A Selected Ion Monitoring Assay for Chlorhexidine in Medical Waste Water

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We have developed a method for determination of chlorhexidine, a disinfectant, in medical waste water by selected ion monitoring (SIM). Our method consists of the following procedures. (1) Chlorhexidine in the medical waste water was extracted and purified by an Extrelut® column with ethyl acetate. (2) The eluted chlorhexidine was converted to a triazine derivative with trifluoroacetic anhydride. (3) The triazine derivative was subjected to on-column methylation on a gas chromatographic column. (4) Finally, the triazine *N*-methyl derivative of chlorhexidine was identified and determined by SIM. Recoveries of the compound added to medical waste water were about 90% in amounts ranging from 1 to 10 μg. In the waste water from our medical waste water treatment plant, chlorhexidine concentrations were found to be 0.085–1.94 mg l^{-1}. The present method is superior to the pre-existing ones in its quick separation, specificity and sensitivity.

INTRODUCTION

Many drugs and chemical reagents are always present in waste water from medical centers. Disinfectants are used daily and discharged into medical waste water. From the results of investigation on disinfectants consumed in Hamamatsu University Medical Center we found that larger quantities of cresol, benzethonium chloride and chlorhexidine were used as compared to other preparations.[1] Chlorhexidine with four guanido groups in its chemical structure has strong and wide antimicrobial ability. This compound is generally employed as a disinfectant for hands and instruments and discarded directly into medical waste water. Therefore, its effects on the environment and activated sludges are suspected to cause problems.

Several researchers have reported colorimetric,[2–5] gas chromatographic[6] and high pressure liquid chromatographic[7,8] determinations of chlorhexidine in disinfectants. However, there are few reports on the method applied to waste and environmental waters.[9,10] In this paper, we report a new SIM assay for chlorhexidine in medical waste water, which includes Extrelut® column chromatography and on-column methylation.

EXPERIMENTAL

Water samples

Samples from our medical waste water treatment plant were collected on 22 July, 19 August, 16 September, 21 October, 18 November and 16 December, 1981.

Reagents

Extrelut columns were obtained from E. Merch, Darmstadt, FRG; trifluoroacetic anhydride from Wako Pure Chemical Industrial Ltd., Osaka; 0.2 M trimethylanilinium hydroxide in methanol from Pierce Chemical Co., Rockford, Illinois; and 1% OV-1, OV-17 and OV-25 on Chromosorb W, 60–80 mesh, from Nihon Chromato Works Ltd., Tokyo. Other common chemicals used were of the highest purity commercially available. Free chlorhexidine was prepared by the following liquid–liquid partition procedure from chlorhexidine-2HCl (ICI Pharma Ltd., Osaka). About 2g of chlorhexidine-2HCl was dissolved in 1 N HCl solution, and 10 N NaOH solution was added to it to adjust its pH value to more than 12.5. The solution was extracted three times with ethyl acetate, washed with distilled water and then dehydrated with anhydrous sodium sulphate. It was crystalized by evaporating ethyl acetate in a rotary evaporator. Finally, the compound was recrystallized in methanol. The purity of the free chlorhexidine was checked by mass spectrometry and gas chromatography mass spectrometry (GC/MS), and no peaks due to impurities were observed in SIM. Both standard stock solutions of free and the HCl salt of chlorhexidine were prepared.

Apparatus

A combined gas chromatograph mass spectrometer (JEOL D-300) equipped with a computer (JMA 2000) was used.

RESULTS

Extraction

Either buffer or NaOH solutions were added to 1–10 μg chlorhexidine of both free and HCl salt forms and their volume was adjusted exactly to 20 ml. These solutions were applied to the Extrelut columns, and eluted with different organic solvents such as ethyl acetate, ethyl ether, chloroform and dichloromethane to investigate the percentage of elution of chlorhexidine. The elution

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CCC–0306–042X/84/0011–0203 $02.00

