DRUGS, COSMETICS, FORENSIC SCIENCES

Planar Chromatography for the Multiclass, Multiresidue Screening of Chloramphenicol, Nitrofuran, and Sulfonamide Residues in Pork and Beef

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A method is described for multiclass and multiresidue qualitative detection of chloramphenicol, nitrofuran, and sulfonamide residues in animal muscle. The drugs are extracted from 1 g tissue with 2 mL ethyl acetate and purified by silica solid-phase extraction. After elution of the cartridge, the collected solution is evaporated, and the residue is dissolved in methanol and chromatographed on a Si_{60} high-performance thin-layer chromatography plate. After evaporation of solvent, nitrofurans are visualized first by their specific UV photochemical reaction with pyridine. Then chloramphenicol is reduced to its amino derivative, and this derivative and the sulfonamides are visualized by long-wave UV after reaction with fluorescamine. Chloramphenicol, nitrofurans, and sulfonamides are detected at residue level of 10, 5, and 100 μg/kg, respectively, or less in pork and beef.

To prevent food containing unacceptable levels of drug residue from entering the food supply, health authorities have set up systems of surveillance. Specific and sensitive analytical methods are needed for such surveillance.

Improving the quality/cost ratio of such surveillance systems involves 2 approaches: First is establishment of multiresidue methods allowing detection and quantitation of as many different analytes as possible in a single analysis. Some multiresidue methods now permit single-run analysis of several compounds of a particular chemical class. However few methods involving multiclass and multiresidue techniques are available (1–12), and these generally lack sensitivity to detect analytes at prescribed maximum residue limits (MRLs).

The second approach is a 2-stage strategy based on use of a screening method and a confirmatory method. The screening method is designed to be as inexpensive as possible. Such a method has to be optimized for sample throughput, low level of false-positive results, and, ideally, no false-negative results. Its sensitivity must be in accordance with MRLs. A screening method may only be qualitative. The confirmatory method establishes the concentration of the drug with sufficient specificity with regards to analyte identity. If a drug is not allowed for use (no MRL), the confirmatory method may also be only quantitative, but it must have the highest possible level of specificity.

A method for simultaneous extraction and quantitation of chloramphenicol (CAP), nitrofurans, and sulfonamides has been described (2), but the method lacks sensitivity for the 2 first classes of drugs at their MRLs (3). The present paper describes a 2-stage strategy for these 3 classes of drugs. Planar chromatography (modern thin-layer chromatography) is good for screening because of its practicality (cost, sample throughput. etc.). Because of its intrinsic characteristics, such as post chromatographic revelation, the method also permits derivatization and successive visualization of different drugs. Our objective was to establish a qualitative screening method for residues involving planar chromatography with visual detection of a derivative by fluorescence. The developed method allows qualitative detection of CAP, nitrofurazone, nitrofurantoine, furaltadone, furazolidone, sulfamethazine, sulfadimethoxine. sulfadoxine, and sulfamethoxypyridazine with a sensitivity of ≤10, 5, and 100 μg/kg for CAP, nitrofurans, and sulfonamides, respectively.

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METHOD

Pork and beef were purchased at a local supermarket.

Apparatus

(a) *Chromatographic chamber and sprayer.*—Camag (Merck, Nogent sur Marne, France).

(b) *UV box.*—Table type, 4 × 6W

(c) *Nitrogen evaporator.*—With aluminum block.

(d) *Solid-phase extraction system.*

(e) *Adaptors.*—8 mL reservoirs and taps (Analytichem, Prolabo, Paris, France).

(f) *Solid-phase extraction cartridge.*—Silica Sep Pack, 1 mL (Millipore, Waters, St Quentin en Yvelines, France).

(g) *Vacuum manifold.*—Prolabo.

(h) *Water purification system.*—MilliRo (Millipore, Waters).

(i) *Vortex mixer.*—With multitube rack (Prolabo).

(j) *Oven (20°–220°C) Binder.*—Thouzard et Matignon, Vitry/Seine, France.

(k) *Automatic pipettes.*—P 100 and P 20, Gilson, Villiers le Bel, France.

Reagents

(a) *Deionized water.*

(b) *Ethyl acetate, pyridine, acetonitrile, dioxane, acetic acid, stannous chloride, phenolphthalein, boric acid, sodium hydroxide, hexane, methanol, dimethylformamide, and sodium sulfate (reagent grade).*—Merck.

