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Determination of the Salivary Retention of Hexetidine In-vivo by High-performance Liquid Chromatography

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Abstract

The non-antibiotic antimicrobial agent hexetidine is widely used at a concentration of 0·1% w/v as an oral rinse to reduce the number of viable microorganisms within the oral cavity. However, following use, the available concentration of hexetidine in the oral cavity declines with time, thus compromising the resultant antimicrobial activity. It is, therefore, desirable to determine the persistence of the agent in the oral cavity by quantification of the drug concentration in saliva, thus enabling prediction of its antimicrobial activity in the oral environment.

A rapid reverse-phase HPLC method was therefore developed and validated for hexetidine in aqueous solution (Oraldene) and in saliva samples collected from volunteers post-rinsing with 15 mL of hexetidine oral rinse for 30 s. The HPLC assay was sufficiently sensitive to accurately detect hexetidine in saliva up to 25 min after in-vivo use of a commercial oral rinse. Furthermore, it was possible to detect hexetidine below the published minimum inhibitory concentrations (MICs) for a selection of microorganisms. From these data a first-order elimination rate constant of hexetidine from the oral cavity was determined post-rinsing in each of six volunteers.

The validated HPLC assay method presented is useful for the assay of hexetidine in the oral cavity both at and below MICs. The first-order elimination rate constant shows significant variation between volunteers.

Certain microorganisms can enzymatically convert fermentable sugars, via glycolysis, to acidic compounds such as lactic acid. In the oral cavity, where microbially mediated acid production is associated with dental plaque, a common consequence of this process is dental caries. The use of non-antibiotic antimicrobial agents in oral rinses has been shown to be of clinical benefit in this situation (Wile et al 1986), reducing the number of microorganisms in the oral cavity after rinsing.

Hexetidine (Figure 1) is a non-antibiotic antimicrobial agent, which is commercially available at a concentration of 0·1% w/v as Oraldene mouthwash and is indicated for oral hygiene applications (Wile et al 1986; Jones et al 1997). Despite its widespread use, there is comparatively little published information on the antimicrobial properties of hexetidine, although the efficacy of this agent in the reduction of plaque and on microbial viability within the oral cavity has been reported (Wile et al 1986; Williams et al 1987). More recently, we have demonstrated its in-vitro and ex-vivo anti-adherence properties and in-vitro suppression of morphogenesis of blastospores of *Candida albicans* (Jones et al 1997). We suggested a clinical role for hexetidine in both the treatment and prophylaxis of infection within the oral cavity. In both applications, salivary retention of non-antibiotic antimicrobial agents within the oral cavity is particularly significant, and has been determined for agents such as chlorhexidine, cetylpyridinium chloride and cetrimide (Bonesvoll & Gjermo 1978). Importantly, the superior retention of chlorhexidine, a cationic bisbiguanide, within the oral cavity has been reported. Despite its clinical use, there have been no reports concerning

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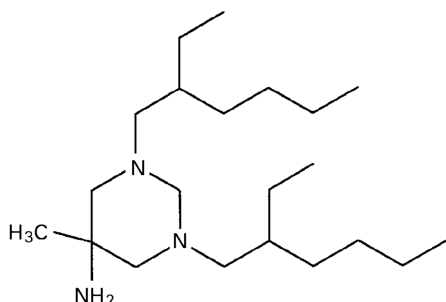

Figure 1. Molecular structure of the non-antibiotic antimicrobial agent, hexetidine.

the biopharmaceutical properties of hexetidine within the oral cavity following its administration as an oral rinse. One potential reason for this is the unavailability of a rapid and sensitive analytical method to quantify hexetidine. Therefore, in this study, a validated HPLC method is presented and employed to quantify the concentration of hexetidine both in a commercial oral rinse formulation and in saliva samples collected from volunteers post-rinsing with this solution. Thus, an elimination rate for hexetidine from the oral cavity was determined in-vivo. This information is of importance in determining an optimum treatment regimen for hexetidine in the oral cavity and may be used as a basis for formulation modification to improve persistence of the agent at the site of action.

Materials and Methods

Materials

Acetonitrile and methanol (both HPLC grade) were purchased from Labscan Ltd (Dublin, Ireland). Hexetidine and Oraldene were kindly donated by Warner Lambert Ltd, Dartford, UK. All other chemicals used were of analytical-reagent grade.

