Gas chromatographic–mass spectrometric determination of oxolinic acid in fish using selected ion monitoring

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ABSTRACT

A gas chromatographic–mass spectrometric (GC–MS) method is described for the determination of oxolinic acid in fish tissues. Oxolinic acid is reduced with sodium tetrahydroborate to permit GC analysis. The sample is homogenized with phosphate buffer (pH 6) and extracted with ethyl acetate. The extract is partitioned between sodium hydrogencarbonate solution and the aqueous phase is acidified and re-extracted with ethyl acetate. The residue from the ethyl acetate extract is dissolved in methanol and reduced with sodium tetrahydroborate. The reduction product is extracted with diethyl ether and analysed by GC–MS in the selected ion monitoring mode for the ions at m/z 204, 219 and 176. The detection limit is 0.001 mg/kg and the recoveries were 95.6% [relative standard deviation (R.S.D.) 7.7%] at 0.1 mg/kg and 72.9% (R.S.D. 13.3%) at 0.01 mg/kg fortification levels in fish.

INTRODUCTION

In recent years in Japan, fish for use as food have been artificially cultivated on a large scale in both fresh water and in sea water, and drugs are used to prevent and to treat disease. Oxolinic acid (1-ethyl-1,4-dihydro-6,7-methylenedioxy-4-oxo-3-quinolinecarboxylic acid) (Fig. 1, a), is an antimicrobial agent which is widely used in the cultivation of fish such as salmon, rainbow trout, sweetfish, carp, eel and yellowfish.

Drug residues in cultivated fish may cause problems with regard to human safety, and in Japan the Food Safety Law established a zero residual level for all
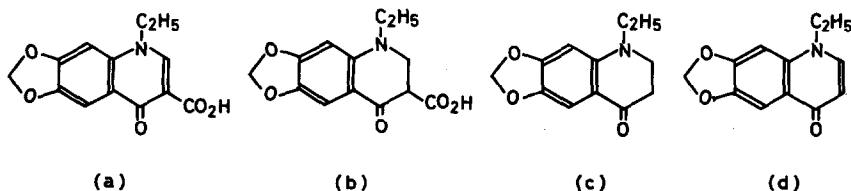

Fig. 1. Structures of (a) oxolinic acid, (b) reduction intermediate, (c) reduction product and (d) oxidation product of c.

antimicrobial agents in foods in 1971. Hence routine screening for oxolinic acid residues in cultivated fish is necessary, but the methods of analysis available are very limited. Endo *et al.* [1] used the microbial assay of oxolinic acid in animal tissues, but this method lacks sensitivity and specificity. Kasuga *et al.* [2] reported a liquid chromatographic method, and the Japanese official method also uses a liquid chromatographic method, but its detection limit of 0.05 mg/kg is not sufficient and identification that relies solely on retention data is not specific enough to support regulatory action.

        Gas chromatography–mass spectrometry (GC–MS) is the most reliable and sensitive technique for residue analysis, and we have been developing GC–MS methods for drug residue analysis. Methyl and *n*-butyl oxolinate were not suitable for GC because they are very polar and did not produce clear peaks on gas chromatograms [3]. We therefore tried other derivatives and found that sodium tetrahydroborate reduction of oxolinic acid smoothly gives a reduction product, 1-ethyl-1,2,3,4-tetrahydro-6,7-methylenedioxy-4-oxoquinoline (Fig. 1,c). This compound has good GC characteristics and gives a sharp, symmetrical peak on gas chromatograms. We therefore developed a GC–MS method with selected ion monitoring (SIM) using this reduction product for the determination of oxolinic acid residues in fish. This method is rapid, sensitive and is specific enough to support regulatory action, and is useful for daily screening and also for the identification of residual oxolinic acid.

