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Assay of stability, free and total concentration of chlorhexidine in saliva by solid phase microextraction

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Abstract

This paper presents the development and application of a solid phase microextraction method for the full investigation of chlorhexidine interaction with saliva during a pharmacokinetic study: chemical stability, binding to proteins, free concentration, total concentration and kinetics of elimination after oral administration. Only 0.1 mL sample were needed for each time point and the concentration of salivary proteins was determined as well. It was shown that chlorhexidine remained stable in the oral cavity for at least 9 h and high concentrations of the drug (2 μg/mL total) were still present even 8 h after mouthrinsing. Supplementary facts were uncovered: while the total concentration followed first-order elimination kinetics, the free concentration remained almost constant for several hours; this showed that the oral cavity acted like a reservoir that slowly released the drug. It was also revealed that following oral administration of chlorhexidine, the normal composition of saliva changed for a few hours, probably as a physiological response to the bitter taste of the medicine. The method had a wide linear range (0.1–40 μg/mL free chlorhexidine) that was perfectly suitable for the study of chlorhexidine retention in the oral cavity. Separation and quantitation were achieved by liquid chromatography coupled to mass spectrometry; no interference from endogenous compounds was observed. This selective and sensitive solid-phase microextraction (SPME) approach for monitoring the free and total concentration of a drug, as well as the concentration of proteins that bind that drug, should prove to be more useful for pharmacokinetic studies than classic methods that only provide the total concentration as a final result.

Keywords: Solid-phase microextraction; Free concentration; Total concentration; Saliva; Binding constant; Chlorhexidine

1. Introduction

Solid-phase microextraction (SPME) is a well-known technique for analysis of volatile and semivolatile substances as a result of simplicity, reliability, flexibility and possibility to eliminate solvent usage during sample preparation. Recently, this sample preparation method has begun to receive more and more attention for applications involving nonvolatile polar compounds from biological samples [1–3].

Biological samples are usually complex mixtures that contain a wide variety of different components. The analysis of this kind of samples frequently requires several sample preparation steps that are necessary in order to increase the concentration of analyte and to reduce interference from other sample components. SPME is a new alternative to classic sample preparation methods that not only provides simplicity but also allows the investigation of supplementary parameters, like the binding constant or the concentration of binding matrix (proteins, humic materials, etc.) in a sample.

Due to its wide spectrum of bactericidal and antiviral activity, chlorhexidine is used to a large extent in various formulations ranging from skin disinfectants to antiplaque or anticariogenic agents, both in human and veterinary medicine. The presence of two symmetrically positioned basic chlorophenyl guanide groups attached to a lipophilic hexamethylene chain aids in rapid absorption through the outer bacterial cell wall, causing irreversible bacterial membrane injury, cytoplasmatic leakage and enzyme inhibition [4].

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The methods that are currently used for analyzing chlorhexidine in saliva involve lengthy and complex sample pre-treatment procedures, especially targeted at eliminating the salivary proteins and they provide as a final result only the total concentration of chlorhexidine. Liquid chromatography is the most widely used method for analysis of chlorhexidine; UV detection around 250 nm is used for quantitative assays [4–6], while for the detection of impurities, mass spectrometry or photodiode array detectors are employed [7,8]. Other methods reported in the literature include fluorometry [9] and direct UV spectroscopy [10]; both of them have several disadvantages: lack of sensitivity, serious interference from compounds naturally present in saliva and inability to compensate for the high variability of saliva composition. Radiolabelled chlorhexidine (^{14}C) was also used for studies on the retention of chlorhexidine in the mouth [11] and no interference from saliva components was observed, but it is not applicable to humans.

This paper presents a full investigation of chlorhexidine interaction with saliva during a pharmacokinetic study: chemical stability, binding to proteins in saliva, free concentration, total concentration and elimination kinetics after oral administration.

2. Theoretical approach for the study of chlorhexidine binding to salivary proteins

Chlorhexidine is well-known for binding to buccal epithelial cells, proteins (mucin and albumin) and teeth (hydroxyapatite), a phenomenon which explains its long period of retention in the mouth. Once released into saliva, chlorhexidine is in equilibrium with the matrix of the saliva, consisting mainly of mucin, albumin and some salts. This interaction is very important because only the free concentration is available for antimicrobial effect. Since the composition of the saliva is not unitary and it continuously changes even in short intervals of time, the free concentration of chlorhexidine may change as well, along with its antibacterial activity.

