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Integrated strategy for the qualitative and quantitative analysis of residues of antimicrobial substances in food products

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SUMMARY. An efficient drug residue monitoring should involve screening as well as confirmatory methods. A demonstration of the feasability of an integrated strategy for the qualitative and quantitative analysis of residues of antibacterial substances in foodstuffs of animal origin (edible tissues) was aimed by the running project. Screening methods - high performance thin layer chromatography for sulfonamide residues and enzymatic immunoassays for tetracyclines and aminoglycosides (neomycin), followed by confirmation by means of high performance liquid chromatography with mass-spectrometric detection, were carried out. The screening methods used were all simple and relatively low-cost, and provided reliable results. Out of the samples tested, 16, 81, 13 % of kidney and 31, 75, 9 % of muscle samples were positive for tetracyclines, sulfonamides or neomycin.

KEYWORDS: antibiotics; residues; foods of animal origin; screening

An increased availability and use of veterinary drugs in livestock production is paralleled by a rising concern to the presence of their residues in the food supply. Residue monitoring is an issue of great interest of health authorities, food producers, distributors, and consumers as well. Regarding the residues, antimicrobial drugs represent a particularly important group of drugs. These compounds may be used in food producing animals for the treatment of diseases or, additionally, to prevent their outbreak, and to promote growth. The illicit use (e. g. over-dosage, improper withdrawal time) of the drugs may result in the presence of their residues in animal derived foodstuffs.

Residues of antimicrobials could represent a risk for consumer health (toxic effects, allergic reactions, potential development of resistant microbial

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strains). But they could be harmful to the treated animal, too, and cause technological problems in the fermentative processing of some foods as well.

To safeguard human health, the European Union (EU) has set safe maximum residue limits (MRLs) for residues of veterinary drugs in products of animal origin entering the human food chain. The establishment of MRLs in the EU is governed by Council Regulation 2377/90 EEC [1]. This regulation includes lists of compounds that have a fixed MRL (Annex I), that need no MRL (Annex II) or which have a provisional MRL (Annex III). Compounds prohibited in veterinary use are listed in Annex IV.

Monitoring of veterinary drug residues is governed by national surveillance schemes, established under Council Directive 96/23/EC [2]. Criteria that define the performance expected of both screening and confirmatory methods for residues have been established in Commission Decision 93/256/EEC [3].

In the USA, the Food Safety and Inspection Service (branch of the United States Department of Agriculture) has developed „Compound evaluation and analytical capability national residue program plan" to monitor the presence of antimicrobials in tissues from food animals and to set their governmental tolerances [4].

For controlling the application of the European directives and regulations, a program of control of residues, mainly substances illegally used as growth promoters and veterinary drugs, including unauthorized substances which could be used as veterinary drugs, must be put into place annually and executed by member states of the EU after approval by the Commission. Besides the controls performed at random and planned in the framework of the national plan, there are also directed controls based on indications of illegal use or in case of forced slaughter [2].

For antibiotics and other growth inhibiting substances, a suitable control system should consist of, at least, these stages:

- prescreening at the level of the slaughterhouses by means of microbiological tests for the presence of growth inhibiting substances;
- selective screening of the such identified positives by means of conventional analytical methods (e. g. immunoassays, high performance thin layer chromatography - HPTLC) to pursue identification of the group of growth inhibitors, namely sulfonamides, β–lactams, tetracyclines, aminoglycosides, chloramphenicol and macrolides;
- chemical identification within the group of individual growth inhibitors by means of gas chromatography - GC, GC with mass-spectrometric detection, high performance liquid chromatography with fluorescence or tandem mass-spectrometric detection - LC/MS/MS;

- quantitative assay of the identified residues in view of the established MRLs.

The latter three stages are not yet operational, due to the lack of an integrated analytical strategy.

A demonstration of the feasability of such an „integrated strategy for the qualitative and quantitative analysis of residues of antibacterial substances in foodstuffs of animal origin“ was aimed by the running project. Harmonization of analytical methods in the food product sector finally should allow a better protection of consumer's health and would offer better guaranties for the quality of the product.

Material and methods

Bovine and porcine kidney samples were collected in the directed control at the slaughterhouses with about 6 % of positives in microbiological prescreening. Random kidney samples, taken out from these positives, together with the tissue samples of the suspected carcasses, as well as blank kidney, were collected and transferred to the laboratories for the development of the pilot analytical methodology including selective (group) screening, identification and quantification of active substances.

The selective screening stage was carried out in two phases. The first series of methods was looking for sulfonamides and tetracyclines, the second set looking for aminoglycosides (neomycin) was carried out only with samples negative for the two previous groups.

