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Determination of chlorhexidine (CHD) and nonylphenolethoxylates (NPEO*n*) using LC-ESI-MS method and application to hemolyzed blood

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Abstract

Rapid and reliable methods for identification of chlorhexidine (CHD) and nonylphenolethoxylates (NPEO*n*) in antiseptic and hemolyzed blood using electrospray ionization mass spectrometry (ESI-MS) were developed. Fragmental analysis provides accurate evidence for the presence of CHD in the samples. For the determination of CHD in hemolyzed blood, the method was also developed using LC-ESI-MS. Linearity of calibration curve was obtained over the concentration range of 0.1–11 μg/mL with residuals from −4.3 to 6.7%. We applied the methods to the case of suicidal injection of antiseptic and successfully detected CHD and NPEO*n* from hemolyzed blood. The CHD concentration was 352 μg/mL.

Keywords: Chlorhexidine; Nonylphenolethoxylates; LC-ESI-MS; Human; Blood; Forensic science; Forensic medicine; LC-ESI; Disinfectant; Toxicology; Suicide; Antiseptic

1. Introduction

A case of accidental intravenous injection to a patient, a fatal case of suicidal injection, and a case of mixing these compounds into beverages have been reported [1–3]. Therefore, antiseptics and surfactants are important compounds from a forensic toxicological point of view.

Chlorhexidine (CHD), 1,1′-hexamethylenebis [5-(4-chlorophenyl) biguanide], is one of the most widely used antiseptics. In addition, it is also used as ingredient of healthcare products such as mouth rinses, contact lens cleaners, burn cream, and cosmetics. Some products of CHD contain surfactants such as nonylphenolethoxylates (NPEO*n*) as additives. So, the detection of these additives supports the identification of CHD containing preparats.

There are many reports about the determination of CHD in biological fluids using high performance liquid chromatography with a UV detector (HPLC-UV)[1,4,6–10] and gas chromatography mass spectrometry (GC-MS) [5].

LC-ESI-MS has higher sensitivity and selectivity than HPLC-UV and gives more information for identification. For the detection of surfactants in samples such as beverages, the methods using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) and LC-ESI tandem mass spectrometry (LC-ESI-MS/MS) have been reported from standpoint of forensic chemistry [2,3]. But there is no report investigating for CHD and NPEO*n* in human fluids using these methods.

Electrospray ionization is a suitable technique for the detection of the compounds which have the lack of volatility, thermal stability, and the presence of quaternary amine moieties [11–19]. Therefore, it is very suitable to use ESI-MS technique for determination of CHD and NPEO*n*. In an analysis of CHD and NPEO*n* by ESI-MS, specific molecular-related ions would be measured. Furthermore, fragmental analysis would strongly identify these compounds. In this study, appropriate extraction and ESI technique was applied to a surely identification of CHD and NPEO*n* in hemolyzed blood.

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2. Materials and methods

2.1. Chemicals and reagents

CHD gluconate solution (20%) was purchased from Wako pure chemical industries, Ltd. (Osaka, Japan) and stored at 4 °C in the dark. Acetonitrile was of LC-MS grade, and perchloric acid (60%) was of analytical grade from Wako pure chemical industries, Ltd. (Osaka, Japan) ammonium acetate and trifluoroacteic acid were purchased from Kanto chemical co Ltd. (Osaka, Japan). Extrelut® NT3 was purchased from Merck (Darmstadt, Germany). Product of CHD, STERICLON® solution 5 (5% CHD gluconate), was purchased from Kenei pharmaceutical co., Ltd. (Osaka, Japan). Other chemicals used were of analytical grade.

2.2. Sample preparation and extraction procedure

Stock solution of CHD standard was prepared in deionized water (120 μg/mL) and stored in a plastic vessel at 4 °C in the dark, because CHD is known to be absorbed to glassware [4]. To obtain the calibration standards for CHD at the concentration range of 0.11–11.0 μg/mL, the working solution was spiked appropriately into drug-free hemolyzed blood in polypropylene microtubes.

The extraction of CHD from hemolyzed blood was performed according to the method of Kudo et al. [1] with minor modification. Briefly, 200 μL of hemolyzed blood was mixed with equal volume of deionized water in a polypropylene microtube. One hundred microliters of 10% perchloric acid was added to the mixture and mixed vigorously for 30 s. Then the mixture was centrifuged at 1630 × *g* for 5 min and the supernatants were transferred to another microtube. Potassium carbonate (50 μL) was added to neutralize and remove the excess of perchloric acid from the supernatant. An aliquot of 10 μL of the supernatant was injected onto the column.

