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Note

Gas–liquid chromatographic determination of chlorhexidine in pharmaceutical formulations

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Chlorhexidine (1,1′-hexamethylenebis[5-(*p*-chlorophenyl)biguanidel]) (Fig. 1), a common antiseptic, was described by Davies *et al.*[1] in 1954.

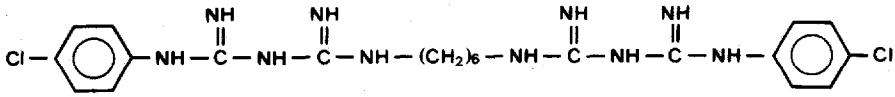

Fig. 1. Structural formula of chlorhexidine.

Various analytical techniques have been employed for the quantitative analysis of chlorhexidine. Among them, non-chromatographic methods, spectrophotometry[2,3], colorimetry[4,5], polarography[6] and gravimetry[7], were used. Other methods utilizing thin-layer chromatography (TLC)[8,9], high-performance liquid chromatography (HPLC)[10–15] and gas–liquid chromatography (GLC)[16–17] were also described.

In the GLC methods, the derivatization takes place on a fragment obtained by degradation of the chlorhexidine molecule. Chlorhexidine is hydrolysed by 25% NaOH to *p*-chloroaniline. This molecule is then transformed into *p*-chloroiodobenzene which is analysed by GLC with electron capture detection. Although the method is relatively sensitive and linear, it cannot be used for stability testing because the analysed molecule is a degradation product of chlorhexidine concentrations in pharmaceutical forms, using derivatization of the whole molecule.

The quantitative analysis was completed by the structural determination of the silyl derivative of chlorhexidine by mass spectrometry (MS) and nuclear magnetic resonance (NMR) studies[18].

MATERIALS AND METHODS

Reagent and standards

All chemicals and solvents were of analytical grade. The reagents for silylation,

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N,O-bis(trimethylsilyl)acetamide (BSA) and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), were obtained from Pierce. The standard of chlorhexidine digluconate (20% in water) was obtained from Lederle (Oullins, France). The internal standard was dibutyl phthalate. The pharmaceuticals tested were creams containing 1.1% of chlorhexidine digluconate.

Apparatus

A 5710 A gas chromatograph (Hewlett-Packard) equipped with a 2 m × 2 mm glass column packed with 3% OV-101 on Chromosorb W HP (80–100 mesh) and a flame ionization detector was used. The output signal was integrated and the results were calculated using a Hewlett-Packard 3385 A electronic integrator. The column was conditioned at 250°C for 16 h with a nitrogen flow-rate of 50 ml/min. Separations were performed with oven temperature programming from 120°C to 240°C at 8°C/min. The injector and detector temperatures were 150°C and 250°C respectively. Nitrogen was used as carrier gas at a flow-rate of 30 ml/min.

