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Short communication

Determination of chlorhexidine in saliva using high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic method for the determination of chlorhexidine in saliva is reported. The method developed includes a simple and short sample preparation with a one-step extraction procedure and a short total chromatographic run time of 5 min. In a preliminary pharmacokinetic study with a healthy volunteer the chlorhexidine concentration found in saliva after 12 h was 0.8 μg/ml.

1. Introduction

Chlorhexidine is regarded as the most effective antiplaque agent in dentistry [1]. Its effect is obviously based on an instant bacteriocidic effect and a prolonged bacteriostatic effect when it is slowly released from mouth cavities [2]. The minimum inhibitory concentration (MIC) of chlorhexidine against most oral streptococci was found to be about 2 μg/ml [3]. The amount of chlorhexidine in saliva has thus interested researchers. The first method to determine the chlorhexidine concentration in saliva was published by Jensen and Christensen in 1971 [4]. The two-step method based on UV-spectrophotometry was, however, rather laborious. The determination was also disturbed by endogenous components from the saliva matrix. Thereafter a fluorimetric method [5] and a method based on the use of radiolabelled ^{14}C [2] have been published. Using the latter method Bonesvoll et al. [2] were able to detect chlorhexidine in saliva 24 h after administration. They were also able to use a non-centrifuged, i.e. whole, saliva sample. In contrast, the reported fluorimetric method [5] did not enable analysis of whole saliva samples. Recently, two different liquid chromatographic (HPLC) methods have been reported [6,7]. The first [6] enabled analysis of whole saliva samples but included a time-consuming evaporation of the organic phase and reconstitution of the dried sample before injection, and thus was not very well suited for routine determination of a large number of samples. The second method [7] enabled both the analysis of very small saliva samples and the use of an internal standard. However, the need for a dual-wavelength UV absorbance detector and an isothermal column temperature (55°C) together with the quantitation limit of 2 μg/ml can be regarded as dis-

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advantageous. The present paper describes a rapid and simple HPLC method designed for the routine determination of chlorhexidine in large numbers of whole saliva samples.

2. Experimental

2.1. Reagents

Chlorhexidine diacetate p.a. was purchased from Sigma (St. Louis, MO, USA). The chlorhexidine diacetate used in the preparation of the tablet for the pharmacokinetic study was pharmacopoeial grade (BP).

Reagents used in the extraction procedure and in liquid chromatography were phosphoric acid, sodium hydroxide, disodium hydrogen phosphate dihydrate p.a., acetonitrile, LiChrosolv (E. Merck, Darmstadt, Germany), 1-heptanesulfonic acid (Sigma), triethylamine p.a. (Fluka Chemie, Buchs, Switzerland), methanol (HPLC-grade, Mallincrodt, Paris, KY, USA) and distilled water. All liquids used in liquid chromatography were filtered and degassed before use.

2.2. Chromatography

For the HPLC assay the following instruments were used: a Hewlett-Packard 1050 Series quaternary pump and variable-wavelength detector operating at 260 nm (Hewlett-Packard, Waldbronn, Germany). The standards and samples were injected through a 20-μl injection loop. The peak areas determined with an 3396A integrator (Hewlett-Packard, Avondale, PA, USA) were used for quantitation.

Reversed-phase HPLC was performed at ambient temperature. The column used was a LiChrospher 100 RP-18 (125 × 4 mm I.D.) from Hewlett-Packard (Avondale, PA, USA) with an average particle diameter of 5 μm. The mobile phase was acetonitrile–buffer (0.1 *M* disodium hydrogen phosphate, 0.005 *M* 1-heptanesulfonic acid and 0.05 *M* triethyleneamine) (35:65, v/v). The pH of the buffer component was adjusted to 2.5 with phosphoric acid.

2.3. Extraction procedure

A non-centrifuged 200-μl sample of clear saliva was introduced into a test tube and 400 μl of 4.5 *M* NaOH and 400 μl of acetonitrile were added. The tube was vortex-mixed for 1 min and centrifuged for 1 min at 14 000 *g*, after which 200 μl of the organic phase was transferred into a new test tube and mixed with 370 μl of the buffer component of the mobile phase. A 20-μl aliquot was injected onto HPLC-system.