In the frame of this study, two commercially available immunoassay kits for tetracycline screening were employed: Ridascreen Tetracyclin kit (Product No. R 3501, Lot No. 01010 and 01330) by R-Biopharm GmbH, Darmstadt, Germany and LacTek TC by IDEXX Laboratories, Westbrook, Maine, USA. At this stage, 80 samples of porcine and bovine kidney and 84 muscle samples submitted by IVK have been analysed for presence of tetracyclines by means of Ridascreen Tetracyclin. High performance liquid chromatography - tandem mass-spectrometric method (by means of the LC/MS/MS system Water Alliance 2690 HPLC system from Waters, Milliford, Massachusetts, USA coupled to a Quattro LCZ mass spectrometer from Micromass UK, Altrincham, Chesire, UK) with on-line extraction and sample clean-up was used for confirmation of residues of tetracyclines in evaluated samples [5].

Since only single-compound immunochemical commercial kits are available until now, the qualitative analysis of sulfonamides in meat and kidney samples was performed by an ethylacetate extraction of homogenized tissue followed by SPE on C18 columns. A 30-times concentrated eluate in acetonitrile/methanol (9/1, v/v) was subjected to HPTLC with a mobile phase of chloroform/*n*-butanol (8/2, v/v) followed by visualization with fluorescamine at 366 nm [6]. We performed the screening of 21 pairs of porcine and bovine kidney and muscle samples (in 42 samples) for sulfamethazine, sulfadimethoxine, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethoxazole, sulfaquinoxaline and sulfapyridine. During this procedure, there was a co-elution of some interfering matrix components resulting in a poor resolution of particular compounds. We have evaluated another extraction and purification method for tissue samples described by Haagsma et al. [7,8]. Other 73 porcine and bovine muscle samples and 59 kidney samples were analyzed for sulfamethazine, sulfadimethoxine, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethoxazole, sulfaquinoxaline and sulfapyridine. A high performance liquid chromatography - tandem mass-spectrometric (LC/MS/MS) method with on-line extraction and sample clean-up was used for confirmation of residues of sulfonamides in evaluated samples [9].

Screening of samples for neomycin as a representative of the group of aminoglycosides was performed by means of a conventional immunoassay kit Neomycin EIA (Euro-Diagnostica B. V., Arnhem, The Netherlands; lot No. BN 5469). In total, 46 meat samples and 47 kidney samples which were negative for either of sulfonamides and tetracyclines in screening procedures, were processed by neomycin EIA.

Results and discussion

Tetracycline immunoassay

Tetracyclines, derived from *Streptomyces* sp., are active against a broad range of Gram-positive and Gram-negative bacteria. They act by inhibiting protein biosynthesis through their binding to the 30S ribosome.

Chlorotetracycline (CTC), doxycycline (DC), oxytetracycline (OTC) and tetracycline (TC) are the most frequently used tetracyclines for veterinary purposes. According to EU legislation, the sum of parent drug and its 4-epimer in the case of each of CTC, OTC and TC should not exceed 100 $\mu g.kg^{-1}$ in muscle and 600 $\mu g.kg^{-1}$ in kidney of all food-producing animal species. For DC, the MRLs are set at levels of 100 $\mu g.kg^{-1}$ in bovine and porcine muscle, and 600 $\mu g.kg^{-1}$ in kidney [1].

For the screening of tetracycline residues in animal tissues, a number of physicochemical methods have been described. All of them require extensive sample clean-up: liquid-liquid extraction, solid-phase extraction (SPE), matrix solid phase dispersion (MSPD), metal chelate affinity chromatography (MCAC). Quantitative analysis is usually carried out by means of high performance liquid chromatography - HPLC, followed by the spectrometric or fluorimetric detection.

Immunoassays, based on the antibody - antigen interaction, are highly selective and theoretically enable analytical procedures to be carried out without sample pretreatment. However, non-specific binding of matrix components could be a distinct problem. Therefore, some form of sample pretreatment, mainly liquid-liquid extraction or SPE, is necessary.

31 % of the meat samples and 16 % of the kidney samples tested by means of Ridascreen Tetracyclin contained tetracyclines at levels higher than set MRLs (Table 1).

Tab. 1. Screening of tissue samples for antibiotics.
Tab. 1. Skríning vzoriek živočíšnych tkanív na obsah antibiotík.

Antibiotic[1]	Tetracyclines[2]		Sulfonamides[3]		Neomycin[4]	
Tissue[5]	t	p [%]	t	p [%]	t	p [%]
Kidney[6]	80	16	59	81	47	13
Muscle[7]	84	31	73	75	46	9

t - number of samples tested, p - samples positive for antibiotics.

t - celkový počet analyzovaných vzoriek, p - vzorky obsahujúce antibiotiká.
1 - antibiotikum, 2 - tetracyklíny, 3 - sulfónamidy, 4 - neomycín, 5 - tkanivo, 6 - obličky, 7 - svalovina.

