ANALYTICA CHIMICA ACTA 637 (2009) 259–264

available at www.sciencedirect.com

ScienceDirect

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

Improved screening method for the detection of a range of nitroimidazoles in various matrices by optical biosensor

Colin S. Thompson, Imelda M. Traynor, Terence L. Fodey, Steven R.H. Crooks*

Agri-Food and Biosciences Institute, Veterinary Sciences Division, Stormont, Belfast BT4 3SD, Northern Ireland, UK

ARTICLE INFO

Article history:
Received 20 June 2008
Received in revised form
4 September 2008
Accepted 9 September 2008
Published on line 21 September 2008

Keywords:
Screening
Detection
Nitroimidazoles
Optical biosensor

ABSTRACT

An immunobiosensor assay was developed for the multi-residue screening of a range of nitroimidazole compounds in various species and sample types including porcine, bovine and ovine kidney, avian liver, serum and eggs and bovine milk. A polyclonal antibody which binds at least seven of the major nitroimidazoles and their metabolites was raised in a sheep after inoculation with a metronidazole protein conjugate. Sample homogenates were extracted into acetonitrile and subjected to micro-centrifugation prior to biosensor analysis. Validation data obtained from the analysis of 20 fortified samples has shown that the method has a detection capability (CCβ) of less than 1 $\mu g\,kg^{-1}$ (or $\mu g\,L^{-1}$) for dimetridazole (DMZ) in all species and matrices investigated. In addition, cross-reactivity data and the analysis of a small number of fortified samples have shown that the method will also detect a range of other major parent nitroimidazoles and their metabolites including ronidazole (RNZ), ipronidazole (IPZ), metronidazole (MNZ), hydroxymetronidazole (MNZOH), hydroxydimetridazole (DMZOH) and hydroxyipronidazole (IPZOH). The cross-reactivity profile and validation data for the detection of these nitroimidazoles are presented together with the results obtained following the analysis of a small number of incurred samples using the developed method.

1. Introduction

Nitroimidazoles are antiprotozoal drugs that have been primarily used in veterinary medicine to treat histomoniasis and trichomoniasis in poultry and haemorrhagic enteritis in pigs. Growth promotion and improvement of feed efficiency were noted side effects following administration of the drugs. They are now suspected to have genotoxic, carcinogenic and mutagenic properties [1] and their use has been banned within the European Union (EU) by Council Regulations 2377/90 and 2205/2001 [2,3].

Evidence of the misuse of these drugs has been detected in a number of countries which export to the EU [4]. In order to monitor for any such illegal use, there is a requirement to have robust and reliable screening and confirmatory tests capable of low level detection of nitroimidazole residues. While physicochemical procedures (HPLC and LC–MS/MS) can detect the drugs at concentrations in the low $\mu g\,kg^{-1}$ (or $\mu g\,L^{-1}$) range [5–8], there are significant drawbacks to be encountered when employing these methods due to time-consuming sample extraction procedures and the cost of the analytical equipment required.

The application of biosensor technology as a tool for rapid and reliable drug residue testing has now been established for a number of years and has been incorporated into both developmental work and the statutory national testing schemes

* *Corresponding author*. Tel.: +44 28 90525866; fax: +44 28 90525840.
E-mail address: colin.thompson@afbini.gov.uk (C.S. Thompson).
0003-2670/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2008.09.040

of many laboratories worldwide [9–15]. In this study, an optical biosensor-screening assay was initially developed for the detection of nitroimidazoles in porcine, bovine and ovine kidney tissue, as this is the matrix specified by the United Kingdom National Surveillance Scheme (UKNSS). The method was further adapted to include avian liver, serum and eggs and bovine milk.

In the absence of an established minimum required performance limit (MRPL) or reference point for action for the nitroimidazoles, the EU's Community Reference Laboratories (CRLs) have published guidelines on the "recommended concentrations" which all Community Laboratories should be able to detect and confirm for the presence of residues. These recommended concentrations have no legal force, but serve to define the minimum standard expected of an analytical method. The recommended concentration for the nitroimidazoles is 3 $\mu g\,kg^{-1}$ (or $\mu g\,L^{-1}$) in all matrices apart from IPZ and IPZOH for which no recommended concentration has yet been defined [16].