EXPERIMENTAL

Materials and chemicals
        Pesticide-grade and analytical-reagent grade chemicals were used as received. Pesticide-grade ethyl acetate, methanol, acetone and anhydrous sodium sulphate and analytical-reagent grade $NaH_2PO_4 \cdot 2H_2O$, NaOH, $NaHCO_3$, NaCl, $NaBH_4$ and HCl were obtained from Wako (Osaka, Japan). Pesticide-grade diethyl ether and methylene chloride (Wako) were used after alumina column chromatography just before use. Alumina was obtained from E. Merck (Darmstadt, F.R.G.) and a silica gel cartridge column (Sep-Pak silica) from Waters Assoc. (Milford, MA, U.S.A.). Oxolinic acid was obtained from Sigma (St. Louis, MO, U.S.A.).
        Solutions were prepared as follows: for phosphate buffer solution (pH 6), 31.2 g of $NaH_2PO_4 \cdot 2H_2O$ were dissolved in 600 ml of water, the pH was adjusted to 6 by adding 1 M NaOH and the volume was made up to 1 l with water; for sodium hydrogencarbonate, 40 g of $NaHCO_3$ were added to 500 ml water and mixed and the supernatant was used; for 5 M HCl, 417 ml of HCl were added to 500 ml of water and the volume was made up to 1 l with water.

Standard solutions
        A stock standard solution (100 mg/l) was prepared by dissolving 10.0 mg of oxolinic acid in 100 ml of methanol in a volumetric flask. Working standard solutions were prepared by diluting the stock solution with methanol to 0.1–1.00 mg/l. These solutions were stored in a refrigerator.

Instrumental
        A Biotron BT 10 20 350D homogenizer (Biotrona, Kussnacht, Switzerland),

a Model 8-1-W wrist-action shaker (Yayoi, Tokyo, Japan) and a Model N-1 rotary evaporator (Tokyo Rikakikai, Tokyo, Japan) were used. A DB-5 capillary GC column (15 m × 0.25 mm I.D., film thickness 0.25 μm) (J&W Scientific, Folsom, CA) was used. A Model 5890 Series II gas chromatograph with a split/splitless injection port was coupled to a Model 5970B mass-selective detector (quadrupole mass spectrometer) and were operated using 59970 MS Chemistation computer software (Hewlett-Packard, Englewood, CO, U.S.A.). The capillary column (DB-5) was directly interfaced to the mass spectrometer ion source.

The operating conditions were as follows: splitless injection; purge on time, 2 min after injection; injection port temperature, 270°C; column temperature, 100°C, held for 2 min, increased to 200°C at 20°C/min, then to 270°C at 10°C/min; carrier gas, helium at a column head pressure of 5 p.s.i. (flow-rate 4.8 ml/min); transfer line temperature, 260°C; electron ionization, 70 eV; electron multiplier, 2600 V; selected ion monitoring, detection ions of m/z 204, 219 and 176.

Extraction and clean-up

A 10.0-g comminuted sample was homogenized with 20 ml of phosphate buffer solution (pH 6) in a 100-ml centrifuge tube for 5 min. The homogenizer shaft was washed with 40 ml of ethyl acetate and the washings were added to the centrifuge tube. The mixture was shaken for 10 min and centrifuged at 1000 g for 10 min. The upper ethyl acetate extract was pipetted into a 100-ml separating funnel. A 20-ml volume of ethyl acetate was added to the residue in the centrifuge tube and mixed again. The second ethyl acetate extract, separated by centrifugation at 1000 g, is added to the separating funnel. The combined ethyl acetate solution was extracted three times with sodium hydrogencarbonate solution (10, 10 and 5 ml) and the aqueous layer was collected in another 100-ml separating funnel.

A 6-ml volume of 5 M HCl was added to the hydrogencarbonate extract, mixed well and the pH was ascertained to be 1–2. If it was more basic, 5 M HCl was added to make the pH 1–2. After addition of 2 g of NaCl, the aqueous solution was re-extracted twice with ethyl acetate (30 and 20 ml) and the combined ethyl acetate extract was washed with 3 ml of phosphate buffer solution (pH 6). The pH of the washings must be 5–6; if it was more acidic, washing was repeated with a further 3 ml of phosphate buffer (pH 6). The ethyl acetate solution was dried over anhydrous Na_2SO_4, filtered into a 100-ml pear-shaped flask and evaporated to dryness using a rotary evaporator at 36°C. The residue was quantitatively transferred into a 10-ml test-tube with 1.0 and 0.5 ml of methanol.

