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Note

Analysis of Chlorhexidine via gas–liquid chromatography

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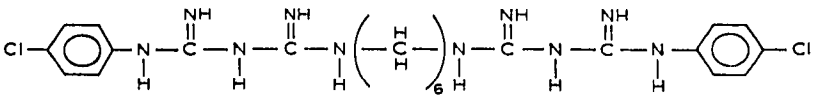
The germicidal attributes of Chlorhexidine —1,1′-hexamethylenebis[5-(*p*-chlorophenyl)-biguanide]— are well documented by Davies *et al.*[1], Lawrence[2], and Cancro *et al.*[3].



1,1′-Hexamethylenebis[5-(p-chlorophenyl)-biguanide]

Spectrophotometric methods have been utilized for Chlorhexidine analysis. Holbrook[4] developed a method for Chlorhexidine analysis which depends on the formation of a red-colored complex between Chlorhexidine and sodium hypobromite, and subsequent spectrophotometric analysis in the visible range. A spectrophotometric method utilizing the UV range (250 nm), in which Chlorhexidine is measured directly, is not specific for the compound in question[5]. Chlorhexidine was also determined by hydrolyzing the compound, then complexing it with Marshall's reagent to form an azo dye, which was subsequently measured colorimetrically[6]. However, one detrimental aspect of all the above spectrophotometric methods for Chlorhexidine analysis is their susceptibility to interferences from other compounds that may be present in solution.

Baunok and Geissbuehler[7] carried out analyses of urea herbicide residues, compounds which are structurally similar to Chlorhexidine. Specifically, the herbicide was hydrolyzed to *p*-chloroaniline; the *p*-chloroaniline was derivatized to *p*-chloroiodobenzene, the latter being analyzed by an electron capture detector on a gas chromatograph. Because of the structural similarities of the urea herbicide residues to Chlorhexidine, it was decided to attempt a similar analysis.

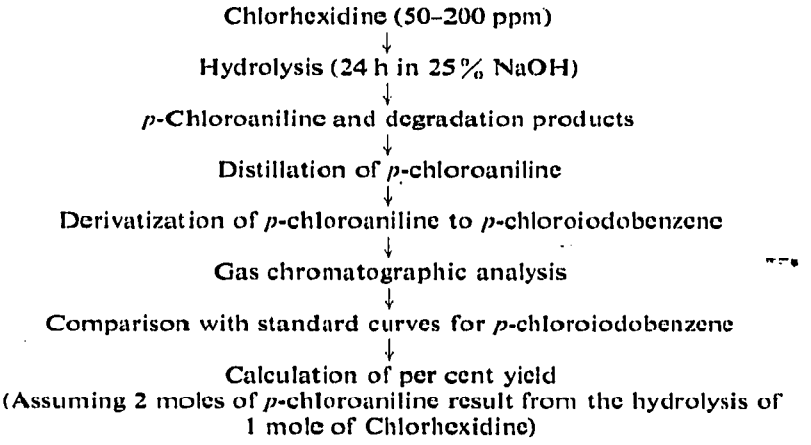


Fig. 1. Scheme used for the GLC analysis of Chlorhexidine.

EXPERIMENTAL

The scheme used for the analysis of Chlorhexidine can be seen in Fig. 1. The initial step involved the base hydrolysis of Chlorhexidine to *p*-chloroaniline; using a custom designed digestion/distillation head the *p*-chloroaniline was distilled into hexane[7]. The hexane extract was treated with 1 *N* HCl to form the *p*-chloroaniline salt; the hexane was then evaporated by a stream of nitrogen; *p*-chloroaniline was derivatized to *p*-chloroiodobenzene and subsequently analyzed by gas–liquid chromatography (GLC). After comparison with previously prepared standard curves for *p*-chloroiodobenzene, the theoretical and actual yields of *p*-chloroiodobenzene from the original quantity of Chlorhexidine were calculated and compared in terms of a percentage yield.

