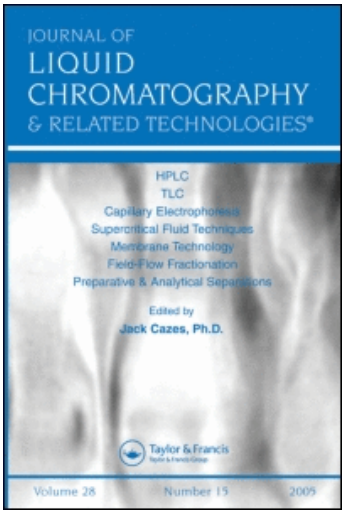

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CONFIRMATION OF RESIDUAL OXOLINIC ACID, NALIDIXIC ACID AND PIROMIDIC ACID IN FISH BY THERMOSPRAY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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ABSTRACT

A thermospray high-performance liquid chromatographic-mass spectrometric (TSP LC-MS) method has been developed for analysis of pylidonecarboxylic acid (quinolone) antibacterials such as nalidixic acid (NA), oxolinic acid (OXA) and piromidic acid (PMA). The LC separation was carried out on an Inertsil ODS-2 (150 x 4.6mm i.d.) using 0.1M ammonium acetate buffer(pH 4.5)-acetonitrile (6:4) as the mobile phase at a flow rate of 0.8 ml/min. The vaporizer temperature was 165 ℃ and the ion source block temperature was 260 ℃. The mass spectra obtained from NA, OXA and PMA were very simple, with base peaks corresponding to the protonated molecule, MH^+. The calibration curve for NA was rectilinear from 1 to 20 ng with a detection limit by selected ion monitoring (SIM) of about 100 pg (signal to noise ratio of 2).

INTRODUCTION

With the development of fish farming involving eel and yellowtail, various kinds of antibiotics and synthetic antibacterials have been widely used for the prevention and treatment of infectious diseases in fish. Concern has arisen as to the presence of drug residues in fish tissues, and demand for a rapid and reliable analytical method of determining them has increased.

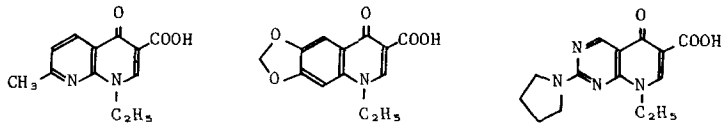
Oxolinic acid (OXA), nalidixic acid (NA) and piromidic acid(PMA) are extensively applied to fish for the treatment of a variety of gram-negative organisms(Fig. 1). The authors previously examined residual OXA, NA and PMA in cultivated fishes and detected OXA at a high rate [1]. In a residual analysis, the identification of detected medical substances is needed to confirm the analytical result. After the residual OXA was aliquoted and purified with high-performance liquid chromatography (HPLC), we confirmed its presence by mass spectrometry (MS) [1]. But, the MS could not be applied in the case of low residual concentrations. Recently, a gas chromatographic-mass spectrometric (GC-MS) method has been reported for the confirmation of OXA in fish [2]. However, this method is complicated and time consuming.

In this study, we investigated and reported the analysis method of OXA, NA and PMA using thermospray liquid chromatography-mass spectrometry (TSP LC-MS), which is directly coupled with HPLC and MS.

MATERIALS AND METHODS

Materials and Reagents

Edible muscle tissues of sweet fish and yellowtail served as samples. NA, OXA and PMA were obtained from Daiichi Pharmaceutical (Tokyo, Japan), Tanabe Pharmaceutical (Osaka, Japan) and Dainihon



Nalidixic acid(NA) $C_{12}H_{12}N_2O_3$=232.2

Oxolinic acid(OXA) $C_{13}H_{11}NO_5$=261.2

Piromidic acid(PMA) $C_{14}H_{16}N_4O_3$=288.3

Fig. 1. Structures of nalidixic acid, oxolinic acid and piromidic acid

Pharmaceutical(Osaka, Japan), respectively. Bond Elut C_{18} cartridges (200 mg) (Varian Associates, Harbor City, CA, U.S.A.) were washed with 5 ml of methanol and then 10 ml of distilled water before use. Hyflo Super-Cel was purchased from Johns-Manville (Denver, CO, U.S.A.). Other chemicals were of reagent grade or HPLC grade.