Sodium tetrahydroborate reduction

A *ca.* 3-mg amount of $NaBH_4$ was added to the methanol solution and reacted at 20°C for 5 min with occasional swirling. After reaction, 2 ml of 0.1 M HCl were added and mixed well. The pH of this solution must be 2–3; if it was more basic, 0.1 M HCl was added to make the pH 2–3. The solution was allowed to stand at 20°C for 5 min, then 2 ml of water were added and the mixture was extracted three times with diethyl ether (2, 2 and 1 ml). The upper ether layer was transfered into a 10-ml erlenmeyer flask using a Pasteur pepette and dried over Na_2SO_4. The solution was filtered into a 20-ml pear-shaped flask and the solvent was evaporated to dryness using a rotary evaporator at 35°C. The residue was dissolved in 3 ml of methylene chloride.

A silica cartridge column was attached to a 5-ml syringe barrel and the above methylene chloride solution was transferred into this syringe. The flask was rinsed with 3 ml of methylene chloride and the rinsings were added to the syringe. The methylene chloride solution was forced gently through column by applying pressure on the syringe plunger. The eluate was discarded. Then 3 ml of methylene chloride–diethyl ether (1:1) were added to the syringe and eluted by gentle pressure. The eluate was collected in a 20-ml pear-shaped flask and the solvent was evaporated to dryness using a rotary evaporator at 35°C. The residue was dissolved in 1 ml of acetone and this solution was ready for injection into the GC–MS system. If GC–MS analysis does not follow immediately, the solutions must be stored under a nitrogen atmosphere in a refrigerator.

Preparation of standard solutions for calibration graph

Volumes of 1 ml of each working solution of 0.1–1.0 mg/l were pipetted into 10-ml test-tubes. A *ca.* 3-mg amount of $NaBH_4$ was added to each test-tube and reacted at 20°C for 5 min. After reaction, 2 ml of 0.1 M HCl were added and mixed well. The pH of this solution must be 2–3. If it was more basic, 0.1 M HCl was added to make the pH 2–3. The solutions were allowed to stand at 20°C for 5 min, then 2 ml of water were added and the solution was extracted three times with diethyl ether (2, 2 and 1 ml). The ether extract was dried over anhydrous Na_2SO_4, filtered into another 10-ml test-tube and the solvent was evaporated to dryness at 35°C with a gentle stream of nitrogen. The residue was dissolved in 1 ml of acetone and this solution was ready for injection into the GC–MS system. This solution must be stored under nitrogen in a refrigerator and must be prepared fresh each week.

Gas chromatographic–mass spectrometric analysis

The GC column was connected to the ion source of the mass spectrometer and the instrument parameters were adjusted. The quadrapole fields were calibrated using perfluorotributylamine as a standard.

A 2-μl volume of each standard solution (0.1–1.0 mg/l) for the calibration graph was injected into the GC–MS system and a calibration graph was constructed by plotting peak areas on the m/z 204 SIM chromatograms against amounts of oxolinic acid. A 2 μl sample solution was subsequently injected into the GC–MS system and the reduction product of oxolinic acid was identified from the retention time of the peaks in the m/z 204, 219 and 176 SIM chromatograms and from the intensity ratio of ions of m/z 219 and 176 to that of m/z 204. The amount of oxolinic acid in a sample solution was calculated by comparison of the peak area at m/z 204 with the calibration graph. The concentration of oxolinic acid in a sample was calculated by dividing the amount of oxolinic acid by the amount of the sample (10.0 g).

