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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CHLORHEXIDINE IN HUMAN SERUM AND URINE

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SUMMARY

A high-performance liquid chromatographic method for the analysis of chlorhexidine in human serum and urine was developed. Chlorhexidine and the internal standard, chlorpheniramine, were extracted into chloroform, containing 5% 2-propanol, and back-extracted into dilute sulfuric acid. Chromatographic separation was achieved on a C_{18} column equilibrated with methanol—water (70:30, v/v), containing 0.005 *M* sodium heptanesulfonate. The sensitivity of the assay was 20 ng/ml of biological matrix, using 0.5-ml samples. The application of this method to monitor chlorhexidine levels in burn patients treated topically with a chlorhexidine-containing burn cream was demonstrated.

INTRODUCTION

Chlorhexidine, 1,1′-hexamethylene-bis[5-(4-chlorophenyl)biguanide], has been widely used since 1953 as a general skin and surface antiseptic agent [1]. It is also an active antibacterial component with a predominately Gram-positive spectrum in a topical burn cream [2].

Absorption of chlorhexidine through adult intact skin has been shown to be negligible [3]. Although low levels of chlorhexidine have been reported in the blood of neonates administered 4% chlorhexidine gluconate [4], topically, further study showed that chlorhexidine did not penetrate through neonate skin and the chlorhexidine found in the previous study was attributed to the contamination of samples [5]. Little is known about the absorption of chlorhexidine through burn-damaged skin. The absorption and disposition of chlorhexidine are related to its physicochemical properties and to the patient's pathophysiologic state. Burn injury not only alters the permeation barriers of

the skin but also produces a wide variety of intricate physiologic changes, which may produce unpredictable changes in the pharmacokinetics of chlorhexidine. Therefore, it is important to assess the degree of absorption and renal excretion of this compound in burn patients.

Three assay methods have been reported in the literature for the determination of chlorhexidine in biological samples [6—8]. One, a gas chromatographic method, is not specific and cannot distinguish chlorhexidine from *p*-chloroaniline, a known breakdown product of chlorhexidine [6]. The detection limit of the others, high-performance liquid chromatographic (HPLC) methods, is in the range 0.1—0.2 μg/ml [7, 8]. A more sensitive method is desirable to monitor chlorhexidine levels in burn patients. We report here a more sensitive HPLC method for the analysis of chlorhexidine in human urine and serum samples. This method was applied to determine chlorhexidine in serum and urine from burn patients treated with a topical chlorhexidine formulation.

EXPERIMENTAL

Reagents and solvents

Chlorhexidine diacetate hydrate was purchased from Aldrich (Milwaukee, WI, U.S.A.). The internal standard, chlorpheniramine maleate, was obtained from Bristol Labs. (Syracuse, NY, U.S.A.). HPLC-grade methanol and 2-propanol, ACS-grade sulfuric acid and certified-grade 2 *M* sodium hydroxide were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). HPLC-grade chloroform (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), sodium heptanesulfonate (Sigma, St. Louis, MO, U.S.A.), screw-cap culture tubes (13 × 100 mm) with PTFE-lined caps (American Scientific Products, McGaw Park, IL, U.S.A.), dichlorodimethylsilane (Eastman Kodak, Rochester, NY, U.S.A.) and human serum (Interstate Blood Bank, Philadelphia, PA, U.S.A.) were obtained from commercial sources. Human urine samples (24 h) were obtained from normal volunteers. Deionized water was generated by a Milli-Q system (Millipore/Continental Water Systems, Bedford, MA, U.S.A.).

High-performance liquid chromatography

The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) 510 pump, a WISP 710B autosampler/injector and a variable-wavelength UV detector (Model 481), set at 260 nm. A 30.0 cm × 3.9 mm μBondapak C_{18} column (10 μm, Waters Assoc.) was used for the analysis. The detector signal output was monitored with an integrator (Model 4270, Spectra-Physics, San Jose, CA, U.S.A.). The mobile phase was methanol—water (70:30, v/v), containing 0.005 *M* sodium heptanesulfonate; the flow-rate was 1.2 ml/min. The injection volume was 20 μl.

Procedures

All the glassware used for this work was silanized with dichlorodimethylsilane.

