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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CHLORHEXIDINE

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SUMMARY

Chlorhexidine, a common antiseptic, has been extracted from blood and urine by shaking with diethyl ether, chromatographed in methanol–water using an ion-pair agent to control k', and detected by ultraviolet absorption. A variety of ion-pair agents have been evaluated. Quantitation was achieved via a calibration curve and a detection limit of 0.2 $\mu g\ ml^{-1}$ proposed. Studies on blood and urine reveal no interference problems. The method has been applied to a preliminary study of post-cystoscopy urine specimens where chlorhexidine had been used as the irrigant, and to the quality control of pharmaceutical preparations.

INTRODUCTION

The bis-biguanide compound chlorhexidine (1,1′-hexamethylene-bis[5-(4-chlorophenyl)biguanide] (Fig. 1) is an antiseptic developed for use in hospital practice (trademark "Hibitane", I.C.I., Alderley Park, Great Britain). It is a strong base readily forming salts from which a range of pharmaceutical preparations have been formulated. For example the skin cleanser "Hibiscrub" (I.C.I.) contains the digluconate salt in aqueous solution. Chlorhexidine is reported to carry four positive charges in strong acid[1] delocalised over all ten nitrogen atoms[2]. However, only two pK_a values of 2.2 and 10.3 have been reported[3]. Thus in neutral or mild alkaline solution the compound is dicationic. At bacteriostatic concentrations the bacterial cell membrane is damaged with irrevocable loss of cell contents and inhibition of enzyme proteins. At higher concentrations, precipitation of cellular proteins and nucleic acids occurs resulting in effective bactericidal action[4–6]. Animal studies indicate that, at

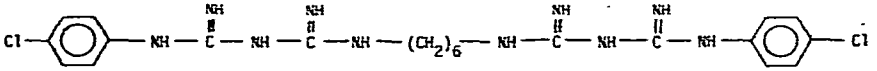

Fig. 1. The structure of chlorhexidine.

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high concentrations, chlorhexidine binds to rat liver microsomes interfering with enzyme metabolism and causing protein precipitation[1]. Such effects have been attributed to the cationic nature of chlorhexidine.

Chlorhexidine-containing preparations are commonly used in hospital practice for disinfection of hands and of the operation site. Further applications involve contact with microsal surfaces other than the intact skin, for example irrigation of the bladder to prevent urinary tract infection, cleansing of post-operative wounds and in the treatment of burns. Little is known of the possible absorption of chlorhexidine administered by such routes. Absorption through intact skin is known to be negligible, even after prolonged use[7], and there is no evidence of systematic toxicity. Low levels of chlorhexidine have been reported in the blood of neonates following topical application of 4% chlorhexidine gluconate[8] or 1% chlorhexidine powder[9]. Further studies of possible systematic absorption of chlorhexidine administered by other routes in urological and obstetric practice are indicated.

The colorimetric method used to estimate chlorhexidine in pharmaceutical preparations[10] lacks the sensitivity required for analysis of body fluids. To date, the only published method fulfilling this requirement for blood samples is gas–liquid chromatography (GLC)[11], involving solvent extraction and hydrolysis to 4-chloroaniline followed by diazotisation and iodination to 4-chloroiodobenzene. This method fails to distinguish 4-chloroaniline resulting from the *in vitro* or *in vivo* decomposition of chlorhexidine prior to analysis. Thermal decomposition occurs slowly at room temperature although increased levels of 4-chloroaniline have been detected in aqueous chlorhexidine solutions autoclaved at 130°C for 30 min[12]. The amount of 4-chloroaniline in pharmaceutical preparations is monitored by a colorimetric method and is unlikely to be sufficiently high to cause toxic side effects or an appreciable loss in antibacterial potency. However for pharmacological and absorption studies, a sensitive method for the independent detection of chlorhexidine and 4-chloroaniline is preferable. Such considerations prompted the investigation of high-performance liquid chromatography (HPLC) as an alternative to the GLC and colorimetric methods. We now report an HPLC assay of chlorhexidine which may be applied to urine and blood with a detection limit of 0.2 μg ml^{-1}. The method may also be used for the rapid determination of chlorhexidine in pharmaceutical preparations.

EXPERIMENTAL

An LC-XPD Model 100 pump (Pye Unicam, Cambridge, Great Britain), loop injection system (Rheodyne 7120 with 50 μl capacity), reversed-phase column (10 μm, ODS Waters Assoc., Cheshire, Great Britain) and ultraviolet detector (Model LC3, Pye Unicam) were used.

