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High-performance liquid chromatographic method for the determination of oxolinic acid residues in crops☆

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ABSTRACT

A procedure for the high-performance liquid chromatographic (HPLC) determination of oxolinic acid in crops was examined. Oxolinic acid was extracted with acidic methanol and re-extracted into dichloromethane. After successive clean-up by liquid–liquid partition and Sep-Pak silica column chromatography, oxolinic acid was determined by HPLC with fluorimetric detection (excitation at 270 nm, emission at 370 nm). The HPLC column was Inertsil ODS and the eluent was 1 m*M* tri-*n*-octylamine in 0.45% citric acid–tetrahydrofuran–acetonitrile (7:1:2, v/v/v) (pH 2.9). The limit of detection was 0.01–0.02 ppm and the recoveries from crops (spiked with 0.5–1.0 ppm, w/w) were 78–95%. The method was shown to be applicable to residue analysis of oxolinic acid in crops sprayed with Starner in fields.

INTRODUCTION

Oxolinic acd (5-ethyl-5,8-dihydro-8-oxo[1,3]-dioxolo[4,5-*g*]quinoline-7-carboxylic acid) is a synthetic antibacterial agent used in human and piscine medicine. Recently, oxolinic acid was found to be effective in agricultural disease control, *e.g.*, *Pseudomonas* and *Erwinia* species in rice crops; it was introduced as a fungicide by Sumitomo Chemical under the trade name Starner (Fig. 1).

Several methods for the determination of oxolinic acid, such as bioassay [1,2], fluorimetry [3] and high-performance liquid chromatography (HPLC) [4–8] have been described. However, these methods have been developed for the assay of pharmaceutical products and/or biological specimens, *e.g.*, plasma, urine and fish; none of the methods has been reported for residue analysis of oxolinic acid in crops.

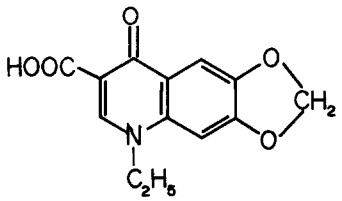

Fig. 1. Structure of oxolinic acid.

This paper reports a simple, sensitive and specific HPLC method for the residue analysis of oxolinic acid in crops.

EXPERIMENTAL

Reagents and materials

Tri-*n*-octylamine, an ion-pairing reagent, was purchased from Tokyo Chemical Industry. A Sep-Pak silica cartridge (Part No. 20520, amount of silica gel 680 mg per cartridge) was obtained from Waters Assoc. Pure standard oxolinic acid was supplied by Sumitomo Chemical. The HPLC column, Inertsil ODS-2, was obtained from GL Sciences and the guard column, Brownlee RP-8, from Applied

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Biosystems. The organic solvents were of pesticide residue grade (or their equivalent) and all the other reagents were of analytical-reagent grade.

Apparatus

A Tri-Rotar SR-1 chromatograph (Japan Spectroscopic) with a fluorescence detector (Hitachi 650-10S) was used for HPLC.

Sample preparation

Brown rice and rice straw. Brown rice grain and chopped rice straw were ground to a powder with a pulverizer (Retsch). A subsample (20 g of brown rice and 10 g of rice straw) was placed in a 300-ml flask and 20 ml of water and 100 ml of methanol were added. After the contents had been blended with a Polytron homogenizer for 1–2 min, 10 ml of 12 *M* hydrochloric acid were added, and the flask was shaken mechanically for 30 min.

Other crops. Crops were homogenized with a mixer. A 20-g subsample was placed in a 300-ml flask, 10 ml of 12 *M* hydrochloric acid and 100 ml of methanol were added and the flask was shaken mechanically for 30 min.