The developed method was assessed to determine that it was capable of meeting this criterion and was subsequently validated for each species and matrix. It was then assessed for its ability to extract and detect parent nitroimidazoles and their relevant metabolites from avian liver and serum samples obtained from treated broilers.

2. Materials and methods

2.1. Instrumentation

An optical biosensor (BIACORE®Q) was obtained from Biacore AB (Uppsala, Sweden). Instrument operation and data handling were performed using BIACORE®Q Control Software (version 3.0.1).

2.2. Reagents and chemicals

HBS-EP buffer, CM5 sensor chips (certified grade) and an amine coupling kit containing *N*-ethyl-*N*′-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), *N*-hydroxy-succinimide (NHS) and 1 M ethanolamine pH 8.5 were all obtained from Biacore AB (Uppsala, Sweden). Reference standards for DMZ, MNZ, RNZ and IPZ were supplied by Sigma–Aldrich Chemical Company Ltd. (Poole, Dorset, UK). The metabolites MNZOH, DMZOH and IPZOH were kindly supplied as gifts by the Community Reference Laboratory for nitroimidazoles (BVL, Berlin, Germany). Carboxymethyl dextran (MW 500) was obtained from Ssens (Hengelo, The Netherlands).

Non-specific binding (NSB) buffer was prepared according to the method outlined by Situ et al. [17]. Briefly, HEPES (0.238 g), sodium chloride (2.922 g), EDTA (0.127 g), carboxymethylated dextran (0.005 g) and 10% Tween 20 (50 μL) were dissolved in deionised water. The pH was adjusted to 9.0 using sodium hydroxide (5 M) and the solution made up to a final volume of 100 mL using deionised water.

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

2.3. Biosensor assay development

2.3.1. Immobilisation of MNZ derivative

A CM5 sensor chip was immobilised as outlined by Connolly et al. [15]. Briefly, the carboxymethyl dextran surface was activated by contact with a 1/1 v/v mixture of 0.2 M EDC/0.05 M NHS for 20 min and the activated surface incubated for 1 h with a 1 M ethylenediamine solution. The ethylenediamine solution was removed and MNZ was coupled to the amine surface via a di-succinimidyl carbonate (DSC) cross-linker [18] by incubating overnight at room temperature. Following this incubation any unreacted sites were deactivated by incubating with 1 M ethanolamine pH 8.5 for 20 min. The chip surface was then washed with deionised water and stored refrigerated in the presence of a desiccant when not in use.

2.3.2. Immunogen and antibody production

An MNZ-DSC-Jeffamine-Bovine thyroglobulin immunogen was produced and anti-MNZ polyclonal antiserum raised in a sheep according to the method outlined in a previously published paper [18].

2.4. Sample preparation and extraction procedure for biosensor analysis

2.4.1. Preparation of kidney, liver, egg and milk samples

Negative and sample tissues were homogenised and weighed (5 g) into glass universal bottles. Negative and sample milks were centrifuged at 3700 rpm for 10 min and the upper layer of fat removed prior to extraction. Aliquots of the defatted milk (5 mL) were pipetted into glass universals. Calibrants (containing 0.01, 0.5, 1.0, 2.0, 5.0 and 10.0 $\mu g\,kg^{-1}$ or $\mu g\,L^{-1}$ DMZ) were prepared by adding working standards (50 μL) to the known negative aliquots to produce a calibration curve (Fig. 1). Control samples were prepared in the same way using the appropriate working controls. All calibrants, controls and samples were allowed to stand for 10 min at room temperature and were then treated identically. Acetonitrile (10 mL) was added to each universal, which was vortexed vigorously for 10 s and then mixed on a roller mixer for 30 min. All universals were centrifuged at 3500 rpm for 10 min at 10 °C and the supernatants decanted into test tubes. The supernatants were evaporated to dryness using a TurboVap® LV sample concentrator at 50 °C