In order to evaluate the interaction of saliva with chlorhexidine (C), saliva is considered to contain a single component that interacts with chlorhexidine (Fig. 1), namely "*M*" (for a list of all abbreviations, please see Table 1). Every molecule of binding matrix may bind *b* molecules of chlorhexidine:

Table 1
List with abbreviations

Abbreviation	Explanation
C	Chlorhexidine
M	Matrix of saliva
SPME	Solid phase microextraction fiber
S_e	Surface concentration at equilibrium
S_a	Active surface (for adsorption)
K^s_{es}	Partition coefficient between fiber and solution
C_f	Free concentration of chlorhexidine at equilibrium (M)
C_t	Total concentration of chlorhexidine (M)
C_m	Total concentration of binding matrix (M)
s	Concentration of chlorhexidine standard (M), in the same volume as the sample
K	Binding constant between chlorhexidine and binding matrix
f_c	Fiber capacity
V	Final volume of solution (L)
d	Dilution factor
b	Number of binding sites per molecule of binding matrix
n	Amount of chlorhexidine extracted by the SPME fiber (moles)

$$b\mathrm{C} + M \leftrightarrows M(\mathrm{C})_b$$

The binding constant *K* for this equilibrium is described by:

$$K = \frac{[M(\mathrm{C})_b]}{[M][\mathrm{C}]^b} = \frac{(C_t - C_f)/b}{(C_m - (C_t - C_f)/b)C_f^b} \quad (1)$$

where $M(\mathrm{C})_b$ represents the complex of chlorhexidine with the binding matrix. Its concentration is equal to the difference between the total concentration (C_t) and the free concentration of chlorhexidine (C_f), divided by the number of molecules of chlorhexidine per molecule of matrix (*b*), since the final concentration of chlorhexidine after binding to the matrix is the free concentration ([C] = C_f). Accordingly, the concentration of free matrix [*M*] is the difference between the total concentration of matrix (C_m) and the concentration of $M(\mathrm{C})_b$.

A new equilibrium occurs after introducing the SPME fiber into the sample (adsorption of chlorhexidine onto the fiber):

$$\mathrm{SPME} + \mathrm{C} \leftrightarrows \mathrm{SPME(C)}$$

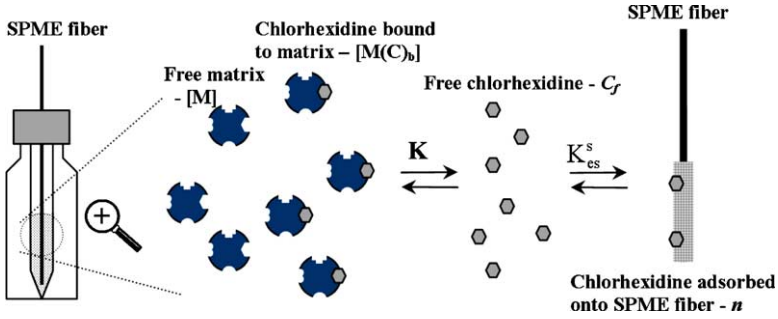


Fig. 1. Schematic representation of experimental setup and equilibrium between chlorhexidine, proteins and SPME fiber.

In the presence of the SPME fiber, an amount n will be extracted from the solution and this amount that is on the fiber will be in equilibrium with the free concentration. Since the extraction phase consists of a solid sorbent, the resulting equilibrium is characterized by K^{s}_{es}, the partition coefficient between fiber and solution [3]:

$$K^{s}_{es} = \frac{S_e}{C_f} \tag{2}$$

where S_e represents the surface concentration at equilibrium. S_e can be expressed as the ratio between the amount extracted, n, and the active surface of the fiber, S_a:

$$S_e = \frac{n}{S_a} \tag{3}$$

By combining Eqs. (2) and (3), the free concentration C_f can be obtained:

$$K^{s}_{es} = \frac{S_e}{C_f} = \frac{n/S_a}{C_f} \Rightarrow C_f = \frac{n}{K^{s}_{es}S_a} \tag{4}$$

Since K^{s}_{es} and S_a are constants for a certain fiber, their product may be replaced by a new constant, f_c (fiber capacity) that can be easily determined experimentally. The free concentration of chlorhexidine at equilibrium may now be obtained from the amount n extracted by the SPME fiber:

$$K^{s}_{es}S_a = f_c \Rightarrow C_f = \frac{n}{f_c} \tag{5}$$

Because the composition of saliva is variable (the content of water fluctuates), the concentration of binding matrix may be different every time when a sample is collected. In order to get accurate results for the free and total concentration of chlorhexidine and determine the concentration of binding matrix at the same time, the standard addition method is required. Since the volume of the sample is small (50 μL), the dilution d produced by standard addition must be considered.

When an amount n is extracted by the fiber from the solution containing chlorhexidine and binding matrix, after dilution to the final volume V (by adding standard solution or water), K may be calculated as:

$$K = \frac{(C_t/d - n/f_c - n/V)/b}{(C_m/d - (C_t/d - n/f_c - n/V)/b)(n/f_c)^b} \tag{6}$$

This equation is obtained by introducing the final concentrations of chlorhexidine and matrix into Eq. (1): after dilution, C_t becomes C_t/d, C_m becomes C_m/d, the free concentration C_f is related to the amount extracted by SPME according to the relation $C_f = n/f_c$ and the final total concentration of chlorhexidine in solution is decreased by the amount n extracted with the SPME fiber.

