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Three for the price of one! Immunodetection of three amphenicols in foodstuffs using a universal standard curve

Inna A. Galvidis,[a] Sergei A. Eremin[b] and Maksim A. Burkin*[a]

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Antibiotics from the amphenicol family are widely used to treat farm animals; however, the regulation of their residues in livestock production varies significantly and depends on the type of animal and the type of particular amphenicol. In this regard, different requirements are imposed on immunoanalytical systems for their identification. In the present study, direct and indirect ELISA formats were developed for simultaneous determination of florfenicol (FF), its main metabolite florfenicol amine (FFA), and thiamphenicol (TAP) using antibodies against FFA conjugated to succinylated BSA. Among a panel of heterologous conjugates, a glutaraldehyde linked FFA-coating antigen and tracer were the only ones which provided equal recognition of FFA, FF, and TAP. Owing to equal 100% cross-reactivity, these analytes could be quantified in chicken muscle at their MRL level using a single universal standard curve. Recovery of amphenicols was in the range 77.2–98.8% with variation no more than 9.6%. The prohibited drug chloramphenicol was undetectable (<0.1%) and had no influence on the assay developed for quantification of approved amphenicols.

Introduction

Thiamphenicol (TAP), florfenicol (FF) and chloramphenicol (CAP) are practically important representatives of a class of amphenicols. These synthetic antibiotics have similar structures composed of three functional units: the phenyl ring substituted in the *para*-position and the dichloroacetyl group connected through a 2-amino-propanol chain with hydroxyl or fluorine at C3 (Fig. 1). The capability of amphenicols to block the peptidyl transferase on the 50S ribosome subunit of bacteria provides a wide spectrum of antimicrobial activity against most Gram-positive and Gram-negative aerobic and anaerobic bacteria, chlamydia, spirochetes and *Rickettsia*.[1]

CAP was discovered initially as a natural product isolated from *Streptomyces venezuelae* in 1947, and from 1949 it has been introduced in practice as synthetic medicine.[2] Being a valuable anti-infective agent,[3] CAP, nevertheless, has a number of serious side effects on hematopoiesis causing bone marrow suppression, aplastic anaemia, and leukemia. CAP is metabolized in the organism into an inactive form by glucuroconjugation. Thus, CAP–glucuronide may serve as a marker for the administration of CAP, but it does not exhibit antimicrobial activity.

TAP is a *p*-methylsulfonyl analogue of CAP and 2.5 to 5 times more potent but has never been associated with aplastic anaemia.[1] It is mainly a veterinary antibiotic; however, it is prescribed to humans in China, Morocco, Brazil and Italy. TAP is not readily metabolized in cattle, poultry, sheep, or humans, so it is excreted predominantly unchanged. In pigs and rats, the drug is excreted both as a parent drug and as a TAP glucuronide (FAO, 1997).

FF is a derivative of TAP with substitution of C3 hydroxyl by fluorine. Such derivation increases antibacterial activity and resistance to bacterial acetylases. FF has been developed for use in veterinary medicine. A number of its metabolites are known including FF amine, FF alcohol, FF oxamic acid, FF amine glucuronide and monochloro-FF.[4] However, the significant metabolite that exhibits antimicrobial activity is FF amine (FFA). Thus, it is established as a marker substance of FF administration.[5]

Amphenicols are widely used not only in human medicine and veterinary practice but also in livestock farming for treating and prophylaxis of infections in dairy and meat producing animals, poultry and fish. To provide food safety and to avoid non-target antibiotic intake and possible side effects on consumers, the permissible residue levels of amphenicols in food products of animal origin are legislated (Table 1).

The contaminants of amphenicols can be revealed by three types of methods: (1) detecting functional (antimicrobial) activity using sensitive microorganisms;[6] (2) detecting physicochemical features by chromatographic methods;[7] and (3) recognizing the spatial image of the analyte as a result of affinity interaction with antibodies, molecularly imprinted polymers, aptamers, or a specific receptor.[8,9] Immunoassays

[a]Mechnikov Research Institute for Vaccines and Sera, Moscow 105064, Russia. E-mail: burma68@yandex.ru; Fax: +7 495 9172753; Tel: +7 495 9172753

[b]Faculty of Chemistry, M. V. Lomonosov Moscow State University, Moscow, 119991, Russia

