Case Report

Toxicological Analysis of Chlorhexidine in Human Serum using HPLC on a Polymer-Coated ODS Column

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Abstract

A simple and reliable high-performance liquid chromatographic (HPLC) method for analyzing chlorhexidine in human serum was developed. After the addition of an internal standard, levomepromazine, 0.2 mL serum was deproteinized with 10% perchloric acid. The acidic supernatant was neutralized with 1M potassium carbonate solution, and the insoluble salt was removed by centrifugation. An aliquot of the supernatant was applied to HPLC with UV detection (260 nm). HPLC separation was achieved on a polymer-coated ODS column equilibrated with acetonitrile/water containing 0.05% trifluoroacetic acid, 0.05% heptafluorobutyric acid, and 0.1% triethylamine (40:60, v/v). The calibration curve was linear in the concentration range from 0.05 to 50.0 pg/mL, and the lower limit of detection was 0.05 pg/mL. The accuracy and precision of the method were evaluated at concentrations of 0.5 pg/mL and 5.0 pg/mL. The coefficients of variation ranged from 4.0 to 4.5%. The concentration of chlorhexidine in the serum of a patient who died after a suspected intravenous injection of chlorhexidine gluconate was determined.

Introduction

Chlorhexidine, 1,1'-hexamethylene-bis[5-(4-chlorophenyl) biguanide] (Figure 1), is an effective antibacterial agent and has been widely used in hospitals as an antiseptic agent and as a disinfectant. It is a strong base and is stable in the form of its salts such as acetate, hydrochloride, and gluconate. Among these salts, chlorhexidine digluconate is most commonly used because of good water solubility. Although the toxicity of this drug is very low by oral administration (LD50 1800 mg/kg in mice), it is high by intravenous administration (LD50 22 mg/kg in mice) (1). Chlorhexidine is known to induce allergic reactions such as urticaria after skin cleaning and urethral instillation (2) and occasionally induce severe anaphylactic shock (3–5). Cytotoxicity of this drug on blood cells and marked chondrolysis in the knee have been also reported (6,7). Because chlorhexidine gluconate solution can be found in many places in a hospital, accidental intravenous injection of this fluid into a patient is possible (8). Therefore, determination of the level of this drug in human serum has to be done, especially in cases of forensic toxicological examinations.

Chlorhexidine has been analyzed in serum, urine, and saliva using HPLC with a silica-based reversed-phase column, carried out by adding an ion-pair reagent such as sodium heptanesulfonate to the mobile phase (9–11). However, many authors mentioned quantitation problems caused by irreversible adsorption of chlorhexidine onto this column (10,12,13). We developed a more reliable HPLC method using a polymer-coated silica-based reversed-phase column, and we determined the serum level of chlorhexidine of a patient who died after a suspected intravenous injection of chlorhexidine gluconate solution.

Experimental

Reagents

Chlorhexidine dihydrochloride and chlorhexidine digluconate were purchased from Sigma Chemical (St. Louis, MO). Levomepromazine maleate was provided by Yoshitomi Pharmaceutical (Osaka, Japan). Trifluoroacetic acid, heptafluorobutyric, and triethylamine were purchased from Wako Pure

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Figure 1. Structure of chlorhexidine.
Chemical Industries (Osaka, Japan). The other chemicals were of analytical reagent grade.

**Biological samples**

Serum samples were obtained from healthy volunteers, and serum from the patient was obtained at autopsy. All samples were kept at -20°C until analysis.

**Standard solutions**

Chlorhexidine hydrochloride (5.72 mg) was dissolved in methanol, and the volume was adjusted to 5 mL to give a concentration of 1000 ng/µL as free chlorhexidine. This solution was further diluted to the required concentrations. Levomepromazine maleate (6.77 mg) was dissolved in methanol in the same manner.

**Extraction procedure**

The serum sample (0.2 mL) was mixed with 50 µL of 10% perchloric acid and 5 µL internal standard solution (5 µg levomepromazine) in a polypropylene conical tube (1.5 mL) and mixed vigorously for 30 s. The mixture was then centrifuged at 2000 × g for 5 min. Twenty microliters of 1M potassium carbonate was added to the supernatant to neutralize the solution, and insoluble salts were removed by centrifugation at 2000 × g for 5 min. An aliquot of the supernatant (20–50 µL) was used for HPLC determinations.

**Conditions of HPLC**

The apparatus used was a model LC100 HPLC system (Yokogawa, Tokyo, Japan) with a UV detector. The UV wavelength was set at 260 nm. The analytical column was a Capcell Pak C18 MG (3.0 mm × 250 mm, 5 µm, Shiseido, Japan). The column temperature was maintained at 40°C. The column was eluted with acetonitrile/water containing 0.05% trifluoroacetic acid, 0.05% heptafluorobutyric acid, and 0.1% triethylamine (40:60, v/v) at a flow rate of 0.8 mL/min.

**Preparation of calibration curves**

Serum samples were prepared so as to contain chlorhexidine at concentrations of 0.5, 1.0, 5.0, 10.0, and 50.0 µg/mL, each containing 25 µg internal standard. These samples were extracted in the same manner as previously described. The calibration curve was obtained by plotting the peak-area ratio of chlorhexidine to internal standard versus the amount of chlorhexidine.

