Talanta 172 (2017) 120–125

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Screening method for the detection of residues of amphenicol antibiotics in bovine, ovine and porcine kidney by optical biosensor

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ARTICLE INFO

Keywords:
Screening
Detection
Amphenicols
Optical biosensor

ABSTRACT

An immunobiosensor assay was developed for the multi-residue screening of the parent amphenicols, thiamphenicol and florfenicol, along with the metabolite florfenicol amine, in bovine, ovine and porcine kidney. A polyclonal antiserum raised in a rabbit after inoculation with a florfenicol amine-protein conjugate was employed in the assay. Sample homogenates were extracted directly into acetonitrile, reconstituted in buffer and diluted prior to biosensor analysis. Validation data obtained from the analysis of fortified samples has shown that the method has a detection capability (CCβ) of less than 25 μg kg^{-1} (1/2 MRL) for thiamphenicol in the kidney of all three species, less than 150 μg kg^{-1}(1/2 MRL) for florfenicol and florfenicol amine and less than 250 μg kg^{-1} (1/2 MRL) for florfenicol and florfenicol amine in bovine/ovine and porcine kidney respectively. Intra-assay variation (n=10) was calculated at 4.5% and 2.6% at concentrations of 10 μg kg^{-1} and 150 μg kg^{-1}respectively for bovine kidney while inter-assay variation (n=3) was determined to be 5.0% and 16.5% respectively at the same concentrations. The cross-reactivity profile and validation data for the detection of these amphenicols is presented together with the results obtained following the analysis of florfenicol incurred samples using the developed method.

1. Introduction

Amphenicols are synthetic antibiotics with broad activity spectra which have been used mainly for the treatment of serious bacterial infections in both human and veterinary medicine. They exhibit a wide range of activity against both gram negative and gram positive organisms, including *Streptococcus spp., Staphylococcus spp., Pasteurella spp ., Escherichia coli, Salmonella spp . and Bordetella bronchiseptica* [1,2] which cause respiratory and enteric diseases in livestock, through the inhibition of protein biosynthesis by binding to the ribosomal sub units of susceptible bacteria.

The family of compounds includes chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FF). FF is a 3-fluoro derivative of TAP, which is itself a CAP analogue in which the *p*-nitro group on the aromatic ring is substituted with a methylsulfonyl group (Fig. 1). FF was developed as a substitute for its structural analogs with a fluoro group rather than a hydroxyl group located at C3. This makes it less susceptible to acetylation, and therefore less susceptible to bacterial resistance than its two predecessors. It is active at lower concentrations against many CAP and TAP resistant strains including *Enterobacteria, Haemophilus spp.* and *Pasteurella spp.* [3,4]. While TAP remains largely unchanged in vivo, FF is rapidly metabolised to at least five other intermediate metabolites including florfenicol amine (FFA), florfenicol alcohol (FA), florfenicol oxamic acid (FO), florfenicol amine glucuronide (FFAG) and monochloroflorfenicol (MCF) [5].

The amphenicols have been used in livestock production for many years, being marketed under brand names such as Nuflor®, Resflor Gold®, Florocol® and Selectan®. Their use has, however, been questioned since the mid 1980s over concerns regarding their haematological toxicity and the associated risk to human health of ingesting amphenicol contaminated animal produce. Additionally, the ever growing concerns worldwide regarding the emergence of drug resistant bacteria due to over-prescribing of antibiotics, has led to strictly regulated usage in many countries including the European Union, China and the United States. As a consequence, the use of CAP in animals producing food for human consumption has been banned by many regulatory authorities worldwide including within the European Union (EU), while maximum residue limits have been set at 300 μg kg^{-1}, 300 μg kg^{-1} and 500 μg kg^{-1} for FF, expressed as the sum of FF and its metabolites measured as florfenicol amine (FFA), in bovine, ovine and porcine kidney respectively and at 50 μg kg^{-1} for TAP in all species [6].

