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Short Communication

Sensitive high-performance liquid chromatographic assay for the determination of chlorhexidine in saliva☆

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ABSTRACT

A high-performance liquid chromatographic assay was developed to determine salivary chlorhexidine concentration. Saliva sample (200 μl) was extracted into methylene chloride. Chromatographic separation was achieved with a C_{18} column using a mobile phase of acetonitrile–0.05 *M* sodium acetate and 0.005 *M* heptanesulfonic acid (40:60, v/v). The standard curve was linear from 0.051 to 20.48 μg/ml ($r^2 \geq 0.997$). Intra-day and inter-day coefficients of variation were < 5.5 and $\leq 9\%$, respectively. The assay is rapid, sensitive, simple, and successfully used for quantitating salivary chlorhexidine content released from a chlorhexidine-impregnated resin worn by patients.

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INTRODUCTION

Chlorhexidine, 1,1′-hexamethylene-bis-5-(4-chlorophenyl)biguanidide, is a common antibacterial agent used in clinical dentistry for many years, primarily for prevention of dental plaque formation and treatment of gingivitis resulting from the plaque [1–3]. Its ability to eliminate oral pharyngeal flora also finds application in treatment of stomatitis [4] and recurrent aphthous ulcers [5], and prevention of mucositis [6]. In addition, it is also widely used as a general antiseptic agent for disinfection of hands and operation sites in hospital practice [7] and in the treatment of burns [8].

Several investigators have reported the potentials of acrylics for drug delivery in the mouth [9–11]. Since the plaque inhibition property of chlorhexidine is most likely due to its slow release from a depot in the oral cavity [12], we were interested in the salivary content of chlorhexidine released from an orthodontic acrylic resin worn by patients, and the usefulness of the resin in oral health maintenance. Therefore, a sensitive high-performance liquid chromatographic (HPLC) assay capable of determining low salivary concentrations of chlorhexidine released from the use of the resin is required. There are three reversed-phase HPLC methods reported in the literature for the determination of chlorhexidine in serum and urine [13–15], but none is published for determining salivary concentrations using a small sample size. Jensen and Christensen [16] reported a spectrophotometric method for determining chlorhexidine in saliva. However, the lower limit of the assay is 5 μg/ml and 1 ml of saliva sample is required. In addition, the spectrophotometric assay involves a two-step extraction procedure.

We report here a HPLC assay with ultraviolet detection for determining chlorhexidine in saliva. The assay is extremely rapid, sensitive, simple, and requires only 200 μl of saliva sample. The assay was used to determine the salivary chlorhexidine concentration in patients who have worn an orthodontic acrylic appliance containing 9 mg chlorhexidine.

EXPERIMENTAL

Reagents, chemicals and solvents

Chlorhexidine diacetate salt and heptanesulfonic acid were purchased from Sigma (St. Louis, MO, USA). Analytical-grade potassium hydroxide and HPLC-grade acetonitrile were purchased from Mallinckrodt (Paris KY, USA). HPLC-grade methylene chloride was obtained from Baxter (Muskegon, MI, USA). Analytical-grade sodium acetate was obtained from Baker (Phillipsburg, NJ, USA). ACS-grade glacial acetic acid was purchased form EM Science (Gibbstown, NJ, USA). Deionized water suitable for HPLC work was produced by a Milli-Q UF system (Millipore, Bedford, MA, USA).

Chromatographic conditions

The HPLC system consisted of a Model 510 pump, a WISP 710 autosampler, a Model 484 variable-wavelength ultraviolet detector set at 260 nm and a Model 745 data module integrator (all Waters Assoc., Milford, MA, USA). A 15 cm × 4.6 mm I.D. Beckman Ultrasphere ODS C_{18} column (5 μm) was used for the analysis. Chromatographic separation was carried out at room temperature. The mobile phase consisted of acetonitrile–0.05 *M* sodium acetate buffer and 0.005 *M* heptanesulfonic acid (40:60, v/v), adjusted to pH 5 with glacial acetic acid. The mobile phase was degassed by vacuum filtration and helium sparging. The flow-rate of the mobile phase was 1.0 ml/min with a back-pressure of 50 bar.