(c) *Chromatographic plates.*—Si_{60} with a concentrating zone and without fluorescent indicator, 10 × 10 or 20 × 10, (Merck).

(d) *Potassium chloride.*—Prolabo.

(e) *Fluorescamine, nitrofurazone, furazolidone, furaltadone, nitrofurantoine, chloramphenicol (CAP), sulfamethazine, sulfadimethoxine, sulfadoxine, and sulfamethoxypyridazine.*—Sigma Chemicals (La Verpilliere, France).

(f) *Solution for CAP reduction.*—Dissolve 0.4 g stannous chloride in 10 mL 5% acetic acid in deionized water and add 0.1 mL 5% phenolphthalein in dioxane. The reagent is unstable and should be prepared just before use.

(g) *Buffer, pH 8.3.*—Dissolve 19 g boric acid and 19.75 g potassium chloride in 0.8 L water, adjust pH with concentrated NaOH, and dilute to 1 L.

(h) *Visualizing reagent.*—Dissolve 50 mg fluorescamine in 500 mL acetone. The solution is stable for at least 12 months when stored frozen.

(i) *CAP solution.*—Dissolve 100 mg CAP in 100 mL methanol to get solution C0. Dilute solution C0 $1/100$ with methanol to get solution C1.

Dilute solution C1 $1/10$ with methanol to get solution C2 (1 μg/mL). Dilute solution C1 $1/100$ with water to get solution C3 (0.1 μg/mL). Solution C0 should be prepared freshly every month, and solutions C1, C2, and C3 must be prepared every week. Solutions must be stored in a refrigerator.

(j) *Nitrofuran solutions.*—Dissolve 100 mg nitrofuran in 10 mL dimethylformamide and make up to 100 mL with methanol. Dilute $1/100$ with methanol to obtain solution F1 for furazolidone, solution F2 for furaltadone, solution F3 for nitrofurazone, and solution F4 for nitrofurantoine (10 μg/mL). Transfer 10 mL of solutions of F1, F2, F3, and F4 in a 100 mL volumetric flask and make up to the volume with methanol to obtain solution F5 (1 μg of each nitrofuran/mL).

Dilute solution F1 (then solutions F2, F3, and F4 respectively) $1/10$ with methanol to obtain solutions F′1, F′2, F′3, and F′4 (1 μg/mL). Dilute solutions F′1, F′2, F′3, and F′4 $5/100$ with water to obtain solutions F″1, F″2, F″3, and F″4 (0.05 μg/mL). Nitrofurans are light sensitive. Care must be taken to avoid sunlight and fluorescent lighting as much as possible during the assay by using brown flasks or protecting them with aluminum foil. Solutions F1–F4 can be stored for 1 month at –18°C, but diluted solutions must be freshly prepared every week. These solutions must be stored in a refrigerator.

(k) *Sulfonamide solutions.*—Dissolve 100 mg sulfonamide in 100 mL methanol to obtain solution Sa0 for sulfamethazine and solutions Sb0, Sc0, and Sd0 for sulfadimethoxine, sulfadoxine, and sulfamethoxypyridazine, respectively. Dilute solutions S0 $1/10$ with methanol to obtain solutions Sa1, Sb1, Sc1, and Sd1 (100 μg/mL). Dilute solutions S1 $1/100$ with methanol to obtain solutions Sa2, Sb2, Sc2, and Sd2 (1 μg/mL). Dilute solutions S2 $1/100$ with water to obtain solutions Sa3, Sb3, Sc3, and Sd3 (1 μg/mL).

The concentrated solutions can be stored for several months in a freezer, but the diluted solutions must be freshly prepared every 2 weeks. These solutions must be stored in a refrigerator.

Sample Preparation

Blend animal tissue in a Waring blender to pulplike consistency. To prepare fortified samples, add 100 μL of the appropriate solution in water—0.1 ng/μL for CAP (C3), 0.05 ng/μL for nitrofurans (F″1, F″2, F″3, and F″4), and 1 ng/μL for sulfonamides (Sa3, Sb3, Sc3, and Sd3)—to 1 g drug-free ground tissue.

Extraction

Weigh 1 ± 0.05 g blended tissue sample in a 12 × 10 mm test tube. Add 0.25 mL ethyl acetate and stir for 1 min. While continuously stirring, add 0.25 mL fractions of ethyl acetate every minute for 7 min (final volume of ethyl acetate, 2 mL). Then place the tube in an ultrasonic bath for 10 min. Centrifuge the sample for 10 min at 3500 rpm (ca 2300 × *g*). Collect the supernatant in a 10 mL centrifuge tube, add 5 mL hexane, and shake on Vortex mixer. Centrifuge again for 10 min at 3500 rpm.

