solution was diluted to 100 ml with 45:55 (v/v) acetonitrile–water mixture.

For the chlorhexidine assay, the CHP sample solution (2.5 mg ml^{-1}) in DMSO was diluted five-fold with DMSO. The sample for analysis was then prepared from this dilute solution to contain 0.05 mg ml^{-1} CHP and 0.33 mg ml^{-1} ethyl benzoate using methanol as the diluent.

Results and Discussion

Solubility and solution equilibria

The solubility of CHP depends on the solvent, pH, temperature and the counter ions in solution. It is more soluble in DMSO (over 100 mg ml^{-1}) and DMF than in water, methanol, 1-propanol, 2-propanol and acetonitrile. In solution, CHP dissociates into chlorhexidine and phosphanilate ions which in turn exist in equilibrium with their multiple acid and base forms depending on the pH of the solution.

Chlorhexidine can exist as free base (C) or its protonated forms CH_2^{2+} and CH_4^{4+}. pK_a values are reported to be 2.2 and 10.3 [9, 10]. Although only one pK_a value of 7.5 is reported for phosphanilic acid [11] it may exist as its protonated form, free acid and mono- and dianionic forms. The approximate pK_a values are estimated from pH titration curves and solubility in strongly acidic solution to be <0.5, 3.5 and 7.1 (C. Zusi, personal communication). Depending on the pH of the medium, different ionic species form in CHP solutions. A schematic view of the equilibria in aqueous solution is shown in Fig. 2. The vertical lines represent points where pH = pK_a. pH regions where the individual species are predominant are shown by horizontal solid lines. The solubility and dissolution rate of CHP are pH-dependent and they become more complex if the pH is favourable to the formation of other sparingly soluble salts (e.g. chlorhexidine monophosphanilate), phosphanilic acid (free acid) and/or chlorhexidine (free base). In water, the solubility of chlorhexidine is only 0.08 mg ml^{-1} [12] and phosphanilic acid is known to be very sparingly soluble [8]. The common salts of chlorhexidine (e.g. chloride, nitrate, sulphate) have solubilities in the range 0.1–1 mg ml^{-1} [12], hence the presence of simple anions could play a significant role in the dissolution kinetics of CHP. Furthermore, the poor solubility of chlorhexidine, phosphanilic acid and related ionic species and salts may be contributory factors to adsorptive losses during sample preparation and analysis.

Analytical standards

Early in the method development it was considered using well characterized lots of CHP as the reference standard for both chlorhexidine and phosphanilic acid assays by HPLC. However, it was realized later that CHP is not a stoichiometric compound and that more reliable standards of chlorhexidine and phosphanilic acid were needed.

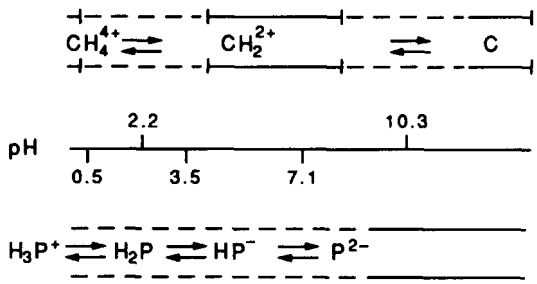

Figure 2
pH-dependent equilibria of chlorhexidine and phosphanilic acid in aqueous solutions.

Among the chlorhexidine salts, chlorhexidine acetate was selected as the analytical standard. It is commercially available in pure form and can be easily purified further by recrystallization from water. Typically, it assayed better than 99% (after correction for water) by non-aqueous titration with 0.1 N perchloric acid in glacial acetic acid [13]. Our data show that it contained about 3.2% m/m moisture (Karl Fischer titration) but it is not hygroscopic at 80% relative humidity. Phosphanilic acid was available at 98.5% m/m purity as shown by titration with nitrate [14], and by HPLC (peak area normalization). Thus a suitable correction was applied in calculations.