Water was double distilled before use. Methanol (Willot Industrial, Bristol, Great Britain), acetonitrile (BDH, Poole, Great Britain), and acetone (Chas. Tennants, London, Great Britain) were used as received, as were sodium lauryl sulphate, sodium cyclamate, toluene-4-sulphonic acid, sodium acetate, Pic B-7 (Waters Assoc.), pentadecafluorooctanoic acid and acetic acid (glacial). The mobile phase was degassed by ultrasonic vibration under vacuum (17 mmHg) for 15 min prior to use and stirred continuously during use.

Optimised chromatographic parameters were: mobile phase 1000 μg ml^{-1} toluene-4-sulphonic acid in methanol–water (65:35); flow-rate, 1.5 ml min^{-1}; injection volume 50 μl; detector wavelength 238 nm; sensitivity 0.04 a.u.f.s. into 10 mV f.s.d. (unless otherwise stated).

To extract 1 ml blood, serum or urine 1 ml of 1 *N* NaOH was added followed by 10 ml diethyl ether. Mechanical shaking (30 min) and centrifugation (15 min, 1110 *g*) was followed by decantation of the organic layer (*ca.* 9.5 ml). This was then blown to dryness (under nitrogen) and redissolved in mobile phase (300 μl) to give a suitable sample for injection.

Chlorhexidine base powder, Hibitane (20% chlorhexidine gluconate solution), Hibiscrub (4% chlorhexidine gluconate) and "Corsodyl" mouthwash (0.2% chlorhexidine gluconate) were obtained from I.C.I. 0.02% chlorhexidine acetate (for irrigation) was obtained from Travenol, (Thetford, Great Britain) and solutions of 0.2% aqueous chlorhexidine gluconate and 0.5% chlorhexidine gluconate in 70% methylated spirit were obtained as dispensed from the Pharmacy Department, Bristol Royal Infirmary, Great Britain.

RESULTS AND DISCUSSION

Optimisation of chromatography

Chlorhexidine itself, usually referred to as chlorhexidine base, is exceedingly insoluble in water (0.008%) and is only slightly soluble in organic solvents such as hexane and acetone. Solubility of the salts is variable with the gluconate salt being the most soluble. Thus in aqueous medium (pH $\approx$ 7) the chlorhexidine moiety exists as the di-cation and thus readily forms ion pairs. These considerations dictated the selection of a reversed-phase column (ODS) and the use of an ion-pair agent possessing an organic anionic moiety.

The mobile phase originally investigated consisted of acetonitrile–water mixtures but impurity problems prompted a change to methanol–water. A relative proportion of 75:25 was selected after studying the chromatography of standard compounds at various relative proportions. In view of the ionic nature of chlorhexidine the effect of pH of the mobile phase was investigated. The mobile phase consisting of methanol–20 m*M* aqueous sodium acetate (75:25) was adjusted to the required pH with acetic acid. The resultant capacity factors (k') suggested that, for the pH range 3.8–5.5, 4-chloroaniline was poorly retained ($k' = 0.2$). For chlorhexidine k' varied from 0.2 (pH 3.8), to 3.0 (pH 5.5) but the peak shape was unacceptable. For these experiments the acetate ion may be considered as an ion-pairing agent albeit a very poor one. A change of mobile phase proportion to methanol–water (pH 4.0) (50:50) raised k' (chlorhexidine) from 0.4 to 10.3. Thus control of k' (chlorhexidine) may be achieved by control of the methanol–water ratio and pH but peak shape (and thus column efficiency) are poor.

The addition of ion-pairing agents to the mobile phase for reversed-phase chromatography of ionic species increases their retention on the column. Various possible ion pair agents were studied, using a constant methanol–water ratio (75:25) at pH 4. In a search for a large organic counter-ion sodium lauryl sulphate and sodium cyclamate were used at 10, 100 and 1000 μg ml^{-1} concentrations. Whilst variation of k' for chlorhexidine was achieved peak shape remained rather broad. In

contrast 0.005 *M* heptanesulphonic acid (Pic B-7, Waters Assoc.) provided excellent peak shape with a k' (chlorhexidine) of 1.2 (Fig. 2a). The argument against the use of this reagent is financial and hence heptanoic acid was evaluated. This proved unsuccessful.

