

MEASUREMENT OF FOUR **NITROFURAN** METABOLITES WITH ELISA KITS USING MULTI-ANALYTE REAGENTS

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Introduction

Nitrofurans have been used in veterinary practice as antibacterial agents and their use in food producing animals is prohibited in many countries. Nitrofurantoin, furazolidone, furaltadone and nitrofurazone are the four main nitrofurantoin antibiotics. Studies have shown

them to be rapidly metabolised *in vivo*, however the tissue bound metabolites, AHD, AMOZ, AOZ and SEM can persist for at least 6 weeks. The development of convenient methods enabling their detection is of interest for monitoring and regulatory applications.

We report the development of ELISA kits for the determination of nitrofurantoin metabolites using multi-analyte reagents. This is of value as a convenient analytical tool for the screening of these compounds.

Materials and Methods

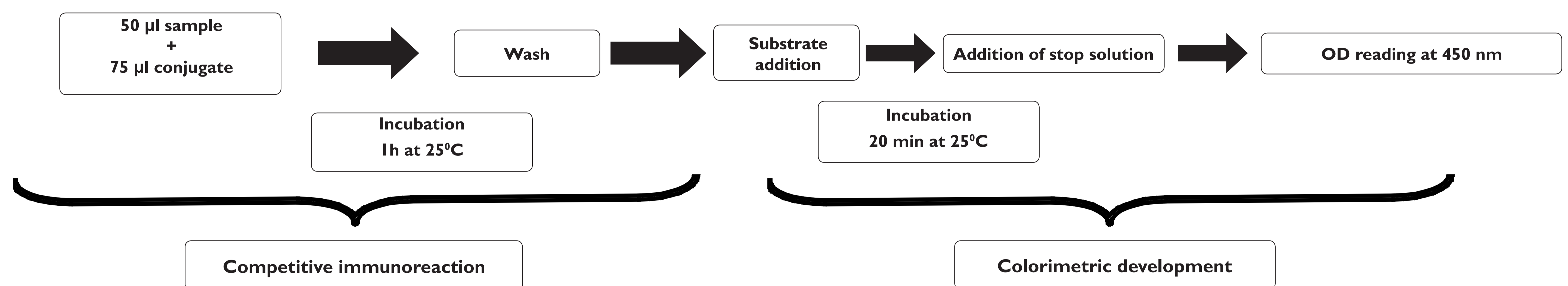
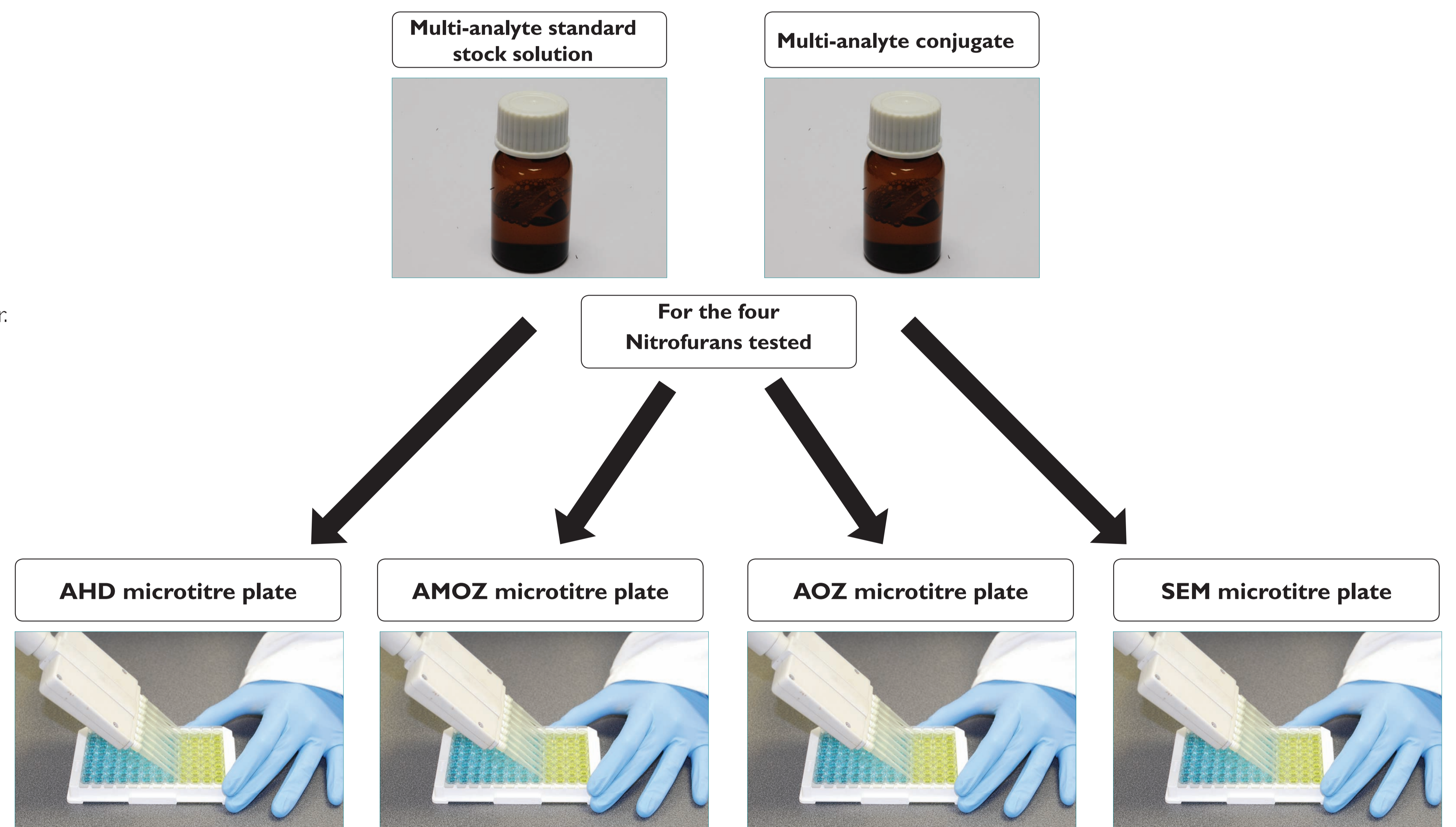
Tissue Sample Preparation (prawn, beef, pork, poultry):