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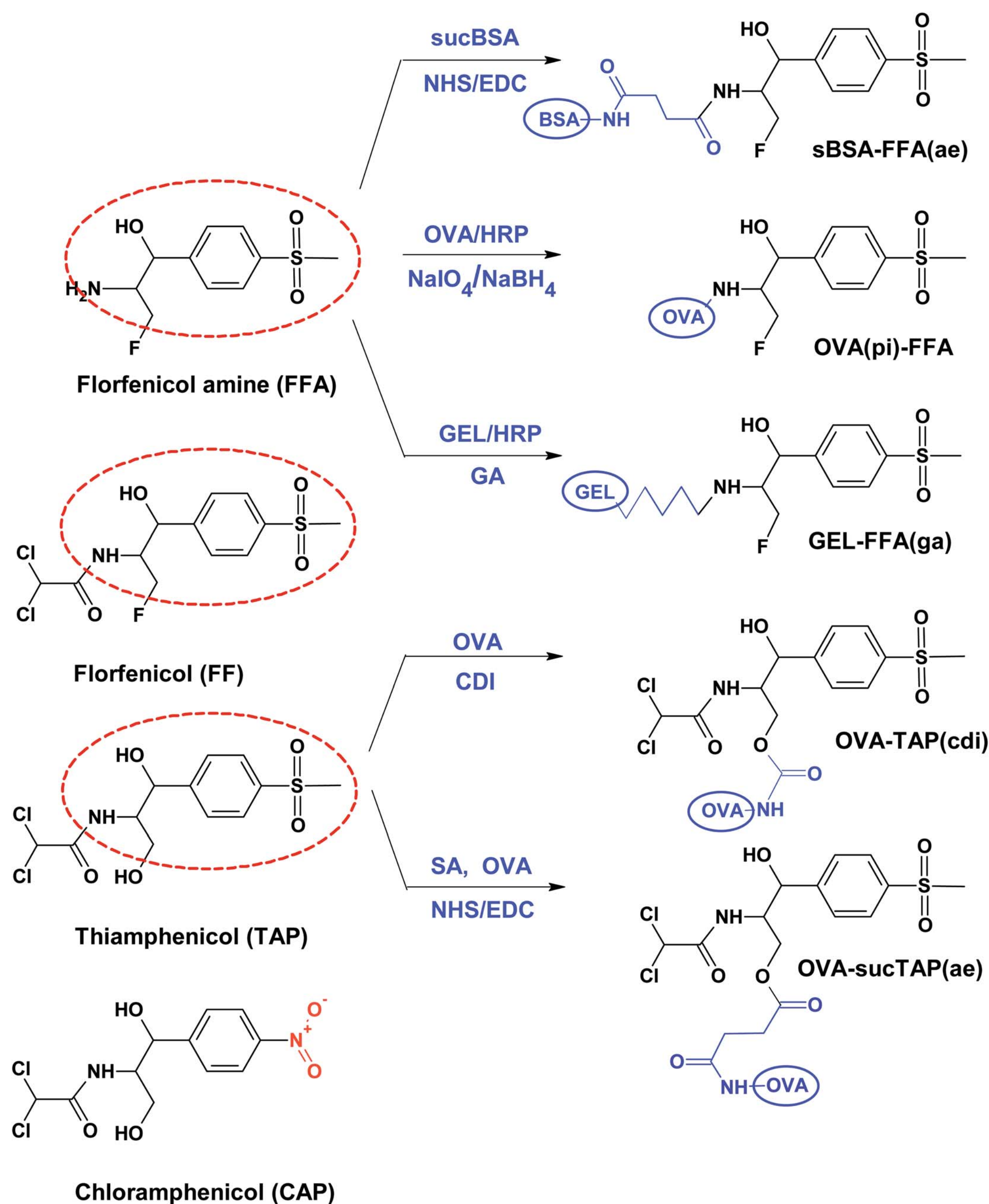

Fig. 1 The structures of amphenicols and scheme of conjugated antigen synthesis.