Mass spectrometric (MS) analysis was carried out first on a VG Micromass

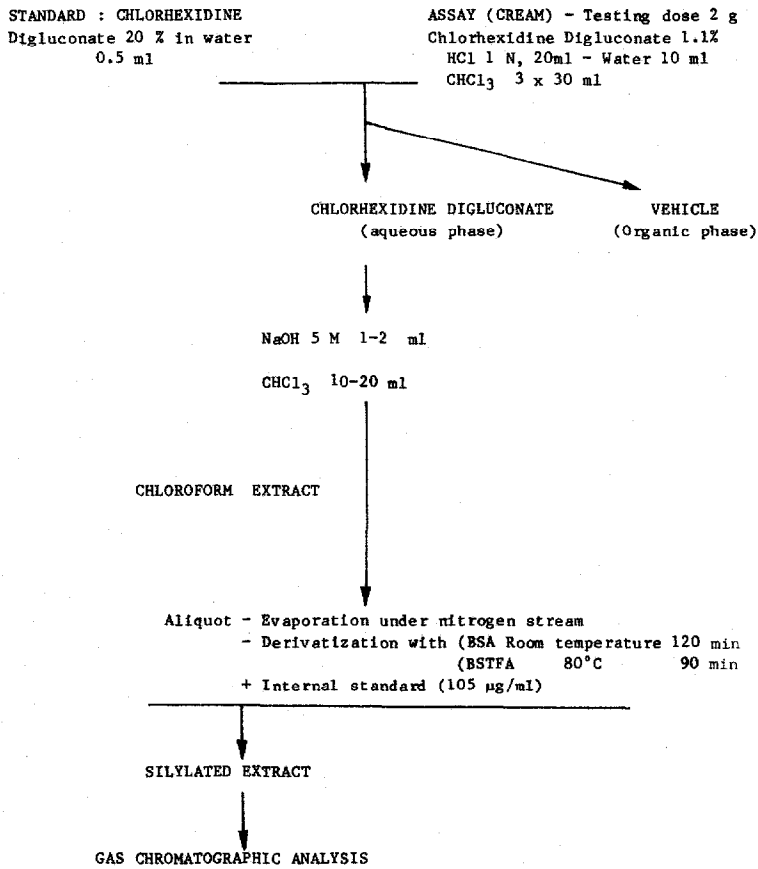

Fig. 2. Scheme of the method used for the GLC analysis of chlorhexidine from creams.

305F using chemical ionization (CI) (NH_3) and desorption chemical ionization (DCI). In order to prevent extensive fragmentation, spectra were also obtained with a fast atom bombardment (FAB) source on a ZAB-HF mass spectrometer. Samples of the silyl derivative were introduced into the source by the direct introduction probe. For DCI and CI analysis, the trap current was set at 500 μA. For FAB analysis on the ZAB mass spectrometer, the acceleration voltage was set at 8 kV.

NMR analyses were carried out on a Bruker spectrometer operating at room temperature and 80 MHz. Samples were dissolved in deuterochloroform.

Extraction

The scheme used for the chlorhexidine assay in pharmaceuticals (cream) is shown in Fig. 2. The initial step involves the vehicle elimination by extraction with chloroform (3 × 30 ml) in an acidic medium (1 *M* hydrochloric acid, 20 ml). Chlorhexidine is then extracted from the alkalinized aqueous phase (5 *M* sodium hydroxide solution, 1 ml) with chloroform. An aliquot of the extract is evaporated to dryness under a stream of nitrogen and submitted to derivatization. Silylation can be carried out either with BSA at room temperature for 120 min of with BSTFA at 80°C for 90 min. The internal standard (dibutyl phthalate) was added at a concentration of 105 μl/ml to the extract with silylation reagents. The silyl derivative is quite stable and can be stored at 4°C until GC analysis. A 0.5-μl aliquot of the silylation product is then injected into the chromatograph. When cream was assayed for chlorhexidine determination, 2 g of the preparation containing 1.1% chlorhexidine digluconate were extracted.

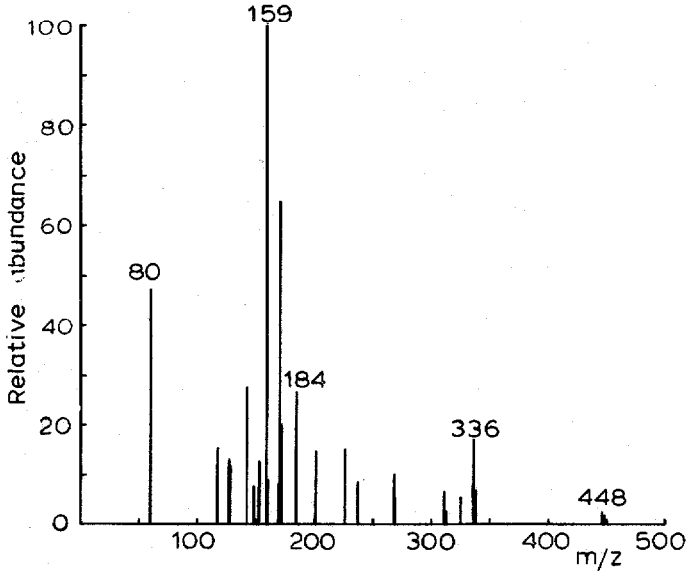

Fig. 3. CI mass spectrum of pure chlorhexidine.