Preparation of serum standards and samples

Chlorhexidine diacetate hydrate standard solution (1 mg/ml) was prepared

in deionized water. A 25-μl aliquot of the stock solution was diluted to 10 ml in a volumetric flask with control serum (pooled from ten subjects). This chlorhexidine diacetate stock solution in serum (2.5 μg/ml) was used as the stock for preparing standards for HPLC analysis. Standards were prepared in duplicate by adding chlorhexidine diacetate stock solution (5–200 μl) to a screw-cap culture tube (13 × 100 mm). Appropriate volumes of control serum (495—300 μl) were added to bring up the volume to 0.5 ml and to give the following serum concentrations: 20.2, 40.4, 80.8, 121.2 202, 404 and 808 ng/ml chlorhexidine. Blank control samples were prepared by pipetting 0.5 ml of control serum to a 13 × 100 mm screw-cap tube. These standard and blank solutions were processed through the extraction procedure described below.

Preparation of internal standard

The internal standard, chlorpheniramine solution (0.05 mg/ml), was prepared in deionized water.

Extraction procedure

A 0.5-ml sample of serum was pipetted into a 13 × 100 mm screw-cap tube. To each sample and standard tube, 50 μl of the internal standard solution were added, followed by the addition of 100 μl of 2 *M* sodium hydroxide and 2.5 ml of chloroform containing 5% 2-propanol. The mixture was shaken vigorously for 20 min on a shaker. The aqueous and organic phases were separated by centrifugation for 5 min at 700 *g*. After discarding the top aqueous layer, the organic layer was poured into a clean 13 × 100 mm screw-cap tube. To each tube, 100 μl of 0.05 *M* sulfuric acid were added, and the contents were vigorously mixed for 10 min on a shaker. The aqueous layer was separated from the organic layer by centrifugation and was transferred to a glass WISP vial.

Preparation of urine standards and samples

The procedure to prepare urine standards and samples was the same as for serum standards and samples, except that urine was used instead of serum.

Validation procedures

Possible interference from endogenous serum and urine was evaluated by analyzing 0.5 ml of serum and urine pre-dose samples from five burn patients. Benzyl alcohol, an excipient in the burn cream, as well as its metabolite, benzoic acid, *p*-chloroaniline, a possible metabolite of chlorhexidine, and phosphanilic acid, a component of the burn cream, were evaluated for their possible interference.

The limit of detection of the assay was determined as follows: two 495-μl aliquots of serum or urine samples from each of ten normal subjects were transferred into twenty separate tubes. To one tube of each pair (subject), 5 μl of serum or urine containing 10 ng of chlorhexidine were added and mixed (spiked sample). The other tube received 5 μl of serum or urine from the same subject (blank). All of the samples were extracted and analyzed. The measured response, peak height in mm, for each blank and spiked sample was recorded and the difference in response for spiked versus blank sample pairs was evaluated using the Student's *t*-test for paired samples.

Seven standards in the concentration range 20.2—808 ng/ml chlorhexidine in human serum or urine were prepared, in duplicate, and analyzed using the described assay procedure. Assay linearity was evaluated using least-squares linear regression analysis of the response ratio (chlorhexidine peak height/chlorpheniramine peak height) versus chlorhexidine concentration data.

The stability of chlorhexidine in human serum or urine was evaluated by analyzing spiked serum or urine samples (323 ng/ml chlorhexidine). These samples were stored at −20°C in 3-ml aliquots in silanized culture tubes, and groups of six samples each were analyzed at various times.

Extraction recovery of chlorhexidine was determined by comparing the slope of a standard curve of extracted chlorhexidine standards in human serum or urine to the slope of a standard curve obtained with chlorhexidine standards in 0.05 *M* sulfuric acid.

Within-day accuracy and precision were determined as follows: ten samples of each of two concentrations of chlorhexidine (96.2 and 808 ng/ml for serum samples and 117 and 808 ng/ml for urine samples) were prepared and submitted as unknown samples. The samples were analyzed using the described method. The observed mean, percentage relative standard deviation (R.S.D.), and percentage theoretical recovery were calculated.