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http://dx.doi.org/10.1016/j.talanta.2017.05.047
Received 3 April 2017; Received in revised form 12 May 2017; Accepted 15 May 2017
Available online 16 May 2017

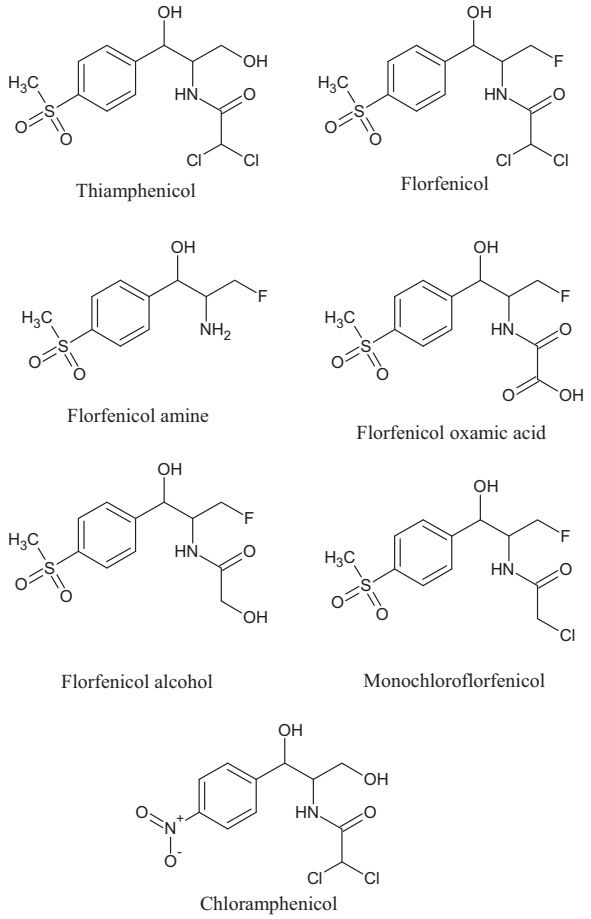

Fig. 1. Structures of chloramphenicol, thiamphenicol, florfenicol and the metabolites of florfenicol.

A significant number of procedures have been reported for the determination of amphenicols in a variety of matrices and are largely based around such physicochemical procedures as HPLC and LC-MS/MS [7–12] with a much smaller number being published based upon immunochemical methods of detection [13–15]. While the physicochemical procedures are undoubtedly extremely sensitive and are able to detect the drugs at concentrations in the low μg kg^{-1} range, they do have the disadvantage of being expensive and time-consuming when applied to the analysis of a large number of samples. Furthermore, it has recently been reported that the use of a solvent extraction alone as part of these physicochemical methods without the inclusion of whole tissue hydrolysis will, in all probability, result in a significant underestimation of FF related content and the subsequent potential for false negative results [16]. In contrast, biosensor technology offers the advantages of allowing rapid and simple sample preparation, as well as being reliable and cost effective and has been established as a screening tool for drug residue testing for many years. This technology has been widely incorporated into both the developmental work and the statutory national testing schemes of many laboratories worldwide [17–20].

The current study describes a simple and cost effective approach to amphenicol screening utilising biosensor technology which requires minimal sample preparation and has no requirement for the acid hydrolysis procedures which must be employed as a prerequisite by physicochemical methods.

An experimental trial was also undertaken to ascertain if the developed procedure could be successfully applied to incurred material and to determine the rate of depletion of FF in porcine kidney following administration of the drug. Biosensor results obtained following the analysis of FF incurred porcine kidney, along with the corresponding UPLC-MS/MS confirmatory results following acid hydrolysis of the same incurred samples, are presented.

2. Materials and methods

2.1. Instrumentation

An optical biosensor (BIACORE®Q) was obtained from GE Healthcare/Biacore (Uppsala, Sweden). Instrument operation and data handling was performed using BIACORE®Q Control Software (Version 3.0.1).

2.2. Reagents and chemicals

CM5 sensor chips and an amine coupling kit containing N-ethyl-N′-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS) and 1 M ethanolamine were obtained from GE Healthcare (Little Chalfont, England). Reference standards for FF, FF-Amine, TAP and CAP were supplied by Sigma-Aldrich Chemical Company Ltd. (Poole, Dorset, U.K.). Nuflor premix was purchased from Devenish Nutrition (Belfast, U.K.). Biosensor assay buffer contained HEPES pH 7.4 (0.01 M), sodium chloride (0.15 M), EDTA (3 mM) and Tween 20 (10%). The solution was degassed and filtered prior to use.

