ANALYSIS OF ANTHELMINTIC RESIDUES IN LIVER BY MULTIPLEX SCREENING APPROACH: COMPARISON OF A BIOCHIP ARRAY VERSUS LC-MSMS APPROACH

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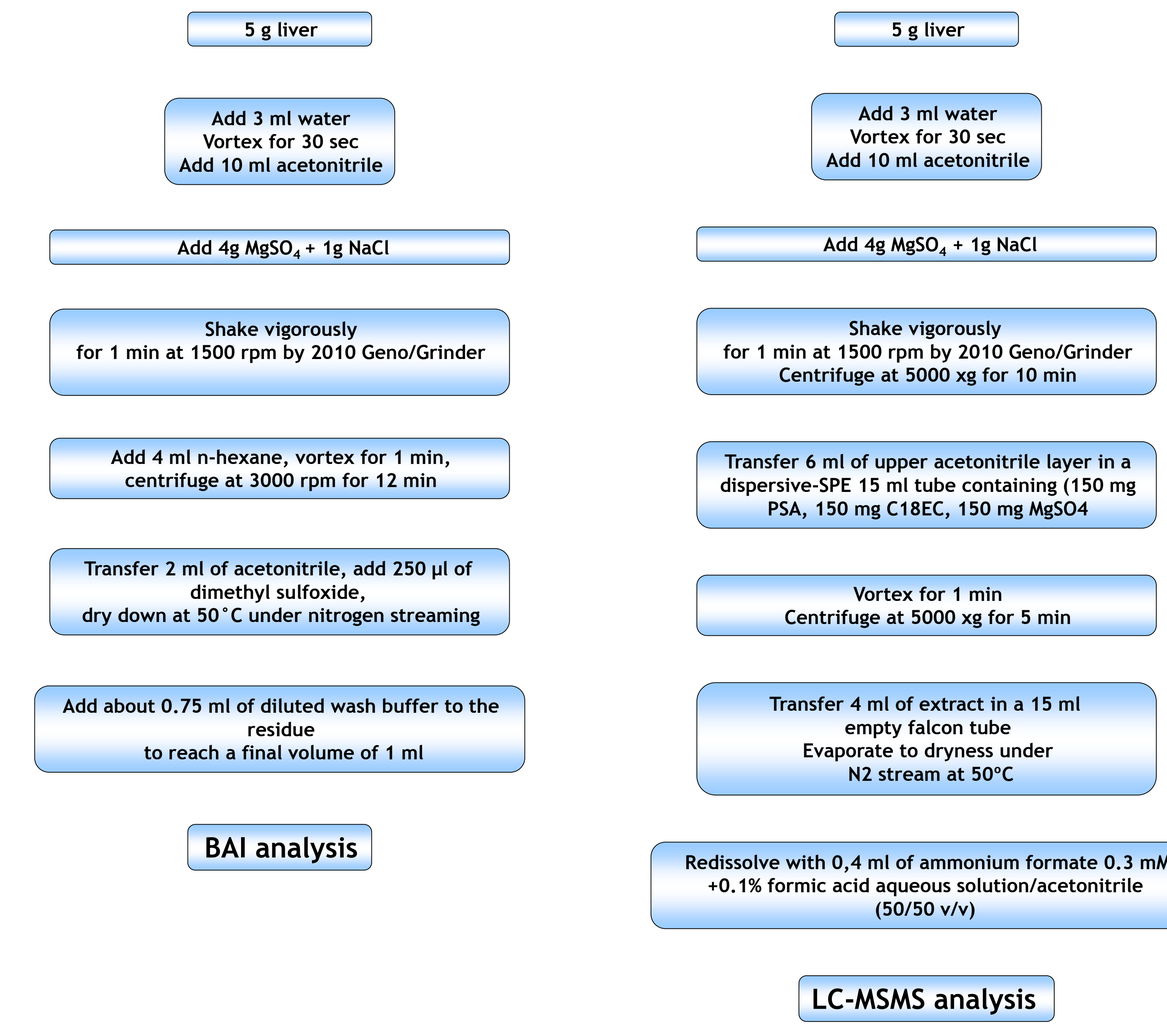
INTRODUCTION

Anthelmintic drugs are used in clinical and veterinary practices for the treatment of infections caused by parasitic worms. These substances belong to different chemical classes: macrocyclic lactones (avermectins/milbemicyn), benzoimidazoles, imidazothiazoles. According to the Italian Residue Control Plan (PNR), several hundreds of liver samples are yearly collected to be analysed for such three distinct classes, and official laboratories usually adopt three distinct class-specific analytical methods based on chromatographic techniques.
The emerging trend in residue analysis is the development of methods that are capable of monitoring in each sample, a wide variety of compounds, representative of different classes of veterinary drugs, by means of one single analytical protocol.
This new approach could represent the basis for an evolution both for future monitoring plans and for supporting producers to better investigate the critical points related to their production chain.
The goal of this project was to develop, validate and apply a multiclass method based on Biochip Array Immunoassay (BAI) for the determination of anthelmintics residues in liver samples and to evaluate the potentiality of this technique as an alternative to LC-MSMS-based analytical methods in terms of applicability and productivity.