HPLC

The HPLC system comprised an LKB 2150 pump and 2151 variable wavelength detector (LKB Biochrom Ltd, Cambridge, UK), together with a Waters model auto-injector (injection volume 20 μL). Chromatograms were recorded on a Perkin-Elmer recorder (Beaconsfield, Bucks, UK). The HPLC column (25 cm × 4·6 mm i.d.) was packed with Hypersil CPS 10 μm (Hypersil, Cheshire, UK). The mobile phase was methanol (20%), acetonitrile (60%) and 0·02 M dibasic ammonium hydrogen phosphate (20%), with the final pH adjusted to 7·0 by dropwise addition of phosphoric acid (85%). The flow rate was 2 mL min^{-1}, with detection at 205 nm and detector sensitivity set at 0·04 aufs.

Collection of saliva

An early morning, unstimulated saliva sample was collected from each of six healthy adults, none of whom were receiving medication. Each subject then rinsed his or her oral cavity with a volume of Oraldene (15 mL) for 30 s before supplying a second saliva sample. Further samples were collected at 1-min intervals for 10 min and subsequently at 5-min intervals up to 30 min post-rinsing. Subjects were not permitted to consume food or liquids during this 30-min period. For each subject, sampling was performed in triplicate.

Extraction of hexetidine from saliva

Equal volumes (1 mL) of saliva and methanol were mixed at high speed for 1 min using a vortex mixer, centrifuged at 14 000 rev min^{-1} for 10 min and the supernatant carefully collected by aspiration with a Pasteur pipette. The hexetidine content of the supernatant was then determined using the aforementioned HPLC method. Due to the difficulty of identifying a suitable internal standard for the analysis, the hexetidine concentration in saliva was determined by comparing sample peak heights with the peak height of standard hexetidine solutions. The elimination rate of hexetidine from the oral cavity was determined for each subject from the gradient of a log (hexetidine concentration) versus time plot. Linearity of this relationship was confirmed using linear regression analysis (Statview 5.0, SAS Institute Incorporated, Cary, NC).

Assay validation

To overcome the poor water solubility of hexetidine, a 1% solution of Tween 20 in phosphate buffered saline, pH 7·4, was employed to prepare aqueous solutions of hexetidine. A calibration graph was constructed for hexetidine solutions (0–90 μg mL^{-1}), correcting for hexetidine density of 0·89 g mL^{-1}. Each solution concentration was determined in triplicate. Accuracy and precision were determined by analysis of five replicate samples from a hexetidine solution of corrected concentration 44·50 μg mL^{-1}. Based on a signal : noise ratio of 3 : 1, the limit of detection for hexetidine in aqueous solution was determined.

By dilution of Oraldene with methanol, a series of solutions of varying hexetidine concentration were prepared in triplicate. Since the concentration of hexetidine in Oraldene is expressed as % w/v, no density adjustment was required. A calibration

graph was constructed over the range 0–100 $\mu g\,mL^{-1}$ hexetidine. Five replicate solutions of hexetidine 50 $\mu g\,mL^{-1}$, as Oraldene, were analysed to determine the accuracy and precision of the assay.

To validate the assay of hexetidine, as Oraldene, in saliva, 5 individual samples of saliva (1·0 mL) were spiked with Oraldene to give a hexetidine concentration of 100 $\mu g\,mL^{-1}$. Following 1 : 1 dilution with methanol and centrifugation, the hexetidine concentration of each sample was determined.

Statistical analysis

The effects of each subject on hexetidine elimination rates were analysed statistically using a one-way analysis of variance, in which $P < 0{\cdot}05$ indicated significance. Post-hoc comparison of mean values was performed using Fischer's least significant difference (Statview 5·0, SAS Institute Incorporated, Cary, NC).

Results

For hexetidine aqueous solutions, the calibration graph was linear over the range 7·20–80·10 $\mu g\,mL^{-1}$ ($y = 2{\cdot}716x - 3{\cdot}501$; $r^2 = 0{\cdot}999$). Data obtained from five replicate determinations of hexetidine aqueous solution gave a mean percentage recovery of 99·92 ± 0·94. The lower limit of hexetidine quantification was 7·20 $\mu g\,mL^{-1}$ and the limit of detection was 5·43 $\mu g\,mL^{-1}$. A representative chromatogram for hexetidine in 1% Tween 20 is shown in Figure 2.

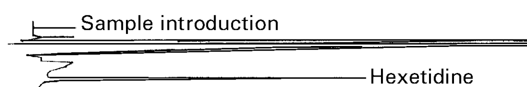

Figure 2. Chromatogram of hexetidine (53·40 $\mu g\,mL^{-1}$) in 1% Tween 20 solution.

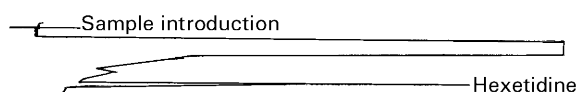

Figure 3. Chromatogram of hexetidine in saliva collected from subjects immediately after a 30-s oral rinse with Oraldene. The concentration of hexetidine in this sample was 161·92 $\mu g\,mL^{-1}$.