2.4. Calibration curve

Calibration curves for chlorhexidine diacetate in the range of 0.5–100 μg/ml were prepared in four replicates using non-centrifuged, i.e. whole, drug-free saliva. Calibration curves were generated by least-squares regression of chlorhexidine peak area vs. the chlorhexidine concentration. For the preparation of standards various volumes of the working standard (10 μg/ml of chlorhexidine diacetate salt in methanol) were pipetted into test tubes. No chlorhexidine was added into the blank sample tube. Methanol was evaporated under a gentle stream of air and dried residues were reconstituted in 200 μl of saliva to generate eight different concentrations ranging from 0.5 to 100 μg/ml. Standards were extracted in the same manner as the samples.

2.5. Recovery

The recovery of chlorhexidine from saliva was determined in four replicates at three different concentrations within the range of the calibration curve (1, 10 and 50 μg/ml). Saliva samples spiked with known amounts of chlorhexidine were extracted in the same manner as the samples. Recovery was assessed by comparing the peak area with the peak area resulting from direct injection of the chlorhexidine standard.

2.6. Precision and accuracy

Intra-day precision was assessed from four replicate analyses of spiked saliva on the same day. Inter-day precision was evaluated from the

slopes of the calibration curves that were analyzed on three different occasions.

Drug-free saliva was spiked with chlorhexidine to yield three concentrations (1, 10 and 50 μg/ml). Samples were assayed and concentrations were derived from the calibration curve. The accuracy was evaluated by comparing the estimated concentration with the known concentration of chlorhexidine.

2.7. Pharmacokinetic study

A male volunteer with no ongoing medication chewed an experimental tablet preparation containing 10 μg of chlorhexidine diacetate [8] and flushed his mouth cavity with the suspension formed for one minute. After that, the suspension was spilled out. Saliva samples were collected 1, 15, 60, 120, 240, 480, 720 and 1440 min after flushing. A blank sample was taken before tablet administration. During the first 8 h no drinking (except water), eating or smoking were allowed.

3. Results and discussion

3.1. Calibration curve

The calibration curve was linear in the range of 0.5–100 μg/ml of chlorhexidine diacetate (coefficient of correlation >0.9998).

The limit of quantitation of the present HPLC method was 0.5 μg/ml. This is clearly lower than the chlorhexidine concentration required to inhibit the growth of streptococcal strains present in the mouth cavity [3]. A lower limit of quantitation could be achieved by evaporation of the organic phase and reconstitution of the dried residue before injection. This is, however, laborious. Besides, a lower limit of quantitation is not required for routine determination of chlorhexidine in saliva samples, e.g. in the in vivo studies.

De Vries and Arends [5] showed that interindividual variation in saliva composition disturbed the chlorhexidine determination when plain UV spectrophotometry is used. In the present study the observed endogenous peaks of saliva always eluted before the chlorhexidine peak and did not disturb the quantitation of chlorhexidine (Fig. 1).

3.2. Recovery

The recovery of chlorhexidine was 95.2%, 99.9% and 103.0% at concentrations of 1, 10 and 50 μg/ml, respectively. Chlorhexidine recovery was quite similar to that observed by Lam et al. [6] for saliva samples but greater than that observed by Huston et al. [9] for urine and blood samples. Jensen and Christensen [4], using chloroform as an extraction solvent and an external standard, found a slightly over 100 percent recovery for chlorhexidine. They deduced that the efficient recovery resulted from the slope of the standard curve.

