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Short communication

High-performance thin-layer chromatographic method for the fluorescence detection of three nitroimidazole residues in pork and poultry tissue

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Abstract

A high-performance thin-layer chromatographic method with fluorescence detection was developed for the qualitative determination of ronidazole, dimetridazole and their major metabolite, hydroxydimetridazole, in pork and poultry muscle. After extraction with dichloromethane and evaporation, the nitroimidazoles are redissolved in ammonium acetate buffer. The buffer phase is washed with hexane. The sample is cleaned-up by solid-phase extraction and the eluate evaporated. The final extract is resuspended in methanol and then spotted on an HPTLC plate. After multiple development with methanol and ethylacetate, the plate is dried, sprayed with pyridine and observed on an UV box (312 nm). The detection limits of this method are about 2 μg/kg for ronidazole, 5 μg/kg for dimetridazole and less than 5 μg/kg for hydroxydimetridazole. Validation was performed to levels of 10 μg/kg for dimetridazole, 5 μg/kg for ronidazole and 5 μg/kg for hydroxydimetridazole.

Keywords: Food analysis; Nitroimidazoles; Ronidazole; Dimetridazole; Hydroxydimetridazole

1. Introduction

Nitroimidazoles have been used for control of blackhead in poultry and for protection against protozoal and certain microbial infections in pigs. These compounds are suspected carcinogens. Therefore, the European Community Commission requires the monitoring of their residues in food of animal origin to protect the consumer's health. The use of ronidazole and dimetridazole is now prohibited and this decision requires the development of a sensitive and specific method for the screening of nitroimidazole residues.

Several analytical procedures have already been developed for measuring dimetridazole and hydroxydimetridazole in food. Some of them use HPLC with UV [1–6] or electrochemical detection [7] and the others gas chromatography [8,9]. Our screening method uses clean-up steps similar to those in the HPLC method described by Patel et al. [1].

Few methods exist for the determination of ronidazole [10,11] in animal feed or in tissues. The best mode of detection to achieve sufficient sensitivity in TLC is fluorescence but no analytical method using fluorescence detection has as yet been described for nitroimidazoles. Therefore, we used a pyridine detection method which we had previously developed [12]. No nitroimidazole multiresidue

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screening methods are currently available. All existing methods require a relatively long time for sample preparation, can only detect one compound (sometimes with its metabolite) and cannot be used for large numbers of samples. Our aim was, therefore, to develop a rapid and sensitive multiresidue screening method, able to detect the lowest possible levels of ronidazole, dimetridazole and hydroxydimetridazole.

We succeeded in developing such a method and validated it for concentrations of 10 μg/kg for dimetridazole, 5 μg/kg for RNZ and 5 μg/kg for hydroxydimetridazole according to the statistical procedure established by McClure [13].

2. Experimental

2.1. Chemicals and solutions

All solvents and reagents were of analytical grade. Methanol, dichloromethane, ethylacetate and pyridine were obtained from Merck and acetic acid from Prolabo. Ammonium acetate buffer (0.05 *M*, pH 4.3) was obtained by dissolving ammonium acetate (Merck) in demineralized water and adjusted to the required pH by addition of acetic acid.

2.2. Antibiotics

Dimetridazole was obtained from Sigma, ronidazole and hydroxydimetridazole from Merck Sharp and Dohme.

Two reference standard solutions were prepared: one by dissolving 50 mg ronidazole and 50 mg hydroxydimetridazole in 50 ml methanol in the same flask to obtain concentrations of 1 mg/ml; the other by dissolving 100 mg dimetridazole in 50 ml methanol to obtain a concentration of 2 mg/ml. These solutions were stored at −20°C in the dark and freshly prepared every two weeks. The working standard solutions were prepared by diluting the reference standard solutions in methanol to obtain concentrations of 1 ng/μl for ronidazole and hydroxydimetridazole and 2 ng/μl for dimetridazole. The fortification solutions were prepared by diluting the reference standard solutions in demineralized water to obtain concentrations of 0.1 ng/μl for ronidazole and hydroxydimetridazole and 0.2 ng/μl for dimetridazole. The working standard solutions and fortification solutions were stored at +4°C in the dark for 24 h.