Chromatography development

In reversed-phase chromatography with μBondapak C_{18} column, CHP behaved as a mixture of two components (chlorhexidine and phosphanilic acid). With weak mobile phases, only one peak due to phosphanilic acid was observed at or near the solvent front. With stronger mobile phases, chlorhexidine peak also showed up generally as a broad peak. It was impossible to retain phosphanilic acid while eluting chlorhexidine in a reasonable time. Similar behaviour of CHP was also observed using μBondapak phenyl column. Only with mobile phases of low pH (about 2.5) did chlorhexidine show a sharp peak. At pH 2.5, which was needed for the optimal peak shape of chlorhexidine, it was not possible to move the phosphanilic acid peak away from the solvent front by ion-pairing. Hence, it became necessary to develop separate assay methods for chlorhexidine and phosphanilic acid.

The best mobile phase for chlorhexidine using μBondapak C_{18} column, was methanol–

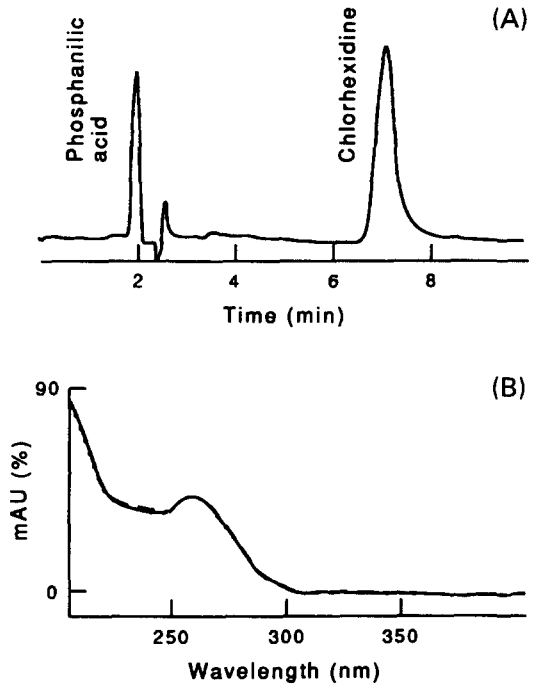

Figure 3
HPLC chromatogram (A) of chlorhexidine phosphanilate and UV spectra (B) at the upslope, apex and downslope of chlorhexidine peak.

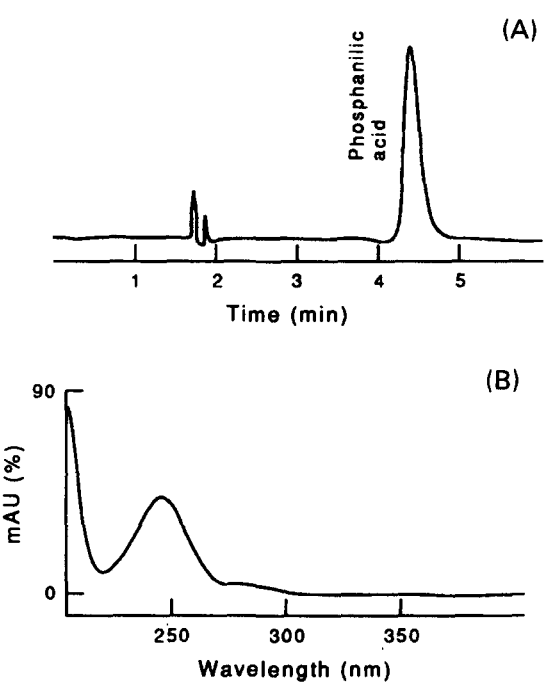

Figure 4
HPLC chromatogram (A) of chlorhexidine phosphanilate and UV spectra (B) at the upslope, apex and downslope of phosphanilic acid peak.

THF–0.1 M sodium sulphate (4:1:5, v/v/v), with the pH adjusted to 2.5 with sulphuric acid (Fig. 3). The use of methanol, instead of acetonitrile, is advantageous since CHP is more soluble in methanol (>0.14 mg ml^{-1}) than in acetonitrile. The addition of THF and sodium sulphate resulted in better separation of chlorhexidine from its impurities. The inclusion of THF as a mixed organic solvent system improved the peak shape. Sodium sulphate acts as an ionic suppressor.

Phosphanilic acid gave a peak with minimal tailing and a reasonable retention time using μBondapak amine column (Fig. 4). A simple mobile phase of acetonitrile–0.012 M ammonium dihydrogen phosphate buffer (1:9, v/v) and the absence of ion-pairing agents gave good separation of phosphanilic acid and the internal standard, salicylic acid. An increase in resolution was observed with lower phosphate concentration but with a corresponding increase in retention times and analysis time. However, by lowering the phosphate concentration and increasing acetonitrile concentration, the useful life of the amine column can be extended especially for CHP product analysis where potential deactivation of the column can be caused by excipients present in the formulation.