Standard and sample preparation

A chlorhexidine solution (5.12 μg/ml) was prepared in methanol and used as the stock for preparing standards. This chlorhexidine stock solution was stored at −20°C. To prepare the seven salivary standards of chlorhexidine (concentrations ranging from 0.05 to 20.5 μg/ml), different volumes (2–800 μl) of the stock solution were pipetted into screw-cap polypropylene tubes (Becton Dickinson, Lincoln Park, NJ, USA) and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with 200 μl of drug-free saliva obtained from healthy vol-

unteers. The chlorhexidine standards were freshly prepared before each analysis.

Saliva samples from subjects who have worn the chlorhexidine-impregnated acrylic resin were stored at −20°C until analysis. On the day of analysis the saliva samples were thawed and centrifuged at 200 *g* before a 200-μl aliquot of the supernatant was pipetted into a screw-cap polypropylene tube. To each salivary standard and sample, 200 μl of 5 *M* KOH were added followed by vortex-mixring for 30 s. Methylene chloride (2 ml) was then added and the mixture shaken vigorously on a horizontal shaker for 10 min. The aqueous and organic phases were then separated by centrifugation at 200 *g* for 10 min. The aqueous phase was removed by aspiration and the organic phase evaporated under a gentle stream of nitrogen. The residue was reconstituted with 100 μl of mobile phase. A 60-μl aliquot was injected into the HPLC system.

Validation procedures

Control samples of chlorhexidine were made up in drug-free saliva and stored at −20°C. Intra-day precision was established by performing four replicate analyses of a low control and high control sample of known concentrations on the same day. The same low control and high control samples were also analyzed on four different days to evaluate inter-day precision. Intra-day and inter-day coefficients of variation (C.V.) were then determined. Extraction efficiency of the assay was determined by comparing the area response of extracted chlorhexidine samples containing specific concentrations (2 and 20 μg/ml) to the area response obtained with unextracted chlorhexidine samples containing identical concentrations in methanol. In addition, the slope of a calibration curve of extracted chlorhexidine standards was also compared to the slope of a calibration curve of unextracted chlorhexidine standards in methanol.

Calculations

Calibration plot (chlorhexidine peak area *versus* chlorhexidine concentration) was constructed using the salivary standards (0.05–20 μg/ml) prepared as described above. Linearity of the assay was determined by linear regression analysis. Detection limit of the assay was established by comparing the peak area obtained with the lowest standard to that obtained with an extracted saliva blank.

RESULTS AND DISCUSSION

Jensen and Christensen [16] reported that after rinsing the mouth with 10 ml of a 0.2% chlorhexidine digluconate solution, the mean chlorhexidine concentration in saliva based upon a first-order elimination from the oral cavity was 3 and 0.78 μg/ml at 6 and 8 h after the mouthwash, respectively. In our evaluation of the usefulness of the orthodontic acrylic resin impregnated with 9 mg of chlorhexidine, subjects who have worn the acrylic resin overnight were instructed to collect their saliva samples when they woke up in the morning. With this study design, the concentration of chlorhexidine in the saliva is expected to be very low. The aim of the present study was to develop a simple and sensitive HPLC assay to quantify salivary concentrations of chlorhexidine.

Chlorhexidine is a dicationic compound with pK_a values of 10.3 and 2.2. Therefore, at the general operating pH range of 2–7 with reversed-phase chromatography, it would be primarily ionized and retained poorly by the non-polar stationary phase. However, addition of an ion-pairing agent (heptanesulfonic acid) increases the retention of chlorhexidine on the analytical column. Initially, methanol was used as the organic solvent for the mobile phase. Significant tailing of the chlorhexidine peak was observed with 60–70% of methanol concentration. Therefore, acetonitrile was used instead which eliminated the tailing problem of the chlorhexidine peak. The optimized chromatographic condition of acetonitrile–0.05 *M* sodium acetate buffer and 0.005 *M* heptanesulfonic acid (40:60, v/v), adjusted to pH 5 with glacial acetic was found to provide the best peak shape, a short retention time and no endogenous interference. Examples of typical chromatograms of extracted saliva blank, patient's