Aliquots (5 μl) of each working standard solution were spotted on both ends of the plate to allow comparison. For the validation, 250 μl fortification solution was added directly to 5.0 g of control minced muscle to produce fortified samples of 10 ng/g dimetridazole, 5 ng/g ronidazole and 5 ng/g hydroxydimetridazole.

2.3. Apparatus

The following equipment was used: rotary shaker Model Rheax II (Heidolph); electric shaker Model Top-mix (Bioblock); ultrasonic bath Model Bransonic 220, 120 W (Brandt); refrigerated centrifuge Model GR4-12 (Jouan); solid-phase extraction system (Touzart et Matignon) and BondElut C_{18} cartridges, 3 ml, 500 mg (Varian); nitrogen evaporator (Prolabo); HPTLC silica gel 60 plates, 10×10 cm or 20×10 cm, without a concentrating zone and without fluorescence indicator (Merck); twin-through chambers Model Camag (Merck); vacuum pump 0.4 bar, 12 W (Bioblock) and a table-type UV 312 nm Model FTX 20MC, 6×15 W (Prolabo).

2.4. Method

Minced tissue (5 g) was put in a centrifuge tube. A 10-ml volume of dichloromethane was added, the tissue was dispersed by vortex-mixing and placed in an ultrasonic bath for 10 min. After centrifugation for 10 min at 3500 rpm and elimination of the water film (by sucking up) and meat, the tube was rested at −20°C for 30 min to congeal the remaining water. Thereafter, the contents were rapidly transfered to a glass tube, which was evaporated to dryness under a stream of nitrogen (an oily residue often remained in the tube). Carefully, 3.5 ml ammonium acetate buffer and 7 ml hexane were added. The tube was placed in a rotary shaker for 2 min at slow speed (about 30 rpm) to minimize any formation of emulsion. Finally, after centrifugation for 10 min at 4000 rpm the hexane fraction was discarded.

The extract was passed through the C_{18} cartridge (previously conditioned with 2×2 ml methanol and 2×2 ml ammonium acetate buffer) with an approxi-

mate flow-rate of 1 drop/s (a vacuum pump can be used). Do not dry the cartridge during these steps. Subsequently, the cartidge was washed with 5 ml ammonium acetate buffer, and the wash was aspirated and discarded. After elution with 2 ml ammonium acetate buffer–methanol (1:3, v/v) the eluate was evaporated to dryness ("just") at 60°C under nitrogen flow. A 40-μl volume of methanol was added as soon as the eluate had evaporated (this evaporation must be surveyed carefully).

2.5. Chromatography and visualization

Whole extracts were spotted on a HPTLC plate and 5 μl of each working standard solution on both ends of the plate. The spots should be placed at least 1 cm apart and 1 cm from the lower plate edge. The plate was eluted with methanol for 3 mm (from the spotting place) and dried under a stream of warm air. Then, it was eluted with ethyl acetate for 4 cm (from the spotting place) and again dried under a stream of warm air. The plate was sprayed evenly with pyridine. When doing so, be careful not to wet the plate. The plate was carefully screened from light and dried under a stream of cold air. Then, the plate was monitored under 312 nm UV light and interpreted after an approximately 30 s wait, because of the very slow photochemical reaction between nitroimidazoles and pyridine. If not all the standards were visible after about 1 min, the plate was sprayed again with pyridine.

3. Results and discussion

During irradiation, nitroimidazoles appear in the following order: ronidazole (as a bluish spot) and then dimetridazole and hydroxydimetridazole (as bluish and yellowish spots). The migration distances are about 25 mm for ronidazole and 21 mm for dimetridazole and hydroxydimetridazole. These distances are only given for guidance but may vary because of the multiple development mode. A pink or yellow spot (possibly due to an endogenous substance) may be observed above the ronidazole. This does not affect results interpretation because of the greater migration distance. Note that this method allows the extraction and detection of metronidazole too (pinkish spot; migration distance of about 18 mm). This compound is usually not used in France in animal species destined for human consumption.