Table 1 Maximum residue limits (μg kg^{-1}) established for amphenicols in edible animal tissues in the EU[5]

	TAP	FF + FFA	CAP
Muscle	50	100–200–300[a]	No
Fish	50	1000	No
Milk	50	No	No
Eggs	No	No	No

[a] Poultry–bovine–porcine muscles (respectively).

are recognized widely as methods alternative to insufficiently specific and time-consuming microbiological tests and to laborious and expensive chromatographic methods. Most of the immunoassays for the determination of low molecular weight compounds are based on the principle of competitive interaction between the antigen or tracer and the analyte in the sample for binding to specific antibodies. The specificity of antibodies can be towards individual substances and provide identification of analytes. The other possibility for analysis can be provided by antibodies against common (group) epitopes. This allowed the detection of any representative from the group of analytes.

From Table 1 it can be seen that analysis of different kinds of foodstuff requires legally used amphenicols distinguishable from unauthorized ones. For example, CAP is a prohibited substance and should not occur in any kind of foodstuff. Thus, CAP-specific immunoassay can be applied for control of every matrix.[10] TAP and FF are used in poultry, but not administered for egg-producing birds, so an assay that recognizes all the amphenicols would be ideal for control of eggs.[11] There are different regulations for meat/fish products and milk. However, the residuals of FF and FFA, the main compound and the metabolite, should be determined in total.

For the total quantitative determination of FF + FFA and to avoid substantial underestimation of drug concentration, physicochemical methods must include an acid hydrolysis step as a prerequisite within the extraction procedure both to release tissue bound FF and other FF metabolites present in samples and to convert them to the stable end product, FFA.[4] Using immunoassay for this purpose, it is necessary either to determine FF and FFA individually and then summarize concentrations or these analytes should be recognized equivalently. In other words, they should exhibit the same cross-reactivity.

Thus, the present work aimed at the development of immunoassay capable of group determination of amphenicols TAP, FF and its main active metabolite FFA and differentiating them from the banned antibiotic CAP and using the test for muscle analysis.

Methods

Chemicals

Bovine serum albumin (BSA), ovalbumin (OVA), gelatine (GEL), horseradish peroxidase (HRP), dimethylformamide (DMF), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), succinic anhydride (SA), sodium periodate (pi), sodium borohydride, glutaraldehyde (GA), dimethyl adipimidate (DMA), 1,1-carbonyldiimidazole (CDI), and trimethylamine (TMA) were analytical grade reagents purchased from Chimmed (Moscow, Russia).

Preparations of immunogens and coating antigens

sucBSA(ae)–FFA; sucOVA(ae)–FFA; BSA(ae)–FFA; OVA(ae)–FFA. Solutions of BSA and OVA (100 nmol, 6.7 and 4.5 mg, respectively) each in 1 mL of 0.05 M carbonate-bicarbonate buffer (CBB, pH 9.5) were added dropwise with succinic anhydride (10 mg, 100 μmol) in 0.3 mL DMF. The mixtures were stirred at room temperature for 3.5 h under control of pH maintaining at 9.5 using 10% sodium hydroxide. The excessive anhydride was removed by dialysis against several changes of 5 L distilled water.

Solutions of succinylated proteins (sucBSA and sucOVA) and unmodified BSA and OVA (90 nmol of each) were treated with a mixture of EDC and NHS (20 μmol of each) in DMF for 1 h at room temperature under permanent stirring. Then, the activated proteins were added dropwise with FFA from 5 mg mL^{-1} in DMF and stirred for another 2 h. Molar ratios between sucBSA (BSA) and FFA were taken as 1/50, and those for sucOVA (OVA) were 1/7 and 1/30.

OVA(pi)–FFA, HRP(pi)–FFA. OVA (9.0 mg, 200 nmol) in 1.7 mL and HRP (4 mg, 100 nmol) in 0.7 mL of 0.01 M acetic buffer (AB, pH 5.0) were combined with 0.3 mL of sodium periodate solution (2.14 mg, 10 μmol), stirred for 20 min, and then dialyzed overnight at 4 °C against 5 L of AB to remove excessive sodium periodate. The oxidized glycoproteins (OVA(pi) and HRP(pi)) were divided into two portions of half volume and mixed with 10- and 50-fold molar excess of FFA in DMF and incubated while stirring for 3 h. Sodium borohydride (50 μL, 1.9 mg mL^{-1}) was added to stabilize conjugates and 1 h later the dialysis against 2 × 5 L of water was conducted.