Extraction

The contents were filtered through a filter-paper under suction. The filter cake and the flask were washed with 30 ml of methanol–12 *M* hydrochloric acid (10:1, v/v) and the washings were filtered. The filtrates were pooled and transferred into a 500-ml separating funnel. To the funnel, 200 ml of 5% sodium chloride solution were added, and oxolinic acid was extracted twice with 50 ml of dichloromethane by shaking for 5 min. The dichloromethane extract was filtered through a Whatman 1PS filter-paper and the filtrate was evaporated to dryness under reduced pressure in a water-bath at <40°C. The dried residue was dissolved in 5 ml of 4 *M* potassium hydroxide solution–methanol (1:3, v/v) and transferred into a 300-ml separating funnel. To the funnel, 50 ml of 10% sodium chloride solution and 50 ml of dichloromethane were added and the funnel was shaken for 5 min. After the dichloromethane layer had been discarded and the residual aqueous solution acidified wih 30 ml of 1.2 *M* hydrochloric acid, oxolinic acid in the aqueous solution was extracted twice with 50 ml of dichloromethane by shaking for 5 min. The dichloromethane extract was filtered through a Whatman 1PS filter-paper and the filtrate was evaporated to dryness under reduced pressure in a water-bath at <40°C.

Clean-up

The dried residue was dissolved in two 5-ml portions of dichloromethane and passed through the Sep-Pak silica cartridge. The cartridge was washed with 8 ml of acetone. After the cartridge had been acidified with 0.8 ml of 8.5% phosphoric acid, oxolinic acid in the cartridge was eluted with 15 ml of dichloromethane.

The eluate was evaporated to dryness under reduced pressure in a water-bath at <40°C. The dried residue was dissolved in 4 ml of 0.25 *M* potassium hydroxide solution–methanol (1:9, v/v) and a 10-μl aliquot (equivalent to 25 or 50 mg of the sample) was subjected to HPLC.

Samples used for the stability study of oxolinic acid in frozen samples were prepared as follows: crop homogenate or ground sample (10 or 20 g) was placed in a 300-ml flask and spiked with 10 μg of oxolinic acid. The flask was stoppered, shaken thoroughly and then stored in a freezer at -20°C. After storage, the samples were thawed in a water-bath at 22°C for 10 min, then analysed immediately.

HPLC determination

The HPLC conditions were as follows: column, Inertsil ODS-2 (150 mm × 4.6 mm I.D.); guard column, Brownlee RP-8 (30 mm × 4.6 mm I.D.); eluent, 1 m*M* tri-*n*-octylamine in 0.45% citric acid–tetrahydrofuran–acetonitrile (7:1:2, v/v/v) (pH 2.9); flow-rate, 1.0 ml/min; column oven temperature, 40°C; excitation wavelength, 270 nm; emission wavelength, 370 nm; and sensitivity setting, range 1, fine 5.

Standard solutions of oxolinic acid were prepared as follows: 50 mg of pure standard material were accurately weighed into a 100-ml volumetric flask and the flask was filled up to the mark with 0.25 *M* potassium hydroxide solution. This solution (0.5 mg/ml) was diluted in 0.25 *M* potassium hydroxide solution–methanol (1:9, v/v) to provide standard solutions of concentrations in the range 0.5–3.0 μg/ml.

A 10-μl aliquot of the standard solutions was subjected to HPLC. A calibration graph was pre-

pared by plotting the peak heights against the amounts of oxolinic acid injected. The amount of oxolinic acid in the sample extract was determined by comparing the observed peak height with the calibration graph.

RESULTS AND DISCUSSION

HPLC conditions

Oxolinic acid in the HPLC eluent has strong fluorescence with maximum emission at 370 nm, when excited at 270 and 340 nm. Excitation at 270 nm gives a 2–3 times higher sensitivity than at 340 nm when determined by HPLC.