Preparation of standard solutions

Each standard(20 mg) was weighed accurately into a 100-ml volumetric flask and diluted to volume with 0.02M aqueous sodium hydroxide -methanol (2:8). Subsequent dilutions were made with the HPLC mobile phase.

Apparatus

The HPLC system consisted of a Shimadzu LC-6A solvent-delivery system (Kyoto, Japan) with a Rheodyne 7125 injector valve (Cotati, CA, U.S.A.) fitted with a 20μl sample loop. The separation was performed on an Inertsil ODS-2 column(5 μm, 150 x 4.6 mm i.d.,) (GL Sciences, Tokyo, Japan) with 0.1 M ammonium acetate buffer(pH 4.5)-acetonitrile (6:4) as the mobile phase at a flow-rate of 0.8 ml/min at 35℃. The mass spectrometer is a Shimadzu LCMS-QP 1000 quadruple mass spectrometer equipped with a Vestec thermospray interface (Houston, TX,

U.S.A.). The filament-on mode (ionization by an electron beam) was used for ionization. The vaporizer temperature was set at 165℃, the ion source block temperature was set at 260 ℃, and the repeller potential was kept at 0 V. The electron multiplier voltage was 3000 V and ionization potential was 1000 eV. The mass spectrometer was operated in the positive ion mode. The measurement mass range was *m/z* 150-400 in 2 s.

Sample Preparation

Sample preparation was based on a previous paper [1]. A 5 g sample was homogenized at high speed for 2 min with 100 ml of 0.2% metaphosphoric acid -methanol(2:1) as a deproteinizing extractant. The homogenate was filtered through *ca.* 2 mm Hyflo Super-Cel coated on a suction funnel. The filtrate was evaporated under reduced pressure at 50℃. Evaporation was interrupted when *ca.* 30 ml of solution remained in the flask. The flask contents were applied to a Bond Elut C_{18} cartridge. After washing with 20 ml of distilled water, the cartridge was eluted with 10 ml of methanol. The eluate was evaporated to dryness under reduced pressure and the residue dissolved in 1 ml of HPLC mobile phase; 20 μl of the solution were injected into the TSP LC-MS system.

LC-MS Analysis

Standards at concentrations of 0.05, 0.1, 0.2, 0.5 and 1.0 μg/ml of each drug were prepared from stock standard solutions. A 20 μl volume of these solutions was injected into the TSP LC-MS system. Calibration graphs were constructed by plotting peak areas on the *m/z* 262, *m/z* 233 and *m/z* 289 SIM chromatograms against the amount of OXA, NA and PMA, respectively. The amount of OXA in a sweet fish sample

was calculated by comparison of the peak area at *m/z* 262 with the calibration graph.

RESULTS AND DISCUSSION

Establishment of TSP LC-MS System

A residual silanol group and metal impurities in the column packing materials are known to be the cause of tailing in reversed-phase liquid chromatography [3-5]. In a previous paper [3], we reported that the use of an oxalic solution in a mobile phase could be used for the purpose of masking metal impurities and inhibiting tailing. But, in the case when an oxalic solution was used as the mobile phase of the TSP LC-MS, it was found unsuitable for analyzing a residual drug having a poor ionization efficiency and containing a trace amount of the target compound. Consequently, an end-capped ODS column based on pure silica-gel with low level metal impurities was examined to determine whether it could inhibit tailing. The use of the Inertsil ODS-2 column [6], which was made of 99.999% pure silica gel, resulted in a reduction of tailing without the oxalic solution in the mobile phase.