All other chemicals were HPLC grade and were supplied by BDH (Poole, Dorset, U.K).

2.3. Biosensor assay development

2.3.1. Immobilisation of florfenicol amine

FFA was immobilised on the surface of a CM5 sensor chip. Briefly, the carboxymethyl dextran surface was activated by contact with a 1/1 mixture of 0.2 M EDC/0.05 M NHS (50 μL) for 30 min. Reactants were removed and FFA (3 mg) dissolved in 1 mM borate buffer pH 8.5 (1 mL) was applied to the prepared surface (50 μL). This was allowed to react overnight at room temperature. Any unreacted sites were deactivated by incubating with 1 M ethanolamine pH 8.5 (50 μL) for 30 min. The chip surface was then washed with deionised water and dried with nitrogen gas. The immobilised sensor chip was stored refrigerated in the presence of a desiccant when not in use.

2.3.2. Immunogen and antibody production

A FFA-BTG (bovine thyroglobulin) immunogen was produced and anti-FFA polyclonal antiserum raised in a rabbit according to the method outlined in a previous publication [21].

2.3.3. Antibody specificity and selectivity

The ability of the polyclonal antibody to cross-react with the compounds of interest and representative compounds from other antibiotic families was assessed by production of calibration curves and determination of the cross-reactivity using the formula:

$$\text{Cross Reactivity} = \frac{IC_{50}\text{ of Florfenicol Amine}}{IC_{50}\text{ Other Compounds}} \text{x } 100$$

If significant cross-reactivity of a compound in buffer was observed then the cross-reactivity was evaluated when fortified biological matrix was subjected to the developed extraction procedure.

2.3.4. Sample preparation and extraction procedure for biosensor analysis

Negative and sample tissues were homogenised and weighed (2.5 g) into glass universal bottles. Calibrants (containing 0, 2.5, 10, 50, 150 and 300 μg kg^{-1} FFA) were prepared by adding working standards (50 μL) to the known negative aliquots to produce a calibration curve. All calibrants and samples were allowed to stand for 10 min at room temperature and were then treated identically. Acetonitrile (5 mL) was

added to each universal, vortexed vigorously for 10 s and mixed on a roller mixer for 30 min. All universals were then centrifuged at 3500 rpm for 10 min and the supernatants decanted into test tubes. Supernatants were evaporated to dryness using a TurboVap® LV sample concentrator at 60 °C under a stream of nitrogen gas. The resulting extracts were reconstituted immediately in biosensor assay buffer (500 µL) by vortexing vigorously for 1 min. Reconstituted extracts were diluted 1:10 by adding 100 µL extract to 900 µL biosensor buffer and vortexing for 1 min prior to being transferred (170 µL), in duplicate, to the wells of a microtitre plate.

2.3.5. Production of florfenicol incurred material

Sixteen pigs (approximately 30 kg bodyweight), known to be free from exposure to amphenicols, were obtained from a government controlled farm and fed with unmedicated meal during an acclimatisation period. A control animal was euthanised immediately prior to commencement of medication. The medicated group received FF medicated feed at a target therapeutic dose of 10 mg kg^{-1} bodyweight per day for a period of five days (allowing for a consumption rate of 1.25 kg feed per animal per day this is equivalent to 7.5 g Nuflor premix or 300 mg FF per animal per day). At the end of this time animals were returned to unmedicated feed and randomly euthanised at days 0, 3, 6, 10, 12 and 14 post-withdrawal of medicated meal and experimental samples taken. These were stored frozen at −20 °C until analysed.

3. Results and discussion

3.1. Antibody characterisation

The ability of the rabbit polyclonal antiserum to cross-react with the compounds of interest was assessed along with representative compounds from other antibiotic families. If significant cross-reactivity of a compound in buffer was observed then the cross-reactivity through the whole method was evaluated in the presence of biological matrix. The antiserum displayed significant cross-reactivity for FF, FFA and TAP. CAP displayed a more limited cross-reactivity however it was insufficient to meet the MRPL requirement for this compound (Table 1).

The cross-reactivity achieved with FFA was particularly important as the marker residue for FF is described as the sum of FF and its metabolites measured as FFA [6].