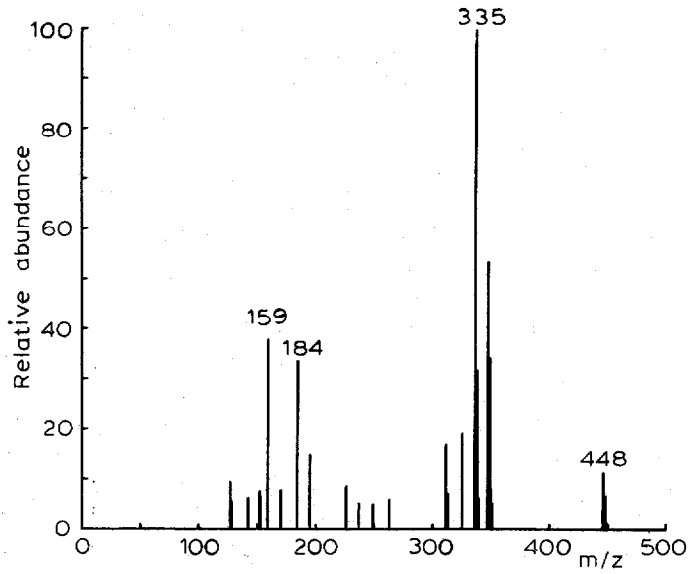

Fig. 4. DCI mass spectrum of pure chlorhexidine.

RESULTS

Structural analysis of derivatives

CI and DCI mass spectra of pure chlorhexidine are given in Figs. 3 and Fig. 4 respectively. These spectra only differ in the relative abundances of the fragment ions due to the difference between the ionization energies in CI and DCI modes. The molecular ion at *m/z* 504 cannot be seen, but a spectrum recorded after FAB ionization showed an ion $M + H^+$ at *m/z* 505. According to the ionization process, the two main fragment ions are recorded at *m/z* 159 and *m/z* 336. They are the base peaks of the mass spectra in CI mode and DCI mode respectively. The ion at *m/z* 159 is formed via the rearrangement of a chlorine atom and corresponds to the C_6H_4–NH–CNH–NH–CN moiety. The ion at *m/z* 336 has one chlorine atom as can be seen by the isotopic cluster (*m/z* 336–338) and corresponds to the N≡C–NH–$(CH_2)_6$–NH–CNH–NH–CNH–NH–C_6H_4–Cl moiety. Then, the loss of a fragment of 152 a.m.u., N≡C–NH–C_6H_4–Cl, gives rise to the fragment ion at *m/z* 184: HN–CH–NH–$(CH_2)_6$–NH–CNH–NH_2. So the main fragmentation pathway corresponds to the successive losses from the molecular ion of two fragments of 170 a.m.u. and 152 a.m.u. respectively. The intense fragmentation of the molecule either in CI modem or in DCI mode shows that the chlorhexidine molecule is rather fragile.

The spectra of the silyl derivative of chlorhexidine are shown in Fig. 5. The first spectrum was recorded 55 sec after introducing the probe into the mass spectrometer source; the second spectrum (Fig. 5b) was recorded 1 min after the sample introduction.

The mass spectrum of the derivative after FAB ionization showed two peaks at *m/z* 505 and 407. The base peak of the first spectrum is at *m/z* 326, and corresponds to the loss of two fragments of 170 and 152 a.m.u. from a molecule containing two