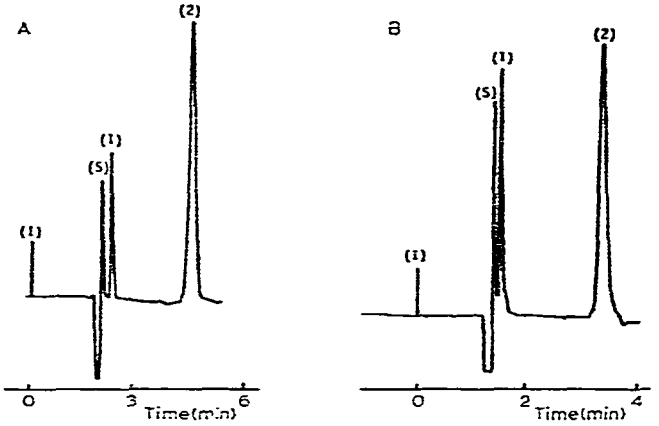

Fig. 2. (A) Chromatogram of chlorhexidine (2) and 4-chloroaniline (1) with Pic B-7 as ion-pair agent. (Detector 254 nm, sensitivity 0.04 a.u.f.s.). (B) Chromatogram of chlorhexidine (2) and 4-chloroaniline (1) with toluene-4-sulphonic acid as ion-pair agent. (Detector 238 nm; sensitivity 0.04 a.u.f.s. I = injection point; S = solvent front).

Attention was thus turned to aromatic sulphonic acids. At first sight the use of an aromatic constituent of the mobile phase when an ultraviolet detector is employed appears self-defeating but examination of the ultraviolet spectrum of toluene-4-sulphonic acid revealed a significant *minimum* in the main absorption band. This minimum was centred at about 242 nm. Chlorhexidine absorbs strongly between 215 nm and 260 nm and thus monitoring the column eluate at approximately 242 nm to coincide with the absorbance minimum for toluene-4-sulphonic acid was apparently possible. Mobile phase was thus prepared containing 1000 μg ml^{-1} of this ion-pair agent. The background absorption of the solvent was increased by the ion-pair agent but was still acceptable. Fine adjustment of the detector-wavelength setting located the point of minimum background absorbance (238 nm). Under these conditions chlorhexidine was eluted very shortly after the solvent front with a capacity factor of 0.2. Changing the mobile phase to methanol–water (65:35) eluted chlorhexidine as a single sharp symmetrical peak (k' = 1.0; Fig. 2B). Using this solvent system a "ghost" peak associated, possibly, with the use of toluene-4-sulphonic acid was observed. It is suggested that this peak is caused by the different solubilities of the sulphonic acid between methanol and water. Injection of a mixture of methanol–water other than in the ratio of the mobile phase causes a discontinuity in the background absorbance due to the change in the concentration of the acid in the eluent (caused by re-quilibration). This "ghost" peak interferes with the chlorhexidine peak when a methanol–water (60:40) mobile phase ratio is employed but is of no significance provided a mixture such as methanol–water (65:35) is maintained. Alternatively use of the mobile phase for the preparation of samples (rather than water) eliminates the discontinuity. Column efficiency using these conditions is com-

parable with that achieved with heptanesulphonic acid. Efficiency decreases with increasing flow-rate. The compromise selected was 1.5 ml min^{-1} to give reasonable chromatographic analysis times.

Thus, although toluene-4-sulphonic acid functioned successfully as an ion-pair agent for chlorhexidine its use provided a high background absorbance.

From these experiments it was apparent that a suitable ion-pair agent should be difunctional, that is it should possess a non-polar part with which to interact with the stationary phase and a polar ionic part with which to interact with the solute. Secondary considerations were solubility in the mobile phase, a low UV absorbance at the detection wavelength, low cost and easy availability. These criteria are satisfied by perfluorocarboxylic acids of which pentadecafluorooctanoic acid is probably the most common.

Used as an ion-pair agent at a concentration of 1000 μg ml^{-1} pentadecafluorooctanoic acid permitted the elution of chlorhexidine as a sharp symmetrical peak ($k' = 1.2$).

The ion-pair agents studied divide into two groups. The successful ones were heptane sulphonic acid, toluene-4-sulphonic acid and pentadecafluorooctanoic acid. The less successful ones were sodium acetate, sodium cyclamate and sodium lauryl sulphate even when used at pH 4 or lower. It would thus appear that the counter ion has either a beneficial (H^+) or deleterious (Na^+) effect. A possible explanation is that the mobility of solvated sodium ions is significantly less than that of solvated hydrogen ions. The presence of sodium ions thus obstructs the association–dissociation mechanism of the chlorhexidine molecules with the ionic sites of the ion pair agent.