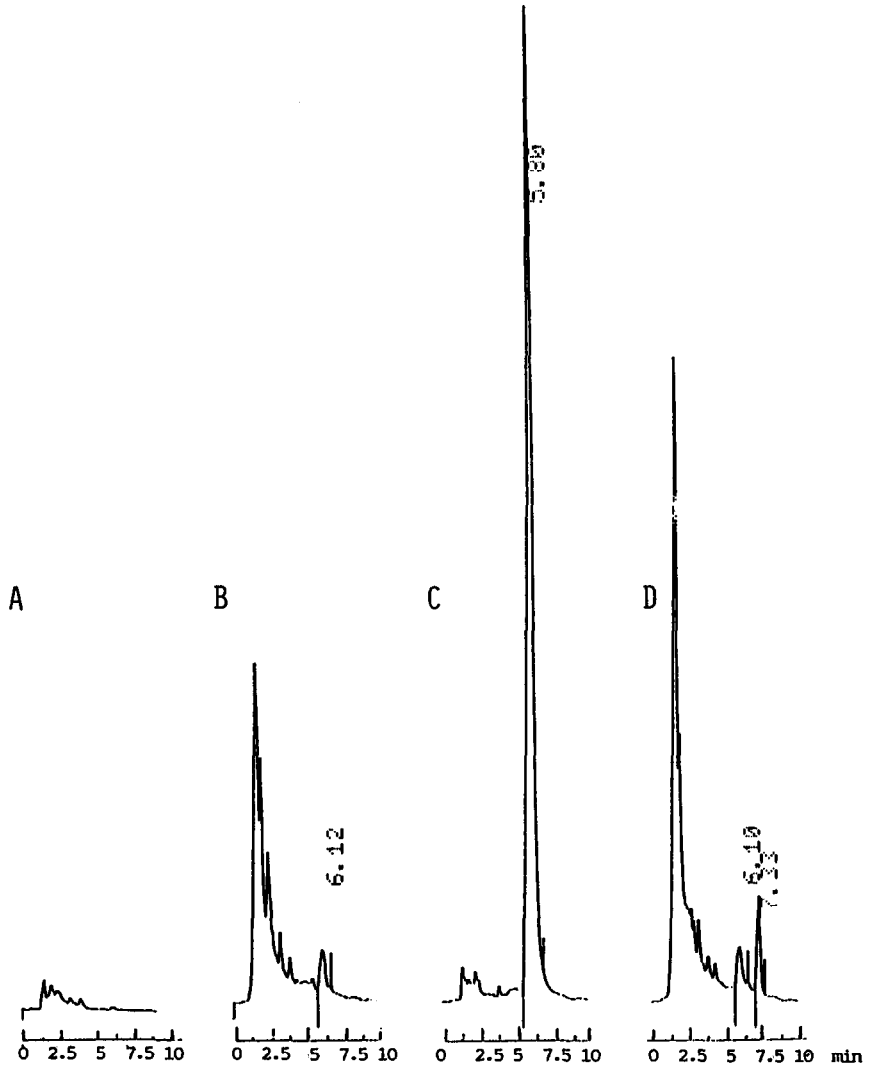

Fig. 1. Representative chromatograms of chlorhexidine. (A) Pooled drug-free blank saliva used to make up chlorhexidine standards; (B) pooled drug-free blank saliva spiked with 0.26 μg/ml chlorhexidine; (C) pooled drug-free blank saliva spiked with 2.05 μg/ml chlorhexidine; (D) saliva from a patient who have worn an orthodontic appliance impregnated with 0.009 g of chlorhexidine for 8 h prior to saliva collection. The peak at 7.33 min is an endogenous substance present in that patient. Sensitivity, 1.00 a.u.f.s. into 1 V f.s.d., detection 260 nm.

sample, and spiked saliva sample are shown in Fig. 1. The retention time for chlorhexidine is 5.8–6.1 min. No interfering peak was present in chromatograms of blank saliva. Possible interferences from drugs that are likely used in dental practice were evaluated. Solutions of erythromycin, lidocaine (xylocaine), and tetracycline were directly injected into the HPLC system. There was no interference from any of these compounds.