MATERIALS AND METHODS

ANALYTICAL METHOD FOR SAMPLE PREPARATION [scheme]



VALIDATION APPROACH

The developed methods were fully validated as qualitative screening methods as described in Commission Decision 2002/657/EC and in CRL Guidelines for the Validation of Screening Methods for Residues: specificity, ruggedness, and detection capability (CCß) were evaluated.

For BAI validation, fenbendazole sulphone (FEN-S), 2-amino-flubendazole (NH2-FLU), 5-hydroxythiabendazole (OH-TIA), triclabendazole-sulphoxide (TRICLA-SO), levamisole (LVM), moxidectin (MXD), doramectin (DOR) were selected as reference compounds for the validation study since they exhibit the worst cross-reactivity compared to other anthelmitics. The mentioned reference compounds belong to benzimidazoles (BZS), amino benzimidazoles (ABZ), thiabendazole (TBZ), triclabendazole (TCBZ), levamisole (LVM), moxidectin (MXD) and avermectins (AVM) class respectively. The level of interest for each reference analyte was chosen considering maximum residue limits (MRLs), detection capabilities (CCßs) recommended in the PNR and preliminary results achieved during method development. This level was chosen as equal to 50 µg kg^{-1} for FEN-S (BZS class) and for TRICLA-SO (TCBZ class), 12.5 µg kg^{-1} for NH2-FLU (ABZ class), 25 µg kg^{-1} for OH-TIA (TBZ class), 60 µg kg^{-1} for LVM (LVM class), 15 µg kg^{-1} DOR (AVM class), 20 µg kg^{-1} MXD (MXD class).

For LC-MSMS method validation, analytes selected for avermectin/milbemycin class were: abamectin (ABA), DOR, emamectin (EMA), ivermectin (IVER), MXD; for benzimidazoles/salicylanilide class were: albendazole (ALB), febantel (FEB), fenbendazole (FEN), FEN-S, flubendazole (FLU), mebendazole (MEB), oxfendazole (OXF), oxibendazole (OXI), thiabendazole (TIA), albendazole-sulphone (ALB-S), albendazole-sulphoxide (ALB-SO), albendazole-2-amino-sulphone (ALB-2NH2-S), NH2-FLU, 2-aminomebendazole (NH2-MEB), hydroxymebendazol (OH-MEB), OH-TIA, triclabendazole (TRICLA), triclabendazole-sulphone (TRICLA-S), TRICLA-SO, and closantel (CLO); for imidazothiazole class was: LVM. Internal standars (ISs) were used for determination of ALB (ALB-D3), ALB-S (ALB-S-D3), ALB-SO (ALB-SO-D3), FEB (FEB-D6), FEN (FEN-D3), OXF (OXF-D3), CLO (CLO-13C6) and LVM (tetramisole-D5, TETRA-D5). The level of interest for each analyte was chosen considering MRLs, CCßs recommended in the PNR and preliminary results during method development. This level was established at 10 µg kg^{-1} for avermectin/milbemycin class, 25 µg kg^{-1} for benzimidazoles/salicylanilide and imidazothiazole class.

Specificity and Detection capability (CCß)
A qualitative approach was used to determine the performance parameter CCß as described in the CRL Guidelines. For both procedures a set of at least twenty blank liver samples from different species (bovine, swine, poultry) were analysed for specificity test; the same samples spiked at the concentration of interest were analysed for ß error verification. The analyses were carried out in within-laboratory reproducibility conditions (different days and operators). A threshold value T and a "cut-off factor" Fm were calculated starting from signals results for BAI (expressed as relative light unit, RLU) and from concentration results for LC-MSMS by following equations:
T = B-1.64 SDb (in the signal domain, for BAI); T = B+1.64 SDb (in the concentration domain, for LC-MSMS)
where B is the mean signal or concentration calculated from blank liver samples and SDb the calculated standard deviation.
Fm = M + 1.64 SDs (in the signal domain, for BAI); Fm = M - 1.64 SDs (in the concentration domain, for LC-MSMS)
where M is the mean signal or concentration calculated from spiked liver samples and SDs the calculated standard deviation.
According to CRL Guidelines for the Validation of Screening Methods for Residues the following equations were verified: Fm < B; Fm < T (in the signal domain, for BAI); Fm > B; Fm > T (in the concentration domain, for LC-MSMS)

RESULTS AND CONCLUSIONS

For benzimidazoles (corresponding to BZS, ABZ, TBZ, TCBZ class of BAI) and milbemycins for both procedures the level of interest chosen for ß-error verification was far below the MRLs in liver.
For imidazothiazole the molecule selected was LVM for both procedures and the level of interest was far below the MRL for LC-MSMS and equal to 60% of the MRL for BAI.
For avermectins, the level of interest was set to 10 µg kg^{-1} for LC-MSMS and to 15 µg kg^{-1} during BAI method development considering DOR as reference analyte. These values are well below the MRLs established for DOR, EPRI, IVER but the level of interest chosen for BAI procedure is higher the MRLs established for ABA in poultry and swine and for EMA in poultry. For this reason, 6 blank swine liver spiked with ABA at 10 µg kg^{-1}, 6 blank poultry liver spiked with ABA at 10 µg kg^{-1} and 6 blank poultry liver spiked with EMA at 10 µg kg^{-1} were analysed. All of samples were recognized as non-compliant giving a ratio RLU/RLU_0 < Fm of AVM.