The extraction of NPEO*n* from hemolyzed blood was performed on Extrelut® NT3 cartridge. Hemolyzed blood (200 μL) was mixed with 2.5 mL of deionized water microtube and the mixture was applied to the Extrelut NT3 cartridge. After wards, the cartridge was held for 5 min. Then NPEO*n* was eluted with 5 mL × 3 of chloroform. After total evaporation of chloroform under a nitrogen stream at 40 °C, the residue was reconstituted with 200 μL of deionized water. An aliquot was directly analyzed by infusion analysis (direct ESI-MS in positive mode).

2.3. Infusion analysis (direct ESI-MS)

Infusion analysis was used for identification of CHD and NPEO*n* in the infused liquid and of NPEO*n* in the blood extracts. The infused liquid and a standard compound in antiseptic were diluted thousand fold with deionized water, and this diluent was directly introduced to a mass spectrometer. A syringe pump used was a Model 11 single syringe (Harvard Apparatus, Inc. USA). Flow rate was set at 10 μL/min. The syringe was connected with a fused silica capillary to a ZMD 4000 mass spectrometer (Micromass, UK) with electrospray ionization probe. The capillary and cone voltage were set at 3.00 kV and ± 20 V, respectively. The source block and the desolvation temperatures were set at 100 and 150 °C, respectively. The nebulizing gas flow rate was set at 200 L/h. MS data were collected as total ion current (TIC).

2.4. LC-MS condition

For LC-MS system, the following instrumentation was used: HPLC system used was a NANOSPACE SI-II (Shiseido, Japan). Reversed-phase chromatography was performed on a TSKgel ODS-100 V (TOSOH, Japan 2.0 mm × 50 mm, 5 μm) with a guard cartridge CAPCELL PAK MG II (Shiseido, Japan; 2.0 mm × 10 mm, 3 μm) using isocratic elution with acetonitrile/water/trifluoroacetic acid (65/35/0.1, v/v/v) at a flow rate of 200 μL/min. The column temperature was set maintained at 22 °C. The injection volume was 10 μL. This HPLC system was

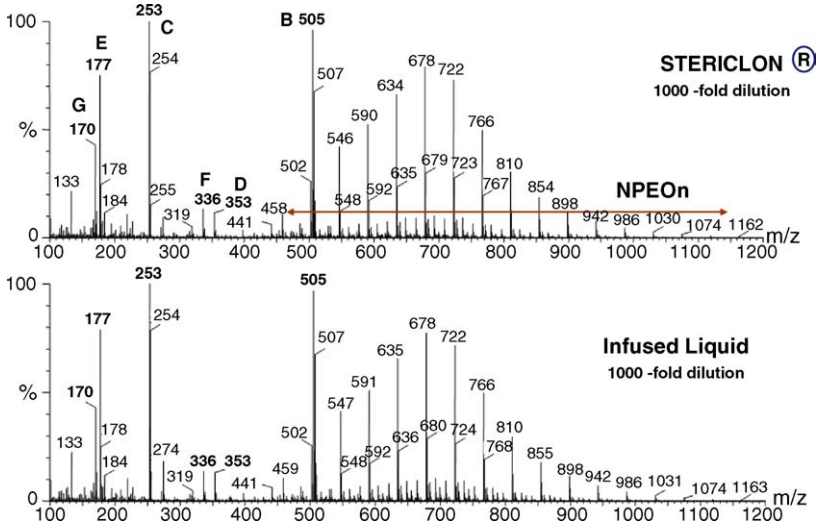

Fig. 1. Comparison of mass spectra (infusion analysis) obtained for antiseptic (upper) and infused liquid (bottom) in positive ion mode. A series of the peaks different by 44 mass units is typical of polyoxyethylene-related surfactant.

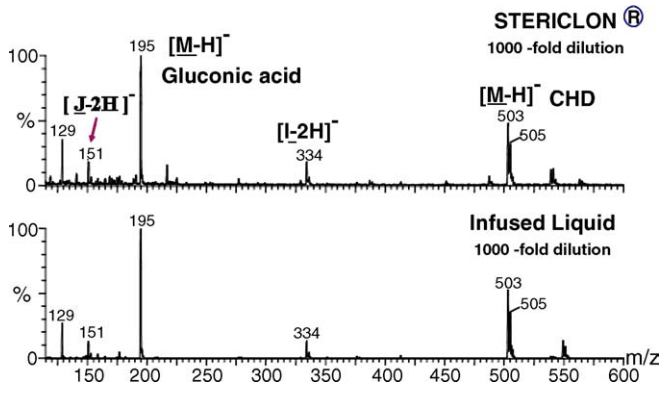

Fig. 2. Comparison of mass spectra obtained for antiseptic and infused liquid in negative ion mode.

coupled with a ZMD 4000 mass spectrometer (Micromass, UK) equipped with an electrospray ionization probe. The capillary and cone voltage were set at 3.00 kV and 20 V, respectively. The source block and the desolvation temperatures were set at 130 and 400 °C, respectively. The nebulizing gas flow rate was set at 400 L/h. MS data were collected as either TIC in positive and negative mode or selected ion monitoring (SIM) at *m/z* 253 and *m/z* 505 (the both of them are molecular-related ions of CHD).

