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IMPROVED METHOD FOR THE DETERMINATION OF CHLORHEXIDINE IN URINE

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SUMMARY

A high-performance liquid chromatographic method is described which is suitable for the determination of the common antiseptic chlorhexidine in urine. The method employs Sep-Pak cartridges to remove chlorhexidine from the urine matrix. Chromatographic separation was achieved on a C_{18} reversed-phase column using a mobile phase of methanol—20 m*M* sodium acetate solution (60 : 40) adjusted to pH 5 with glacial acetic acid. An ion-pair agent (pentadecafluorooctanoic acid) was used at a concentration of 100 μg ml^{-1}. 3-Bromobenzophenone was used as chromatographic standard (k' = 4.0). 4-Bromobenzophenone (k' = 3.9) or dibenzal hydrazine (k' = 4.4) may also be used. A series of urine samples was analysed and no interferences were observed. The method is simple and rapid with a total analysis time of ca. 30 min.

INTRODUCTION

Chlorhexidine (1,1'-hexamethylene-bis-5-(4-chlorophenyl)biguanide) was one of a series of polybiguanides first synthesised in the late 1940's. Its antibacterial activity [1] was found to be more potent than other established cationic antiseptics such as cetrimide and hence it was selected for use under the trade name of "Hibitane".

Being a dicationic compound (in neutral or mild acid or alkaline solutions) it is usually formulated as the digluconate to enhance the solubility in water. Its

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mode of action is via adsorption onto bacterial cell walls, a process which is favoured at higher pH values [2].

Methods currently available for the determination of chlorhexidine in pharmaceutical preparations where it is present in high concentrations are based on colorimetry [3] or high-performance liquid chromatography (HPLC) [4, 5]. The colorimetric method lacks sensitivity and is prone to interference. Of the two HPLC methods, one [4] is based on normal phase chromatography employing a silica column with an acetonitrile—0.04 *M* sulphuric acid mobile phase and detection at 254 nm. Bearing in mind that chlorhexidine is dicationic [2, 6] under these conditions it is likely that the separation is based on an ion-exchange process with the silica providing the stationary ionic sites. The other HPLC method [5] is based on a reversed-phase separation employing a C_{18} column and a methanol—water mobile phase containing an ion-pairing agent. Detection was at 238 nm. None of these methods is particularly suited to the study of chlorhexidine in body fluids where relatively low concentrations may be encountered. The HPLC method which employs the silica column suffers from poor analyte peak shape and hence low sensitivity. The reversed-phase HPLC method relies on a solvent extraction step of only moderate efficiency coupled with quantitation by calibration curve.

A sensitive method for the determination of chlorhexidine using gas—liquid chromatography (GLC) has been reported [7]. This method would appear to be very sensitive but, in addition to the extraction procedure from biological samples, a derivatization of the chlorhexidine involving hydrolysis to 4-chloroaniline followed by diazotisation and iodination to 4-chloroiodobenzene is required. Thus this procedure fails to distinguish between 4-chloroaniline resulting from the in vitro or in vivo decomposition of chlorhexidine prior to analysis and that derived from chlorhexidine by the analytical method. Recently [8], 4-chloroaniline has been detected and quantitated in pharmaceutical preparations of chlorhexidine and hence results obtained from this GLC method must be viewed with suspicion.

The physico-chemical properties of chlorhexidine indicate that HPLC with ultraviolet (UV) detection should be the analytical technique of choice. We now report an improved method for the determination of chlorhexidine in urine in which an efficient extraction step and quantitation via a chromatographic standard are combined to yield significant improvements over our previous method [5].

EXPERIMENTAL

Reagents and chemicals

The mobile phase was methanol (Willot Industrial, Bristol, U.K.)—20 m*M* sodium acetate buffer in double distilled water (60:40), adjusted to pH 5 with glacial acetic acid and modified with pentadecafluorooctanoic acid; 100 μg ml^{-1}, (BDH, Poole, U.K.). Degassing of the mobile phase was achieved by ultrasonication for ca. 10 min under reduced pressure (15 mmHg). All mixed solvents were stirred slowly but continuously whilst the chromatography was running.

Chlorhexidine was of analytical quality (97.6%; Bristol Royal Infirmary).