Purification

Deposit extract in a barrel connected to a silica Sep Pack Vac 1 cc cartridge. (Generally, the solution elutes through the cartridge by gravity flow. When vacuum is used, care must be taken to maintain a flow of ca 1 drop/s.) Rinse the cartridge successively with 2 mL ethyl acetate–hexane (3 + 1, v/v) and 2 mL acetonitrile–methanol (95 + 5, v/v), and discard eluates. Elute the column with 2 mL acetone–hexane (2 + 1, v/v). Evaporate the collected organic phase to dryness under a nitrogen flow. Dissolve the residue in 50 μL methanol.

Chromatography

Spot the whole volume of extract (by using automatic pipets) on a Si_{60} silica gel plate. Also spot 2 other tracks on both edges of the plate: tracks 1 and 1′, 4 ng of each of the nitrofurans (4 μL of solution F5); track 2, and 2′, 4 and 8 ng, respectively, of CAP (4 and 8 μL of solution C2); and overspot tracks 1, 1′, 2, and 2′, 4 ng of each sulfonamide (4 μL of solutions Sa2, Sb2, Sc2, and Sd2). Air dry the plate and then elute with ca 4 mL ethyl acetate–hexane (2 + 1, v/v) to 5 cm above the concentrating zone.

Detection of residues

(a) *Derivatization 1, visualization of nitrofurans.*—Spray the plate evenly with a very small amount of pyridine. (Two

passes of sprayer for ca 1 s each time is sufficient. If not sufficient, standard spots are not detectable under UV light). Examine the plate under UV at 366 nm. After a few seconds, the furans appear as yellow fluorescent spots on a purple background. At very low amounts, the spots of furans appear blue. The rf value of nitrofurazone is ca 0.2 (measured from the beginning of the chromatographic zone), and the subsequent order of elution (in increasing rf) is furaltadone, furazolidone, and nitrofurantoine.

(b) *Derivatization 2, visualization of sulfonamides and CAP.*—Place the plate for 10 min in an oven set at 110°C to eliminate pyridine. (*Caution*: Pyridine is toxic; care must be taken against inhalation of its vapors). Cool and spray evenly with stannous chloride solution until the plate appears gray (do not wet the plate). Let the plate stand for 15 min in the dark and then place it for 15 min in a 110°C oven. Cool the plate and then spray carefully with a small volume of sodium hydroxide solution until the plate becomes ever so slightly pink. Spray sodium borate buffer until the plate becomes slightly gray. Dry the plate again in the oven for 15 min. When cool, spray the plate with fluorescamine solution. CAP and sulfonamides appear as yellow spots on a purple background when viewed under UV at >366 nm. The rf value of chloramphenicol is ca 0.4, and those of sulfonamides range from 0.75 to 0.90.

Results and Discussion

A previously described validation (13) process was used. This process consisted of a series of n blind experiments, each involving 5 samples of drug-free control and 5 samples fortified with the drug of interest at a specified level. Raw results are coded as follow: good results (fortified sample found positive and blank samples found negative) = 1 and erroneous results (fortified sample found negative and blank samples found positive) = 0. A χ^2 analysis allows to test the homogeneity of the n experiments. When the calculated χ^2 value exceeds the value in the table for $(n - 1)$ degree of freedom (df), one (or several) experiment(s) gives results that significantly differ from those of the other experiments. In that case, it is possible to separate the experiments into 2 classes: one defined as an outlier and the other as a nonoutlier. It is then possible to test whether the proportions of good results are the same for both groups. When the new calculated χ^2 value exceeds the χ^2 value for df = 1, the results significantly differ. In that case, it is of interest to test whether the results are homogeneous in both groups. When it is demonstrated (by χ^2 analysis) that results are homogeneous, it is then possible to calculate the method's performance for each particular drug (for details of calculation, *see* reference 14).

Table 1. Validation results for chloramphenicol (6 experiments, n = 60)

Parameter	Value
Sensitivity	1
Specificity	1
False-positive rate	0
False-negative rate	0

Certain concepts require definition: Sensitivity is the probability of finding positive a portion incurred at the specified level of concentration (it is calculated by dividing the total number of positive results among the fortified samples by the total number of fortified samples). Specificity is the probability of finding negative a blank portion under the same conditions (it is calculated by dividing the total number of negative results among the blank samples by the total number of blank samples).