As oxolinic acid is an organic acid, adjustment of the HPLC eluent is required for its determination. Acidification of the eluent (ion suppression) was not satisfactory as this resulted in tailing peaks, giving non-reproducible elution volumes and poor sensitivity (Fig. 2A). Addition of tetrabutylammonium phosphate or tri-*n*-octylamine to the eluent as an ion-pairing reagent resulted in a sensitive and reproducible determination (Fig. 2B and C). Tri-*n*-octylamine was better than tetrabutylammonium phosphate; the latter, widely used as an ion-pairing reagent, gave broad peaks resulting in lower sensitivity. Further, tetrabutylammonium salts are likely to be deposited in the HPLC line because of lack of solubility, hence requiring conditioning before use and washing after use.

The effect of the alkyl chain length of the trialkylamine on determination was investigated (Table I). Among the trialkylamine compounds tested as ion-pairing reagents, longer alkyl chain compounds

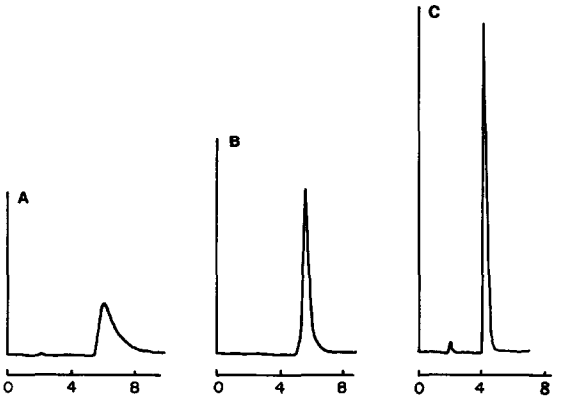

Fig. 2. HPLC determination of oxolinic acid. Detected peak: 20 ng of oxolinic acid standard. HPLC eluent: (A) 0.45% citric acid–tetrahydrofuran–acetonitrile (7:1:2 v/v/v) (pH 2.9); (B) 5 m*M* tetrabutylammonium phosphate/citric acid–tetrahydrofuran–acetonitrile (7:1:2 v/v/v) (pH 2.9); (C) 1 m*M* tri-*n*-oxtylamine/citric acid–tetrahydrofuran–acetonitrile (7:1:2 v/v/v) (pH 2.9). Retention times in min.

gave sharp and symmetrical peaks of oxolinic acid, resulting in higher sensitivity. It was thought that longer alkyl chain compounds formed ion pairs with oxolinic acid, which behaved like a lipophilic compound and prevented irreversible adsorption to residual silanol. Adding tetrahydrofuran to the eluent improved the solubility of tri-*n*-octylamine in the eluent.

Clean-up

For clean-up of the crude extract, liquid–liquid partitioning and column chromatography were used.

TABLE I

EFFECT OF ALKYL CHAIN LENGTH OF ION-PAIR REAGENT ON HPLC DETERMINATION OF OXOLINIC ACID

Ion-paired reagent	Carbon number of alkyl chain	Content[a] (m*M*)	Relative peak height[b]	Retention time (min)[b]
Triethylamine	C_2	2	33	4.4
Tri-*n*-hexylamine	C_6	1	52	4.2
Tri-*n*-octylamine	C_8	0.5	76	4.2
		1	96	4.2
		2	100	4.2

[a] Content in HPLC eluent, 0.45% citric acid–tetrahydrofuran–acetonitrile (7:1:2, v/v/v) (pH 2.9).
[b] 20 ng of oxolinic acid standard were detected.

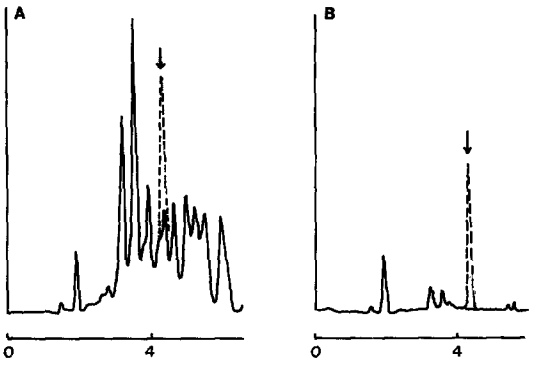

Fig. 3. Chromatograms of rice straw extract (A) before and (B) after clean-up with a Sep-Pak silica cartridge. A 25-mg rice straw sample was injected. Solid lines, control sample; dashed lines, control ground sample spiked at 1.0 ppm.