Quantitation was via the use of a chromatographic standard. Several compounds with suitable chromatographic characteristics were found including 4-bromobenzophenone (Aldrich, Dorset, U.K.), 3-bromobenzophenone and dibenzal hydrazine (Aldrich). All sample manipulations were carried out with conventional glassware. Small volume measurements were made with Hamilton 250-μl syringes. For calibration purposes standard solutions of chlorhexidine diacetate, incorporating the internal standard were prepared from methanolic stock solutions (1000 μg ml^{-1} of active ingredient) by serial dilution with the mobile phase.

Samples were collected routinely in screw-top plastic containers by staff at the Bristol Royal Infirmary and were stored at 4°C until analysed. Samples were extracted using C_{18} Sep-Paks (Waters Assoc., Northwich, U.K.) by a process detailed below.

The liquid chromatograph consisted of an LC-XPD Model 100 pump (Pye Unicam, Cambridge, U.K.), a Rheodyne Model 7010 loop injector (PhaseSep, Clwyd, U.K.) with 20-μl sample loop, a μBondapak (C_{18}) column (30 cm × 4 mm I.D., 10 μm particles) (Waters Assoc.) and an LC—UV detector fitted with 8-μl cell (Pye Unicam, Cambridge, U.K.). Output was to a recorder (10 mV f.s.d.) or a suitable integrator (e.g. Minigrator, Erba Science, Swindon, U.K.). Other chromatographic parameters were: flow-rate 1.5 ml min^{-1}; back pressure 90 bar; detector wavelength 260 nm.; sensitivity of the detector 0.02 or 0.04 a.u.f.s. into 10 mV f.s.d.

Sample preparation

A C_{18} Sep-Pak was attached to a 20-ml glass syringe and methanol (2 ml) passed slowly through it to remove any adsorbed material and cleanse the cartridge prior to use. Water (2 ml) was then passed through the cartridge to remove any residual methanol. Urine (10 ml) was placed in the syringe barrel (which was clamped in a vertical position and was forced slowly (ca. 5 ml min^{-1}) through the Sep-Pak using the syringe barrel. After passage of the urine, water (2 ml) was passed through the Sep-Pak to remove unwanted polar materials. Subsequently passage of methanol (2 ml) desorbed the chlorhexidine and this eluate was collected. The chromatographic standard (3-bromobenzophenone in methanol) was added (using a 250-μl syringe) to the methanol eluate to give a final concentration (in 5 ml) of 10 μg ml^{-1}. The volume of eluate was then standardised to 5 ml using mobile phase. From this standardised volume (5 ml) an aliquot (20 μl) was injected onto the chromatograph.

RESULTS AND DISCUSSION

Chromatographic aspects

Chlorhexidine absorbs UV radiation strongly between 215 nm and 270 nm with an absorbance maximum at 260 nm ($\epsilon = 3.1 \cdot 10^4$ l mol^{-1} cm^{-1}). Previously we have selected the detector wavelength as 238 nm because of the restriction imposed by the use of toluene-4-sulphonic acid as the ion-pair agent. The use of an alternative, non-absorbing, ion-pair agent (see below) permits the detector wavelength to be set at 260 nm providing an increase in sensitivity of the order of 40%.

The column used for the determination of chlorhexidine was a μBondapak C_{18} column (Waters Assoc.). Several other reversed-phase materials were also evaluated to ensure general applicability of the method. From this evaluation it was clear that only reversed-phase materials which are fully end-capped are suitable for the elution of chlorhexidine. Columns which are not de-activated in this way should not be used. On such columns chlorhexidine is either completely retained or eluted with very poor peak shape.

Based upon previous results [5] a capacity factor (k') for chlorhexidine of 2 would be adequate to completely resolve the compound of interest from all likely interferences whilst maintaining an acceptable analysis time. However, an ion-pair agent is required to promote the retention of chlorhexidine on ODS columns. Hence the value of k' may be varied by adjusting both the methanol—water ratio and the concentration of the ion-pairing agent. Previously [5] we have used pentadecafluorooctanoic acid (PDFOA) as an ion-pairing agent. An arbitrary concentration of PDFOA was selected (100 μg ml^{-1}) and the water—methanol ratio varied. An 8 μg ml^{-1} standard solution of chlorhexidine was used. From these experiments a mobile phase consisting of methanol—water (60 : 40) containing PDFOA (100 μg ml^{-1}) was selected. This yielded a k' value for chlorhexidine of 1.6.