Sulfonamide HPTLC

Sulfonamides are bacteriostatic antimicrobials acting by competing with *p*–aminobenzoic acid in the enzymatic synthesis of dihydrofolic acid. This leads to a decreased availability of reduced folates that are essential for purine synthesis, methionine synthesis, etc. Their residues in food of animal origin are of great concern because of a possibility that exposure to these drugs could suppress the effectiveness of human therapeuticals and because of a potential carcinogenity of some sulfonamides.

For control purposes, all substances belonging to the group of sulfonamides need to be determined and the total amount of the residues of parent molecules should not exceed 100 μg.kg^{-1} in muscle and in kidney of all food-producing animal species [1].

For the determination of sulfonamides in animal tissues, a variety of analytical methods have been already described. All of them require various extraction and sample clean-up procedures. Liquid-liquid extraction, SPE and MSPD are mostly employed. HPLC with fluorescence or mass-spectrometric detection is usually utilized for the quantitation of sulfonamides in tissues. Besides several single-compound immunochemical assays, a new immunochemical screening method utilizing polyclonal antisera to 8 different sulfonamides has been developed recently.

38 % of the meat samples and 62 % of the kidney samples were positive for sulfonamides when tested by HPTLC according to Posyniak et al. [6]. Results obtained by HPTLC according to Haagsma et al. [7,8] showed a very good resolution of particular sulfonamides. The limit of detection for standard solutions and spiked kidney samples was 15 μg.kg^{-1}, i. e. 0.3 ng of total sulfonamides per spot on the HPTLC plate. The described method was improved by an adaptation of the composition of the mobile phase of chloroform/*n*-butanol (Merck) to 9/1 (v/v). The R_f-values of particular sulfonamides are presented in Table 2.

Visual evaluation of HPTLC plates with a concentration gradient of 5, 10, 15, 20, 25, 50, 75, 100, 200 μg.kg^{-1} of mixes of the 8 sulfonamide standards

TAB. 2. R_f-values of 8 sulfonamides on HPTLC silica gel plates (eluent: chloroform/*n*-butanol, 9/1).
TAB. 2. R_f-hodnoty 8 sulfónamidov na silikagélovej HPTLC platničke (vyvíjacia sústava: chloroform/*n*-butanol 9:1).

Compound[1]	R_f-value[2]
Sulfadimethoxine	0.65
Sulfamethoxazole	0.57
Sulfquinoxaline	0.55
Sulfamethazine	0.52
Sulfamerazine	0.47
Sulfadiazine	0.42
Sulfapyridine	0.35
Sulfathiazole	0.22

1 - zlúčenina, 2 - R_f-hodnota.

has been done either by trained or untrained laboratory staff. Set MRLs (100 μg.kg^{-1}) could be reliably recognized even by untrained personnel.

75 % of the analyzed meat samples and 81 % of the kidney samples were positive for sulfonamides (Table 1).

Confirmation of sulfonamide residues in meat and kidney samples

From 13 analysed samples of meat, screened by HPTLC according to Posyniak et al. [6], 23 % was found false positive and 8 % false negative in the screening procedure. From 17 meat samples, screened by an improved HPTLC according to Haagsma et al. [7,8], 6 % was found false negative and 6 % false positive. According to these results, the latter HPTLC method seems more suitable for screening purposes.

Neomycin immunoassay

Streptomyces spp. and *Micromonospora* spp. produce aminoglycosides, broad-spectrum antibiotics consisting of an aminocyclitol ring connected to 2 or more aminosugars in a glycosidic linkage. The antimicrobial activity of aminoglycosides is based on their ability to inhibit bacterial protein synthesis.

In veterinary therapy, neomycin, gentamicin, kanamycin, streptomycin, and dihydrostreptomycin are the most commonly used aminoglycosides. Within the EU, provisional MRLs for neomycin have been fixed at 5.0 mg.kg^{-1} in kidney and 0.5 mg.kg^{-1} in muscle tissue of all food-producing animals [1].

For screening purposes, aminoglycosides have been analysed in animal tissues by radioenzymatic assays and radioimmunoassays, by enzyme immunoassays and fluorescence polarization immunoassays.

Neomycin EIA kits used facilitated recovery rates varying from 17 to 53 % for meat samples and from 32 to 78 % for kidney. 9 % of meat and 13 % of kidney samples were found positive for neomycin (Table 1).