Eqs. (1) and (6) are subsequently used in the experimental part for the calculation of the binding constant, free and total concentration of chlorhexidine, matrix concentration and the number of binding sites.

3. Experimental

3.1. Chemicals and reagents

Chlorhexidine diacetate, chlorhexidine digluconate and *p*-chloroaniline were purchased from Sigma (Ont., Canada); 98% formic acid was obtained from BDH Inc. (Toronto, Ont., Canada); HPLC grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). SPME fibers (CW-TPR 50 μm, PDMS-DVB 60μm and PA 85 μm for HPLC) were obtained from Supleco (Bellefonte, PA, USA). Deionized water was obtained using a Barnstead/Thermodyne NANO-pure ultrapure water system (Dubuque, IA, USA).

3.2. Apparatus and analytical conditions

LC–MS analyses were performed using an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum solvent degassing unit, a binary high pressure gradient pump, an autosampler, a column thermostat and a variable wavelength UV–vis detector coupled on-line with an Agilent 1100 series MSD single quadrupole instrument with atmospheric pressure electrospray ionization. High purity nitrogen, used as nebulizing and drying gas, was generated by means of a Whatman nitrogen generator (Whatman, Haverhill, MA, USA).

Chromatographic separations were carried out on a Zorbax Eclipse extra-densely bonded 150 mm × 4.6 mm i.d. column packed with 3.5 μm C18 particles (Agilent), guarded by an on-line filter (0.5 μm). Data were collected and analyzed using the CHEMSTATION software from Agilent Technologies.

LC and ESI–MS conditions were as follows: column temperature 25 °C, mobile phase acetonitrile:water (both with 0.01% HCOOH, 15:85, v/v) and linear gradient to 50:50 over 4 (for quantitation) or 60 min (for identification of impurities), flow rate 0.6 mL/min, nebulizer gas N_2 (35 psi), drying gas N_2 (13 L/min, 350 °C), capillary voltage 3500 V, fragmentor voltage 90 V, quadrupole temperature 100 °C, positive ionization mode. For optimization and identification experiments scan mode in the range 120–510 amu was used; for quantification experiments selected ion monitoring (170.1 and 505.2) was used, with a scan time of 0.42 s/cycle and a dwell time of 199 ms. All other parameters of the mass-selective detector were automatically optimized using a calibration standard.

3.3. SPME conditions

The technique for coupling fiber SPME with HPLC was developed previously [12–14]. Briefly, a homemade interface consisting of a Valco zero-volume tee piece with an enlarged thru-hole was used as a desorption chamber for the SPME fiber. Twenty microliters of mobile phase (50:50) were used for desorption.

New fibers were conditioned by exposing them to the mobile phase flow for 30 min or until they were free of contaminants; the fibers were conditioned in mobile phase each day before performing analyses.

Aliquots of 100 μL sample or standard solution were extracted with a SPME fiber from 200 μL polyethylene tubes; the height of the liquid column was 12 mm so that the whole length of the fiber (10 mm) was immersed in solution. After the SPME fiber was inserted into the polyethylene tube, the assembly was placed in a suitable support and swirled on a shaking bed. When equilibrium was reached (15 min), the fiber was introduced into the desorption chamber under ambient pressure. The autosampler was programmed to switch mobile phase flow through the interface either immediately (for dynamic desorption) or 2 min after inserting the fiber (static desorption), allowing for elution of the desorbed compounds. Upon completion of the analysis, in order to minimize carryover, the fiber and the desorption chamber were flushed with 400 μL mobile phase.

3.4. Standard solutions, saliva collection and sample preparation

Stock solutions of chlorhexidine diacetate (1 mg/mL as base) were prepared in water and kept refrigerated at 4 °C; further dilutions were made with water.

For stability studies, stock solutions were exposed to different stress conditions: increased temperature (100 °C for 24 h or 60 °C for 1 week), low pH (10 mL sample mixed with 0.1 or 1 mL concentrated HCl), exposure to light (1 month) and to oxidants (H_2O_2). Before analysis, they were diluted 1:10.

Saliva was obtained with consent from a healthy volunteer that did not receive any medical treatment prior to these experiments; samples were collected as stated by the guidelines for saliva collection. A blank sample was obtained before chlorhexidine administration. The volunteer rinsed his mouth for 1 min with 10 mL solution of chlorhexidine diacetate (1 mg/mL as base) and saliva was collected at the following time points: 0.25, 0.5, 1, 1.5, 2, 4 and 8 h after rinsing (without consuming food or beverages in this period, except limited amounts of water after 2 h). After collection, 50 μL salive were diluted with 50 μL of water, while another 50 μL were mixed with 50 μL standard solution of chlorhexidine (standard addition) in 200 μL polyethylene tubes; accordingly, 0.1 mL saliva were required for each time point. All samples were analyzed immediately after collection.