- Addition of H₂O, HCl and 4-nitrobenzaldehyde (in DMSO) to 1g of sample.
- Vortexing and incubation (2h, 50°C)
- Addition of K₂HPO₄ and NaOH
- Vortexing, centrifugation and decantation
- Addition of K₂HPO₄ to the pellet
- Vortexing, centrifugation and decantation
- Combination of supernatants and filtration
- Addition of ethyl acetate
- Vortexing, centrifugation, transfer and evaporation to dryness of the upper layer.
- Resuspension in diluent/wash buffer and vortexing

The sample is now ready for application onto the microtitre plate.

Competitive ELISA

In-house made capture antibody was immobilised and stabilised on 96 well microtitre plates. The nitrofurantoin assays are based on a competitive reaction where any free analyte contained in the standards/samples competes for binding sites of the capture antibody with horseradish peroxidase labelled conjugate. Following the incubation and washing steps, enzyme substrate is added. Measurement of the optical density is carried out at 450nm once the colour reaction is stopped producing a colour change from blue to yellow. Colour intensity is inversely proportional to the concentration of the analyte present.



Nitrofurans Accessory Kit (NF3464, the kit contains the multi-analyte standard stock solution, multi-analyte conjugate and derivatisation agent), AHD ELISA kit (NF3463), AMOZ ELISA kit (NF3462), AOZ ELISA kit (NF3465) and SEM ELISA kit (NF3461) were used (Randox Laboratories, Crumlin, UK) and their analytical performance was assessed.

Analytical parameters

Limit of Detection (LOD)

LOD was defined as mean concentration of negative samples + 3SD.