RESULTS AND DISCUSSION

Methyl or *n*-butyl oxolinate was not suitable for GC, so we tried other possible derivatives and found that sodium tetrahydroborate reduction of oxolinic acid proceeded smoothly to give a fluorescent product. The structure of this product was proved to be 1-ethyl-1,2,3,4-tetrahydro-6,7-methylenedioxy-4-oxoquinoline (Fig. 1, c) from the mass spectrum (Fig. 2) (M^+, m/z 219; $M^+ - CH_3$, m/z 204; $M^+ - CH_3 - CO$,

m/z 176). This compound (c) is considered to be formed by decarboxylation of an intermediate product (b) (Fig. 1, b), which is produced by Michael-type addition of hydride ion (H⁻) to oxolinic acid (a). Compound c is smoothly eluted from GC columns, and gives a sharp, symmetrical peak at moderate temperatures, so it is well suited for GC analysis. We decided to use this reduction product (c) for the GC–MS determination of oxolinic acid residues.

Compound c gradually changed to another compound (d) when the diethyl ether solution was exposed to air at room temperature. This had a longer GC retention time analysis and its mass spectrum showed distinct ions at *m/z* 217 (M⁺), 202 and 174, which indicated that the structure is a dehydrogenated product, 1-ethyl-1,4-dihydro-6,7-methylenedioxy-4-oxoquinoline (Fig. 1, d). This seems to be formed by air oxidation of c. Therefore, c must be analysed by GC–MS as quickly as possible, and the solutions must be stored under a nitrogen atmosphere in a refrigerator.

The detailed experimental conditions for this reduction were investigated with oxolinic acid solution. The time course of the reduction with sodium tetrahydroborate in methanol was followed by GC–MS-SIM. Fig. 3 shows the results, which indicate that the optimum reaction time was 5 min at 20°C. Reduction at ice-cooled temperature was fairly slow. After reduction, the reaction mixture was treated with HCl to decompose excess of sodium tetrahydroborate. When the amount of HCl was not sufficient to make the solution acidic, the yield of c decreased considerably. The pH of the reaction mixture after HCl addition is critical and should be carefully established with a pH test paper to be 2–3 in each reduction. The reaction time of acid treatment also affected the yield of c, and the optimum was 5 min at 20°C. The diethyl ether used for extraction of c must be column chromatographed on alumina just before use, because aged diethyl ether considerably lowered the yield. Compound c is very sensitive to oxidation, as described above.

The ions used for GC–MS-SIM detection are three distinct ions of c (Fig. 2), *i.e.*, those at *m/z* 204, 219 and 176. The injection volume is 2 μl in the splitless mode. Identification is based on the retention time and on the intensity ratio of the ions of *m/z* 219 and 176 to that of *m/z* 204. Sphone [4] pointed out that three ions are necessary for
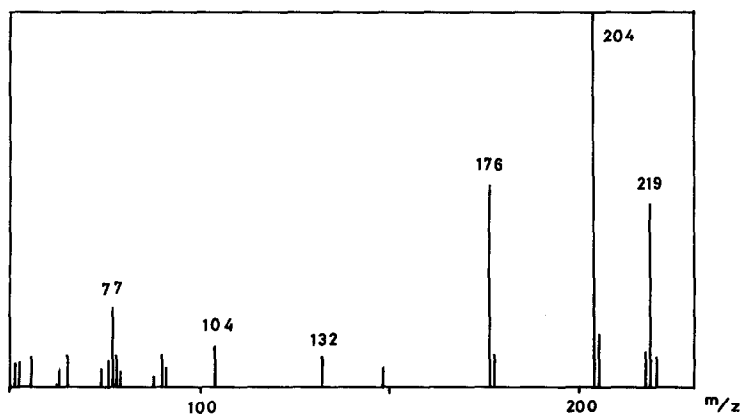