4. Results and discussion

4.1. Choice of SPME coating

Initial investigations were performed with three different coatings: CW-TPR, PA and PDMS-DVB. Maximum sensitivity was obtained using the CW-TPR coating, which was used for further experiments.

4.2. Dynamic versus static desorption

The choice of dynamic versus static desorption depends on the desorption rate: for fast desorbing analytes dynamic mode should be used, while for slow desorbing analytes better peak shapes are obtained using static desorption. The best results for chlorhexidine and *p*-chloroaniline (the final degradation product) were obtained with 2 min time of static desorption.

4.3. Chromatographic conditions

The mobile phases described so far for the determination of chlorhexidine consist of methanol or acetonitrile mixed with buffer (ammonium acetate pH = 5.0 or 3.6, disodium hydrogen phosphate pH = 2.5 or sodium phosphate pH = 3.0) [4–8]. To the authors' best knowledge, no mobile phase was reported that allows detection of all chlorhexidine impurities or degradation products by LC–MS or LC–UV, because some compounds do not absorb in UV, while others do not ionize efficiently (for example, *p*-chloroaniline). Since it is known that amines tend to form stable salts with acetic acid and ammonium acetate, a mobile phase consisting of acetonitrile and water acidified with formic acid (pH approximately 3.2, with 0.01% HCOOH) was proposed; this mobile phase allowed for sensitive detection of all impurities by electrospray MS.

Chlorhexidine is a dicationic compound with $K_{a1} = 6.3 \times 10^{-3}$ and $K_{a2} = 5.0 \times 10^{-11}$ and is almost completely ionized (1+) at pH = 3.2, allowing sensitive detection by electrospray-MS. Because chlorhexidine and some of its degradation products are strongly basic, they are intensely retained on most silica-based reversed phase columns; the difficulty was overcome by using a doubly end-capped, extra-densely bonded C18 column and an acid mobile phase (pH = 3.2), without the need to use ion-pairing reagents in the mobile phase, as these reagents are not always compatible with electrospray LC–MS. It is known that chlorhexidine is quite stable at this low pH (some HPLC methods use pH = 2.5). In order to check the stability of chlorhexidine impurities at this low pH, a concentrated sample was diluted with mobile phase instead of water and several chromatograms were recorded at 0.5, 1, 1.5 and 16 h after preparation; even after 16 h, no significant change in peak area was observed.

4.4. Extraction time profile for chlorhexidine and p-chloroaniline

While the concentration of the sample analyzed by SPME has no impact on the concentration time profile and the equilibration time, the agitation conditions, coating thickness (especially for liquid coatings), distribution constant and diffusion coefficient of the analyte play a very important role in equilibration time. Sample temperature is important as

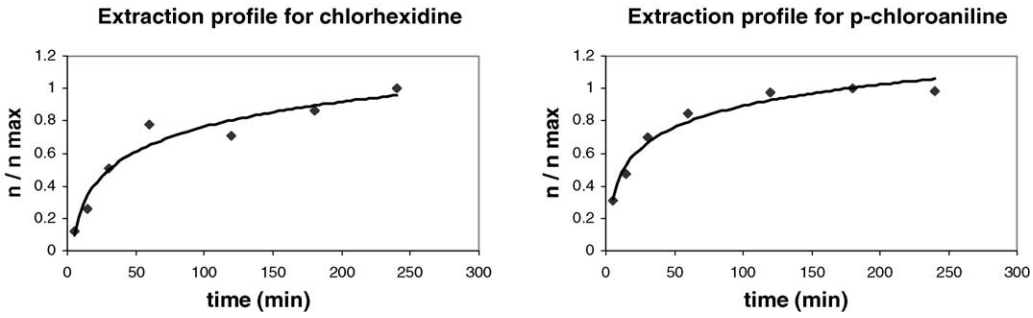


Fig. 2. Extraction time profile for chlorhexidine and *p*-chloroaniline, without agitation.

well, since it has a great influence on the distribution constant and diffusion coefficient. Also, in the case of very small volumes (and less than perfect agitation) the equilibration time should be shorter since the distance that the analyte has to travel through the solution is smaller. It was found that even with small volumes of sample, agitation still plays an important role in reducing the equilibration time, since with non-agitated samples the time necessary to reach equilibrium is more than 4 h (Fig. 2).

Employing 100 μL of sample and vigorous shaking, the equilibration time for chlorhexidine and *p*-chloroaniline is about 15 min (Fig. 3). This extraction time was used in all subsequent experiments. The same equilibration time was observed when extracting chlorhexidine from saliva. As reported before, adsorption of proteins onto the fiber was negligible [15].

4.5. Linearity, precision and limit of detection

For the assay of chlorhexidine stability (extraction from water or buffer), the method proved to be linear in the range 0.05–40 μg/mL, with $r^2 = 0.9945$. In the case of extraction from saliva, the method was linear in the range 0.10–40 μg/mL (expressed as free concentration of chlorhexidine). The limits of detection were 0.01 and 0.02 μg/mL, respectively, while RSDs ranged from 2.3 to 9.6%.