The control of mobile phase pH by the addition of buffers may be used to encourage (or discourage) the retention of ionic compounds on reversed-phase columns. Chlorhexidine is ionic under the chromatographic conditions used here and thus a complex set of equilibria exist which govern its chromatographic behaviour. These equilibria can be shifted by varying the mobile phase pH. This was done by using 20 m*M* sodium acetate as the aqueous part of the mobile phase and adjusting its pH between 3.0 and 5.0 with glacial acetic acid with a constant concentration of PDFOA (100 μg ml^{-1}). From this set of experiments an optimised pH of 5.0 was selected. At lower pH values (4.5 and 4.0) a second much smaller peak was observed to interfere with chlorhexidine. This peak only occurs when the injected solvent is not closely matched in composition to the mobile phase and presumably is the "system" peak [9] for this particular chromatographic system. It arises because PDFOA has a small absorbance at 260 nm (ϵ = 3.3 l mol^{-1} cm^{-1}) due to part of a very broad, weak band most probably arising from a $n \rightarrow \pi^*$ transition of the carbonyl group. Confirmation of the assignment of this peak to the system was achieved by first injecting water (a positive peak resulted) and then methanol from which a negative peak with an identical k' value was observed. For a detailed explanation of this phenomenon, which only occurs when one of the components of the mobile phase (in this case PDFOA) has a significant absorbance at the wavelength selected for detection, see reference [9].

In order to promote wide applicability alternative ion-pair agents were evaluated. As well as PDFOA, heptafluorobutyric acid (HFBA) and heptanesulphonic acid (HSA) were studied. The variation of k' (chlorhexidine) with concentration is shown graphically in Fig. 1. Clearly the concentration of PDFOA has a marked effect on retention when compared with either HFBA or HSA at concentrations above 100 μg ml^{-1}. There is little point in using more ion-pair agent than is necessary to achieve the desired separation. Quite apart from the cost, excess back pressure may be generated. Although all three ion-

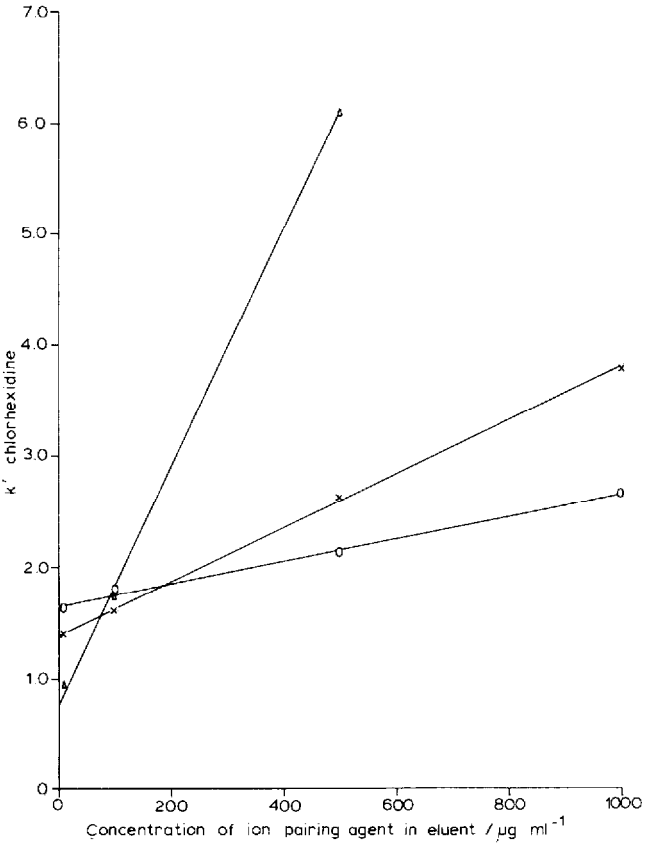

Fig. 1. Plot of k' (chlorhexidine) vs. concentration of various ion-pairing agents in methanol—aqueous acetate buffer (pH 5.0) (60:40) eluent. (○), HFBA; (×) HSA; (△) PDFOA.

pair agents may be used satisfactorily the low cost and wide availability of PDFOA justify its use.