Oxolinic acid is an organic acid compound, so washing of the alkaline solution, which contained oxolinic acid, with dichloromethane, then extraction into dichloromethane after acidification of the aqueous layer was effective for the removal of co-extractives.

Further clean-up, however, was needed prior to HPLC determination. Silica gel column chromatography (Sep-Pak silica cartridge) was evaluated. Organic solvents were unsuccessful as eluents; oxolinic acid was adsorbed to tightly to the adsorbent that it could not be eluted with acetone or methanol. It was found that oxolinic acid could be eluted from silica gel with dichloromethane when the cartridge was acidified. The chromatographic procedure was as follows: the sample extract (dichloromethane solution) was loaded on the column and the column was washed with 8 ml of acetone. After the column had been fortified with 0.8 ml of 8.5% phosphoric acid, oxolinic acid on the silica gel was eluted with 15 ml of dichloromethane. This method was very effective for purification of the extract; acetone could be used for washing the column, so almost all co-extractives were eliminated (Fig. 3), while oxolinic acid was recovered quantitatively. For acidification of the Sep-Pak silica cartridge, 0.8 ml of acid solution was sufficient to fortify the adsorbent uniformly.

We have successfully applied this chromatographic method to the residue analysis of some acidic metabolites of pesticides.

Recovery and limit of detection

A known amount of oxolinic acid was added to the crop homogenate (or ground sample) and determined by the proposed procedure. The recoveries were between 78 and 95% at fortification levels of 0.5 or 1.0 ppm (Table II), and the limit of detection was 0.01–0.02 ppm; the minimum detectable amount was 0.5 ng and 25–50 mg of the sample was chromatographed. The method was also reproducible (Table II). Typical chromatograms are shown in Fig. 3B. Almost the same chromatograms were obtained for other samples; a few co-extractive peaks were observed on the chromatogram but the oxolinic acid peak was clearly separated from them.

TABLE II

RECOVERIES OF OXOLINIC ACID ADDED TO CROP HOMOGENATES OR GROUND SAMPLES

Crop	Fortification (ppm)	Recovery[a] (%)	Crop	Fortification (ppm)	Recovery[a] (%)
Lettuce	0.5	90 ± 6.4	Onion	0.5	95 ± 0.0
Chinese cabbage	0.5	87 ± 5.9	Japanese radish, leaves	0.5	88 ± 3.6
White potato	0.5	88 ± 5.3	Japanese radish, root	0.5	90 ± 3.6
Cucumber	0.5	91 ± 5.8	Konnyaku	0.5	87 ± 3.1
Melon	0.5	87 ± 3.6	Brown rice	0.5	81 ± 5.1
Broccoli	0.5	82 ± 2.7	Rice straw	1.0	81 ± 3.3
Cabbage	0.5	78 ± 0.9			

[a] Mean ± standard deviation (n = 4–6).

Stability of oxolinic acid in crop homogenates (or ground samples) frozen during storage

For residue analysis, samples are often stored in a freezer before analysis. Therefore, it is important to check the stability of pesticides in samples frozen during storage [9,10]. The stability of oxolinic acid was examined when crop homogenates (or ground samples) were stored at −20°C. It was found that oxolinic acid was fairly stable in crop homogenates (or ground samples) when stored at −20°C; recoveries above 84% were obtained for every sample (listed in Table II) after storage for 16–326 days.

Starner is used for a wide variety of crops, and the proposed simple, sensitive and selective method is applicable to residue analysis of oxolinic acid on crops sprayed with Starner in fields. The principle of this method has been applied to the official method notified by the Environmental Agency of Japan.

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