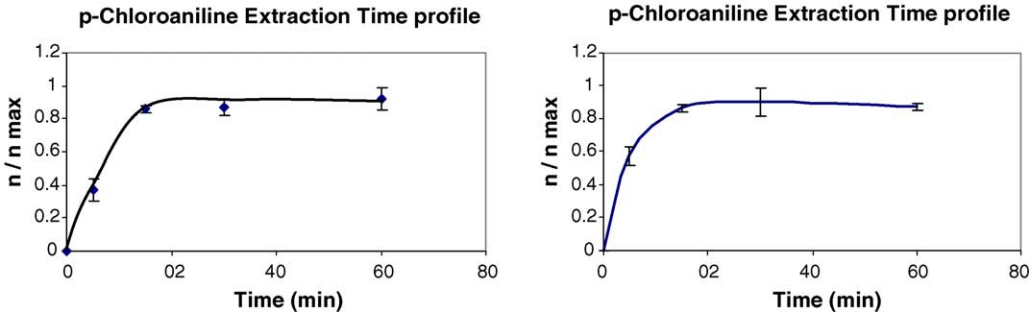


Fig. 3. Extraction time profile for chlorhexidine and *p*-chloroaniline, with vigorous shaking (three replicates).

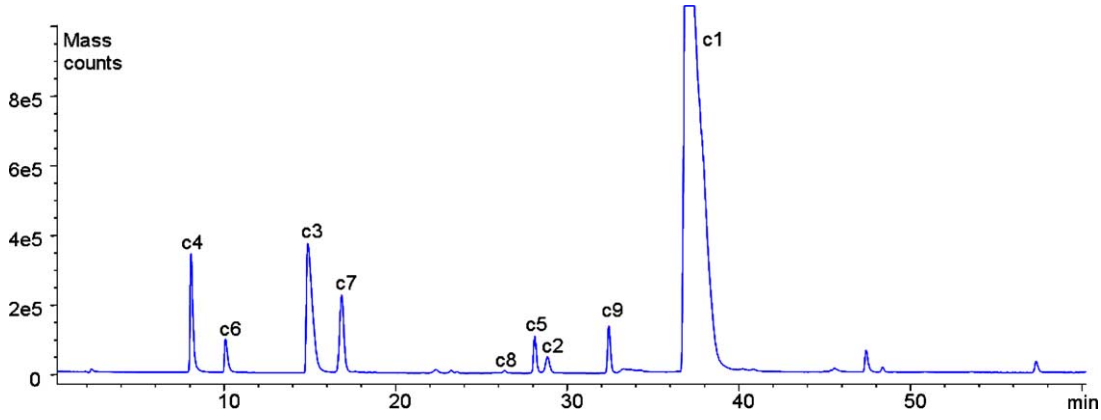
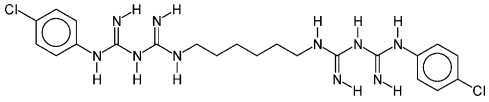
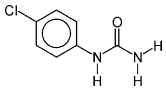
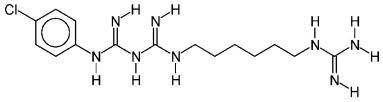
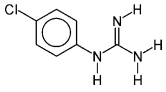
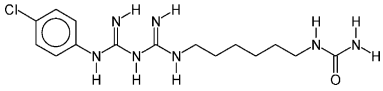
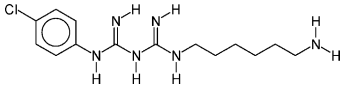
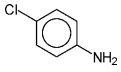
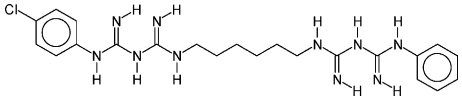
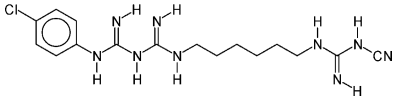


Fig. 4. SPME–HPLC–MS analysis of a chlorhexidine sample exposed to accelerated decomposition conditions. A detailed description of peak identities can be found in Table 2.

Table 2
Structures of chlorhexidine and degradation products

Compound	Formula	*m/z* for molecular ion
c1 (chlorhexidine)		505.20
c2		171.10
c3		353.15
c4		170.10
c5		354.15
c6		311.10
c7		128.05
c8		471.10
c9		378.25

4.6. Study of chlorhexidine stability in saliva

Synthesis of chlorhexidine results in several by-products that are difficult to separate; prolonged exposure to light, heat or acids can also result in several degradation products (Table 2) [7,8]. All these compounds were found by extraction with the SPME fiber from samples of standard chlorhexidine diacetate exposed to accelerated decomposition conditions (Fig. 4). The same compounds were sought afterwards in saliva, in the first 9 h following mouthrinsing with chlorhexidine solution (1 mg/mL), using the mobile phase gradient that was proposed for the detection of impurities. No chromatographic interference from saliva components was observed and no detectable amounts of degradation products were found during the investigated period of time. As there was no need to separate a large number of degradation products from saliva, a shorter analysis time was subsequently used for the quantitative determination of chlorhexidine (Fig. 6).