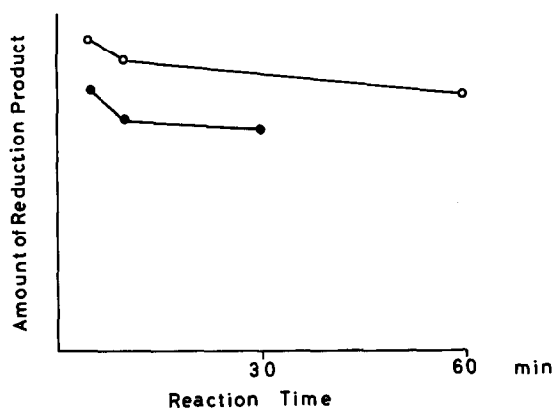Fig. 2. Mass spectrum of the reduction product of oxolinic acid.

Fig. 3. Reaction time of sodium tetrahydroborate *vs.* yield of reduction product. $\bigcirc$ = 20°C; $\bullet$ = ice-cooled.

the qualitative identification of an endogeneous drug residue by use of low-resolution MS.

Quantification was effected by comparing the peak area on the m/z 204 SIM chromatogram with a calibration graph. The calibration graph was constructed by plotting the peak areas on the m/z 204 SIM chromatogram of the reduction product of oxolinic acid against the amount of oxolinic acid. A straight-line graph was obtained in the range 0.2–2.0 ng with a correlation coefficient γ = 0.9983. The SIM chromatograms of c at 0.1 mg/l concentration are shown in Fig. 4. Fig. 5 shows the SIM
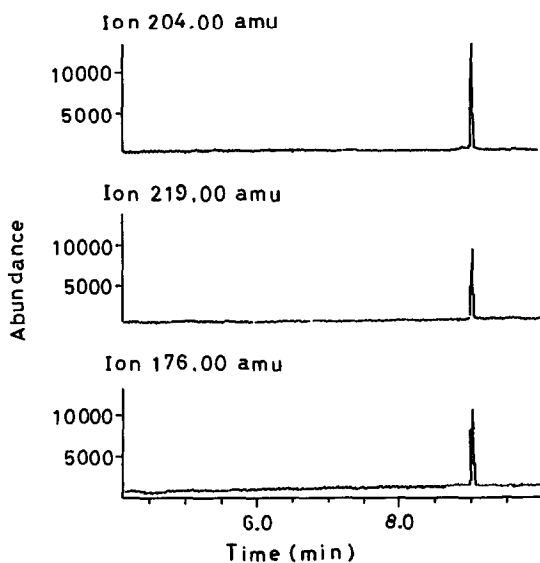

Fig. 4. SIM chromatograms at m/z 204, 219 and 176 of standard oxolinic acid reduction product (oxolinic acid, 0.2 ng).
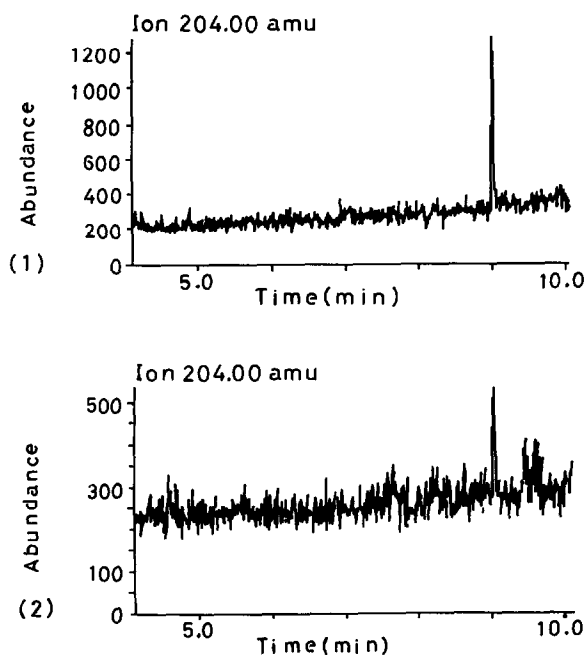
Fig. 5. SIM chromatograms at *m/z* 204 of standard oxolinic acid reduction product. (1) Oxolinic acid, 20 pg; (2) oxolinic acid, 6 pg.

chromatograms based on the ion of *m/z* 204 (most sensitive) at the detection limit concentration. A concentration of 0.003 mg/l was detectable with a standard solution, but 0.01 mg/l is the detection limit with the sample extract solution, which corresponds to 0.001 mg/kg in the sample.