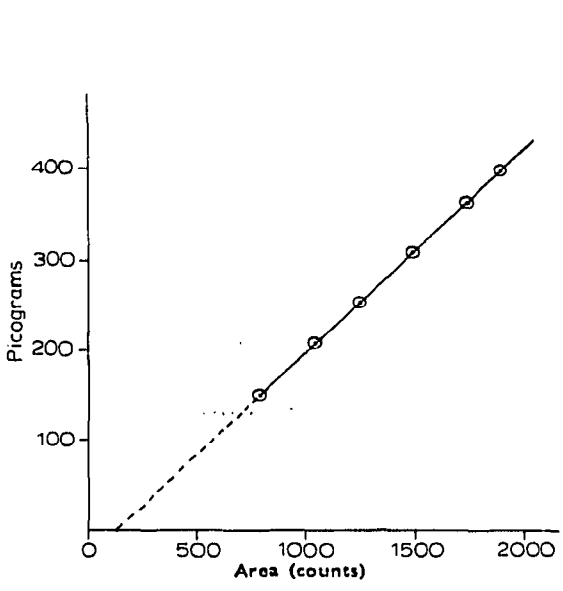
Chlorhexidine in the form of Chlorhexidine diacetate was obtained, compliments of Imperial Chemical Industries (Macclesfield, Great Britain), and used throughout the study. Throughout this note the term Chlorhexidine will be used to designate the actual substance used —Chlorhexidine diacetate.

Hydrolysis of Chlorhexidine and distillation of p-chloroaniline

The initial step for Chlorhexidine analysis involves the hydrolysis of Chlorhexidine to *p*-chloroaniline and subsequent distillation of *p*-chloroaniline into hexane. The effects of pH on this reaction have been previously determined by Jaminet *et al.*[8]. It was found that as the pH value increases, the rate of hydrolysis increases. Thus, it was decided to use 25% NaOH as the hydrolyzing agent. A time/rate study, to be discussed later, indicated that maximum yields of *p*-chloroaniline would be obtained upon 24-h hydrolysis. (The 24-h hydrolysis was adopted for the procedure.) At each concentration of Chlorhexidine investigated (10–200 ppm) the experiments were carried out in quadruplicate. A blank, consisting of all ingredients but Chlorhexidine was carried through the procedure along with each set of experiments.

Derivatization of p-chloroaniline to p-chloroiodobenzene

The hexane solution containing *p*-chloroaniline was treated in a beaker with



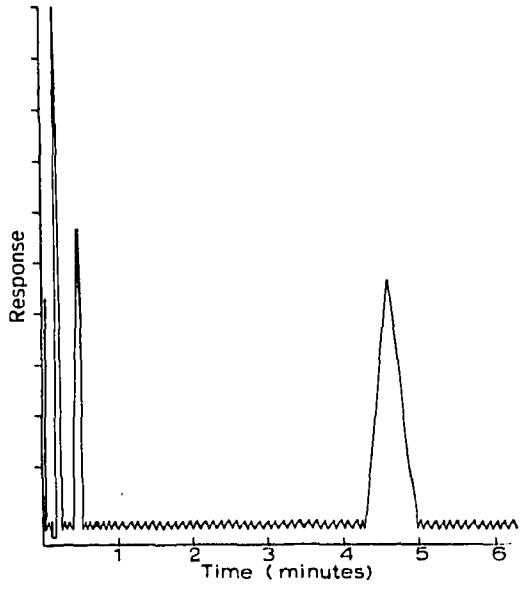


Fig. 2. Standard curve for *p*-chloroiodobenzene via GLC analysis.

Fig. 3. Gas–liquid chromatogram of Chlorhexidine (50 ppm). For parameters, see Table I.