**Results and Discussion**

**Extraction procedure**

We first extracted chlorhexidine with methylene chloride under alkaline conditions using the method reported by Lam et al. (9). However, the yield varied considerably, especially for the internal standard, and quantitation results were not reproducible. Tsuchiya et al. (14) used the acidic deproteinization procedure with perchloric acid for analyzing chlorhexidine in saliva. When we used their method, a constant recovery for both chlorhexidine and internal standard was obtained. However, acidic extracts quickly damaged the column, and a significant tailing of the chlorhexidine peak was observed on the chromatogram. These problems were overcome by neutralizing the acidic supernatant with 1M potassium carbonate solution, and insoluble salts were removed by centrifugation. The colorless clean extracts gave sharp and symmetrical peaks of chlorhexidine and did not damage the column.

**Selection of internal standard**

A number of authors have mentioned the difficulties of selecting IS, using solvent extraction procedures, and they analyzed chlorhexidine without the use of an internal standard (9,11). We examined several compounds, including diphenhydramine, chlorpheniramine, chlorpromazine, levomepromazine, and some benzodiazepines, as the internal standard in

![Figure 2. HPLC chromatograms of chlorhexidine at two different column temperatures.](https://academic.oup.com/jat/article/26/2/119/739225)
our extraction procedure; we found that every compound could be reproducibly extracted. Among these compounds levomepromazine was selected as the internal standard because of its good separation from chlorhexidine and good peak shape.

**HPLC conditions**

Chlorhexidine is a dicatonic compound with $pK_a$ values of 10.3 and 2.2. Therefore, at the general operating pH range of 2–7 with reversed-phase chromatography, it would primarily be ionized and retained poorly by the non-polar stationary phase. For a proper retention of chlorhexidine on the column, an ion-pairing agent such as heptanesulfonic acid is often added to the mobile phase of HPLC. When we used a silica-based ODS column, no peak appeared, perhaps because of chlorhexidine adsorption onto the silanol residue of the column. Ha and Cheung (15) reported the usefulness of a polymer-based reversed-phase column. When we used this column, the chlorhexidine peak appeared on the chromatogram but with significant tailing. There was also a limitation for setting the flow rate because of the lower pressure limit than that seen with use of the silica-based column. These problems were overcome using a polymer-coated silica-based column. The peak shape was sharp, and sensitivity was higher.

The peak shape for chlorhexidine was also influenced by the ion pair reagent and column temperature. Volatile organic acids which have been used as ion-pair reagents for LC–MS (16) yielded a better peak shape for chlorhexidine than did heptanesulfonic acid. The peak shape was significantly improved by increasing the column temperature, as shown in Figure 2. The optimal conditions were obtained by adding organic acids, trifluoroacetic acid and heptafluorobutyric acid, as ion-pair reagents and setting the column temperature at 40°C.

**HPLC determination of chlorhexidine in human sera**

HPLC–UV chromatograms of extracts from blank serum and spiked serum containing 5 µg/mL of chlorhexidine and 25 µg/mL of internal standard are shown in Figure 3. Each peak was clearly separated, and there were no interfering peaks in blank human serum. The calibration curve was linear in the concentration range from 0.05 µg/mL to 50.0 µg/mL with correlation coefficients of 0.999. The lower limit of detection, at a signal-to-noise ratio of 3, was 0.2 ng on column and 0.05 µg/mL in serum with 0.2 mL sample volume. The absolute recoveries of chlorhexidine in serum at concentrations of 0.5 and 5.0 µg/mL were determined by comparing the peak area of chlorhexidine in samples with those in standard solutions directly injected onto the column; the calculated recoveries were 35 and 41%, respectively. When the precision of this method was examined at serum concentrations of 0.5 and 5.0 µg/mL, the coefficients of variation were 4.0 and 4.5%, respectively ($n = 5$); hence, good reproducibility of the method was confirmed.

**Toxicological examination**

A 58-year-old Japanese woman with articular rheumatism underwent synovectomy on her left middle finger. The next morning she was injected via the indwelling venous catheter with antibiotics followed by 10-mL saline solution containing heparin sodium anticoagulant by a nurse. Immediately after the injection, she felt uncomfortable, and cardiac arrest occurred 20 min later. Intensive cardiopulmonary resuscitation was unproductive, and she died 101 min after the injection. During investigation of the cause of death, the possibility was raised that the patient was injected with 20% chlorhexidine gluconate solution prepared for another patient with diabetic gangrene. Our team was requested to do a toxicological examination. Serum was collected from the right heart at the time of autopsy 27 h after death. The sample was preserved at –20°C until analysis.

Chlorhexidine was clearly detected by our method with no interfering peaks as shown in Figure 4, and the UV spectrum of the peak was identical to that of standard chlorhexidine. The concentration of chlorhexidine in the serum of the patient was 39.5 µg/mL.

Concentrations of chlorhexidine in blood were determined by
at least two groups of investigators. Nilsson et al. (17) detected chlorhexidine in sera from 34 of 96 patients, the levels being 0.010 to 0.083 μg/mL following vaginal washing with chlorhexidine solution of 2 g/L during obstetrical delivery. Brougham et al. (10) also detected chlorhexidine in sera from 2 of the 9 patients treated with a chlorhexidine-containing burn cream, the levels being 0.128 and 0.0205 μg/mL. Although no study have been performed on the postmortem redistribution of chlorhexidine, the level in the case we studied (39.5 μg/mL) was much higher. Therefore, injection of the 20% chlorhexidine gluconate solution (10 mL) into the patient was confirmed.

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