3.2. Biosensor assay validation

The developed immunobiosensor assay was validated in accordance with Commission Decision 2002/657/EC [22].

Calibration curves were extracted from bovine, ovine and porcine kidney which had been fortified with FFA at a range of concentrations and these were then overlaid on a single plot (Fig. 2). The data obtained showed that there were no species differences after extraction using the developed method and that all kidney samples could be analysed together in a single assay. As the MRL for bovine and ovine kidney is the same, it was decided to combine the determination of the cut-off point for these two species.

Twenty one known negative bovine kidneys were analysed in three batches of seven on three successive days (Table 2). Ten of these bovine kidneys along with a further ten known negative ovine kidneys were then analysed in four batches on four successive days fortified separately with FF, FFA and TAP at half their respective MRLs and the results used to determine the cut-off value (Table 3).

To enable comparison of the responses in fortified and unfortified samples between days it is necessary to transform the raw data obtained from the biosensor analysis which is expressed in terms of "response units" (RU). To do this an "index score" is calculated. This takes into account both the day to day variability in response (in the same way that calculation of B/B_0 does) and also the dynamic range of the test (being the difference in response between the negative and positive calibration standards).

The index score is defined by the formula:

$$\text{Index Value} = \frac{\text{Mean RU Negative Calibrant} - \text{Mean RU Sample}}{\text{Mean RU Negative Calibrant} - \text{Mean RU Positive Calibrant}} X$$

$$100$$

This formula gives an index score of zero for the negative calibrant and an index score of 100 for the positive calibrant. Index scores were calculated following the analysis of unfortified bovine kidneys and fortified bovine and ovine kidneys (Tables 2 and 3).

The cut-off point of the test is defined as the response that indicates a sample contains a compound at or above the level of interest (in this instance half the MRL). A sample with a response greater than the cut-off point is deemed to be "screen positive" which triggers a confirmatory analysis of the sample. Calculation of the value of the cut-off point is statistically based to take account of the performance of the method and is calculated as the mean index score of the fortified samples minus 1.64 standard deviations of the population mean (1.64 corresponding to a level of confidence of 95% in a one sided *t*-test).

The calculated values were 98.4 for FFA, 107.1 for FF and 101.4 for TAP. Two samples (BOV POS 9 and BOV POS 10) gave index values of less than 98.4 which corresponds to a 10% β-error at the level of interest (which is greater than the 5% acceptable level for screening assays as determined by Commission Decision 2002/657/EC). To increase the level of confidence to 98%, the cut-off value was recalculated using the mean index score minus 1.96 standard deviations (1.96 corresponding to a level of confidence of 98% in a one sided *t*-test) to produce a value of 97.4. All positive samples were above this new cut-off point and therefore meet the criteria of 5% as an acceptable false negative rate. This selected cut-off value of 97.4 for FFA was therefore adopted which, being lower, will give enhanced assurance of

Table 1
Rabbit polyclonal antiserum cross-reactivity in buffer and biological matrix.

Compound	% Cross reactivity in buffer	IC50 buffer (µg kg^{-1})	% Cross reactivity in bovine kidney	IC50 bovine kidney (µg kg^{-1})
Florfenicol Amine	100	0.83	100	10.86
Florfenicol	213	0.39	621	1.92
Thiamphenicol	244	0.34	366	2.97
Chloramphenicol	33	2.49	21	56.50
Chlortetracycline	0	Not Assessed	Not Assessed	Not Assessed
Erythromycin	0	Not Assessed	Not Assessed	Not Assessed
Lincomycin	0	Not Assessed	Not Assessed	Not Assessed
Kanamycin	0	Not Assessed	Not Assessed	Not Assessed
Cephalexin	0	Not Assessed	Not Assessed	Not Assessed
Sulphamethazine	0	Not Assessed	Not Assessed	Not Assessed
Enrofloxacin	0	Not Assessed	Not Assessed	Not Assessed
Ampicillin	0	Not Assessed	Not Assessed	Not Assessed