BAI validation results

Class	Reference analyte/Cross reactivity (%)	Level of interest (µg kg^{-1})	B (RLU/RLU_0) (%)	T (RLU/RLU_0) (%)	F_m (RLU/RLU_0) (%)	Lowest MRLs (µg kg^{-1})	ß error
BZS	FEN-S/ 14	50	88.0	77.7	25.7	200	≤ 5% for FEN-S, MEB, FLU, parbendazole, OXF, OXI, ALB-SO, ALB, ALB-S
ABZ	NH2-FLU/ 99	12.5	89.6	80.0	12.9	400	≤ 5% for NH2-FLU, ALB-2NH2-S, NH2-MEB
TBZ	OH-TIA/ 91	25	83.2	74.2	63.0	100	≤ 5% for OH-TIA, TIA, cambendazole
TCBZ	TRICLA-SO/ 40	50	97.7	77.0	69.2	250	≤ 5% for TRICLA-SO, TRICLA, keto-triclabendazole
LVM	LVM/ 100	60	77.5	68.4	51.6	100	≤ 5% for LVM
MXD	MXD/ 100	20	103.5	93.4	77.0	100	≤ 5% for MXD
AVM	DOR/ 75	15	101.1	86.9	69.4	10	≤ 5% for DOR, IVER, ABA, EPRI, EMA

LC-MSMS results

Class	Reference analyte	Level of interest (µg kg^{-1})	B (µg kg^{-1})	T (µg kg^{-1})	F_m (µg kg^{-1})	Lowest MRLs (µg kg^{-1})	ß-error
Benzimidazoles (corresponding to BZS, ABZ, TBZ, TCBZ class of BAI)	ALB	25	0.05	0.09	21.8	1000 as sum	≤ 5%
	ALB-SO	25	0.04	0.06	21.5		≤ 5%
	ALB-S	25	0.04	0.11	21.9		≤ 5%
	ALB-2NH2_S	25	0.02	0.04	13.8		≤ 5%
	FEN	25	0.11	0.56	22.2	500 as sum	≤ 5%
	FEN-S	25	0.01	0.08	20.7		≤ 5%
	FEB	25	0.03	0.06	22.0		≤ 5%
	OXF	25	0.10	0.24	21.9		≤ 5%
	OXI	25	0.01	0.02	20.0	200	≤ 5%
	MEB	25	0.02	0.14	18.9	400 as sum	≤ 5%
	OH-MEB	25	0.07	0.21	19.8		≤ 5%
	NH2-MEB	25	0.01	0.06	10.6		≤ 5%
	FLU	25	0.02	0.05	18.6	400 as sum	≤ 5%
	NH2-FLU	25	0.01	0.07	10.8		≤ 5%
	TIA	25	0.03	0.15	18.0	100 as sum	≤ 5%
	OH-TIA	25	0.004	0.01	12.0		≤ 5%
	TRICLA	25	0.04	0.12	18.5	250 as sum	≤ 5%
	TRICLA-SO	25	0.35	1.46	8.8		≤ 5%
	TRICLA-S	25	0.06	0.15	10.7		≤ 5%
Salicylanilides	CLO	25	0.63	2.48	19.2	1000	≤ 5%
Imidazothiazoles (corresponding to LVM class of BAI)	LVM	25	0.02	0.08	21.5	100	≤ 5%
Milbemycins (correspondig to MXD class of BAI)	MXD	10	0.01	0.04	5.5	100	≤ 5%
Avermectin s (correpsonding to AVM class of BAI)	ABA	10	0.01	0.03	7.6	10 (poultry, swine)	≤ 5%
	DOR	10	0.01	0.02	7.0	100	≤ 5%
	EMA	10	0.01	0.02	7.6	10 (poultry)	≤ 5%
	EPRI	10	0.01	0.02	7.5	1500	≤ 5%
	IVER	10	0.03	0.20	6.0	100	≤ 5%

Concerning the comparison of the proposed analytical approaches, it can be concluded that: (1) the sensitivity of LC-MSMS is better than the sensitivity of Biochip Array when referring to the entire group of analytes. (2) sample preparation is similar, but Biochip Array enables to capture images of up to 9 samples per carrier, thus reducing the total time required for the analysis of a consistent batch of samples; (3) The major drawbacks related to Biochip Array are represented by the fact that CLO is not detectable, the cost of the dedicated kit, and a false suspect ratio for TCBZ (18%) observed during the implementation of this approach on routinary samples, not confirmed by subsequent confirmatory analysis.

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