Aminoglycosides exhibit physicochemical properties that impair the development of suitable confirmatory methods. They are basic and very hydrophilic, making extraction from complex biological matrices difficult; and they are thermally labile, making analysis by gas chromatography impossible. Ion-pair chromatography with the volatile fluorinated carboxylic acids as counterions seems to be the most appropriate chromatographic method for their analysis. LC/MS should be also a suitable tool to analyse them.

Development of a method for the extraction of antibiotics from biological matrices suitable for screening, confirmation and quantification at once

seems to be a crucial step to reach an integrated strategy for the analysis of drug residues in food products. From another point of view, particular attention should be paid to the extraction of different groups of antibiotics to get one extract for confirmative analysis of total antibiotic residues by LC/MS/MS.

Conclusion

Currently, it can be concluded that an integrated strategy for the qualitative and quantitative analysis of residues of antimicrobial substances in foods of animal origin can be conducted successfully.

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References

1. Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down the examination of the animals, meat and meat products for residues of veterinary drugs and the establishment of their maximum residue limits. Official Journal of the European Community, 1990, No. L 224, p. 1-10.
2. Council Directive 96/23/EC of 29 April 1996 laying down the national surveillance schemes for monitoring of veterinary drug residues. Official Journal of the European Community, 1996, No. L125, p. 10-18.
3. Commission Decision 93/256/EEC of 14 April 1993 laying down the methods to be used for detecting of hormones. Official Journal of the European Community, 1993, No. L118, p. 64-74.
4. AGARWAL, V. K.: Analysis of antibiotic/drug residues in food products of animal origin. New York and London : Plenum Press, 1992. 263 p.
5. VAN EECKHOUT, N. - PEREZ, J. C. - CLAEREBOUDT, J. - VANDEPUTTE, R. - VAN PETEGHEM, C.: Determination of tetracyclines in bovine kidney by liquid chromatography/tandem mass spectrometry with on-line extraction and clean-up. Rapid Communication in Mass Spectrometry, *14*, 2000, p. 280-285.
6. POSYNIAK, A. - NIEDZIELSKA, J. - SEMENIUK, S. - ZMUDSKI J.: Isolation and determination of sulfonamide residues in animal tissues by solid phase extraction and chromatographic techniques. In: HAAGSMA, N. - RUITER, A. (Eds.): Proceedings of Euroresidue III, Conference on Residues of Veterinary Drugs in Food. Veldhoven, The Netherlands : RIVM, Bilthoven, 1996, p. 804-807.
7. HAAGSMA, N. - DIELEMAN, B. - GORTEMAKER, B. G. M.: A rapid thin-layer chromatographic screening method for five sulfonamides in animal tissues. Veterinarian Quarterly, *6*, 1984, No. 1, p. 8-12.

8. HAAGSMA, N. - PLUIJMAKERS, H. G. J. M. - AERTS, M. M. L. - BEEK, W. M. J.: Rapid sample preparation methods for analysis of residues of sulfamethazine and its N^4-acetyl- and desamino-metabolites in swine tissue by HPLC. Biomedical Chromatography, *2*, 1987, No. 1, p. 41-45.
9. VAN EECKHOUT, N. - VAN PETEGHEM, C.: Determination of eight sulfonamides in bovine kidney by liquid chromatography/tandem mass spectrometry with on-line extraction and sample clean-up. In: VAN GINKEL, L. A. - RUITER, A. (Eds.): Proceedings of Euroresidue IV, Conference on Residues of Veterinary Drugs in Food. Veldhoven, The Netherlands : RIVM, Bilthoven, 2000, p. 426.

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Integrovaná stratégia kvalitatívnej a kvantitatívnej analýzy reziduí antimikrobiálnych látok v požívatinách

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SÚHRN. Efektívne monitorovanie reziduí liečiv by malo zahŕňať metódy skríningové aj konfirmačné. Názorná demonštrácia možnosti integrovanej stratégie kvalitatívnej a kvantitatívnej analýzy reziduí antibakteriálnych látok v živočíšnych potravinách (svalovina, vnútornosti) bola cieľom rovnomenného projektu. Testovala sa kombinácia skríningových metód (vysokoúčinnej tenkovrstvovej chromatografie na analýzu reziduí sulfónamidov a enzýmové imunometódy na analýzu tetracyklínov a aminoglykozidov - neomycínu) a konfirmačnej vysokoúčinnej kvapalinovej chromatografie s hmotnostnospektrometrickou detekciou. Použité skríningové metódy boli jednoduché, relatívne lacné a dostatočne spoľahlivé. Zo skúmaných vzoriek obsahovalo 16, 81, 13 % obličiek a 31, 75, 9 % svaloviny reziduá tetracyklínov, sulfónamidov, resp. neomycínu.

KĽÚČOVÉ SLOVÁ: antibiotiká; reziduá; potraviny živočíšneho pôvodu; skríning