Day-to-day accuracy and precision were determined as follows: human serum or urine samples containing 80.8 and 404 ng/ml chlorhexidine were prepared and analyzed in duplicate on four different days using the described method. The observed mean, percentage relative standard deviation, and percentage theoretical recovery were calculated.

Collection of patient samples

In a safety and tolerance study, nine patients, with burns ranging from 3.5 to 24.75% total body surface area and second- and/or third-degree injuries, were treated with a cream (58—966 g) containing 2% chlorhexidine phosphanilate, vehicle, or Silvadene® once daily for four days, according to a randomized, double-blind, cross-over design. Blood samples were collected once each day, starting before the first application of study medication and before each subsequent application through the fifth study day. After centrifugation, serum was transferred to a silanized screw-cap glass tube and kept frozen at −20°C until analyzed. Complete 24-h total urine samples were collected during study days 3 and 4 and kept frozen at −20°C until analyzed.

Calculations

The regression of the weighted (1/concentration) peak-height ratio of chlorhexidine/chlorpheniramine versus chlorhexidine concentration in the serum or urine standards was calculated by least-squares linear regression analysis, and the concentration of chlorhexidine in the samples was estimated by the inverse prediction technique [9].

RESULTS AND DISCUSSION

Method development

A solid-phase extraction technique, using a C_{18} Sep-Pak cartridge to clean-up

samples for the determination of chlorhexidine in human urine, has been reported [8]. However, this approach requires a large sample size (10 ml) for sample preparation; hence it is not suitable for the analysis of chlorhexidine in serum. We investigated liquid–liquid extraction using diethyl ether, chloroform, chloroform with 5% 2-propanol, methylene chloride, and ethylene dichloride as extraction solvents. It was found that diethyl ether, and also chloroform with 5% 2-propanol, gave the highest extraction efficiency; however, the extraction with chloroform containing 5% 2-propanol gave cleaner blanks. Therefore, chloroform containing 5% 2-propanol was chosen as the extraction solvent. Further sample clean-up/sample concentration was achieved with the back-extraction with 0.05 *M* sulfuric acid.

Chlorhexidine is an amine compound. In a simple reversed-phase HPLC system, it is retained poorly; hence, the ion-pairing approach [7] was used. The best peak shape, and reasonable retention time, for chlorhexidine and *p*-chloroaniline was observed using methanol–water (70:30, v/v) containing 0.005 *M* sodium heptanesulfonate. The following compounds were tested to select an internal standard for the assay: diphenhydramine, phenacetin, pheniramine, prednisone, hydroquinidine, and chlorpheniramine. Chlorpheniramine, hydroquinidine, and diphenhydramine gave the desired retention time and optimum peak shape. Chlorpheniramine was selected as an internal standard due to its high extraction recovery (~100%).

Method validation

Typical chromatograms of extracted serum and urine blanks and spiked samples are shown in Figs. 1 and 2, respectively. The retention times of *p*-