The standards and samples for injection for both chlorhexidine and phosphanilic acid methods were matched approximately to have a similar medium (solvents, pH) which is compatible with the mobile phase. Because of the complex equilibria which exist in CHP solutions and the poor solubilities of several components in equilibria, this matching is considered important. A similar observation was made by others [5, 6] in the assay of chlorhexidine gluconate in ophthalmic solutions. The solvents, pH, concentrations (chlorhexidine, phosphanilic acid, CHP) and the sequence of dilutions were selected in a manner to minimize any losses due to precipitation or adsorption on surfaces. Sudden perturbations in equilibria due to sudden changes in pH and concentrations of counter ions could lead to loss of analyte or distortion of the peaks in chromatographic separations.

Chlorhexidine method validation

Using solutions of chlorhexidine acetate standard with the internal standard (ethyl benzoate), the linearity of the chlorhexidine

response ratio was studied. Both peak height and peak area ratios showed good linear response in the chlorhexidine concentration range 0.013–0.053 mg ml^{-1} (correlation coefficient >0.999). A low negative *y*-intercept by peak height ratio, 2.3% compared to response at 0.033 mg ml^{-1} chlorhexidine, suggests that a single point standard may be used in the assay instead of a calibration curve with multiple standards. The *y*-intercept by peak area ratio was somewhat higher (5.1%).

Specificity of the method was demonstrated by analysing methanolic solutions of CHP (0.27 mg ml^{-1}) which were force-degraded by heat (60°C) and light (≈1000 foot candles). After 8 days, the chlorhexidine content was found to be 13.0 and 94.9% m/m of the initial in the heat and light degraded samples respectively. No peaks interfering with either the chlorhexidine or the internal standard peak were observed (Fig. 5). *p*-Chloroaniline, a known hydrolytic degradation product of chlorhexidine [15, 16] did not interfere. It elutes on the tail end of the phosphanilic acid peak near the solvent front.

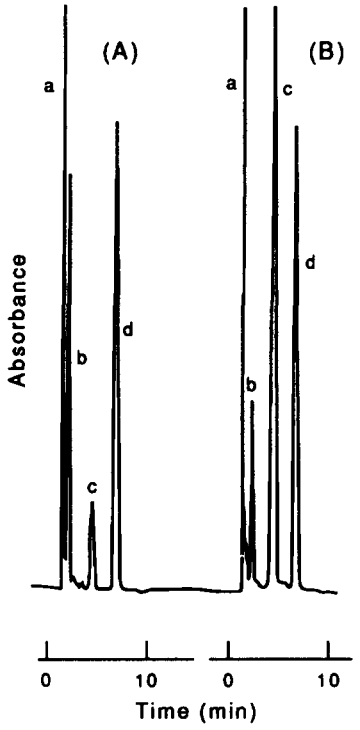

Figure 5
Chromatograms of degraded chlorhexidine phosphanilate solutions by the chlorhexidine assay method. (A) Heat degraded solution (8 days, 60°C). (B) Light degraded solution (8 days, 1000 foot candles). Peak identification: a, phosphanilic acid; b, degradation product; c, chlorhexidine and d, ethyl benzoate.

Purity of the chlorhexidine peak was studied by recording the spectral–chromatographic data using the photodiode array detector. A typical superposition of upslope, apex and downslope spectra is shown in Fig. 3. Good superimposability of spectra from three regions of the peak and also with that of chlorhexidine (acetate) standard provided evidence for the absence of coeluting peak(s) under the chlorhexidine peak.

Four solutions of chlorhexidine acetate spiked with different amounts of sodium phosphanilate were analysed by the chlorhexidine method. Chromatographic responses (chlorhexidine to internal standard peak area ratio) of 1.134, 1.140, 1.120 and 1.120 were observed when the phosphanilic acid to chlorhexidine molar ratio in solutions were 0.88, 1.40, 1.75 and 2.10, respectively. These data demonstrate that the chlorhexidine response is not sensitive to the phosphanilic acid to chlorhexidine ratio.