Gel–FFA(ga), HRP–FFA(ga), Gel–FFA(dma). Gel (16 mg, 100 nmol) was dissolved in 2.0 mL of warm water, cooled to room temperature and divided into two equal portions. Then, the mixtures of Gel and FFA were composed with molar ratios 1/10 and 1/50 and each one was divided into two. Freshly prepared 2.5% GA solution (20 μL, 5 μmol) or DMA (1.23 mg, 5 μmol) was added to the prepared compositions and stirred for 3 h. The following dialysis against 2 changes of 5 L of water was aimed to remove unreacted reagents. The same procedure was conducted for preparation of glutaraldehyde coupling FFA with HRP.

OVA–sucTAP(ae). TAP (3.6 mg, 10 μmol) was combined with SA (1 mg, 10 μmol) and TMA (10 μg, 0.1 μmol) to final volume 1 mL DMF and stirred overnight at room temperature. The solution containing the formed succinylated TAP was then activated with the mixture of EDC (2.9 mg, 15 μmol) and NHS (1.7 mg, 15 μmol) in 0.2 mL DMF for 30 min and added dropwise to Gel solutions (4 mg, 25 nmol) in 0.05 M carbonate-bicarbonate buffer (CBB, pH 9.6). The molar ratios between the hapten and protein were taken as 1/10, 1/30, and 1/100. The composed mixtures were stirred using a magnet stirrer for 3 h.

OVA–FFA(cdi), OVA–FF(cdi), OVA–TAP(cdi), OVA–CAP(cdi). The coupling procedure was conducted according to the description in ref. 12 with modifications. Briefly, 10 μmol quantities of amphenicols, namely FF (3.6 mg), FFA (2.5 mg), TAP (3.6 mg), and CAP (3.2 mg), were dissolved in 1 mL solutions of CDI (3 mg, 18.6 μmol) in acetone and stirred using a magnet stirrer for 3 h at room temperature in an exhaust cabinet until complete evaporation. Then, the residues were dissolved in 0.5 mL DMF and added dropwise to OVA (10 mg, 0.22 μmol) in CBB. The mixtures were composed using the molar ratios between protein and haptens as 1/10 and 1/30 and stirred for 2 h.

After dialysis all the prepared conjugates were supplemented with glycerol and stored as 1 mg mL^{-1} (protein) solutions at −15 °C.

Immunization and preparation of polyclonal antibodies

All experiments with the laboratory animals were performed in accordance with the guidelines for the care and use of laboratory animals in biomedical research and approved by the Ethics Committee of the I. Mechnikov Research Institute for Vaccines and Sera. Chinchilla rabbits (2.0–2.5 kg) were obtained from the Scientific and Production Centre for Biomedical Technologies (Elektrogorsk, Russia). Immunogens (100 μg, BSA(ae)–FFA or sucBSA(ae)–FFA) were emulsified in the complete Freund adjuvant and injected in rabbits subcutaneously at multiple points on the

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back. The same doses of immunogens in the incomplete Freund adjuvant were administered to animals monthly and a week later a blood sample from ear veins was taken for testing. The sera supplemented with equal volume of glycerol were stored at −15 °C.

Competitive indirect ELISA

An estimation of antibody activity and development of analyte assay was conducted in the format of indirect and direct competitive ELISA according to the routine procedure detailed in ref. 13.

Coating antigens were adsorbed on high-binding polystyrene 96-well microplates (Costar, USA) from solutions (10.0–0.1 μg mL^{-1}) in CBB 100 μL per well overnight at 4 °C. Washings were conducted three times after each assay step by filling wells with 300 μL phosphate buffered saline with 0.05% Tween 20 (PBST, pH 7.2) and subsequent empting. Antibody activity was first analyzed on binding of antisera serially diluted in PBST containing 1% BSA. Then, optimal antibody concentration (100 μL per well) was used in competitive assay together with 100 μL of analyte standard (10 000–0.01 ng mL^{-1} and 0 ng mL^{-1}) or samples tested. After 1 h of incubation at 25 °C in a thermostatic shaker chamber (ELMI, Latvia) and washing plate, 100 μL of goat anti-rabbit IgG–HRP conjugate was added to detect the antibody bound (1 h, 37 °C). The enzymatic reaction with TMB-substrate solution was terminated after 30 min with 1 M sulfuric acid and absorbance values were registered using a Stat Fax 2100 reader (Awareness Technologies, Westport, CT, USA).