3. Results and discussion

3.1. Mass spectrum of antiseptic (STERICLON®)

To identify CHD in the samples, the intensive peaks in the spectra were assigned in detail taken in infusion analysis. Fragmental analysis was performed based on mass shift rule which was proposed by Nakata [20,21]. This rule is very useful to predict fragmentations of even-electron organic ions. Refer to the reference for details.

Mass spectra of antiseptic obtained by infusion analysis are shown in Fig. 1 (positive mode) and Fig. 2 (negative mode).

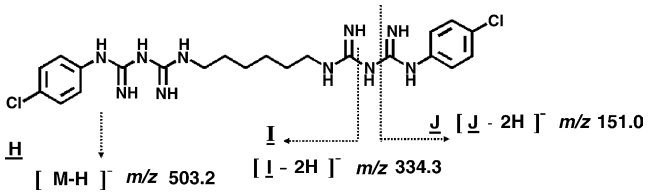

Fig. 4. Proposed fragment pathways of CHD on ESI-MS in negative ion mode. The alphabets in this figure indicate the same samples in Fig 2.

Because CHD has two chlorines in its structure, observed spectrum shows a characteristic isotope ratio. Two molecular-related ions of CHD, protonated molecular ion $[M+H]^+$ (*m/z* 505) and doubly charged molecular ion $[M+2H]^{2+}$ (*m/z* 253), are also discriminating feature. So confirmation of isotope ratio, molecular-related ions, and fragment ions are very important to identify CHD in samples as shown in Fig. 3.

A series of the peaks different by 44 mass units was also observed in positive mode (Fig. 1). This distribution of the peaks different by 44 mass units was typical of polyoxyethylene-related surfactant and identical with molecular-related ions of the NPEO*n* (NH_4 and Na adduct ions). The intense ions observed in the spectrum except for ions from NPEO*n* were reasonably assigned as shown in Fig. 3.

In negative mode, deprotonated molecular ion of gluconic acid ($[M-H]^-$) was observed at *m/z* 195 and also some intense peaks originated from CHD were observed (Fig. 2) and reasonably assigned as shown in Fig. 4.

These intense peaks in positive and negative ion modes were very useful for identification of CHD in the samples.

3.2. LC-ESI-MS

3.2.1. Calibration curve

The calibration curve of seven standards for CHD in hemolyzed blood was obtained by plotting the weighed (1/concentration) peak area against their concentration. Linear response over the concentration range of 0.10–11.0 μg/mL

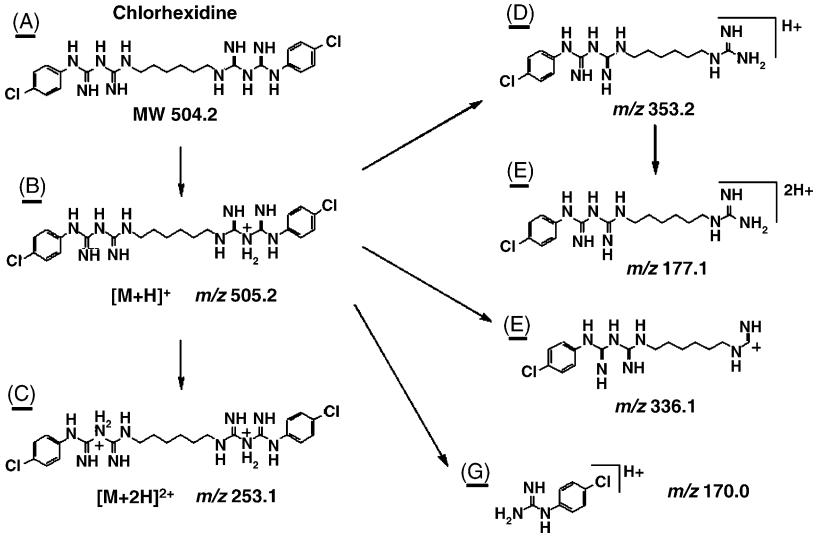

Fig. 3. Proposed fragment pathways of chlorhexidine on ESI-MS in positive ion mode. The alphabets in this figure indicate the same samples in Fig. 1.