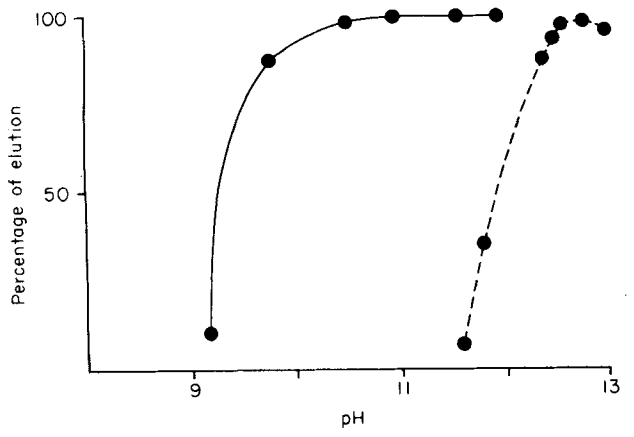

Figure 1. The percentage of elution of chlorhexidine from an Extrelut column with ethyl acetate as a function of pH of the chlorhexidine solution applied to the column. Buffer solutions (–•–) used were 0.1 M NH_4Cl-NH_3 buffer at pH 9.18–11.0, and 0.1 M Na_2HPO_4-NaOH buffer at pH 11.0–12.0. NaOH solutions (- - • - -) of various pHs were prepared by diluting 0.1 N NaOH solution.

percentage with ethyl acetate as a function of various pHs of the initial solutions is shown in Fig. 1. Ethyl acetate was adopted as an extraction solvent since the best recovery of the compound was achieved with this solvent. More than 95% of it was eluted from the columns with 30–60 ml ethyl acetate in pH 10.4–12.0 buffer solutions or in pH 12.5–13.0 NaOH solutions. There was no difference in elution pattern between free and HCl salt forms of the compound.

Derivatization

To 1–10 μg chlorhexidine dissolved in ethyl acetate, was added 0.2 ml trifluoroacetic anhydride to make a triazine derivative[11] of the compound at room temperature. The mass spectrum of this derivative is given in Fig. 2. Chlorhexidine was completely derivatized by mixing it for 1–3 min at room temperature; this was confirmed by mass spectrometry and GC/MS. It was possible to derivatize 600 μg of chlorhexidine with 0.2 ml trifluoroacetic anhydride.

On-column methylation

Since the peaks of the triazine derivative in SIM showed strong tailing due to the four residual imido groups, we methylated these groups on a gas chromatographic column.[12] The triazine derivative of chlorhexidine dissolved in 0.2 M trimethylanilinium hydroxide in methanol was injected into a gas chromatograph. It was confirmed by GC/MS that all chlorhexidine was converted to a triazine *N*-methyl derivative. The peaks determined by SIM using this on-column methylation were sharp and symmetrical.

Gas chromatographic columns

The columns of 1% OV-1, OV-17 and OV-25 all loaded on Chromosorb W 60–80 mesh were tested to determine the retention time of the triazine *N*-methyl derivative