In summary therefore, the optimised chromatographic parameters are a C_{18} reversed-phase column, which is fully end-capped, a mobile phase consisting of methanol—20 m*M* sodium acetate (pH 5.0) (60 : 40) with 100 μg ml^{-1} of PDFOA as ion-pairing agent. The recommended detector wavelength is 260 nm and the recommended flow-rate is 1.5 ml min^{-1}. Using a mobile phase of this complexity requires continual stirring at constant speed to maintain homogeneity and so prevent baseline drift.

Quantitative aspects

An internal standard is desirable in trace analysis of biological samples because it may compensate for some errors likely to occur during isolation and chromatography. Ideally an internal standard should be both chemically similar and chromatographically similar to the analyte species. This latter requirement includes the ability to respond significantly in the detection system. No material is available which fulfils these requirements with respect to chlorhexidine. Benzyl hibitane does fulfil these requirements but is not available

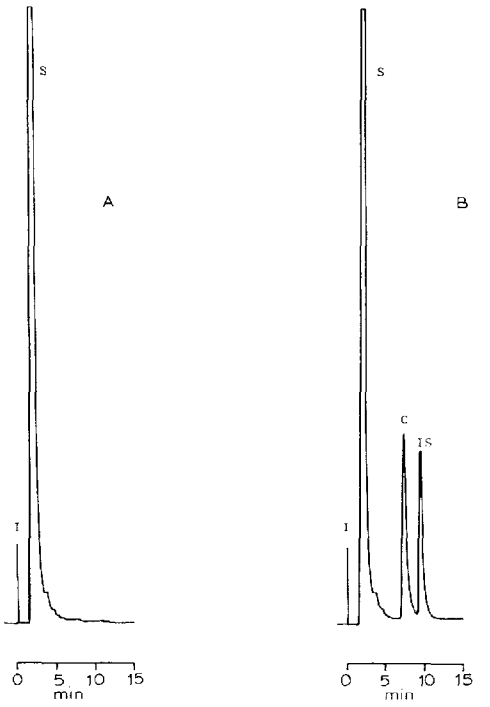

Fig. 2. A, Blank urine; B, urine spiked with chlorhexidine. Peaks: I = point of injection; S = solvent front and co-extracted material; C = chlorhexidine; IS = chromatographic standard. Parameters: column, μBondapak C_{18}; eluent, methanol—sodium acetate buffer (pH 5) (60 : 40) containing 100 μg ml^{-1} PDFOA; flow-rate, 1.5 ml min^{-1}; sensitivity, 0.04 a.u.f.s. into 10 mV f.s.d.; detection, 260 nm.

commercially. Hence a material which is chromatographically similar to chlorhexidine was chosen. Of the many compounds screened as possible chromatographic standards three were found to be suitable. These were 4-bromobenzophenone (k' = 3.9), 3-bromobenzophenone (k' = 4.0) and dibenzalhydrazine (k' = 4.4). 3-Bromobenzophenone was chosen because it was available in this laboratory and because it has a 40% larger extinction coefficient at the analytical wavelength ($\epsilon_{260\ nm} = 1.1 \cdot 10^4$ l mol^{-1} cm^{-1}) than dibenzalhydrazine and is thus similar to chlorhexidine ($\epsilon_{260\ nm} = 3.1 \cdot 10^4$ l mol^{-1} cm^{-1}). Using a mobile phase flow-rate of 1.5 ml min^{-1} both the analyte and the internal standard eluted in ca. 12 min (Fig. 2). 4-Bromobenzophenone (k' = 3.9) may also be used and provides a slight reduction in chromatographic analysis time when compared with 3-bromobenzophenone.

Previously [5] we have relied on quantitation via a calibration curve constructed prior to the determination of a set of samples i.e. external calibration. Use of a chromatographic standard permits quantitation via peak height ratios or peak area ratios. The level of chlorhexidine in real samples (urine) was expected to vary from 0—200 μg ml^{-1}. Calibration lines were constructed for the range 0—10 μg ml^{-1} and thus samples of concentration greater than 10 μg ml^{-1} would require dilution prior to analysis. The results of calibration are presented in Fig. 3. (Note: calibration is linear for the range 0—200 μg ml^{-1} and thus dilution is unnecessary if a reporting integrator is used. For peak height