Because buffer solutions, such as phosphate and sulfate, are slightly volatile and deteriorate ion sources, they are inappropriate as a mobile phase for the TSP LC-MS [7]. Instead of the buffers, volatile buffers, such as ammonium acetate and ammonium formate, has been used [7-11]. Therefore, the investigation concerning the effect of ammonium acetate concentrations on the intensity of the MH^+ ions showed a maximum intensity of ions for OXA at 0.05M, and NA and PMA at 0.1M. Next, the pH of 0.1M ammonium acetate was adjusted in the range of 4.5-7.0 with 0.1 M acetic acid and the effects on the intensity of MH^+ ions were examined. A statistically significant effect did not

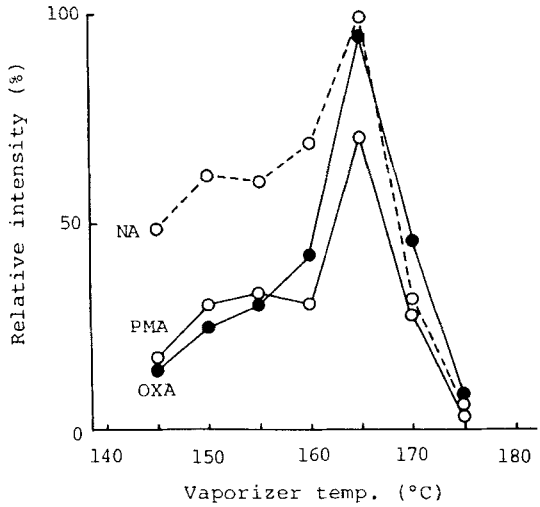


Fig. 2. Effect of vaporizer temperature on the intensity of the MH^+ ions of oxolinic acid(OXA), nalidixic acid(NA) and piromidic acid(PMA). Conditions: column, Inertsil ODS-2(150 x 4.6 mm i.d.); mobile phase, 0.1 M ammonium acetate buffer(pH4.5)-acetonitrile(6:4); flow rate, 0.8 ml/min; column temperature, 35℃; ion source temperature, 260 ℃.

appear within the range, but sharper peaks appeared at lower pHs. Finally, the acetonitrile content was determined at 40% in consideration of the intensity of ions and the mutual separation of each drug.

The ionization efficiency strongly depends on set temperatures such as vaporizer temperatures and ion source block temperatures of the interface [7,9]. Accordingly, the effect on the intensity of the MH^+ ions was examined with varying vaporizer temperatures. As shown in Fig. 2, the intensity of the MH^+ ions strongly depended on vaporizer temperatures; OXA, NA and PMA showed the maximum ionic intensity at 165℃. A similar analysis has been performed for ion source block

temperatures and the optimal temperature was found to be 260℃. Furthermore, vaporizer temperatures affected the intensity of the MH^+ ions more than the ion source block temperatures.

Mass Spectra

As the TSP LC-MS method is a mild ionization method, fragment ions are not usually observed [7, 8, 12]. Only the the MH^+ ions, which gave molecular weight information, were observed in the mass spectra of OXA, NA and PMA. Generally, it is desirable for an identification of the organic compounds that fragment ions, which give molecular and some structural information, are observed. As previously mentioned, the TSP LC-MS method is a fairly gentle ionization technique, and many compounds show only molecular ion species. In order to get more structural information from the mass spectrum, the discharge electrode and repeller have sometimes been used to introduce fragmentation [7, 8, 13]. Consequently, ionization modes and repeller voltages were varied, but good, repeatable ions could not be observed.

Mass Chromatography (MC) and Selected Ion Monitoring (SIM)

Fig. 3 shows the total ion chromatograms (TIC) of OXA, NA and PMA, and the mass chromatograms(MC) comprised of the MH^+ ions of each component. The detection limits by the MC technique were 2 ng for NA, 10 ng for OXA and PMA (signal to noise ratio of 2); these amounts correspond to sample concentrations of 0.02 and 0.1 μg/g, respectively.