4.7. Study of binding to saliva matrix

Eq. (6) may be used to find unknown values of b, K and C_m, by solving it for n or C_t and fitting the solution to a set of experimental data. The data can be obtained by adding known amounts (C_t) of chlorhexidine to aliquots of blank saliva and measuring the amount extracted (n). To obtain a fit for the data, C_t may be expressed as a function of n:

$$C_t = bC_m\left(1 - \frac{1}{1 + K(n/f_c)^b}\right) + \frac{dn(f_c + V)}{f_c V} \quad (7)$$

The fiber capacity, f_c, was determined by analyzing standard solutions of chlorhexidine in buffer with the SPME fiber ($f_c = 2.36 \times 10^{-4}$ L), while V and d resulted from the experimental setup ($V = 10^{-4}$ L and $d = 2$). The amount extracted, n, was determined from the peak area of each corresponding chromatogram, using a calibration curve (n = Area/1.6×10^{17} and correlation coefficient $r^2 = 0.9959$).

Experimental data that were used for fitting Eq. (7) are presented in Table 3 and Fig. 5 (blank saliva was spiked with chlorhexidine). The best fit is obtained for $K = 3.51 \times 10^5$ L mol^{-1}, $C_m = 8.06 \times 10^{-5}$ M and $b = 1$. These results were used to determine the total and free concentration of chlorhexidine as well as the concentration of binding matrix in saliva during a pharmacokinetic study.

While no value for the binding constant between chlorhexidine and salivary proteins is published in the litera-

Table 3
Experimental data for chlorhexidine binding to saliva matrix; blank saliva was spiked with chlorhexidine to obtain the concentration C_t

C_t (μg/mL)	C_t (molar)	n (average)
0.5	9.89E-07	1.23E-12
1.0	1.98E-06	2.00E-12
2.0	3.96E-06	1.00E-11
4.0	7.91E-06	5.57E-11
10	1.98E-05	1.62E-10
15	2.97E-05	2.60E-10
20	3.96E-05	3.73E-10

ture, some results obtained by ultrafiltration indicate that chlorhexidine is 95% bound to mucin [5], corresponding to $K = 3 \times 10^5\,\mathrm{L\,mol^{-1}}$. This value is in good agreement with the current study, which considers all proteins in saliva (the most important ones are mucin and albumin).

4.8. Pharmacokinetic study after mouthrinsing with chlorhexidine

Chlorhexidine concentration in saliva was determined at several time points after washing the mouth with 10 mL solution (1 mg/mL as base, corresponding to 1.27 mg/mL chlorhexidine diacetate or 1.71 mg/mL chlorhexidine digluconate). As stated before, no interference was observed from endogenous compounds (Fig. 6). The free concentration of chlorhexidine and the concentration of binding matrix were determined in addition to the total concentration of chlorhexidine. The values of the total concentration obtained by this method are similar to those published by other authors [6].

Eq. (7) was used to determine the total concentration of chlorhexidine and the concentration of binding matrix. In the first step, 50 μL of sample were analyzed after dilution with 50 μL water and in the second step another 50 μL of sample were mixed with 50 μL standard and analyzed. Eq. (7) can be applied for each stage (n_1 and n_2 represent the amount of chlorhexidine extracted in each case):

$$C_t = bC_m\left(1 - \frac{1}{1 + K(n_1/f_c)^b}\right) + \frac{dn_1(f_c + V)}{f_c V} \quad (8)$$

$$C_t + s = bC_m\left(1 - \frac{1}{1 + K(n_2/f_c)^b}\right) + \frac{dn_2(f_c + V)}{f_c V} \quad (9)$$

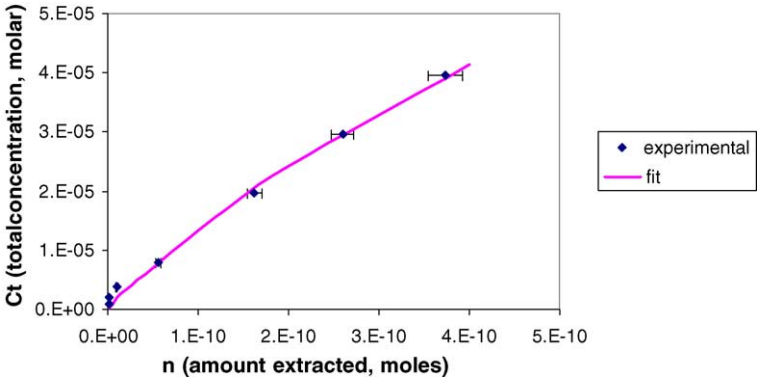


Fig. 5. Nonlinear regression fit of Eq. (7) to experimental data.