The CAP validation was performed at a fortification level of 10 μg/kg (6 blind experiments with n = 60 samples). Results are summarized in Table 1. The nitrofuran drug validation was performed at a fortification level of 5 μg/kg. Results are summarized in Table 2. The sulfonamides were validated at a fortification level of 100 μg/kg. Results are summarized in Table 3.

Modern farming involves use of veterinary drug treatments to prevent or cure animal diseases or to promote growth. To protect the health of consumers, MRLs have been set by national and international organizations such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA). In Europe, use of CAP (for which the MRL in muscle was 10 μg/kg), was banned recently because a definitive MRL could not be established. Use of CAP in food-producing animals in the United States never has been authorized. Nevertheless, the minimum mandatory sensitivity for methods to monitor illegal use is 10 μg/kg (15). The European Community (EC) MRL for nitrofurans in muscle was 5 μg/kg, but use of nitrofurans for treatment of food-producing animals recently was banned. For sulfonamides, the EC MRL in muscle is set at 100 μg/kg.

The planar chromatographic method allows screening of residues in food with good sensitivity and a sample throughput

Table 2. Validation results for nitrofurans

Parameter	Value for			
	Nitrofurazone (3, n = 30)[a]	Furaltadone (3, n = 30)[a]	Furazolidone (2, n = 20)[a]	Nitrofurantoine (2, n = 20)[a]
Sensitivity	1	1	1	1
Specificity	1	1	1	1
False-positive rate	0	0	0	0
False-negative rate	0	0	0	0

[a] Number of experiments and total number of samples.

Table 3. Validation results for sulfonamides, *n* = total number of samples

Parameter	Value for			
	Sulfamethazine (3, $n = 30$)[a]	Sulfadimethoxine (3, $n = 30$)[a]	Sulfadoxine (2, $n = 20$)[a]	Sulfamethoxypyridazine (2, $n = 20$)[a]
Sensitivity	0.93	1	1	1
Specificity	1	1	1	1
False-positive rate	0	0	0	0
False-negative rate	0.062	0	0	0

[a] Number of experiments and total number of samples.

of about 20 samples/analyst/day. Although it involves rather tedious extraction, purification, and visualization steps, it does not require any expensive apparatus. Moreover, unlike liquid chromatography, planar chromatography does not require time-consuming column equilibration. So the analyst may, if necessary, conduct extraction, purification, and chromatography of other analytes at the same time (15). The reported drugs may be screened in meat with sensitivities compatible with consumer health protection: it has been stated that the minimum analytical sensitivity for consumer protection for chloramphenicol is 10 μg/kg (16). The limit used by the U.S. Food and Drug Administration for enforcement purposes for nitrofurans in 1989 was 0.1 mg/kg (17), and the expected limit of sensitivity with the method described here is 0.001 mg/kg. Validation for sulfonamides was performed at the level of MRLs (17).

The detected sensitivity for sulfamethazine differs from 1 because of one false-negative result. To allow elimination of this experiment, more than 10 experiments without a false-negative result is necessary; therefore, we did not try it. However this result helps to make the significance of the calculated sensitivities more comprehensible. These results were obtained for a lot contamination of 50% (fortification of 50% of the samples). It could be expected that for field samples the contamination should be less than 5%. According to McClure (14), the expected sensitivity is <0.00367; that is, for 10 000 samples analyzed negative, only 37 would be expected to be positive with regards to the level of fortification. (For a contamination of less than 5%, the expected false-negative proportion would decrease.)

Moreover the method is of intrinsic security: The higher the drug concentration in the tissue, the greater the probability of detection. But because an actual amount of drug is detected on the plate, a slight increase in concentration involves a larger increase in sensitivity. With its sensitivity—for CAP at 10 μg/kg = 1, for the 4 nitrofurans at 5 μg/kg = 1, for the sulfa drugs at 100 μg/kg = 1 (except sulfamethazine = 0.93)—the described method may ensure consumer health protection. Once trained on the method, an analyst can perform 20 analyses in a working day. This method does not require any expensive apparatus, which can be set aside for confirmatory purposes. Moreover the method allows detection of 3 classes of residues in a single analysis at a sensitivity in accordance with consumer health safety. For these reasons, this planar chromatographic method may be of interest for regulatory control.

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