For hexetidine as Oraldene solutions in methanol, a linear calibration ($y = 2{\cdot}212x - 2{\cdot}000$; $r^2 = 0{\cdot}999$) was obtained. Data obtained from five replicate determinations of hexetidine as Oraldene gave a mean percentage recovery of 100·06 ± 6·25. The lower limit of hexetidine quantification was 5 $\mu g\,mL^{-1}$ and the limit of detection was 3 $\mu g\,mL^{-1}$.

For hexetidine in extracted saliva samples, data obtained from five replicate determinations gave a mean percentage recovery of 93·93 ± 2·17. An example chromatogram of hexetidine in extracted saliva post-treatment with Oraldene is shown in Figure 3.

Quantifiable hexetidine concentrations in saliva samples from six volunteers obtained over a 30-min period post-oral rinsing are presented in Table 1. Each subject exhibited an exponential-like decay in

Table 1. Salivary hexetidine concentrations in healthy subjects, post-rinsing with hexetidine solution (15 mL, as Oraldene).

Time post-rinsing (min)	Salivary hexetidine concentrations ($\mu g\,mL^{-1}$)					
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6
0	144·60 ± 1·04	138·90 ± 1·04	122·10 ± 1·03	173·70 ± 1·56	136·20 ± 1·37	171·90 ± 1·56
1	53·40 ± 1·37	54·00 ± 0·90	48·60 ± 1·56	73·80 ± 0·90	47·40 ± 1·04	55·20 ± 1·04
2	37·80 ± 0·90	31·80 ± 0·52	36·00 ± 1·8	47·10 ± 0·52	39·60 ± 1·56	29·10 ± 0·52
3	26·40 ± 1·04	22·80 ± 0·52	22·80 ± 1·37	14·10 ± 0·52	21·30 ± 0·52	18·30 ± 1·37
4	19·20 ± 1·04	24·90 ± 2·08	24·30 ± 0·90	11·70 ± 0·00	15·60 ± 0·52	14·70 ± 0·52
5	15·00 ± 1·04	26·70 ± 2·27	18·00 ± 0·90	12·30 ± 0·52	5·70 ± 0·52	12·30 ± 0·52
6	12·30 ± 0·51	25·80 ± 1·03	20·70 ± 0·90	9·30 ± 0·51	5·10 ± 0·52	8·70 ± 0·52
7	11·40 ± 0·52	20·10 ± 0·52	18·90 ± 0·00	7·50 ± 0·52	NQ	5·70 ± 0·51
8	7·80 ± 0·52	20·40 ± 1·03	11·40 ± 1·04	5·40 ± 0·00	NQ	NQ
9	5·70 ± 0·51	15·90 ± 0·52	8·70 ± 0·52	5·70 ± 0·52	NQ	NQ
10	NQ	12·30 ± 0·52	10·50 ± 1·04	NQ	NQ	NQ
15	NQ	9·30 ± 0·51	10·50 ± 1·03	NQ	NQ	NQ
20	NQ	6·60 ± 0·51	6·00 ± 0·52	NQ	NQ	NQ
25	NQ	5·40 ± 0·00	NQ	NQ	NQ	NQ
30	NQ	NQ	NQ	NQ	NQ	NQ

Results are expressed as mean ± s.d. NQ = not quantifiable.

Table 2. Hexetidine elimination rates in healthy subjects following a 30-s oral rinse with hexetidine solution (15 mL, as Oraldene).

Subject no.	Elimination rate (min^{-1})
1	$135.33 \pm 0.58 \times 10^{-3}$
2	$73.33 \pm 1.15 \times 10^{-3}$
3	$91.33 \pm 1.15 \times 10^{-3}$
4	$143.00 \pm 3.46 \times 10^{-3}$
5	$165.00 \pm 3.61 \times 10^{-3}$
6	$178.00 \pm 5.57 \times 10^{-3}$

Results are expressed as mean ± s.d.

hexetidine concentration as time progressed, with salivary hexetidine falling below the level of accurate quantification 7–30 min after use of Oraldene. In general, salivary hexetidine concentrations were found to differ significantly between all the volunteers. The elimination rates of hexetidine from the oral cavity, calculated from the slopes of log (hexetidine concentration) versus time plots, are given in Table 2. Significant differences in this parameter were revealed between each of the volunteers.

Discussion

The inclusion of non-antibiotic antimicrobial agents such as hexetidine as active ingredients in oral rinses confers the benefit of a three-pronged defence against infection. The combination of antimicrobial and anti-adherent properties, together with a relatively low level of resistance exhibited by microorganisms to non-antibiotic antimicrobial agents, is advantageous compared with the use of antibiotics. Therefore, a growing interest has emerged in the clinical use of these agents within the oral cavity.