The direct competitive ELISA format meant immobilization of antibodies on plates and reaction with the FFA–HRP conjugate. The optimized concentrations of reagents were found from the results of checkerboard titration and then were used in competitive assay.

Pretreatment and analysis of chicken muscles

Sample pretreatment was conducted according to the procedure described[14] with light modifications. Briefly, tissue samples (antibiotic-free) obtained from private organic-farm hens were homogenized. Homogenates (1 g) were put in tubes and PBST was added up to the 10 mL mark. After vigorous stirring, the tubes were left overnight at 4 °C to complete extraction. For recovery experiments the homogenate portions were spiked with each amphenicol to obtain half, one and double MRL level concentrations. Then, the samples were centrifuged for 10 min at 3000 rpm and supernatants were analysed by ELISA. The standard of amphenicol was prepared in PBST containing 5% skim milk to equilibrate the effect of the muscle matrix on antibody binding. For recovery estimation, the ratio between the concentration measured using a universe standard curve and concentration of fortifying the blank muscle homogenate was calculated.

Results and discussion

Preparation of immunogens and antibodies raised against amphenicols

To develop an immunoassay for equal detection of TAP, FF, and FFA and distinguishing CAP, the generated antibody should be targeted to the common moiety of the molecule, crowned by a methylsulfonyl group, which is the structural difference from CAP. This target region is indicated with a red circle in Fig. 1.

A number of conjugation approaches for the preparation of immunogens have been described in the literature, but few have allowed the production of antibodies with specificity that would satisfy the mentioned goal.

Different amphenicols may be used as haptens for preparation of immunizing conjugates. It was *a priori* clear that CAP should not be chosen as an immunizing hapten if it should not be recognized. Even if the CAP nitro group, its distinguishing feature, was used in coupling and was masked by a carrier protein, the CAP remained recognizable.[15] Moreover, the most represented epitope in such an immunogen was the dichloroacetyl group, and due to its absence, FFA could not be determined.

Among the possible sites for hapten conjugation the hydroxyls from TAP or FF could be considered. TAP bears two hydroxyls, and they could be involved in activation and coupling simultaneously that will result in undesirable multi-presentation haptens on the carrier. A single available hydroxyl in FF allowed site-specific conjugation to be carried out. However, the resultant conjugate will present more advantageous dichloroacetyl groups and fluorine atoms. These substituents are lacking in FFA and TAP, respectively, so these analytes will be poorly detected. The research groups which use FF hydroxyl as the conjugation site[11,16,17] confirmed this suspicion and reported about no detectability of FFA (Table 2).

FFA amine is one more possible functional group for coupling or hapten derivation (Fig. 1). On the one hand this conjugation site is useful for enhanced presentation of the *p*-methylsulfonyl group on the immunogen, and on the other hand it is convenient for masking the lacking dichloroacetyl. Several attempts have been conducted to raise antibodies against FFA-based immunogens, but only a few reached simultaneous detection of FF, FFA, and TAP (Table 2).

For the above-mentioned reasons and known experience, FFA amine was chosen as the most favourable immunizing hapten candidate for development of the group recognition assay of amphenicols except CAP. We used the conjugation method that resulted in the formation of amide bonds for additional imitation of structure FF/TAP. BSA–FFA was prepared using succinic anhydride as the coupling and spacer agent, but instead of synthesizing the succinylated derivative of FFA, followed by isolation and purification,[17] it appeared easier first to succinylate BSA and then conjugate with FFA amine (Fig. 1).

The formation of the immunizing conjugate, sucBSA(ae)–FFA, was confirmed by UV spectra showing the combination of carrier sucBSA and hapten FFA features in the conjugate spectrum (Fig. 2). The comparable conjugate BSA(ae)–FFA prepared without succinic anhydride demonstrated no changes in the UV spectrum and failed to induce anti-FFA antibodies (data not shown). Due to the weak absorbance of amphenicols and lower hapten load in coating conjugates their spectra were not demonstrative. Their successful synthesis was assessed based on the binding activity of antibodies in ELISA.