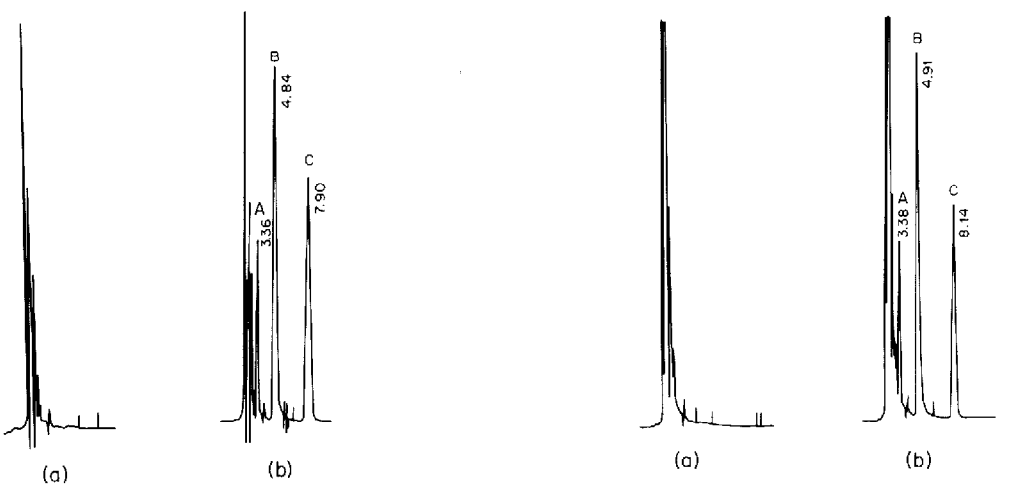

Fig. 1. (a) Typical chromatogram of drug-free human serum (0.5 ml). (b) Typical chromatogram of human serum (0.5 ml) containing 2 μg/ml *p*-chloroaniline, 1 μg/ml chlorpheniramine, and 808 ng/ml chlorhexidine. Peaks: A = *p*-chloroaniline; B = chlorpheniramine; C = chlorhexidine.

Fig. 2. (a) Typical chromatogram of drug-free human urine (0.5 ml). (b) Typical chromatogram of human urine (0.5 ml) containing 2 μg/ml *p*-chloroaniline, 1 μg/ml chlorpheniramine, and 808 ng/ml chlorhexidine. Peaks: A = *p*-chloroaniline; B = chlorpheniramine; C = chlorhexidine.

chloroaniline, chlorpheniramine (internal standard) and chlorhexidine are 3.4, 4.9 and 8.0 min, respectively. No interfering peaks were present in chromatograms of blanks at the retention time for chlorhexidine, *p*-chloroaniline or the internal standard. Pre-dose serum or urine samples from burn patients also showed no interferences. No interferences were observed from direct injections of benzyl alcohol, benzoic acid or phosphanilic acid.

The mean detector responses for the serum and urine samples spiked with 20 ng/ml chlorhexidine were found to be significantly greater than the corresponding blank samples by analysis ($p < 0.005$). Therefore, the 20 ng/ml concentration was chosen as the detection limit of the method. This high sensitivity could not have been achieved without silanized glassware. Silanization of glassware was also required to prevent adsorption of chlorhexidine onto glass in order to obtain a linear detector response at low concentrations of chlorhexidine.

Adsorption of chlorhexidine onto glassware has been reported to be significant [10]. Our studies confirmed this. We also found that the adsorption of chlorhexidine onto glassware was pH-dependent; the higher the pH, the more significant is the chlorhexidine adsorption. Chlorhexidine did not adsorb to polypropylene; however, some adsorption was observed with polystyrene.

The assay was found to be linear in the range of 20.2–808 ng/ml chlorhexidine in human serum and urine. Linear regression analysis of the data of

TABLE I

DAY-TO-DAY REPRODUCIBILITY FOR CHLORHEXIDINE IN HUMAN SERUM

Test date	Concentration spiked (ng/ml)	Concentration found (ng/ml)	Mean concentration found (ng/ml)	Recovery (%)
Day 1	80.8	83.4	81.6	101.0
		79.8		
Day 2	80.8	80.8	82.0	101.5
		83.1		
Day 3	80.8	75.4	80.1	99.1
		84.8		
Day 4	80.8	74.9	77.6	96.0
		80.4		
Mean				99.4
R.S.D. (%)				4.5
Day 1	404	425.0	410.4	101.6
		395.7		
Day 2	404	380.7	396.25	98.1
		411.8		
Day 3	404	388.8	405.2	100.3
		421.7		
Day 4	404	398.3	409.2	100.3
		420.2		
Mean				100.3
R.S.D. (%)				4.1

standard curves obtained on various days showed correlation coefficients of 0.998 or greater for serum standards and 0.994 or greater for urine standards. The mean (± S.D.) slopes for serum (n = 4) and urine (n = 6) standard curves were (6.18 ± 0.47) · 10^{-4} and (5.59 ± 0.54) · 10^{-4}, respectively. The y-intercepts corresponded roughly to 3 ng/ml chlorhexidine.

Frozen stability data of human serum and urine at −20°C in silanized culture tubes showed that chlorhexidine was stable for 40 days in serum, whereas the compound was stable for only 13 days in urine. The difference in stability may be due to a pH effect of the matrix.

The slope for the non-extracted chlorhexidine standard curve was 12.0. The slopes for extracted serum and urine chlorhexidine standard curves were 9.57 and 9.75, respectively. Therefore, based upon the slope ratio, the extraction recovery of chlorhexidine from serum is 80% and the extraction recovery of chlorhexidine from urine is 81%.