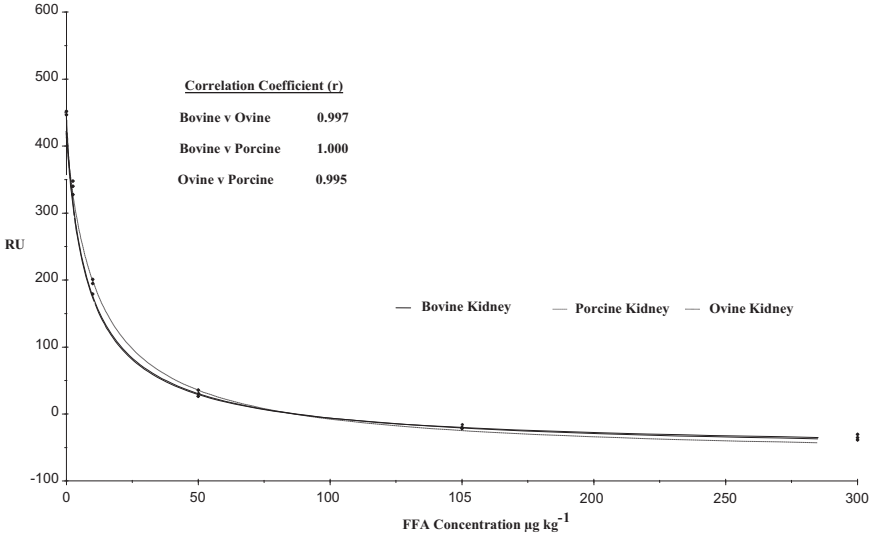

Fig. 2. Overlay of typical calibration curves obtained following analysis of fortified bovine, ovine and porcine kidney.

Table 2
Index score for negative bovine kidneys.

Run 1 Day 1	Sample	RU Diff	Index Score
	BOV NEG 1	62.4	10.3
	BOV NEG 2	61.5	10.2
	BOV NEG 3	51.2	8.5
	BOV NEG 4	51.1	8.4
	BOV NEG 5	52.4	8.7
	BOV NEG 6	85.5	14.1
	BOV NEG 7	62.8	10.4
Run 2 Day2	**Sample**	**RU Diff**	**Index Score**
	BOV NEG 8	−21.3	−3.8
	BOV NEG 9	−33.3	−6.0
	BOV NEG 10	−28.9	−5.2
	BOV NEG 11	1.6	0.3
	BOV NEG 12	−28.0	−5.0
	BOV NEG 13	−27.1	−4.8
	BOV NEG 14	−39.3	−7.0
Run 3 Day3	**Sample**	**RU Diff**	**Index Score**
	BOV NEG 15	−10.0	−1.6
	BOV NEG 16	−6.6	−1.1
	BOV NEG 17	−2.7	−0.4
	BOV NEG 18	−4.1	−0.7
	BOV NEG 19	2.4	0.4
	BOV NEG 20	3.5	0.6
	BOV NEG 21	2.4	0.4

selecting potential positives and avoiding the possibility of false compliant results. The assay is qualitative as it cannot distinguish between the various amphenicols but, as a result of the specificity of the antibody, it does indicate that one or more is present. Any amphenicol detected following biosensor analysis of samples will represent the total concentration of FFA-like immunoreactivity in the sample and given that the purpose of the test is to select samples for confirmatory testing using the criterion that the level of interest is half of the MRL, the method is judged fit for purpose using an index score of 97.4 as the cut-off point.

Twenty porcine kidney samples were analysed in four separate batches on four successive days fortified separately with FF, FFA and TAP at half their respective MRLs and index scores calculated (Table 3). The calculated cut-off values were 106.0 for FFA, 111.2 for FF and 103.4 for TAP and, being the lowest, the cut-off for TAP was adopted to once again reduce the risk of missing a non-compliant sample.

Of the twenty results for these fortified samples, only one (TAP POS 13) gave an index score of less than 103.4 which meets the acceptable false negative rate of 5% and therefore indicates that the CCβ is approximately equal to the fortification concentration of 25 µg kg^{-1} and more than meets the criteria set out in Commission Decision 2002/657/EC. Given that the purpose of the test is to select samples for confirmatory testing using the criterion that the level of interest is half of the MRL, the method is judged fit for purpose using an index score of 103.4 as the cut-off point.