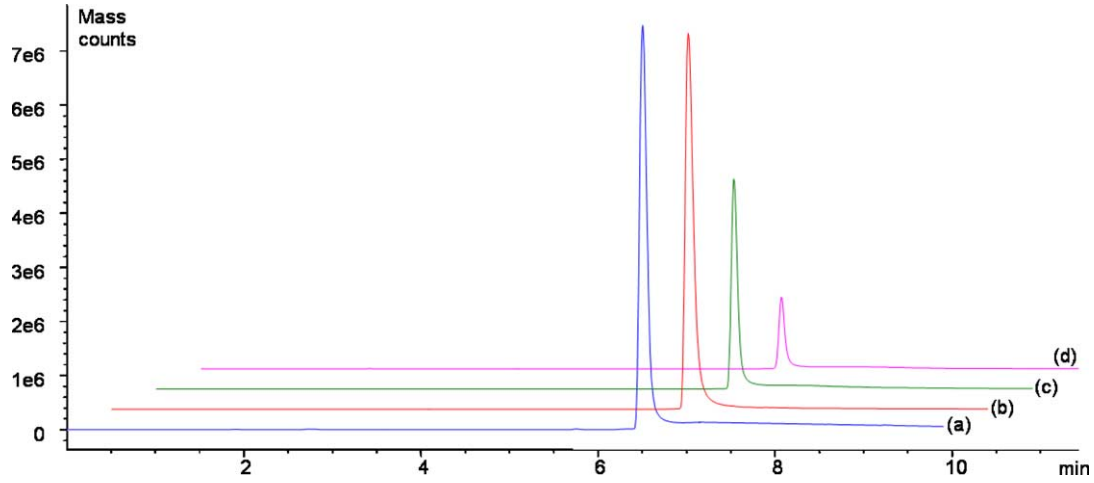


Fig. 6. Chromatograms of saliva samples at different time points after administration of chlorhexidine: (a) 0.25 h, (b) 1 h, (c) 4 h and (d) 8 h.

Table 4
Experimental data for the pharmacokinetic study

Time (h)	C_m (molar) (concentration of binding matrix)	C_t (molar)	C_t (μg/mL) (total concentration of chlorhexidine)	C_f (μg/mL) (free concentration of chlorhexidine)
0.25	1.90E-03	5.84E-04	295.0	0.64
0.5	4.58E-04	1.47E-04	74.25	0.67
1.0	2.32E-04	7.90E-05	39.93	0.72
1.5	2.02E-04	6.47E-05	32.72	0.66
2.0	1.54E-04	4.13E-05	20.88	0.51
4.0	1.28E-04	2.53E-05	12.81	0.34
6.0	4.04E-05	4.22E-06	2.13	0.15
8.0	4.10E-05	3.95E-06	2.00	0.14

C_t and C_m can be obtained by solving this system of two equations and two unknowns:

$$C_t = \frac{1}{f_c V}\left(dn_1(f_c + V) + \frac{(n_1/f_c)^b(1 + K(n_2/f_c)^b)(f_c s V + d(n_1 - n_2)(f_c + V))}{(n_2/f_c)^b - (n_1/f_c)^b}\right)$$

$$C_m = \frac{f_c s V + d(n_1 - n_2)(f_c + V)}{b f_c V}\left(\frac{1}{1 + K(n_1/f_c)^b} - \frac{1}{1 + K(n_2/f_c)^b}\right)^{-1}$$