Quantitation

An internal standard is desirable in the trace analysis of biological samples because it compensates for possible errors in the extraction step. Two possible internal standards were considered of which one, an undefined white powder, available on special request from the manufacturer of chlorhexidine, was rejected because of commercial non-availability in spite of being chromatographically acceptable. The other, benzyl-hibitane, although chromatographically suitable *i.e.* being well resolved from chlorhexidine was rejected because it was only poorly recovered from biological fluids (see below).

Quantitation was thus achieved via use of a calibration curve. A stock solution of chlorhexidine diacetate (1000 μg ml^{-1}) and a set of standards (1–10 μg ml^{-1}) produced daily by serial dilution was used. [Studies of the adsorption of chlorhexidine onto glass suggests that it is of little significance[13] hence daily serial dilution is a precautionary measure only]. For this concentration range and injection of a 50 μl sample response was linear (regression equation $y = 13.9x - 1.27$; correlation coefficient = 0.9997). Linear response extended to at least 20 μg ml^{-1}. Below 1 μg ml^{-1} results became increasingly irreproducible. At high detector sensitivity (0.01 a.u.f.s.; 10 mV f.s.d.) a 1-μg ml^{-1} sample gave almost full scale response on the recorder. However the relative standard deviation (10 injections) was only 5%. We therefore tentatively suggest a detection limit of 0.2 μg ml^{-1} but a sensitivity for the method of only 1 μg ml^{-1}.

Recovery from spiked samples

Blank samples of urine, horse blood and horse serum were spiked with chlorhexidine to give a concentration of 2 μg ml^{-1}. Samples were made alkaline with 1 *N* NaOH and extracted with either dichloromethane, ether containing 3% amyl acetate or ether. Initially benzyl-hibitane was added to all samples as internal standard but it was soon apparent that it was not recovered by any of these three solvents from blood, urine or serum. Hence quantitation via calibration curve was used. A comparison between ether and ether containing 3% amyl acetate for the extraction of chlorhexidine from urine showed that interferents were co-extracted if amyl acetate was used. A similar result was obtained for the recovery from blood. Thus ether was the selected solvent for extraction. Its volatility reinforces its selection permitting easy removal prior to dissolution of the sample in the mobile phase. A typical chromatogram (Fig. 3) of an extract from blood spiked with chlorhexidine indicates that no serious interferences are present. A similar statement is applicable to extracts obtained from spiked urine (Fig. 4).

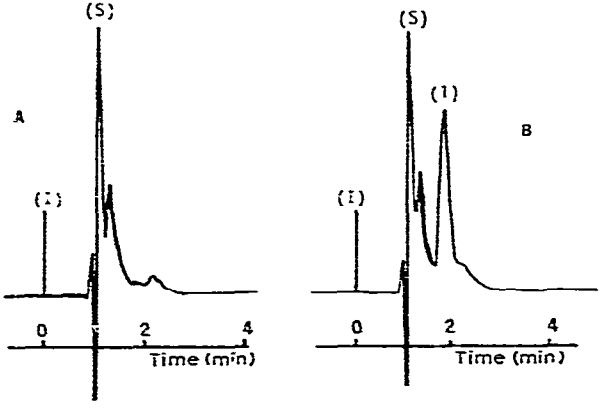

Fig. 3. (A) Chromatogram of blank blood extracted with ether. (B) Chromatogram of spiked blood [chlorhexidine concentration = 2 μg ml^{-1}]. (I = injection point; S = solvent front; I = chlorhexidine; flow-rate 2.5 ml min^{-1}; detector 238 nm; sensitivity 0.08 a.u.f.s.; methanol–water (65:35), 1000 μg ml^{-1} toluene-4-sulphonic acid as ion-pair reagent.

Recovery efficiency from urine was assessed from a standard pool sample spiked to give a concentration of 2 μg ml^{-1}. Ten 1-ml aliquots were removed and extracted. Two methods of agitation were assessed. Use of an ultrasonic bath was found to be less effective than mechanical agitation. The latter procedure is thus recommended. Recovery of chlorhexidine from urine was determined to be 89% with a coefficient of variation for the ten samples of 1.9%. A similar experiment using blood gave a recovery efficiency of 68% (10 aliquots, coefficient of variation 6.3%) at the 2-μg ml^{-1} level. This recovery reduced to 48% (coefficient of variation 7.8%) if ultrasonic agitation was used.