The aim of the developed biosensor test is to allow for the screening of large numbers of samples for possible non-compliance prior to confirmatory analysis and not to determine a definitive concentration of amphenicol present. Therefore, since kidney from all three species can be analysed together, it was decided to use bovine kidney as the choice of matrix for the preparation of calibrants within each statutory run. This runs the risk of a greater number of porcine samples being forwarded for confirmatory analysis than is strictly necessary as porcine kidney has a higher MRL (500 µg kg^{-1}) than bovine and ovine

Table 3
Index score for fortified bovine, ovine and porcine kidneys.

	Florfenicol-Amine		Florfenicol		Thiamphenicol	
Species Sample Numbers & ½ MRL	**Bovine & Ovine (1-20) 150 µg kg^{-1}**	**Porcine (1–20) 250 µg kg^{-1}**	**Bovine & Ovine (1-20) 150 µg kg^{-1}**	**Porcine (1–20) 250 µg kg^{-1}**	**Bovine & Ovine (1-20) 25 µg kg^{-1}**	**Porcine (1–20) 25 µg kg^{-1}**
RU Difference Range	281–628	306–483	342–561	468–558	321–519	428–524
Index Score Range	97.5–108.8	111.3–109.6	103.7–118.3	111.2–115.0	101.7–114.3	101.6–108.0
Index Mean	**103.6**	**110.2**	**112.8**	**115.3**	**106.6**	**108.7**
Index SD	**3.14**	**2.51**	**3.51**	**2.51**	**3.20**	**3.26**
Mean - 1.64SD	**98.4**	**106.0**	**107.1**	**111.2**	**101.4**	**103.4**

Table 4
Comparison of biosensor screening and UPLC-MS/MS confirmatory results for florfenicol incurred porcine kidney samples.

Animal ID/Days post treatment/Standard conc. (μg kg^{-1})	Biosensor RU mean samples 1/10	Biosensor index value	Biosensor result compliant/Non-compliant	Biosensor RU mean samples 1/100	Biosensor result μg kg^{-1} corrected	UPLC-MS/MS μg kg^{-1} FFA
0	502.5	0		549.8		
2.5 FFA				462.1		
10 FFA				319.4		
50 FFA				132.2		
100 FFA				70.1		
250 FFA	2.8	100		16.6		
Control Pig	507.2	−0.9		552.3	0.0	0.0
Pig 1 Day 0	−49.2	110.4	Non-Compliant	13.8	> 2500	1640
Pig 2 Day 0	−59.7	112.5	Non-Compliant	−19.8	> 2500	2454
Pig 3 Day 0	−48.2	110.2	Non-Compliant	13.8	> 2500	1509
Pig 1 Day 3	−46.4	109.8	Non-Compliant	95.3	719	1144
Pig 2 Day 3	−38.5	108.3	Non-Compliant	80.2	863	869
Pig 1 Day 6	−45.9	109.7	Non-Compliant	23.3	2220	1233
Pig 2 Day 6	−31.1	106.8	Non-Compliant	75.4	917	493
Pig 1 Day 10	−34.3	107.4	Non-Compliant	92.1	746	404
Pig 2 Day 10	−27.2	106.0	Non-Compliant	116.0	574	547
Pig 3 Day 10	−40.0	108.6	Non-Compliant	161.7	371	447
Pig 4 Day 10	−34.3	107.4	Non-Compliant	182.1	311	413
Pig 1 Day 12	−30.0	106.6	Non-Compliant	187.7	297	291
Pig 2 Day 12	−18.2	104.2	Non-Compliant	223.7	222	288
Pig 1 Day 14	−10.8	102.7	Compliant	312.1	110	304
Pig 2 Day 14	9.9	98.6	Compliant	348.2	82	245

Note 1: The screening method cut-off index value for porcine kidney=103.4.
Note 2: The MRL for FF/FFA in porcine kidney=500 μg kg^{-1}.

kidney (300 μg kg^{-1}); however it was determined that this policy would be adopted at our own risk and reviewed after six months when analysis data could be assessed to determine the level of false positives. The number of calibrants used for a statutory analysis of real samples is two, namely a negative and a positive at ½ MRL FFA (0 and 150 μg kg^{-1} FFA). FFA was chosen for the biosensor analysis as it displays least cross-reactivity and therefore lessens the chance of incorrectly screening a non-compliant sample.