Our studies initially involved extraction and clean-up steps very similar to those described by Patel et al. [1]. However, some changes were essential to develop a multiresidue screening method by planar chromatography. Such a method had to be applicable to all three nitroimidazoles and also be sufficiently sensitive and rapid. We first reduced the extraction and washing volumes and, hence, the time of evaporation. The nitroimidazole residues were extracted from meat by ultrasound and only one extraction was performed. The end of the procedure was also modified because of differences between planar and liquid chromatography: after evaporation of the extract to dryness, the residue was dissolved in 40 μl methanol and then spotted on an HPTLC plate. Multiple development involving methanol and ethylacetate was used to obtain flat spots. Similar results can be obtained by using plates with a concentrating zone, but studies showed that the dimetridazole spots were largely diffused on the concentrating zone during migration and, therefore, poorly detected on this sort of plate.

Different results indicate that particular attention must be paid to the following points: nitroimidazoles are extremely light-sensitive and care must be taken to protect the solutions from light during the manipulations (all work must be carried out in a dark room and brown glassware should be used). The final evaporation step is also critical: excessive drying reduces dimetridazole recovery. Methanol should therefore be added as soon as drying is complete. These precautions are determinant to obtain correct results.

3.1. Evaluation of the method

The method allows a throughput of about 15–20 analyses per day and per operator. It was validated (with fortified samples) according to the statistical procedure described by McClure [13]. The validation principle is as follows: 10 blank muscle samples are prepared and 5 of them are randomly fortified by a person other than the operator (blind validation). This procedure is reiterated each day for five days (for each compound) and the primary results permit

Table 1
Validation of the nitroimidazole residues screening method in muscle with a contamination rate of 50%: results for samples fortified with 5 μg/kg of ronidazole, 5 μg/kg of hydroxydimetridazole and 10 μg/kg of dimetridazole

	Sensitivity	Specificity	False negative rate	False positive rate
Ronidazole	0.92±0.05	0.92±0.05	0.08±0.05	0.08±0.05
Dimetridazole	1	1	0	0
Hydroxydimetridazole	0.92±0.049	0.92±0.049	0.08±0.045	0.08±0.045

calculation of the following parameters: sensitivity rate, specificity rate, false positive rate and false negative rate. The sensitivity rate is the probability that the method will classify a test sample as positive, given that this test sample is a "known" positive. The specificity rate is the probability that the method will classify a test sample as negative, given that the test sample is a "known" negative. The false positive rate is the number of misclassified "known" negatives divided by the total number of positives (misclassified positives plus the number of correctly classified "known" positives) obtained with the method. The false negative rate is the number of misclassified "known" positives divided by the total number of negatives (misclassified positives plus the number of correctly classified "known" negatives) obtained with the method. Details of the calculation are precisely described by McClure [13]. The procedure was validated to levels of 10 μg/kg for dimetridazole, 5 μg/kg for ronidazole and 5 μg/kg for hydroxydimetridazole in pork muscle. The validation results are given in Table 1 and prove the reliability of the method. The detection limits of this method are about 2 μg/kg for ronidazole, 5 μg/kg for dimetridazole and less than 5 μg/kg for hydroxydimetridazole. These are subjective data because the detection is visual. The suitability of this method to poultry muscle was proved by many experiences on this matrix. The method was tested on 60 poultry muscle samples during 6 days. Ronidazole and hydroxydimetridazole were easily detected to level of 5 μg/kg and dimetridazole to level of 10 μg/kg in poultry muscle. The chromatograms appearance are similar for pork or poultry muscle.

4. Conclusion

Our aim was to develop a rapid and sensitive screening method for nitroimidazole residues. The above described method is easily applicable, rapid (with a sample throughput of about 15–20 samples per analyst per day), sensitive for three different nitroimidazoles, and particularly reliable.

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