Table 2 Immunoassays of amphenicols. Comparison of specificity and sensitivity characteristics[a]

Immunoassay (antibody)	Immunogen//antigen	Matrix	Sensitivity IC_{50} (ng mL^{-1})	Specificity, %	Reference
SPR-immunosensor (rabbit pAb)	KLH–MBS–FFA//Chip(ae)–FFA	Shrimps	FFA > 1000 FF – 1.82 TAP – 7.39 CAP – 1.94	FFA – <0.1 FF – 107 TAP – 26 CAP – 100	Dumont *et al.*, 2006 (ref. 18)
	BSA–sucCAP(ae)//Chip(ae)–CAPbase		FFA – >1000 FF – 245 TAP – 400 CAP – 0.95	FFA – <0.1 FF – 0.4 TAP – 0.2 CAP – 100	
CL-ELISA (rabbit pAb)	BSA–FFA(f)//OVA–glutFF(ma)	Chicken	FFA – 0.15 FF – 0.21 TAP – 3.8 CAP > 1000	FFA – 100 FF – 74.3 TAP – 4.0 CAP – <0.1	Tao *et al.*, 2012 (ref. 19)
CL-ELISA (rabbit pAb)	HSA–sucFF(ae)//OVA–malFF(ma)	Pork	FFA – 50 FF – 0.15 TAP – 0.31 CAP > 180	FFA – 0.3 FF – 100 TAP – 48.4 CAP – <0.1	Tao *et al.*, 2015 (ref. 17)
ic-ELISA, ICA (mouse mAb)	KLH-(diazo)-CAP//OVA-(diazo)-CAP	Milk Honey	FFA – >1000 FF – 2.5 TAP – 0.39 CAP – 0.13	FF – 15.6 TAP – 100 FFA – <1 CAP – 300	Guo *et al.*, 2015 (ref. 15)
ic-ELISA (mouse mAb)	KLH–sucFF(ae)//OVA–sucFFA(ae)	Chicken Swine Fish Feed	FF – 0.21 TAP – 0.35	FF – 167 TAP – 100 FFA – 0 CAP – 0	An *et al.*, 2016 (ref. 16)
SPR-immunosensor (rabbit pAb)	BTG–FFA(ni)//Chip–FFA(ae)	Bovine Ovine Porcine Kidney	FFA – 0.83 FF – 0.39 TAP – 0.34 CAP – 2.49	FFA – 100 FF – 213 TAP – 244 CAP – 33	Thompson *et al.*, 2017 (ref. 20)
ic-ELISA, ICA (mouse mAb)	BSA–sucFF(ma)//BSA–sucFF(ae)	Eggs	FFA > 1000 FF – 0.2 TAP – 0.27 CAP > 50	FFA – <1 FF – 100 TAP – 74 CAP – <1	Lei *et al.*, 2018 (ref. 11)
ic-ELISA (rabbit pAb)	sucBSA(ae)–FFA//Gel–FFA(ga)	Muscle	FFA – 6.6 FF – 6.6 TAP – 6.6 CAP > 10 000	FFA – 100 FF – 100 TAP – 100 CAP – <0.1	Present study

[a] MBS – (3-maleimidobenzoyl-*N*-hydroxysuccinimide ester); ae – active ester method; suc – succinylated; f – formaldehyde; glut – glutaric anhydride; mal – maleylated; ma – mixed anhydride method; ni – not identified; ga – glutaraldehyde.

Analysis of immune response showed that antiserum antibodies reached the maximum titre and provided the highest sensitivity of FFA determination after the fourth booster immunization. The immunoglobulin fraction was isolated from the serum using caprylic acid and sulphate ammonia precipitation[21] and used in indirect and direct formats of ELISA.

Cross-reactivity examination

All the prepared conjugates were examined as coating antigens and demonstrated binding with antibodies. The homologous assay format had poor sensitivity, so several conjugates of heterologous designs were compared. The effect of hapten and spacer arm heterology is able to change the assay specificity by selecting various repertoires of antibodies from the antiserum.[22] This study aimed to search for conjugation approaches which provided antigen design for the best assay parameters. The coupling bond in OVA(pi)–FFA and Gel–FFA(ga) was heterologous to that in the immunogen and differed in the spacer arm – zero- and C5-length, respectively. Heterologous TAP-based antigens, OVA–TAP(cdi) and OVA–sucTAP(ae), were synthesized through different conjugation sites and also differed in the spacer, 1- and 4-atom chains, respectively (Fig. 1).