We used the extraction solvent system reported by Browning and Pratt [5] for the determination of nalidixic acid. Oxolinic acid is amphoteric, so extraction with ethyl acetate after homogenization of the sample with phosphate buffer (pH 6) is necessary.

TABLE I

RECOVERY (%) OF OXOLINIC ACID ADDED TO SILVER SALMON

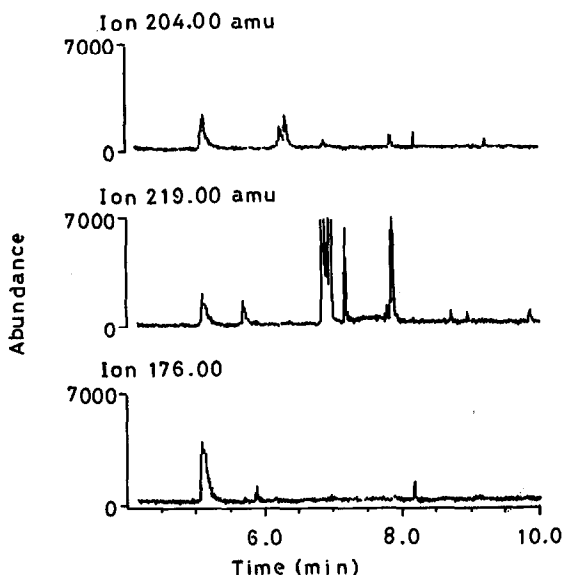|	Amount added (mg/kg)	
	0.1	0.01
	91.2	74.9
	97.6	64.9
	107.6	81.7
	89.6	60.8
	91.8	82.1
Mean	95.6	72.9
R.S.D.*a* (%)	7.7	13.3

a Relative standard deviation.

Fig. 6. SIM chromatograms at m/z 204, 219 and 176 of extract from silver salmon fortified with 0.01 mg/kg of oxolinic acid. The retention time of oxolinic acid reduction product is 9.028 min.

Kasuga et al. [2] also used this extraction solvent for the liquid chromatographic determination of oxolinic acid in fish and obtained a good extraction efficiency (94%).

The ethyl acetate extract from samples contained an oily substance and after sodium tetrahydroborate reduction many interfering peaks were observed in the SIM chromatograms, which made impossible to identify the peak of c. Extraction of oxolinic acid from the ethyl acetate solution with 0.1 M NaOH caused emulsion formation and interfering peaks were not completely eliminated. However, in extraction with sodium hydrogencarbonate solution, no emulsion was formed and interfering peaks were completely removed. Other drugs used for fish, such as tetracycline, ampicillin, sulphamonomethoxine, sulphadimethoxine and thiamphenicol, did not interfere in the determination of oxolinic acid with this procedure. Nalidixic and piromidic acid were reduced with $NaBH_4$ to give reduction products, which could be completely differentiated from that of oxolinic acid (c) by the retention times and SIM detection ions. After sodium tetrahydroborate reduction, the sample extract is passed through a silica gel cartridge column to remove high-boiling polar compounds for protection of the open-tubular GC column.

Recoveries were determined by adding oxolinic acid to blank silver salmon muscle tissue, which had been previously analysed with this method to confirm that no oxolinic acid was detected. Table I gives the results, and the SIM chromatograms are shown in Fig. 6. The detection limit is 0.001 mg/kg in muscle tissues.

This method is sensitive and reliable and the procedure is simple and rapid, and so is useful for both daily screening and qualitative identification of residual oxolinic acid. The application of this method to the determination of nalidixic and piromidic acid is in progress and will be reported later.

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