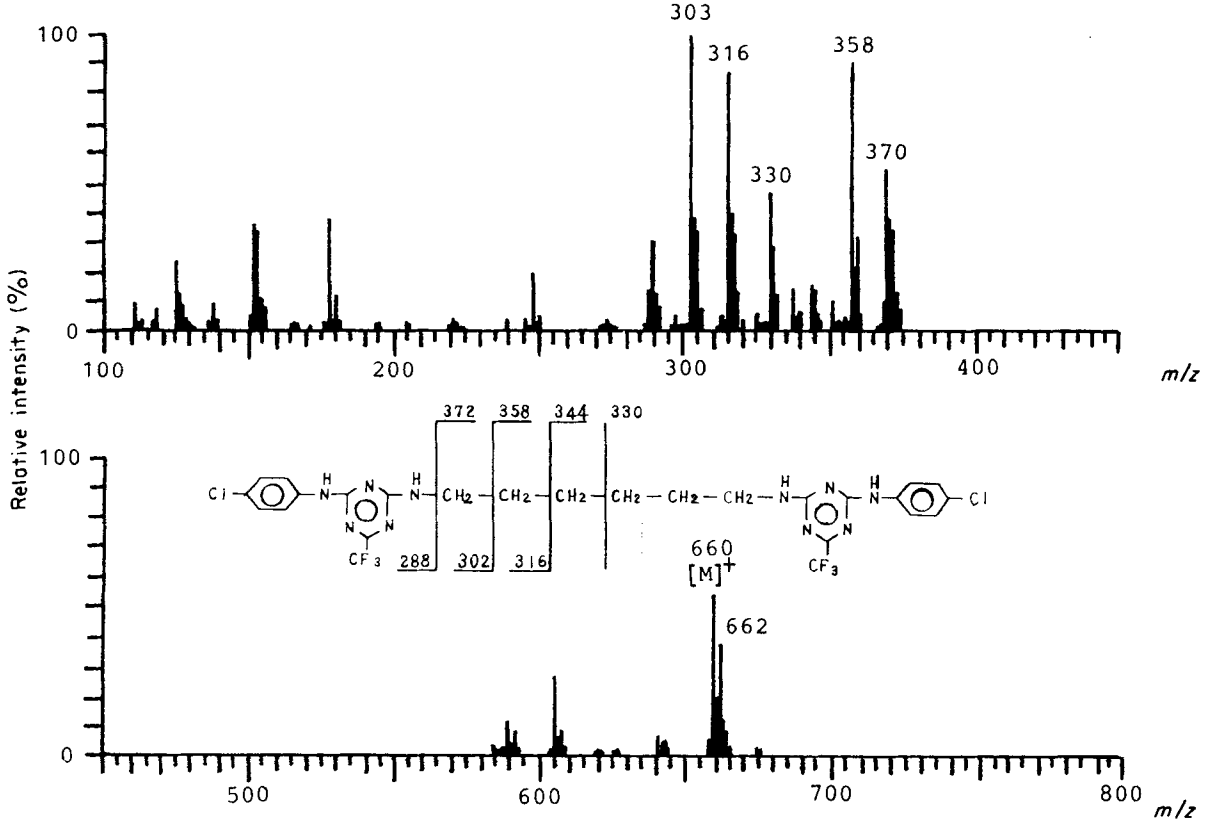

Figure 2. Mass spectrum of the triazine derivative of chlorhexidine.

Table 1. Operational conditions for GC/MS

Column	1% OV-1 on Chromosorb W 60–80 mesh, 1 m × 2 mm i.d.[a]
Column temperature	290 °C
Carrier gas	He 30 ml min^{-1}
Separator temperature	320 °C
Ion accelerating voltage	3.0 kV
Ionizing current	300 μA
Ionization chamber temperature	250 °C
Ionization energy	70 eV

[a] Glass column and solid support treatment: AW-DMCS.

by SIM. Using the OV-1 column, the retention time of the derivative was about 5 min at the column temperature 290 °C. With the OV-17 and OV-25 columns, the time was about 10 min at 330 °C or more. Therefore, the OV-1 column was adopted. Operational conditions for GC/MS are listed in Table 1.

Identification and determination

The mass spectrum of the triazine *N*-methyl derivative obtained from the standard chlorhexidine and the suspected fragmentation mechanism are shown in Fig. 3. There were ions at m/z 718 and 720, and a molecular ion at m/z 716, with an intensity ratio of 9:6:1 (m/z 716:718:720). The base peak of the derivative appeared at m/z 330 and strong fragment ions were observed at m/z 331, 344 and 386. SIM was carried out at m/z 330, 331 and 386. SIM of the derivative obtained from the standard chlorhexidine and the extract of a sample of medical waste water is shown in Fig. 4. There were no interfering peaks of impurities around the chlorhexidine peaks.

The derivative of chlorhexidine was identified on the basis of its retention time and its mass spectrum by direct comparison with those of the standard compound. However, when the amount of the compound was less than 20–30 ng, chlorhexidine was identified only on the basis of its retention time in SIM. The determination was carried out by preparing the absolute calibration curve with the peak area in SIM. The linearity of the calibration curve was obtained from 0.1 to 50 ng of this derivative per the gas chromatographic injection volume. The limit of sensitivity was 50 pg in the injection volume.