5 ml of 1 *N* HCl to form the non-volatile *p*-chloroaniline salt. The hexane was evaporated by a stream of nitrogen. Additional 1 *N* HCl was added to the residue and the volume was brought to 100 ml; the *p*-chloroaniline could now be derivatized to *p*-chloroiodobenzene. A 10-ml aliquot of this solution was placed in a 50-ml screw-capped erlenmeyer flask; the solution was then cooled in an ice–salt-bath to a temperature of 3°. After addition of 2 ml of a 1% $NaNO_2$ solution, the sample was allowed to stand in the ice-bath for 30 min to accomplish diazotization. Excess nitrite was destroyed by adding 2 ml of a 10% ammonium sulfamate solution and shaking the flask vigorously until nitrogen generation had ceased. To the still cold diazonium salt solution, 0.5 ml of KI–I_2 (2.5 g iodine in 50 ml of a 10% potassium iodide solution) was added and the mixture was allowed to stand at room temperature for 30 min. Excess iodine was then destroyed by adding approximately 200 mg of sodium sulfite (the solution turns colorless). The solutions were then made alkaline by adding 1 ml of 50% NaOH. Hexane (20 ml) was also added. After shaking vigorously for 1 min, the layers were allowed to separate and the hexane layer, which contained the *p*-chloroiodobenzene, was poured off into a small vial having a PTFE-lined cap. The sample was now stored at 0° until needed for GLC analysis.

Gas–liquid chromatographic procedure for p-chloroiodobenzene analysis

Table I contains all the operating parameters for the GLC of *p*-chloroiodobenzene. A Hewlett-Packard Model 5750B chromatograph equipped with a ^{63}Ni detector was used for analysis and a Hamilton Model 701N syringe was used to inject 5 μl of sample. Integration of peaks was accomplished by a disc integrator. The column packing (Supelco, Bellefonte, Pa., U.S.A.) was conditioned at 250° for 24 h.

TABLE I

OPERATING PARAMETERS USED FOR THE GLC ANALYSIS OF *p*-CHLOROIODOBENZENE

Column	5 ft. × 1/8 in. stainless steel
Packing	10% OV-3 on Chromosorb W HP (60–80 mesh)
Carrier gas	90% argon–10% methane
Flow-rate	100 ml/min (helium)
Column temperature	130°
Detector temperature	310°
Injector temperature	135°
Pulse rate	150/sec.
Range × attenuation	10 × 16 ($10^{10}\ \Omega$)

Standard curve for p-chloroiodobenzene

A standard curve for *p*-chloroiodobenzene was constructed by dissolving reagent grade *p*-chloroiodobenzene in hexane in varying concentrations of 10–100 pg/μl and injecting 5 μl of each standard. The linear response of the electron capture detector can be seen in Fig. 2. Each point on the curve represents the average of injecting 5-μl samples in triplicate.

Each day GLC determinations of unknowns were intermingled with standard solutions. Any variances in the ^{63}Ni response were corrected. Fig. 3 shows a chromatogram resulting from the analysis of Chlorhexidine (50 ppm). Only one peak can be observed, corresponding to *p*-chloroiodobenzene.

Blanks

A blank consisting of all ingredients, except Chlorhexidine, was hydrolyzed and used through the remainder of the procedure. Contamination by *p*-chloroaniline was an initial problem. However, adsorption of *p*-chloroaniline on the hydrolysis/distillation heads, which was the cause, was corrected by allowing the heads to run with base and hexane overnight. The base and hexane were discarded and the glassware was dried. After this procedure, blank runs showed no significant peaks on subsequent GLC.

RESULTS

Recovery of p-chloroaniline in the derivatization and GLC steps

Two solutions (100 and 10 ppm) of *p*-chloroaniline were carried through the derivatization and subsequent GLC analysis for *p*-chloroiodobenzene. The experiment was carried out in triplicate at both concentration levels. The average recovery of *p*-chloroaniline in both cases was found to be 36.4%.

Time/rate study on Chlorhexidine analysis

To validate thoughts on Chlorhexidine hydrolysis, a time/rate study was initiated. Questions to be answered by such an investigation included: (1) Can the hydrolysis time be significantly decreased? (This would make for an easier laboratory arrangement for hydrolysis.) (2) Does Chlorhexidine degrade in two steps, *viz.* one

p-chloroaniline molecule breaking off Chlorhexidine initially, then the second *p*-chloroaniline breaking off later?