Chlorhexidine in urine

The developed assay has been successfully used in a preliminary study of chlorhexidine in urine. Specimen samples of urine were collected from patients (8) immediately prior to cystoscopy. An instillation of chlorhexidine (200 μg ml^{-1}) was

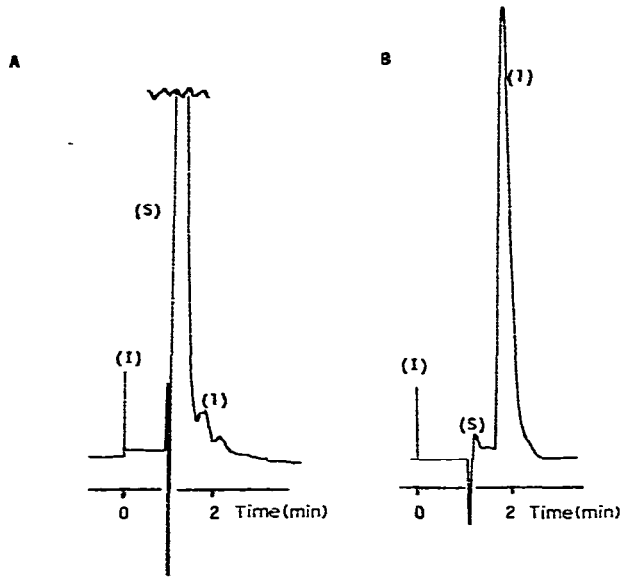

Fig. 4. (A) Chromatogram of blank urine extracted with ether. (B) Chromatogram of spiked urine extracted with ether (conditions as for Fig. 3).

made and the examination carried out. Urine samples were then collected about 5 min after completion of the examination. No trace of chlorhexidine was found in any patient prior to instillation of the chlorhexidine solution. All patients showed relatively high levels of chlorhexidine after treatment with values in the range 112–170 $\mu g\ ml^{-1}$ with a mean value of 153 $\mu g\ ml^{-1}$. After 25 min the level of chlorhexidine in one patient had reduced from 170 to 159 $\mu g\ ml^{-1}$ probably due to dilution by accumu-

TABLE I

THE CHLORHEXIDINE CONTENT OF SOME COMMON PHARMACEUTICAL PREPARATIONS

Pharmaceutical preparation	*Nominal concentration ≡ 100% (w/v)*	*Specific function*	*Chlorhexidine relative to standard (= 100%) (%)*
"Hibitane" (gluconate concentrate)	20	Stock solution for pharmacy use	101
"Hibiscrub" (gluconate in detergent)	4	Skin disinfection	92
Chlorhexidine gluconate in 70% industrial methylated spirit	0.5	Skin disinfection	102
"Hibiscrub" (gluconate in water)	0.2	Skin cleansing	98
"Corsodyl" (gluconate in water)	0.2	Mouthwash	96
Chlorhexidine acetate	0.02	Bladder irrigation	109

lated urine. No interference from other co-extracted compounds was observed for any of the samples.

Chlorhexidine in pharmaceutical preparations

To facilitate accurate comparison between the nominal value of a pharmaceutical preparation containing chlorhexidine and the determined value all preparations investigated were diluted to a nominal 10 $\mu g\ ml^{-1}$. Triplicate injections were made, the average response measured, corrected for the conjugate base if necessary and compared with a freshly prepared 10 $\mu g\ ml^{-1}$ solution of chlorhexidine diacetate. The results are presented in Table I. Hospital preparations diluted in the pharmacy are normally acceptable if the composition is within 10% of the nominal value. In a related study[13] of the shelf life of chlorhexidine we observed no obvious deterioration of concentration for solutions in the range 50–2500 $\mu g\ ml^{-1}$ when stored either in acid-washed or deactivated (silanised) glass over a period of 49 days.

CONCLUSIONS

The method described provides a sensitive and quantitative assay for chlorhexidine in blood, urine and pharmaceutical preparations. The extraction step is short and simple. Recoveries from urine (89%) and blood (68%) are acceptable and reproducible. Heptane sulphonic acid, toluene-4-sulphonic acid and pentadecafluorooctanoic acid may be used as ion-pair agents. The method is sufficiently sensitive to permit the detection of chlorhexidine in body fluids and studies on clinical specimens obtained following urological applications of chlorhexidine are in progress.

Concern has been expressed about the possible toxic consequences of chlorhexidine degradation to 4-chloroaniline. Although 4-chloroaniline may not be determined simultaneously with chlorhexidine by this method, the use of HPLC prevents interference between the two species. A significant advance over the GLC method of determining chlorhexidine is thus achieved.

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