Determination of chlorhexidine in medical waste water

On the basis of the above data, the following procedure was adopted to determine chlorhexidine in medical waste water. To 100 ml of the waste water, was added 1.0–3.0 ml of 1 N NaOH solution to adjust its pH to 12.5–13.0. A 20 ml aliquot of the solution was applied to an Extrelut column and then eluted with 40 ml of ethyl acetate. To the eluate concentrated to about 0.1 ml under a reduced pressure, was added 0.2 ml trifluoroacetic anhydride and mixed vigorously for 2 min at room temperature. The mixture was evaporated to dryness under N_2 and the residue was dissolved in 0.2 M trimethylanilinium hydroxide in methanol. It was subjected to on-column methylation on a gas chromatographic column connected to a mass spectrometer. The

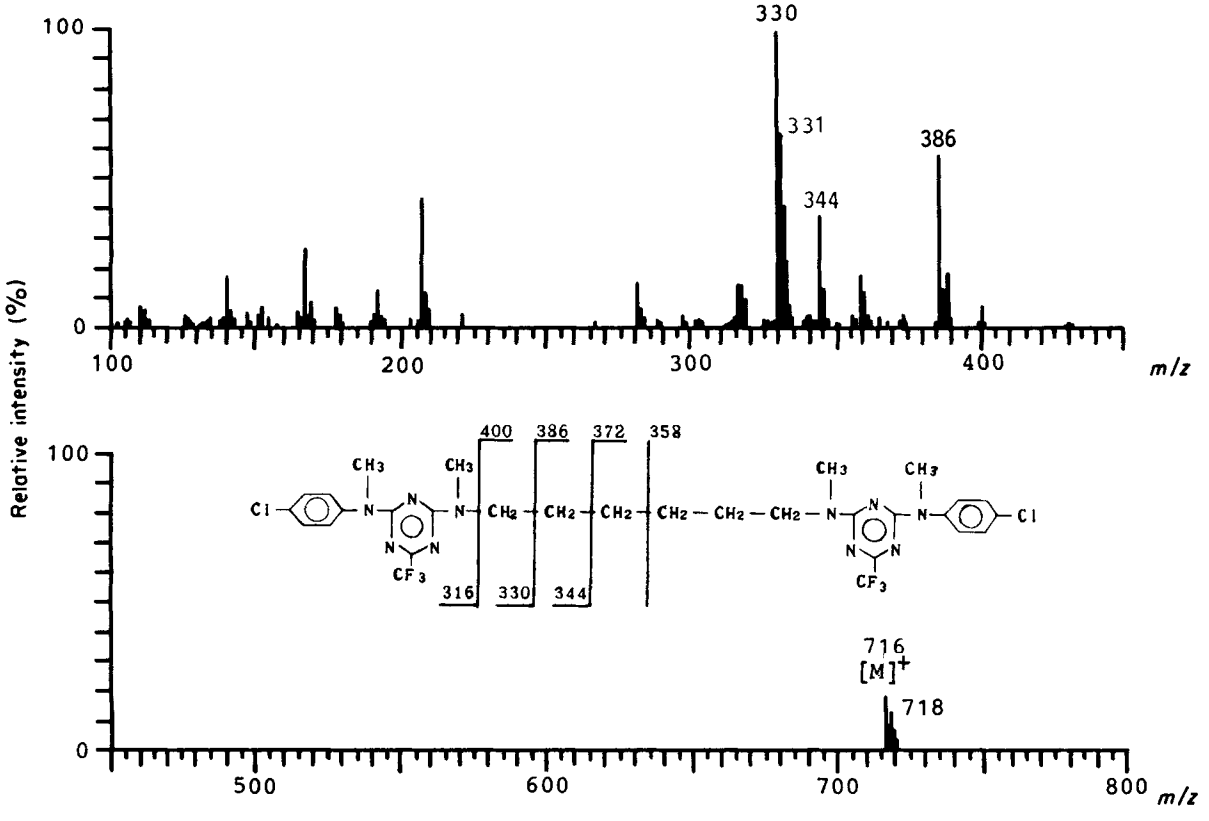

Figure 3. Mass spectrum of the triazine *N*-methyl derivative of chlorhexidine.

Table 2. Chlorhexidine in medical waste water from Hamamatsu University Medical Center

Date	Chlorhexidine[a] (mg l^{-1})
22 July 1981	0.21 ± 0.01 (3)[b]
19 August 1981	0.085 ± 0.01 (3)
16 September 1981	0·23 ± 0.03 (3)
21 October 1981	0.22 ± 0.02 (3)
18 November 1981	1.94 ± 0.05 (3)
16 December 1981	0.51 ± 0.05 (3)

[a] Means ± SD were given.
[b] The number of experiments is in parentheses.

amounts of chlorhexidine were determined by monitoring three ions at m/z 330, 331 and 386 to ensure specificity of this assay.