3.3. Incurred samples

The incurred porcine kidney samples were analysed by both the developed biosensor based procedure and by a UPLC-MS/MS confirmatory method developed within this institute [16]. On this occasion, for the biosensor analysis, a full calibration curve was also extracted in an attempt to provide a more definitive correlation between results obtained from the two technologies in terms of μg kg^{-1} as well as via calculation of index values. Initial analysis of the incurred samples showed that the concentrations obtained were outside the dynamic range of the calibration curve and a further 1/10 dilution of samples in negative extract was required. Table 4 shows results obtained from the initial 1/10 final dilution of samples (index values) as well as those obtained from the 1/100 final dilution (μg kg^{-1} values) together with the corresponding UPLC-MS/MS results.

Of the 15 animals that were fed medicated meal during the trial, all but 2 (day 14 withdrawal) gave biosensor screening results above the designated 103.4 cut-off point i.e. 13 of the 15 samples would have been selected for confirmatory analysis. UPLC-MS/MS analysis of the same samples confirmed 7 of these to be above the 500 μg kg^{-1} MRL while the remaining 6 were above the 250 μg kg^{-1}½ MRL concentration level. The unmedicated animal tested compliant using both methods. Although a number of samples which were screened by the developed method as non-compliant were shown to be at concentrations less than the MRL by confirmatory analysis, it is desirable that a screening test should err on the side of caution. This laboratory is more concerned with eliminating any possibility of false negative results during the screening of samples than with obtaining a small number of false positive ones. It should also be remembered that it is for this very reason that these samples are being screened relative to half the MRL.

4. Conclusions

A screening procedure utilising biosensor technology has been developed which is capable of detecting low concentrations of a range of amphenicols at less than half their respective MRL. The cross-reactivity data obtained has shown that the antiserum is suitable for the detection of at least three of the parent drugs and metabolites.

While many methods have been published for the detection of these compounds in various biological matrices, most employ elaborate and expensive physicochemical procedures such as HPLC and LC-MS/MS. Furthermore, recently published findings have reported that these physicochemical methods must include an acid hydrolysis step as a prerequisite within the extraction procedure to both release tissue bound FF and other FF metabolites present in samples and to convert them to the stable end product, FFA, to avoid substantial underestimation of drug concentration present [16]. The current study has provided a fully validated screening method which has many advantages over other procedures in terms of cost and simplicity of extraction, allowing the complete extraction and analysis of at least 24 samples within 12 h. It has furthermore demonstrated, through the analysis of incurred tissues, that this immunoassay does not require an acid hydrolysis step, with detection of incurred residues at less than half the MRL being easily achieved.

FF metabolism is complex, however, and it is possible that other identified FF metabolites (FO, FA, FFAG and MCF) or conjugates display significant cross-reactivity to the polyclonal antiserum employed in the screening test and are present as a significant portion of the residue concentration present in the extracts from incurred samples. This is supported by evidence from Faulkner et al. [16], who analysed extracts from incurred samples, with and without a hydrolysis step. Their results demonstrated very low levels of free FF or FFA compared to the post hydrolysis totals. The antiserum used in this study was raised to FFA by conjugating the drug via its amine group to a carrier protein and using the resulting complex as an immunogen in

the host animal. Therefore, antibodies would have been produced to bind the remainder of the structure not used in the conjugation reaction, as indicated by the high cross-reactivity obtained for TAP and FF. Consequently, it can be assumed that antibodies have been produced that bind the methylsulfonyl-benzene-alcohol part of the structure, which is also common to the metabolites in question. Lack of availability meant that it was not possible to assess the cross-reactivity of the metabolites themselves but, based on the fact that the only differences between their structures (and FFA) occur at the point of conjugation, it would be expected that it would be considerable. Furthermore it is also possible that in vivo moieties, incapable of being hydrolysed to FFA and which cross-react with the antibody, are present in tissue and are isolated by solvent extraction alone.

Further work will attempt to establish a definitive answer, however it is clear from the experimental results obtained that, when applied to incurred samples, the developed method was shown to be capable of successfully screening potentially non-compliant samples from FF-treated animals. Confirmatory analysis of the same samples by UPLC-MS/MS showed good correlation between the two technologies and demonstrated that the screening test displayed a zero false negative rate for the analysis of the incurred samples.

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