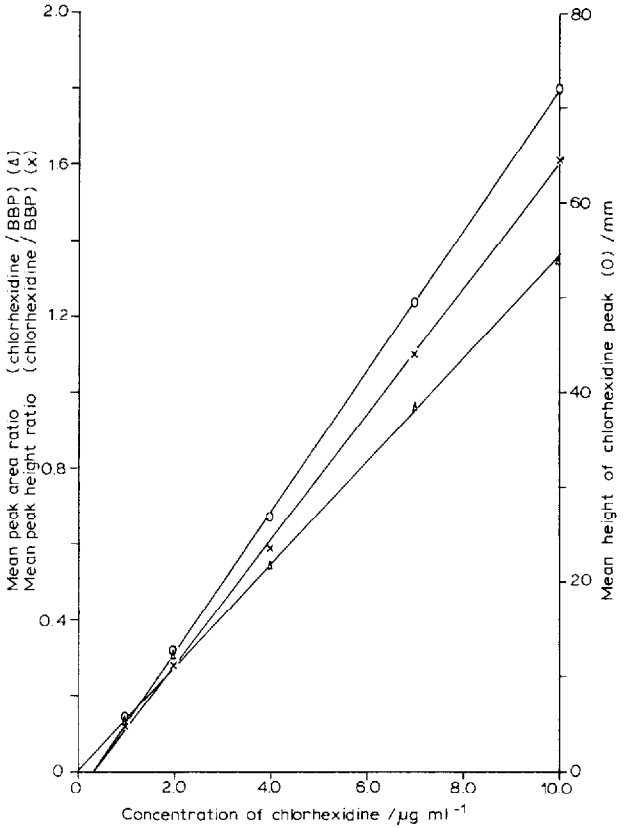

Fig. 3. Calibration graphs of mean peak area ratio (△), mean peak height ratio (×), and mean chlorhexidine peak height (○) vs. concentration for standard solutions of chlorhexidine chromatographed under normal operating conditions.

measurement a narrow range of concentrations should be used to minimise errors of measurement and thus dilution to a particular concentration range is recommended.) Quantitation by peak area ratio measurement resulted in a linear relationship between this parameter and the concentration of chlorhexidine, (regression equation: $y = 0.135x + 0.008$; correlation coefficient 0.9995). Similarly, quantitation by peak height ratio measurement resulted in a linear relationship, (regression equation: $y = 0.166x - 0.056$; correlation coefficient 0.9998). Direct quantitation by chlorhexidine peak height measurement against weight injected (external calibration) also yielded a linear relationship, (regression equation: $y = 7.42x - 2.41$; correlation coefficient 0.9999). Clearly all three methods of measurement are acceptable for quantitative analysis.

Sensitivity and detection limit

For the purposes of this investigation sensitivity was defined as that concentration of chlorhexidine which, when chromatographed, gave rise to a signal-to-noise (S/N) ratio of 20 : 1. Detection limit was defined as that concentration which gave rise to a S/N ratio of 2 : 1. For these experiments the detector sensitivity was set to the maximum, i.e., 0.01 a.u.f.s.: 10 mV f.s.d. The detection limit was determined to be 0.1 $\mu g\ ml^{-1}$ which corresponds to an injected

weight (20-μl sample loop) of 2 ng. Hence the sensitivity was 1.0 μg ml^{-1} or 20 ng weight injected. These values may be improved proportionately if a 50-μl sample loop is used without loss of chromatographic performance. The relative standard deviation (R.S.D.) for ten replicate injections of a 10 μg ml^{-1} chlorhexidine standard solution (with chromatographic standard) chromatographed using a 20-μl sample volume was 3.0%. Huston et al. [5] reported an R.S.D. of 5.0% using a 50-μl sample loop.

Recovery experiments

Chlorhexidine was extracted from urine by use of C_{18} Sep-Pak cartridges (Waters Assoc.). These were used as recommended by the manufacturer. Using a standardised procedure (see Experimental) ten aliquots (10 ml each) of a pooled urine sample spiked to give a concentration of 10 μg ml^{-1} were extracted. A blank urine sample was also extracted. Chromatograms of the blank (A) and spiked (B) samples are shown in Fig. 2. No interference was detected over the k' range of chlorhexidine and the internal standard. (Note: no interference was ever observed from the many extractions of blank urine specimens made during the optimisation of the Sep-Pak procedure.) From these ten extractions the mean recovery was 99.3% with an R.S.D. of 3.0%. Hence, within experimental error, recovery of chlorhexidine is quantitative and no correction need be made for extraction efficiency when assaying clinical samples. This recovery efficiency is significantly better than that obtained by the liquid—liquid extraction procedure [5]. A particular advantage of the use of the Sep-Pak extraction technique is that sample preparation time is reduced to ca. 7—8 min.