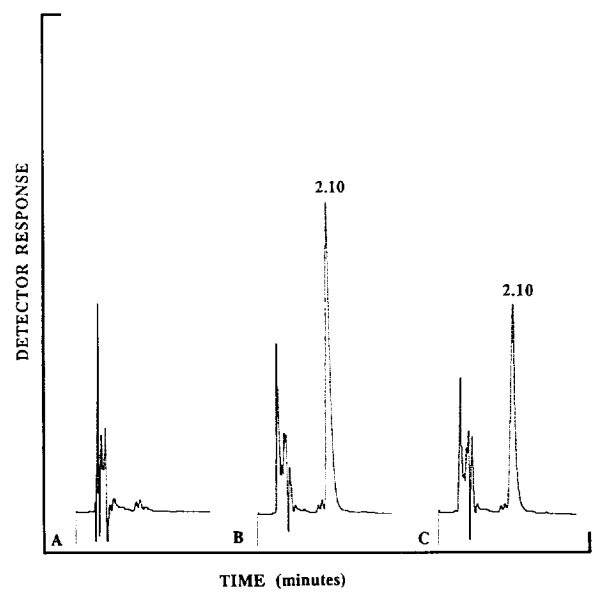

Fig. 1. Chromatograms of blank saliva sample (A), saliva sample spiked with 5 μg/ml of chlorhexidine (B), and saliva sample of the pharmacokinetic study, taken 7 h after administration (C). 2.10 = chlorhexidine peak. Sensitivity is 0.25 mV full scale.

3.3. Precision and accuracy

The coefficient of variation for the intra-day precision over the concentration range 0.5–100 μg/ml was 1.7–5.8%. The inter-day precision calculated from the slopes of the standard curves on three different days was 1.8%. Thus the developed method is precise with respect to both intra- and inter-day assays. The bias based on the accuracy measurement at concentrations of 1, 10 and 50 μg/ml was 17.2%, 6.3% and 1.1%, respectively. The bias was greatest at the concentration of 1 μg/ml. However, the significant concentration range is above the MIC-value for chlorhexidine diacetate salt (3 μg/ml) against oral streptococci [3].

3.4. Pharmacokinetic study

The chlorhexidine diacetate concentration measured 24 h after administration, i.e. 3.2 μg/ml, agreed with the results obtained by the radiolabelling method [2], where the chlorhexidine concentrations for a low- and a high-retention person were found to be 1 μg/ml and 5 μg/ml, respectively.

The elimination of chlorhexidine from saliva, i.e. from the mouth cavity, followed approximately first order kinetics (Fig. 2). The required chlorhexidine gluconate concentration in saliva for the inhibitory effect against oral streptococcal strains is ca. 4 μg/ml (corresponding to about 2 μg/ml of chlorhexidine base and about 3 μg/ml of diacetate salt) [3]. According to Fig. 2 this concentration was maintained in saliva up to about 10 h. The measured chlorhexidine diacetate concentration after 24 h was clearly greater than that after 12 h. The 24-h sample was collected immediately after waking up in the morning. The higher concentration in the 24-h sample is obviously due to the smaller salivary secretion during the night (Fig. 2).

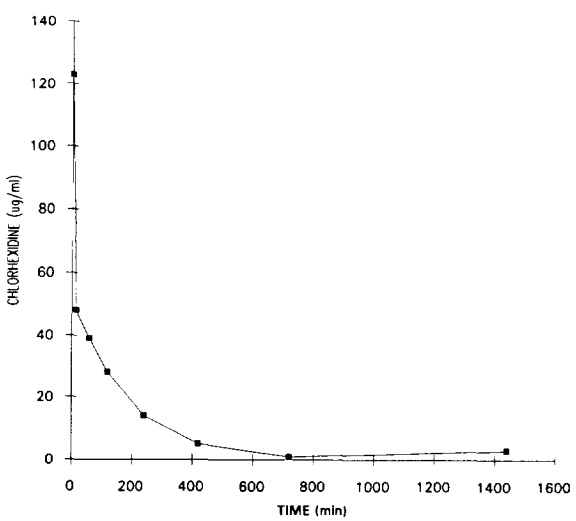

Fig. 2. Chlorhexidine concentration (μg/ml) as a function of time after administration of a tablet preparation containing 10 mg of chlorhexidine diacetate.

4. Conclusions

The reported method for the determination of chlorhexidine from saliva samples includes a rapid and simple sample preparation and a short chromatographic run time. Endogenous components of saliva elute separately and do not disturb the quantitation of chlorhexidine. Owing to the rapid procedure and suitable limit of quantitation the developed method is useful for routine in vivo studies with a large number of saliva samples.

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