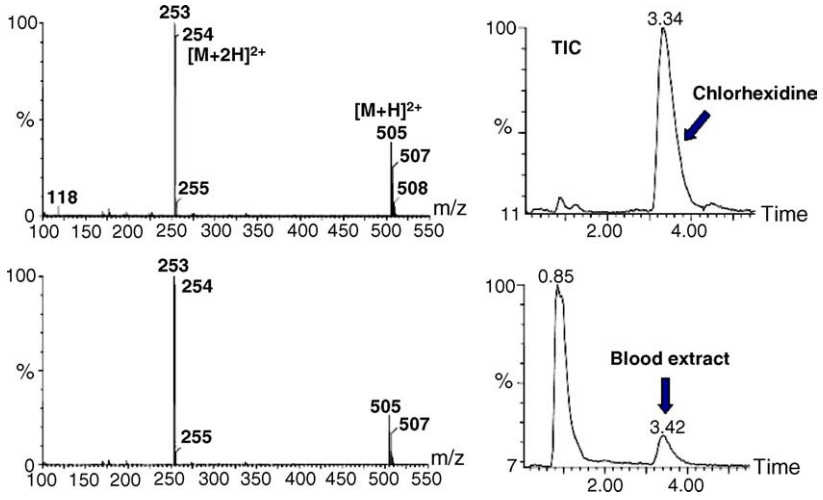

Fig. 5. Total ion chromatograms and mass spectra of standard CHD and the decedent's blood extract.

(0.10, 0.25, 0.50, 1.0, 2.5, 5.0, 11.0 μg/mL) was demonstrated ($r = 0.996$). The recoveries of each point were 96–107%. The limit of detection (LOD) is 18.3 ng/mL and limit of quantitation (LOQ) is 100 ng/mL.

It is well known that CHD is easily adsorbed to the HPLC system including columns [1,4] and carryover peaks are often detected especially in SIM mode. So it was difficult to estimate the reliable values at the low concentration range (below 0.1 μg/mL).

3.2.2. A case of suicide by intravenous injection of antiseptic

A 23-year-old female nurse was found dead in her room with her left arm connected to a drip bag through an intravenous drip line. It was strongly suspected the self-injection of antiseptic because a red colored solution was remained in the drip bag. The decedent's hemolyzed blood and the infused liquid were collected for toxicological analysis.

The methods described in previous section were used to identify CHD and NPEO*n* in the samples.

Infusion analysis (direct ESI-MS in positive and negative modes) was performed to analyze the infused liquid because we thought that there is less interfering matrix in the infused liquid than other samples such as biological fluids. It revealed the presence of CHD and NPEO*n* in the infused liquid (Fig. 1, bottom). The observed peaks in the infusion liquid were identical with control antiseptic (STERICLON® solution).

LC-ESI-MS also revealed the presence of CHD in the blood sample. Fig. 5 shows the chromatograms and mass spectra of standard CHD and the blood extract. Both of data were identical. The concentration of CHD in the blood sample was estimated to be 352 μg/mL. The concentration of the decedent's blood was much higher than that of reported by Kudo et al. [1]. These data provides evidence for the intravenous injection of CHD.

If the decedent used antiseptic of CHD, we must also detect NPEO*n* from the blood sample. Fig. 6 also displays the mass spectrum of the blood sample extract. The mass spectrum showed the distribution of the peaks different by 44 mass units and was identical with the molecular-related ions of the NPEO*n* (NH_4 and Na adduct ions).

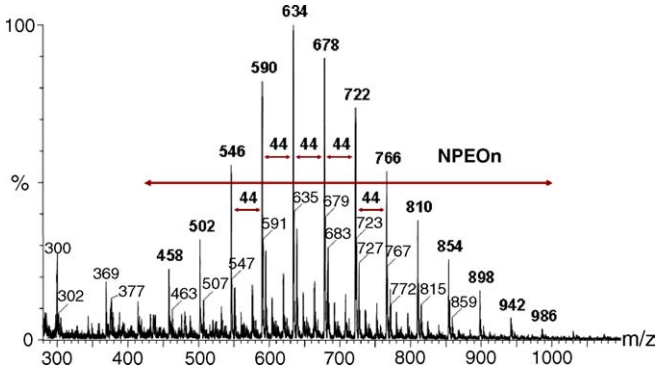

Fig. 6. Mass spectrum of the decedent's blood extract. This distribution of the peaks different by 44 mass units was typical of polyoxyethylene-related surfactant and identical with molecular-related ions of the NPEO*n* (NH_4 and Na adduct ions).

4. Conclusion

We have devised rapid and reliable methods for identification of CHD and NPEO*n* in antiseptic and hemolyzed blood sample using direct ESI-MS or LC-ESI-MS coupled with simple extraction process. The assignments of the peaks derived from CHD and NPEO*n* on ESI-MS in positive and negative modes were made in detail. We applied the methods to the case of suicide, and successfully detected the ingredients of antiseptic, CHD and NPEO*n*, from hemolyzed blood and infused liquid, demonstrating that antiseptic was used for suicidal injection. This is the first report of the LC-ESI-MS method both for CHD and NPEO*n*, and their applications for hemolyzed blood. It is suggested that this method is greatly useful for forensic case analysis.

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