Chlorhexidine (1 and 10 μg) was added to 20 ml water and processed by our method to investigate the recovery of the compound. The experiment was repeated five times. The recoveries were 88.0% for 1 μg and 92.7% for 10 μg of the compound added. The precision was 6.1 and 4.2%, respectively.

The results obtained with samples of medical waste water by this method are shown in Table 2; chlorhexidine concentrations were found to be 0.085–1.94 mg l^{-1}.

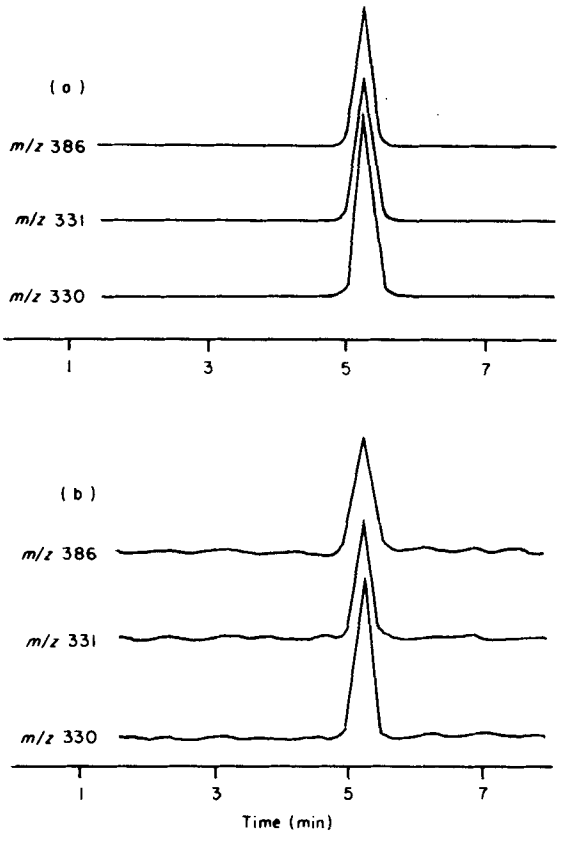

Figure 4. SIM for the standard chlorhexidine (a) and a medical waste water extract (b).

DISCUSSION

For the determination of chlorhexidine in disinfectants, colorimetric,[2–5] gas chromatographic[6] and high pressure liquid chromatographic[7,8] methods have been employed. However, there are few reports on the method applied to waste and environmental waters.[9,10] The colorimetric method for the analysis of chlorhexidine in waste water reported by Yamayoshi *et al.*[9] is not sensitive (detection limit, 0.2 μg ml^{-1}). Although gas chromatography and high pressure liquid chromatography are relatively quick, their disadvantage is the unreliability of its specificity. The present method with SIM is simple, rapid, sensitive, and quite specific for chlorhexidine.

In the mass spectrum (Fig. 3) of the triazine *N*-methyl derivative of chlorhexidine, there were ions at m/z 718 and 720 in addition to the molecular ion at m/z 716 with an intensity ratio of 9:6:1. These ions are due to two chlorines of the derivative. The ion at m/z 331 presumably results from the addition of the proton to the ion at m/z 330.

Yamayoshi *et al.* reported by a colorimetric method that the concentrations of chlorhexidine in medical waste water were in the range of hundreds of μg l^{-1}. Matsushima and Sakurai[10] also determined the compound by gas chromatography and obtained similar values. The results reported by the present method agree well with those in the literature.[9,10]

Miyazawa *et al.*[13] have reported that the concentrations of chlorhexidine capable of affecting the bacterial activity of activated sludges are in the range of hundreds of mg l^{-1} in its digluconate form. Therefore, it is considered that chlorhexidine, at the concentrations of 0.085–1.94 mg l^{-1} (Table 2), in our medical waste water does not affect the activated sludges of our plant.

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Received 6 July 1982; accepted 13 May 1983