Chlorhexidine (100 ppm) was placed under base digestion (25% NaOH) for varying periods of time. The resulting *p*-chloroaniline was derivatized to *p*-chloroiodobenzene and subsequently analyzed by GLC. The concentration of *p*-chloroiodobenzene, resulting from the procedure, was then stoichiometrically related to Chlorhexidine. Using these calculations, it was observed that after a 24-h hydrolysis, the hydrolysis step was 94% complete. The derivatization–GLC steps give a yield of 36.4%. If additional heat is applied to the reaction mixture, derivative yields of 78% could be obtained[9,10]. It was decided for convenience and safety to use the technique without supplemental heat, especially since reproducibility and sensitivity —as seen later— were excellent.

It was decided to utilize a 24-h hydrolysis because of considerations related to efficiency, working hours in an industrial plant, etc. Any hydrolysis period greater than 4 h but less than 20 h would cause serious problems with regard to working hours in industry.

Because two peaks were not observed in a graph of hydrolysis time *vs.* *p*-chloroaniline, the *p*-chloroaniline molecules on either end of the Chlorhexidine molecule hydrolyze from the parent molecule at the same time. Also, the curve is smooth and without steps.

Per cent recoveries of various concentrations of Chlorhexidine upon hydrolysis, distillation, derivatization and GLC analysis

Analyses of various known concentrations of Chlorhexidine by the technique previously described are summarized in Table II. In virtually every case, the experiment consisted of running the hydrolysis of Chlorhexidine at a particular concentration in quadruplicate and an associated blank was carried through the same procedure.

TABLE II

CALCULATED RECOVERIES OF CHLORHEXIDINE UTILIZING HYDROLYSIS, DISTILLATION, DERIVATIZATION AND GLC TECHNIQUES PREVIOUSLY DESCRIBED

Concentration of Chlorhexidine used for hydrolysis (ppm)	*Recovery of Chlorhexidine (%)*	*Average deviation*
200*	24.4	1.0
100*	27.7	2.5
50**	25.8	1.6
10**	7.7	1.0

* Nine hydrolyses, two derivatives per hydrolysis and four GLC injections per derivative.
** Eight hydrolyses, two derivatives per hydrolysis and four GLC injections per derivative.

The per cent recovery of Chlorhexidine was calculated by working out the theoretical yield of *p*-chloroiodobenzene from a particular concentration of Chlorhexidine being hydrolyzed, determining the actual quantity of *p*-chloroiodobenzene obtained via experiment and subsequently comparing the two values. It can be seen that the recovery of Chlorhexidine on analysis is approximately 25% for concen-

trations between 50 and 200 ppm. Considering the number of steps involved, the average deviations are relatively low.

In order to determine the reason(s) for the low recovery of Chlorhexidine at the 10-ppm level, it was decided to carry *p*-chloroaniline through the entire experimental procedure. Solutions containing various concentrations of *p*-chloroaniline (10–200 ppm) were carried through the entire analytical procedure: hydrolysis, distillation, derivatization and GLC analysis. The per cent yields showed no differences between runs at the various concentrations. Thus, the relatively low yield at the 10-ppm Chlorhexidine level cannot be explained.

The technique represents a powerful qualitative and quantitative tool for Chlorhexidine analysis in the range of 50–200 ppm, since only 10 ml of the solution containing 1 *N* HCl and *p*-chloroaniline (total volume 100 ml) was used for derivatization and subsequent GLC analysis.

CONCLUSIONS

A very sensitive GLC technique has been developed for the analysis of Chlorhexidine. Chlorhexidine can be analyzed with a minimum of 50 ppm with a low yield but with good reproducibility. The reproducibility of the technique in the 50- to 200-ppm Chlorhexidine range was ± 1.3%.

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