As can be seen from Table 3, the significant influence of the immobilized antigen on assay specificity was observed concerning the recognition of FFA. Since FFA was an immunizing hapten, its activity was taken in each assay variant as 100%. However, the sensitivity (IC_{50}) of FFA changed 100-fold, from 6.0 to 585 ng mL^{-1}, whereas the IC_{50}-level of FF and TAP was very similar and practically did not change. It should be noted that namely FFA-based coating antigens promoted the better detectability of FFA and group detection of amphenicols. The glutaric spacer in Gel–FFA(ga) appeared to be a necessary element of hapten presentation for equal recognition of FF, FFA, and TAP by the developed antibody. CAP remained unrecognizable despite the type and orientation of the immobilized hapten.

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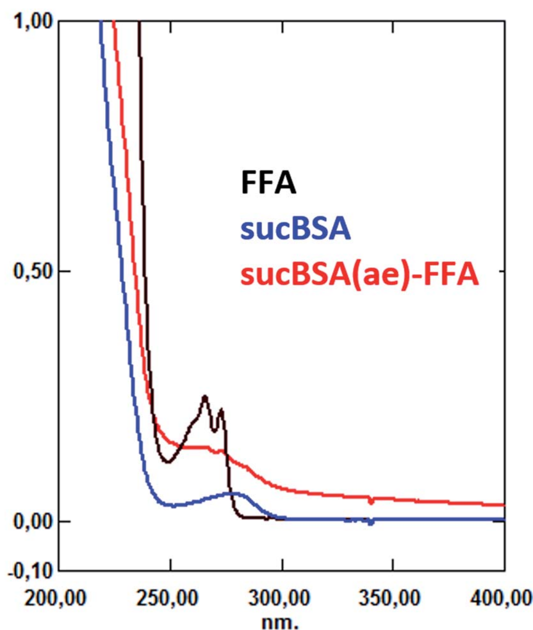

Fig. 2 UV-spectra of the hapten, carrier, and immunogen conjugate (0.1 mg mL^{-1}).

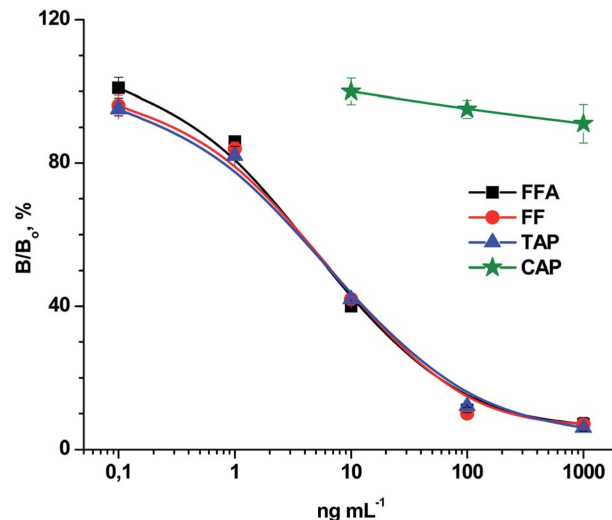

Fig. 3 Standard curves of amphenicols in direct ELISA using the anti-sucBSA–FFA antibody and HRP–FFA(ga). Symbols and error bars represent the average values ($n = 3$) and standard deviations.

Thus, the conditions for group detection of amphenicols except CAP were found. The glutaraldehyde method provided the required presentation of FFA for interaction with anti-sucBSA(ae)–FFA antibodies, so this coupling procedure was chosen for synthesis of tracers and development of direct assay formats.

Determination of amphenicols in chicken muscles and recovery assessment

Two direct formats were developed using HRP(pi)–FFA and HRP–FFA(ga). Both versions of the analysis reproduced the cross-reactivity profiles obtained in indirect ELISAs, confirming that specificity depends on the presentation of the hapten, but not on the type of carrier protein. Thus, the latter variant with 100% cross-reactivity for FFA, FF, and TAP was chosen to test the hypothesis of a universal quantification of analytes recognizable equally (Fig. 3). The limit of amphenicol detection (LOD) taken as IC_{10} was 0.4 ng mL^{-1} and the dynamic range (IC_{20}–IC_{80}) was 1.0–60 ng mL^{-1}.