Phosphanilic acid method validation

The response ratio (phosphanilic acid to salicylic acid peak area ratio) was observed to be linear in the concentration range 0.02–0.06 mg ml^{-1} of phosphanilic acid. A correlation coefficient of 0.9999 and a *y*-intercept of 0.006% (compared to response ratio of 0.04 mg ml^{-1} phosphanilic acid standard) were calculated.

CHP solutions degraded by light and heat, using conditions described under chlorhexidine method validation, were analysed by the phosphanilic acid assay method. Chromatograms in Fig. 6 show the separation of both phosphanilic acid and salicylic acid peaks from the degradation products of CHP. The purity of the phosphanilic acid is demonstrated by the essentially identical spectra recorded on the upslope, apex and downslope portion of the peak (Fig. 4).

The effect of chlorhexidine on the phosphanilic acid response was also studied by analysing sodium phosphanilate solutions spiked with chlorhexidine acetate. Analysis of four solutions with molar ratios of phosphanilic acid to chlorhexidine that varied from 2.06 to 4.96 showed no effect on the phosphanilic response (0.13% RSD).

Composition of chlorhexidine phosphanilate

Sixteen lots of CHP drug substance were analysed separately for their chlorhexidine and phosphanilic acid content. The molar ratio of

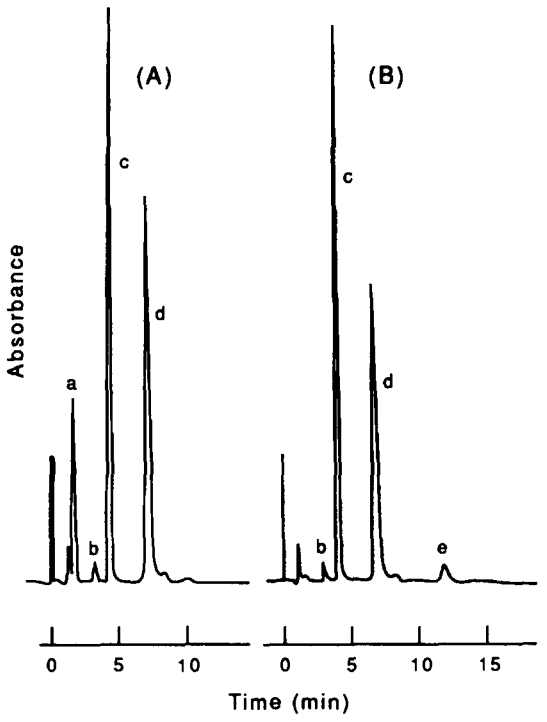

Figure 6
Chromatograms of degraded chlorhexidine phosphanilate solutions by the phosphanilic acid assay method. (A) Heat degraded solution (18 weeks, 60°C). (B) Light degraded solution (18 weeks, 1000 foot candles). Peak identification: a, degradation product; b, system peak; c, salicylic acid; d, phosphanilic acid and e, degradation product.

phosphanilic acid to chlorhexidine was found to be in the range of 1.76–1.88 with a mean of 1.83 (n = 16, RSD = 2.2%). The corresponding molar ratios obtained from elemental analysis data (chlorine and phosphorus assays) are in the range 1.77–2.01 with a mean of 1.92 (RSD = 3.9%). Considering the normal uncertainty in the elemental analysis data, the ratios observed by the two techniques are considered to be in fair agreement. Hence the CHP drug substance is a non-stoichiometric compound with a mean phosphanilic acid to chlorhexidine molar ratio of 1.83.

Conclusions

The complex equilibria of CHP in solution and the poor solubility of CHP and its equilibration products required special care in preparing and handling of samples and standards. Analysis of CHP by two separate stability-indicating HPLC methods for chlorhexidine and phosphanilic acid demonstrated that CHP is a non-stoichiometric compound with phosphanilic acid to chlorhexidine ratio of 1.83.

Acknowledgements — The authors thank the Analytical R&D staff and Dr C. Zusi for their assistance during the course of this work. The contributions of Dr J. Allison, J. Loder, C. Miller and S. Gadde during the preparation of manuscript are also acknowledged.

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[Received for review 29 April 1991; revised manuscript received 15 May 1991]