Although recovery was quantitative at a concentration of 10 μg ml^{-1} from a 10-ml sample (equivalent to a 100-μg loading of the cartridge) it was deemed necessary to determine whether the efficiency of extraction was constant over the range of concentrations likely to be encountered in real samples. To achieve this another series of samples was prepared by spiking blank urine with aqueous chlorhexidine solution to give concentrations of 1 μg ml^{-1} (≡ 10 μg loading), 10 μg ml^{-1} (≡ 100 μg loading), 100 μg ml^{-1} (≡ 1000 μg loading) and 200 μg ml^{-1} (≡ 2000 μg loading). These samples (two aliquots were taken), were extracted by the standard procedure. The recoveries for this range of concentrations indicate that chlorhexidine is recovered quantitatively across this range of concentrations. The concentration of chlorhexidine digluconate commonly used for bladder irrigation is 0.02% (w/v) which is equivalent to 160 μg ml^{-1} of chlorhexidine and thus this value is the highest concentration likely to be encountered with real samples. Considerably higher values may be found in urine collection bags and samples where a high chlorhexidine level is anticipated should be diluted (the red dye used to colour the pharmaceutical preparations provides a useful visual indicator of concentration).

Re-usability of Sep-Pak cartridges

Although this type of extraction cartridge is intended for single use the possibility of re-use was investigated. Twelve identical blank urine samples were spiked with an aqueous solution of chlorhexidine to a concentration of 10 μg ml^{-1}. Two Sep-Paks were used. For the first cartridge six samples were extracted sequentially with no washing of the cartridge between samples. The second

cartridge was washed with methanol (5 ml at 5 ml min^{-1}) and water (5 ml at 5 ml min^{-1}) between sample extractions. The two batches of six samples were then chromatographed.

Extraction was essentially quantitative for all the samples. There was no indication of any deterioration of extraction efficiency with successive extractions nor was any cross-contamination apparent.

Determination of chlorhexidine in clinical samples

Eighteen urine specimens were obtained from four patients who had undergone catheterisation — a process whereby a flexible plastic tube is inserted into the urethra. The catheter tube is connected to a plastic urine collection bag via a non-return value. On fitting a new bag, an aliquot (10 ml) of chlorhexidine digluconate (5%, w/v) is dispensed into the bag to sterilise the urine which accumulates. Concern has been expressed recently over the efficiency of the non-return valves particularly when the urine collection bags are subjected to pressure from, for example, the weight of the patient. In order to evaluate the functioning of the non-return valves urine samples were taken from the catheter tube above the non-return valve and from the urine collection bag below the valve. Samples may thus be categorised as either bag or catheter. As is not uncommon with samples collected routinely in hospitals four of the eighteen samples supplied were unlabelled. The optimised analytical procedure

TABLE I

CHLORHEXIDINE CONCENTRATIONS FOUND IN URINE SAMPLES TAKEN FROM CATHETER TUBING AND COLLECTION BAGS

Patient	Sample No.	Sample origin	Dilution factor	Concentration of chlorhexidine in original specimen ($\mu g\ ml^{-1}$)
A	1	Bag	1:10	89
	2	Unknown	1:1	0.15
	3	Catheter	1:1	0.15
	4	Catheter	1:1	<0.1
B	5	Catheter	1:1	<0.1
	6	Catheter	1:1	<0.1
	7	Bag	1:1	6.0
	8	Unknown	1:100	272
	9	Bag*	1:1	0.10
	10	Unknown	1:10	42
	11	Bag	1:100	135
	12	Unknown	0.3:1	0.48
C	13	Catheter	1:1	<0.1
	14	Catheter	1:1	0.28
	15	Bag	1:1000	1400
	16	Catheter	1:1	0.14
D	17	Catheter	1:1	<0.1
	18	Bag	1:1	274

*Sample probably mis-labelled.