For example, in our previous studies it was shown that macrolides, tylosin (100%) and tilmicosin (103%) could be adequately quantified in different foodstuffs using a universe standard (tylosin) curve.[23] A similar experience was reported concerning quantification of fluoroquinolones, sarafloxacin and difloxacin in group immunoassay.[14] Being equivalent, these analytes could conveniently be measured using a common standard.

Thereby, in the following recovery experiments we fortified the muscle homogenates with each amphenicol around its limitary level and quantified by developed assay using a common FFA standard curve. CAP was spiked in muscle samples at a comparable concentration level to demonstrate the absence of possible influence. The calculated recovery rate for FFA, FF, and TAP was in the range 77.2–98.8% and accuracy error did not exceed 9.6% (Table 4).

In comparison with other anti-amphenicol immunoassays (Table 2) the present study characterized not so high sensitivity as achieved in the previous studies,[16,17,19,20] but sensitive enough for detection of MRL levels established for FF, FFA, and TAP in foodstuffs. The main success of this study lies in the creation of an analytical system for the identical recognition of FFA, FF and TAP, which compares favorably with the assays in the mentioned publications. This feature allowed us to quantify any of these analytes by universal calibration. Besides, the cross-reaction of CAP revealed in some of the reported assays[15,18,20] does not allow distinguishing the approved drugs (FF, FFA, and TAP) from the prohibited one (CAP). This important point was given special attention in this study, and there was no influence of CAP on determination of other amphenicols.

Table 3 The cross-reactivity of anti-sucBSA(ae)–FFA antibodies with amphenicols

	OVA(pi)–FFA		Gel–FFA(ga)		OVA–TAP(cdi)		OVA–sucTAP(ae)	
Analytes	IC_{50}, ng mL^{-1}	CR, %	IC_{50}, ng mL^{-1}	CR, %	IC_{50}, ng mL^{-1}	CR, %	IC_{50}, ng mL^{-1}	CR, %
FFA	6.0	100	6.6	100	585	100	80	100
FF	8.6	70	6.6	100	11	5318	4.7	1702
TAP	10	60	6.6	100	13	4500	5.8	1379
CAP	>10 000	<0.1	>10 000	<0.1	>10 000	<5.9	>10 000	<0.8

Table 4 Recovery of amphenicols from chicken muscles determined in group-specific ELISA using a common standard curve ($n = 4$)

Analyte	Spiked, ng g^{-1}	Measured, ng g^{-1}	Recovery, %	RSD, %
FFA	200	162.6 ± 8.3	81	4.2
	100	90.5 ± 8.9	90.5	8.9
	50	47.9 ± 3.5	95.8	6.9
FF	200	163 ± 8.3	81.5	4.2
	100	95.6 ± 8.4	95.6	8.4
	50	43.6 ± 4.7	87.2	9.4
TAP	100	98.8 ± 8.6	98.8	8.6
	50	42.5 ± 4.8	85.0	9.6
	25	19.3 ± 1.8	77.2	7.2
CAP	200	<LOD	—	—
	100	<LOD	—	—
	50	<LOD	—	—

Conclusions

The equal recognition of several analytes by antibodies or by other receptor structures allows quantifying any of them and using a single reference standard for this. In the present study, to develop an assay with equal recognition of FF, FFA and TAP and avoiding CAP recognition for the analysis of meat products, FFA was conjugated to succinylated BSA and used to generate antibodies in rabbits. A number of ELISAs based on different heterologous antigens were performed on the cross-reactivity profile of amphenicols. The presentation of the FFA-hapten on the coating or enzyme conjugates prepared using the glutaraldehyde method provided the conditions for the same cross-interaction of FFA, FF, and TAP with anti-sucBSA(ae)–FFA antibodies. The developed indirect and direct ELISA formats failed to detect prohibited amphenicol CAP, so the assay was useful for analysis of any approved amphenicols in meat products. The same cross-reactivity of FFA, FF, and TAP allowed quantification of any of them using a universal standard curve. The dynamic range of assay was 1.0–60 ng mL^{-1} and the LOD value was 0.4 ng mL^{-1}. It was demonstrated that the developed test was capable of determining amphenicols about their MRL level in chicken muscles with good recovery 77.2–98.8%.

Conflicts of interest

There are no conflicts to declare.

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