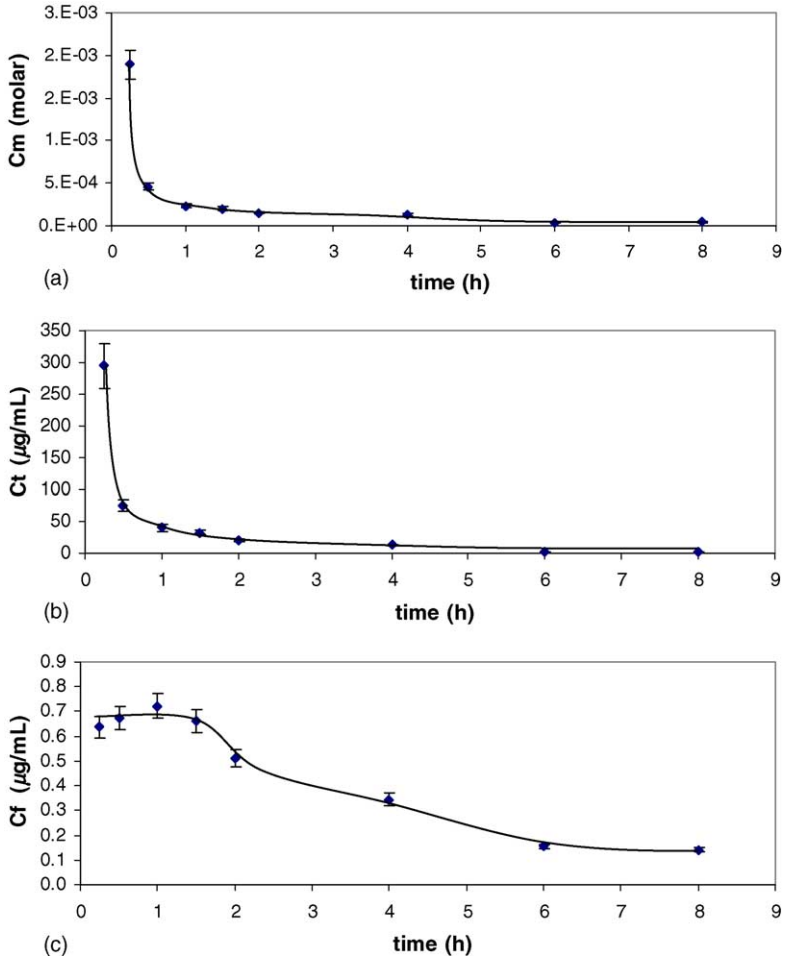


Fig. 7. Variation of concentration in time during the pharmacokinetic study (mouthrinsing with 1.0 mg/mL chlorhexidine base): (a) binding matrix concentration (molar), (b) total concentration of chlorhexidine (μg/mL) and (c) free concentration of chlorhexidine (μg/mL).

Finally, the free concentration of chlorhexidine was determined by solving Eq. (1) for C_f (this is possible since C_t and C_m represent the initial concentrations, before adding standard and it is already known that $b = 1$):

$$C_f = \frac{1}{2K}\left(-1 + C_tK - C_mK + \sqrt{4C_tK + (1 - C_tK + C_mK)^2}\right) \quad (10)$$

The results of the pharmacokinetic study are presented in Table 4 and Fig. 7.

As it can be seen, the total concentration of chlorhexidine followed a first order kinetics of elimination, while the free concentration remained almost constant for the first 2 h and then slowly decreased. The concentration of binding matrix in saliva was quite high at the beginning and reached normal levels approximately 1.5 h after chlorhexidine administration. This observation is consistent with the fact that the volunteer who self-administered chlorhexidine reported an intense sensation of dryness in the mouth for the first 2 h after mouthrinsing; it seems that the bitter taste of chlorhexidine resulted in a change of the normal proportion of water in saliva for a few hours. The concentration of proteins in saliva after 5 h from mouthrinsing (~40 μM) closely matches the normal concentration of salivary proteins as reported in literature [16].

The total concentration of chlorhexidine in saliva required for the inhibitory effect against cariogenic bacteria is 0.8–6.3 μg/mL [5]. According to the graph in Fig. 7(b), this concentration was maintained for at least 8 h.

Only 100 μL of sample are needed for each time point. Additionally, sampling with a SPME fiber in a small volume of sample brings the advantage of a shorter equilibration time and a higher tolerance to interfering substances that could also be extracted [17].

5. Conclusions

The proposed SPME method for analysis of chlorhexidine proved useful for quantitative and qualitative determinations in saliva. In addition to the total concentration of chlorhexidine, this method provided the concentration of free chlorhexidine and of binding matrix (proteins) in saliva.

Coupling of SPME with liquid chromatography and mass spectrometry resulted in high specificity, as no interference was observed: the chromatograms for saliva samples contain only the peak corresponding to chlorhexidine (Fig. 6), unlike those obtained with UV detectors. The method had a wide linear range (0.1–40 μg/mL free chlorhexidine, in the presence of proteins) that was perfectly suitable for the study of chlorhexidine retention in the oral cavity.

It was shown that chlorhexidine is stable in the oral cavity for at least 9 h and high concentrations of the drug (2 μg/mL total) are still present in saliva even after 8 h from mouthrinsing. These results are in good agreement with previous studies.

This new method allowed a full investigation of chlorhexidine elimination during a pharmacokinetic study: while the total concentration followed a first-order elimination kinetics, the free concentration remained almost constant for several hours, showing that the oral cavity acts like a reservoir that slowly releases the drug. Also, it was revealed that following oral administration of chlorhexidine, the normal composition of saliva changes for a few hours, probably as a physiological response to the bitter taste of the medicine.

The calculations of free, total and binding matrix concentration are facilitated as soon as a suitable spreadsheet or program is created.

This selective and sensitive SPME approach for monitoring both the concentration of chlorhexidine (free along with total) and binding matrix (proteins) in saliva should prove to be more useful for pharmacokinetic studies than classic methods that only provide the total concentration, since in addition to the elimination profile, the way that the drug influences the concentration of proteins may be studied, without the need to use a different analytical method. A similar approach should be appropriate for other pharmacokinetic studies, in any biological fluid. As an example, the method seems to be particularly useful for studying the interaction between drugs and specific binding proteins, like receptors and enzymes.

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