For a highly sensitive analysis, the analysis of OXA, NA and PMA by selected ion monitoring (SIM), which selected the MH^+ ions, was examined. Fig. 4 shows the SIM chromatograms resulting from injection of 1 ng. Each calibration graph comprised of the SIM method shows good linearity in the range of 1-20 ng. The limits of detection by

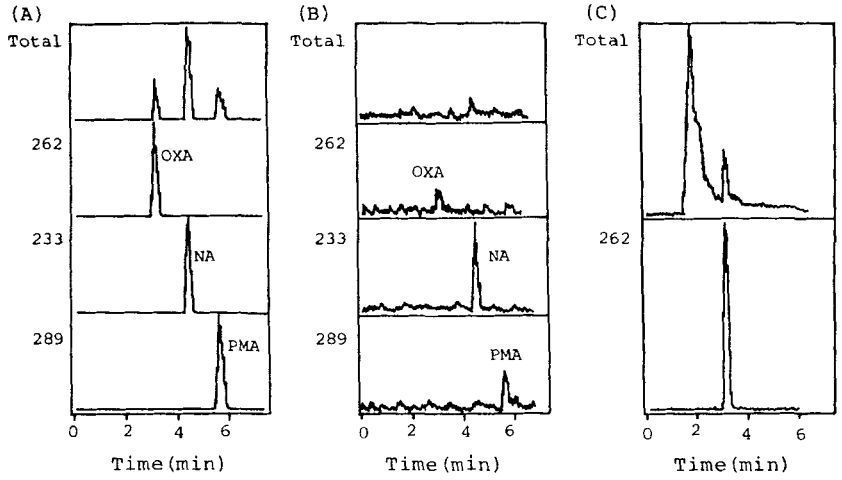


Fig . 3. Thermospray LC-MS total ion chromatograms and mass chromatograms of (A) standard mixture (each 200 ng; equivalent to 2 μg/g), (B) standard mixture (each 10 ng) and (C) sweet fish sample containing 1.9 μg/g incurred oxolinic acid residue. Conditions same as Fig. 2.

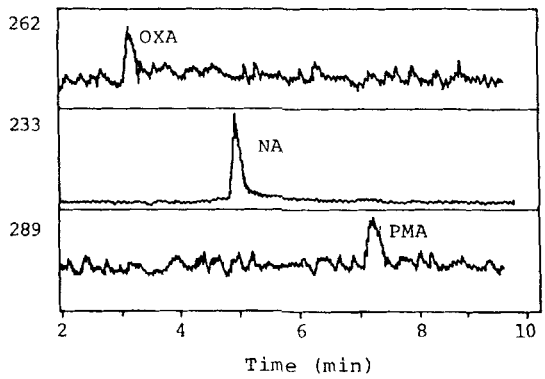


Fig. 4. Thermospray LC-MS SIM chromatograms of oxolinic acid(1 ng), nalidixic acid(1 ng) and piromidic acid(1 ng). Conditions same as Fig. 2.

the SIM method (signal to noise ratio of 2) were 0.1 ng for NA and 1 ng for OXA and PMA. It is possible to detect a minimum concentration of 0.01 μg/g (converted to the sample concentration). 10ng of each standard was measured thrice by the SIM method and the coefficient of variation of the peak area was determined. As a result, each coefficient fell within a range of 15%, showing an almost satisfactory reproducibility. Consequently, it appears that the SIM method has enough sensitivity and reproducibility to be a residual analysis method.

Application

As described in the introduction, it is required in the analysis of residual drugs to confirm the detected drugs. Thus, a sample in which OXA had been detected by HPLC was applied to this method.

Fig. 3(C) shows the TIC of sweet fish sample detected with 1.9 μg/g of OXA by the HPLC method with a UV and a fluorometric detector [1] and the MC comprised of the mass number of OXA added protone. The retention time of the peak comprised of m/z 262 on the MC was the same as that of the standard OXA. Next, this fish sample was subjected to the SIM method. As a result, the data thus obtained was 2.5 μg/g agreed with the previously mentioned one, though they were somewhat higher. Furthermore, the mass spectrum of the peak was in accordance with the standard OXA and the component, which was eluted at about 3.2 min, was confirmed as OXA.

As already mentioned, since only the MH^+ ion, which indicates molecular information, was observed in the mass spectrum, the method was not good enough to be used for identification. However, compared with conventional HPLC depending on retention time, both retention time and molecular weight information can be simultaneously obtained as qualitative data by this method. It seems that there are few

compounds showing the same retention time and molecular weight as those of OXA, NA and PMA. Accordingly, it is considered that this method is usable as a procedure for confirmation of residual OXA, NA and PMA in cultured fish.

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