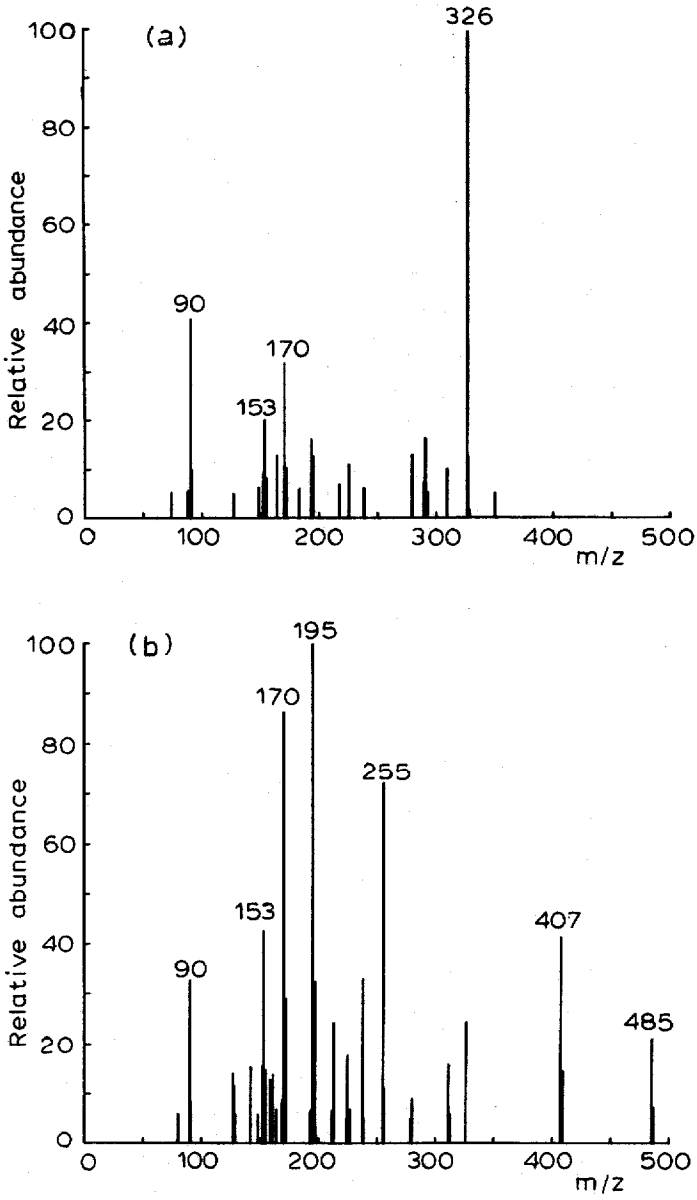

Fig. 5. Cl mass spectra of the disilyl derivative of chlorhexidine: (a) 55 sec after introduction of the probe; (b) 1 min after introduction of the probe.

trimethylsilyl groups (mol.wt. 648). The second spectrum shows fragments at *m/z* 407 and 255 corresponding to the successive loss of 170 a.m.u. and 152 a.m.u. from an ion at *m/z* 576 containing only one trimethylsilyl group. In this spectrum, the base peak at *m/z* 195 corresponds to the fragment Cl–C_6H_4–NH–CNH–NH–CN. These spectra show that chlorhexidine gives a derivative containing two trimethylsilyl

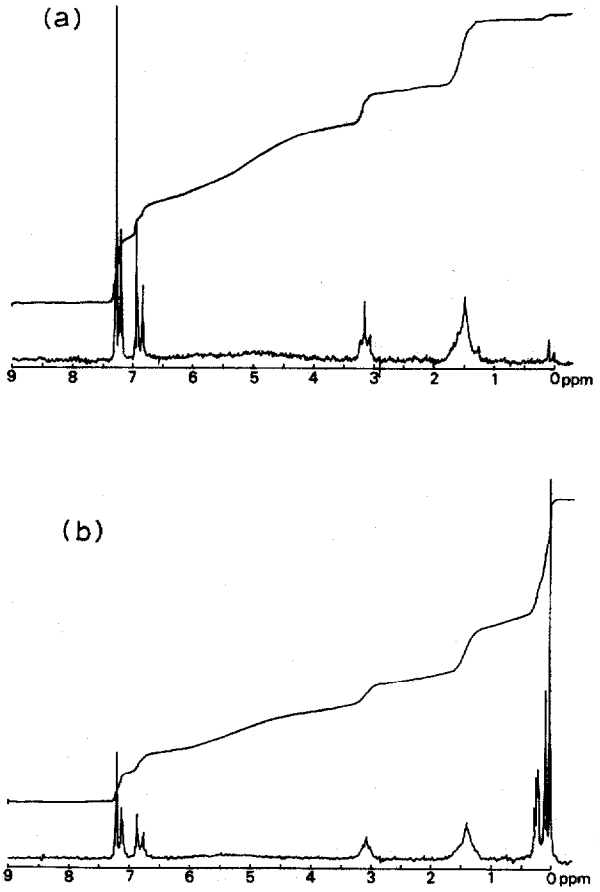

Fig. 6. NMR spectra of pure chlorhexidine (a) and of the disilyl derivative of chlorhexidine (b).

groups and that this compound is labile in the mass spectrometer at the source temperature. NMR spectra confirm the presence of two trimethylsilyl groups in the molecule. Fig. 6a shows the NMR spectrum of chlorhexidine and Fig. 6b the spectrum of the silyl derivative. It can be seen that up to 0.03 ppm, eighteen protons have been added to the molecule, corresponding to two trimethylsilyl groups. In the interval 4–6 ppm, eight hydrogen atoms are present instead of ten in the spectrum of the underivatized molecule. We concluded that the derivative determined by GLC after silylation corresponds to the whole molecule of chlorhexidine containing two trimethylsilyl groups.