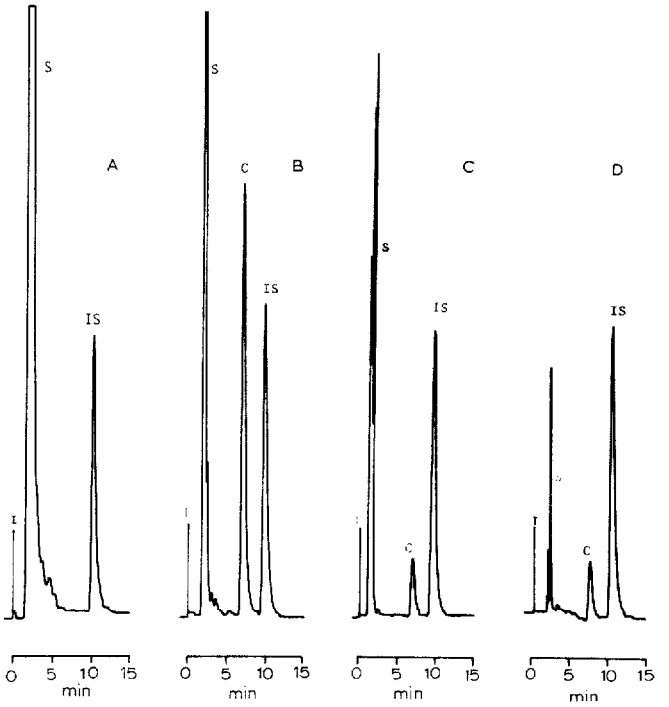

Fig. 4. Some typical chromatograms of urine extracts. A, urine with no chlorhexidine present; B, urine with a large absorbance due to co-extracted material (S) and a high chlorhexidine (C) content; C, urine with a large absorbance due to co-extracted material (S) but a low chlorhexidine (C) content, D, urine containing little co-extracted material (S) and a low chlorhexidine (C) content. For all chromatograms, IS = chromatographic standard and the conditions are as for Fig. 2.

was applied to these samples with appropriate dilution of the bag samples as necessary. Where sample volume was insufficient for a 10-ml aliquot to be extracted a semi-micro extraction procedure was employed. For this case a known volume of urine was passed through the Sep-Pak and the chlorhexidine was then eluted with methanol (2 ml). This methanol eluate was blown to dryness under a stream of nitrogen and then made up to 200 μl with chromatographic standard and mobile phase to give a sample for analysis. The results for the eighteen samples are presented in Table I. Examples of chromatographic traces are shown in Fig. 4. The results of these analyses are quite conclusive. Substantial quantities of chlorhexidine were found in samples taken from the urine collection bags (6—1400 μg ml^{-1}) as was expected, but only negligible amounts of chlorhexidine were detected in samples taken from catheters (< 0.3 μg ml^{-1}). In 63% of the urine samples known to have been taken from catheter tubes no chlorhexidine was detected. The four unlabelled specimens (Nos. 2, 8, 10 and 12) gave chlorhexidine values which fitted well into these two extremes and hence the source of these samples could be assigned with confidence. Specimen 9 was labelled as a sample taken from a urine collection bag but was found to contain only a trace of chlorhexidine. In this case it was suspected that either the specimen was mis-labelled and was actually taken from a catheter tube or that addition of chlorhexidine digluconate to the urine collection bag had been omitted.

The determined chlorhexidine levels in these urine samples indicate that the non-return valves function correctly in these cases. Trace levels of chlorhexidine were found in 37% of the urine samples taken from catheter tubes. This probably arose from detection of the remains of a gel containing chlorhexidine which is used to disinfect the urethra during catheterisation and which may have translocated down the catheter tube.

CONCLUSIONS

Chlorhexidine may be quantitatively extracted from urine using Sep-Paks. The resultant extracts may be chromatographed successfully on a reversed-phase C_{18} column using a methanol—sodium acetate buffer (pH 5.0) (60 : 40) mobile phase and detected by UV absorption at 260 nm. The detection limit is 0.1 $\mu g\ ml^{-1}$. The chlorhexidine present may be quantitated by reference to 3-bromobenzophenone as chromatographic standard with a within-batch precision of 3.0% R.S.D. No between batch precision has been established. No interference from co-eluting compounds has been observed.

The developed method has been used to demonstrate that the non-return valves in urine catheter bags function effectively.

The method is simple, rapid, and suitable for routine hospital use.

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