In the method development process, different extraction solvents including methylene chloride, chloroform, chloroform with 5% 2-propranol, diethyl ether, hexane, hexane with 5% butanol, and ethyl acetate were evaluated. The use of methylene chloride resulted in good recovery of

TABLE I

INTRA-DAY AND INTER-DAY ASSAY VARIABILITY FOR THE DETERMINATION OF CHLORHEXIDINE IN SALIVA

Control 1		Control 2	
Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)
Intra-day variability			
0.22 ± 0.01	4.74	9.08 ± 0.50	5.48
Inter-day variability			
0.28 ± 0.03	9.00	9.45 ± 0.57	6.02

chlorhexidine and cleaner chromatograms. Based upon the area of two chlorhexidine samples containing 2 and 20 μg/ml, the recovery was 93.2 and 96.2%, respectively. Based upon the slope of calibration curves of unextracted *versus* extracted chlorhexidine, the recovery was 96.8%. This extraction efficiency is comparable to that reported in the literature [14–16]. However, unlike other reports [15,16], sample extraction with methylene chloride does not require a back-extraction step with acid for further sample clean-up. Finally, the sample size used (200 μl) is small compared to other extraction methods (up to 10 ml) reported in the literature [13,14].

An internal standard is usually desirable in the analysis of biological samples as it compensates for possible loss of analyte during the extraction procedure. Different internal standards, including diphenhydramine, chlorpheniramine, cyheptamide, and ondansetron, were evaluated. None of these compounds resolved well from the chlorhexidine or produced a good peak shape with our chromatographic conditions. Quantitication of chlorhexidine was thus achieved with the use of a calibration curve. The peak-area response was linear over the concentration range of 0.05–20 μg/ml ($r^2 \geq 0.997$). The same linearity was also achieved with peak-height assessment ($r^2 \geq 0.999$). The variability of the slope of the calibration curve ($n = 6$) over a ten-day period of time was 7.2%. The intra-day C.V. for the assay for low and high control samples were 4.7 and 5.5%, respectively. The inter-day C.V. were 9.0 and 6.0%, respectively (Table I). The mean absolute difference between the theoretical and actual concentrations for the low and high control samples ranged from 0.8 to 12% for the intra-day and inter-day evaluations. Other investigators had also reported the validity of using external calibration for quantitation of chlorhexidine [13,14]. The peak-area response for a saliva sample spiked with 50 ng/ml chlorhexidine was at least two times that for blank saliva sample. Therefore, the detection limit of the assay was 50 ng/ml, which is a significant improvement compared to spectrophotometric detection [16].

Jensen and Christensen [16] have evaluated the stability of chlorhexidine in saliva. They reported that there was no loss of chlorhexidine over a three-day period at either 2°C or room temperature (21°C). Our control samples were prepared in batch and stored at −20°C. We have analyzed the control samples over a two-month period and found no loss of chlorhexidine, which suggest that chlorhexidine is stable in saliva at −20°C for at least two months. Other investigators have reported that chlorhexidine was also stable in other biological fluids such as urine and serum at −20°C for up to forty days [15].

The method described is developed specifically for determination of chlorhexidine in saliva. The assay is reproducible, can be performed without an internal standard, and can detect a concentration as low as 0.05 μg/ml with only 200 μl of sample. Ohter advantages include a short retention time, a short run time, simple sample preparation, and simple extraction procedure with good recovery of chlorhexidine. The developed assay has been sucessfully used for quantitating salivary chlorhexidine concentration as low as 0.21 μg/ml released from the orthodontic appliance.

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