Chromatographic analysis

The retention times for the silyl derivative of chlorhexidine and the internal standard were 5.76 and 10.44 min respectively. Blanks carried through the same procedure and analysed by GLC did not show any interference at the corresponding retention times.

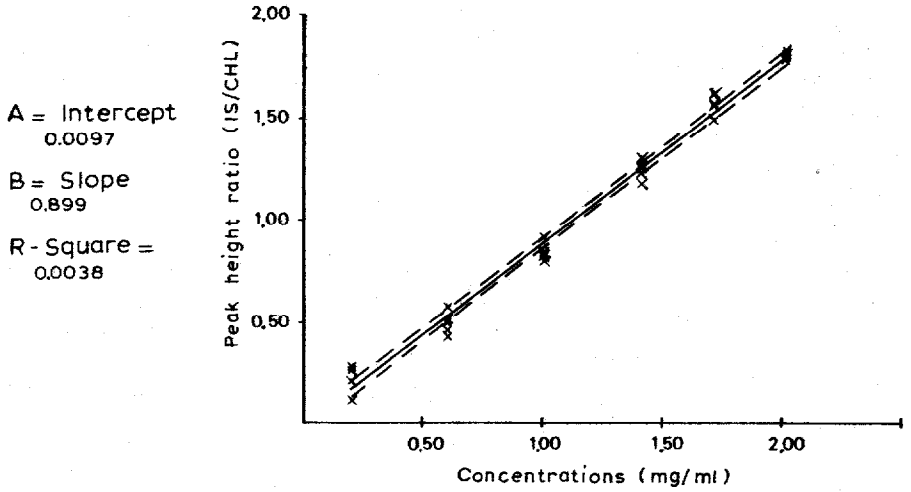

Fig. 7. Calibration curve for GLC analysis of chlorhexidine (CHL) from pharmaceutical creams. IS = Internal standard.

Precision

Precision was studied by determination of the repeatability, carried out by calculation of the relative coefficient of variation from injections of fourteen samples, the injected amount corresponding to the analysis of 2 g of ointment containing 1.1% of chlorhexidine digluconate. The repeatability of the method showed a relative standard deviation of 2.35% (n = 14) which is acceptable for GLC analysis after derivatization.

The reproducibility was studied by analysis of variances on five series of samples. Each series was injected five times. This determination gave the result F = 2.24 and $P\,(F^4_{17})$ = 0.89, with no significant difference between the samples.

Accuracy

The accuracy of the method was determined on five series of samples with concentrations ranges from 0.2 to 2 mg/ml, each series being injected five times. Each result calculated from the standard curve was compared to the real value 100 $\Delta C/C$ = 1.1% for the higher value, which corresponds to an accuracy of 98.9%.

Recovery of chlorhexidine from creams

To study the analytical recovery of chlorhexidine from ointments, six different samples from two batches of cream containing 1.1% of this drug were extracted and analysed according to the described method. For the first batch the mean recovery was 99.36 ± 1.47%; for the second, 99.41 ± 2.67%. So, the percentage of chlorhexidine digluconate in the preparation was 1.093 ± 0.017 and 1.092 ± 0.04 respectively.

Linearity

Linearity was measured under the conditions described above with six different chlorhexidine concentrations ranging from 0.2 to 2 mg/ml. Standardization was per-

formed using the internal standard method. A linear regression analysis of peak height ratios *versus* concentration indicated a good linear fit of the data (Fig. 7): slope 0.899; intercept −0.0097; r 0.993. The same procedure performed with the same concentrations of chlorhexidine in pharmaceutical ointment gave the following results: slope 0.859; intercept −0.044; r 0.992.

CONCLUSIONS

The GLC method described provides a new sensitive, quantitative assay for determining chlorhexidine in pharmaceutical preparations (cream and ointments). The extraction step is rather short and simple. The main advantage of the method lies in the quantitative derivatization of the whole molecule to give a disilyl derivative. The method will allow stability studies in the quality control of the product. It is quite linear, specific, reliable and precise.

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