In a comparison of antiseptic mouthwashes, Roberts & Addy (1981) measured salivary bacterial counts following a single oral rinse with each of four antimicrobial solutions. Numbers of viable bacteria were significantly reduced immediately after rinsing. A return to pre-rinse bacterial levels was observed 7 h after use of a chlorhexidine gluconate (0·2% v/v) rinse, while hexetidine maintained a significantly lower bacterial population for only 90 min. The observation with hexetidine was attributed to its weakly basic nature, which may translate to poor adsorption properties, indicating a relatively lower retention in the oral cavity. The in-vitro antimicrobial activities of proprietary hexetidine and chlorhexidine mouthwashes were found to be essentially similar (Ashley 1984), however. Interestingly, it has been suggested that accumulation of hexetidine in the mouth may occur following multiple dosing, the mechanism involving binding to gingival mucosa, plaque or hydroxyapatite (Roberts & Addy 1981; Wile et al 1986).

Clearly, studies on residual oral retention of hexetidine have been hindered by the lack of a rapid and simple instrumental analytical method for quantification of the agent in saliva. The validated HPLC assay for hexetidine in saliva developed in this study rectifies this problem and thus provides a quantitative description of the first-order decay in salivary hexetidine concentration following rinsing in-vivo with a proprietary hexetidine-containing mouthwash. As presented in Table 1, the fall in salivary hexetidine concentration over the first 25 min post-rinsing is indicative of relatively rapid elimination. Following instillation, hexetidine adsorbs to the various mucus-coated surfaces within the oral cavity. However, due to the lipophilic and weakly basic nature of this agent, the degree of interaction with the anionic mucus will be limited in comparison to, for example, chlorhexidine. Consequently, hexetidine will be rapidly desorbed as a result of mucus shedding and subsequent swallowing. In this study, the concentration of hexetidine in saliva was quantifiable up to 25 min after use of the proprietary hexetidine product, Oraldene. Determination of the concentration of hexetidine in pooled saliva was deliberately chosen to overcome the known regional differences in the location of therapeutic agents (Weatherell et al 1994). Interestingly, the retention time of hexetidine in the oral cavity observed in this study was markedly lower than that described by Roberts & Addy (1981). It must be realised however, that retention of hexetidine was defined as the period after which an increase in viable bacteria was observed within the oral cavity. Therefore, the observed disparity in retention between the previous study and this study may be a result of the time required for bacterial recovery following exposure to hexetidine. The variation in threshold quantification time (i.e. 7–30 min) may be explained by the fluctuating elimination rate of drug from the oral cavity, a factor that appears to be subject dependent (Weatherell et al 1994). The sensitivity of the HPLC analytical method was such that the limit of detection for both hexetidine, alone and as Oraldene, was below the minimum inhibitory concentration (MIC) level for selected microorganisms. For example, the lowest MIC of hexetidine was 11·10 $\mu g\,mL^{-1}$ for *Streptococcus mutans* and *Streptococcus sanguis*, whereas the MICs against other pathogens of the oral cavity (e.g. *Pseudomonas aeruginosa* and *Staphylococcus aureus*) were considerably greater, particularly

whenever present in sessile (biofilm) mode (Wile et al 1986; McGovern et al 1996).

Interestingly, we have shown that the microbial anti-adherence properties of hexetidine persist for at least four hours following use as an oral rinse. It has been previously reported that the anti-adherence properties of many non-antibiotic, antimicrobial agents may be observed both at sub-MICs and, additionally, at concentrations below which monolayer coverage of the substrate occurs (Fowler & Jones 1992; Jones et al 1995; Schep et al 1995). As the adherence of microorganisms to epithelial cells is a surface phenomenon, and in light of the relatively rapid elimination of hexetidine into saliva, it may be postulated that the prolonged anti-adherence effects were due, at least in part, to the retention of hexetidine, albeit at low concentrations, on the surface of epithelial cells. Studies are currently ongoing to confirm the nature of interaction of hexetidine with epithelial cell membranes.

Conclusion

A rapid, simple HPLC method for salivary hexetidine has been developed and validated, allowing detection of the agent at concentrations that are sub-MIC for a range of pathogenic microorganisms. Following use of a commercial hexetidine oral rinse (Oraldene), the HPLC method accurately quantified hexetidine concentrations in human saliva for up to 25 min after rinsing. However, rates of elimination of hexetidine from the oral cavity were found to vary significantly between individuals and the agent may decay to non-quantifiable levels within a much shorter time.

Acknowledgements

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