Specificity/Cross-reactivity

The specificity, expressed as % cross-reactivity

(%CR) was calculated as follows:

$$\%CR = \frac{[IC50 (AHD)] / [IC50 (Cross-reactant)]}{[IC50 (AHD)] / [IC50 (Cross-reactant)]} \times 100$$

$$\%CR = \frac{[IC50 (AMOZ)] / [IC50 (Cross-reactant)]}{[IC50 (AMOZ)] / [IC50 (Cross-reactant)]} \times 100$$

$$\%CR = \frac{[IC50 (AOZ)] / [IC50 (Cross-reactant)]}{[IC50 (AOZ)] / [IC50 (Cross-reactant)]} \times 100$$

$$\%CR = \frac{[IC50 (SEM)] / [IC50 (Cross-reactant)]}{[IC50 (SEM)] / [IC50 (Cross-reactant)]} \times 100$$

The IC50 for each analyte was calculated by taking 50% of the optical density (OD) from the zero calibrator and reading this OD value from the x-axis (concentration in ng/ml) of the respective calibration curve. This concentration corresponded to the inhibitory concentration that produced 50% inhibition.

Precision

Intra-assay precision (n=12) was determined from the mean results corresponding to six different concentration levels within the same run and was expressed as %CV.

Recovery

A negative sample was spiked for a range of concentration levels and further assessed. Results were expressed as % recovery.

Results

AHD		Specificity/ Cross-reactivity (CR)		AMOZ		Specificity/ Cross-reactivity (CR)		AOZ		Specificity/ Cross-reactivity (CR)		SEM		Specificity/ Cross-reactivity (CR)													
Limit of Detection (LOD)		Analyte		Limit of Detection (LOD)		Analyte		Limit of Detection (LOD)		Analyte		Limit of Detection (LOD)		Analyte													
Limit of Detection* 0.3 ng/g				Limit of Detection* 0.2 ng/g				Limit of Detection* 0.3 ng/g				Limit of Detection* 0.6 ng/g															
Recovery				Recovery				Recovery				Recovery															
% Recovery (n=20)				% Recovery (n=20)				% Recovery (n=20)				% Recovery (n=20)															
Typical recovery ranged from 82 to 108% at 3 concentrations.				Typical recovery ranged from 71 to 81% at 3 concentrations.				Typical recovery ranged from 100 to 124% at 3 concentrations.				Typical recovery ranged from 70 to 125% at 3 concentrations.															
Precision				Precision				Precision				Precision															
Intra-assay precision (n=12)				Intra-assay precision (n=12)				Intra-assay precision (n=12)				Intra-assay precision (n=12)															
Analyte	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Analyte	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Analyte	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6							
AHD	%CV	%CV	%CV	%CV	%CV	%CV	AMOZ	%CV	%CV	%CV	%CV	%CV	%CV	AOZ	%CV	%CV	%CV	%CV	%CV	%CV	SEM	%CV	%CV	%CV	%CV	%CV	%CV
	1.4	2.6	4.3	4.9	4.5	6.9		1.9	2.6	3.0	3.8	4.3	7.2		1.5	4.8	3.6	4.2	6.2	8.7		0.9	3.0	2.8	4.7	3.6	2.9

Conclusion

- Data indicate that the developed competitive ELISAs detect 4 nitrofurantoin metabolites with LODs ranging from 0.2 ng/g (AMOZ) to 0.6 ng/g (SEM) in tissue samples. Overall typical recovery was >70%. Intra-precision values were typically %CV<9% over a range of concentration levels.
- Use of multi-analyte calibrators and multi-analyte conjugate simplifies the assay procedure, avoids the use of different calibrators and conjugates for the analysis of 4 nitrofurantoin metabolites from 1 sample preparation.
- After sample preparation, for each assay 40 samples can be screened in 90min.
- These assays represent a valuable and convenient analytical tool for the *in vitro* determination of nitrofurantoin metabolites in tissue samples.