The within-day precision values (R.S.D.) for serum samples were 7.2 and 3.5% for the 96.2 and 808 ng/ml samples, respectively. Recovery values were 91.9 and 100.8% for the 96.2 and 808 ng/ml serum samples, respectively. The precision value for urine samples was 8.1% for both the 117 and 808 ng/ml samples. Recovery values were 91.1 and 93.0% for the 117 and 808 ng/ml urine samples, respectively.

Day-to-day precision and accuracy are reported in Tables I and II. For serum

TABLE II

DAY-TO-DAY REPRODUCIBILITY FOR CHLORHEXIDINE IN HUMAN URINE

Test date	Concentration spiked (ng/ml)	Concentration found (ng/ml)	Mean concentration found (ng/ml)	Recovery (%)
Day 1	80.8	66.2	69.0	85.4
		71.8		
Day 2	80.8	77.1	80.0	99.0
		82.8		
Day 3	80.8	77.0	85.9	106.3
		94.8		
Day 4	80.8	78.9	77.2	95.6
		75.6		
Mean				96.6
R.S.D. (%)				10.7
Day 1	404	421.9	406.0	100.5
		390.0		
Day 2	404	414.5	404.3	100.1
		394.1		
Day 3	404	390.7	382.9	94.8
		375.1		
Day 4	404	405.1	397.8	98.4
		390.4		
Mean				98.4
R.S.D. (%)				3.8

samples, precision was within 5% for the 80.8 and 404 ng/ml samples. Recovery values were 99.4 and 100.3% for the 80.8 and 404 ng/ml samples, respectively. For urine samples, precision values were 10.7 and 3.8% at concentrations of 80.8 and 404 ng/ml, respectively. Recovery values were 96.6 and 98.4% for the 80.8 and 404 ng/ml samples, respectively. These data show that the method is reproducible, precise and accurate.

Determination of chlorhexidine in urine and serum

The HPLC procedure was applied to the analysis of chlorhexidine in serum and urine samples from nine burn patients treated topically with a chlorhexidine-containing burn cream. In serum, chlorhexidine was detected only in two patients. The highest concentration detected was 128 ng/ml. This patient happened to be the one treated with the highest dose of burn cream (966 g). The other concentration detected was 20.5 ng/ml. Chlorhexidine was also detected in the urine of three patients. The highest concentration found in the urine was 646 ng/ml and the lowest concentration was 22.6 ng/ml. Sample chromatograms of burn patient's serum and urine samples are shown in Fig. 3. Due to the cross-over design of the study, the estimation of possible total penetration of chlorhexidine could not be obtained. It was reported that the permeation of chlorhexidine through intact human skin is negligible [1]. Nevertheless, in this study, we observed that chlorhexidine showed some degree of penetration through the burn wound. This might have been expected, since in the burn-damaged skin, the stratum corneum, which controls the transport of a polar permeant such as chlorhexidine, is not intact.

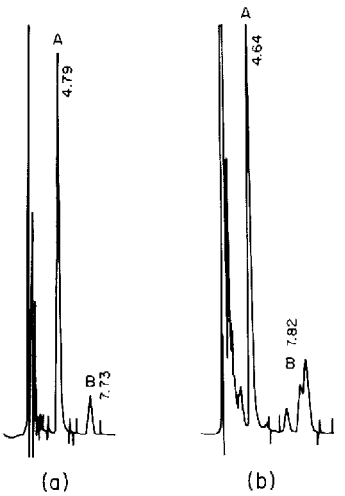

Fig. 3. (a) Chromatogram of a burn patient's serum sample (0.5 ml). (b) Chromatogram of a burn patient's urine sample (0.5 ml). Peaks: A = chlorpheniramine; B = chlorhexidine.

Analysis of p-chloroaniline

In this procedure, *p*-chloroaniline was also extracted; however, recovery was very low (20%). Because of this low recovery, analysis of *p*-chloroaniline was not as precise as was desired, and therefore, quantitative analysis was not attempted. The chromatograms of the burn patients' samples, however,

indicated the presence of *p*-chloroaniline in the urine of two patients in the concentration range 150—800 ng/ml.

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