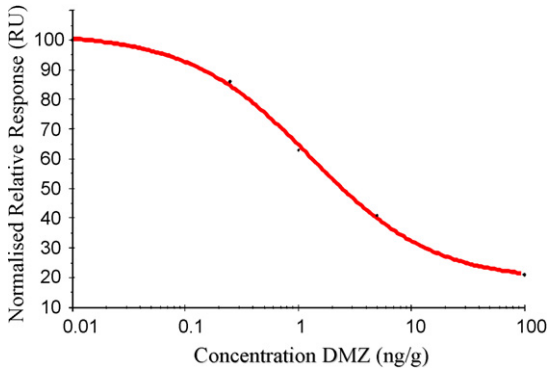

Fig. 1 – Typical calibration curve obtained following biosensor analysis of DMZ residues in porcine kidney.

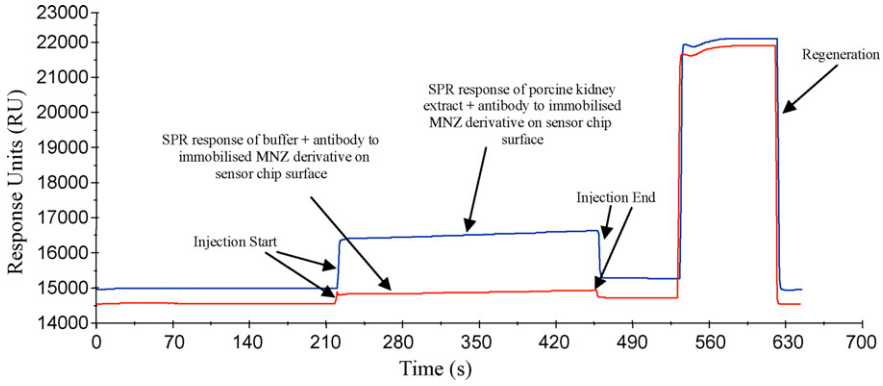

Fig. 2 – Typical sensorgrams obtained during optical biosensor analysis for DMZ residues in buffer and porcine kidney samples.

under a stream of nitrogen gas. The resulting extracts were reconstituted immediately in HBS-EP buffer (250 μL for tissue and milk assays and 500 μL for egg assays) by vortexing vigorously for 1 min and transferred to micro-centrifuge tubes prior to centrifugation at 13,000 rpm for 10 min. For egg assays only, the extracts were passed through 0.22 μm filter units. Extracts for analysis (105 μL) were transferred, in duplicate, to the wells of a microtitre plate.

2.5. *Preparation of serum samples*

Negative and sample sera were pipetted (2 mL) into glass universal bottles. Calibrants (containing 0.01, 0.5, 1.0, 2.0, 5.0 and 10.0 $\mu g\,L^{-1}$ DMZ) were prepared by adding working standards (50 μL) to the known negative aliquots to produce a calibration curve. Control samples were prepared in the same way using the appropriate working controls. All calibrants, controls 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, which were then 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 at 10 °C and the supernatants decanted into test tubes. These were then evaporated to dryness using a TurboVap® LV sample concentrator at 50 °C under a stream of nitrogen gas. The resulting extracts were reconstituted immediately in NSB buffer (250 μL) by vortexing vigorously for 1 min and transferred to micro-centrifuge tubes prior to centrifugation at 13,000 rpm for 10 min. Extracts for analysis were transferred (105 μL), in duplicate, to the wells of a microtitre plate.

2.6. *Biosensor assay*

Extracts were mixed with antibody prepared at a predetermined dilution in HBS buffer (70/30 v/v) and injected (80 μL) over the sensor chip surface at a flow rate of 20 $\mu L\,min^{-1}$. Report points were recorded before and after each injection to measure the response generated by antibody binding to MNZ immobilised on the sensor chip surface (Fig. 2). The chip surface was regenerated with a 1-min injection of 20% acetonitrile in 0.25 M sodium hydroxide at a flow rate of 20 $\mu L\,min^{-1}$.

2.7. *Production of nitroimidazole incurred material*

Eight chickens known to be free from any nitroimidazole treatment were purchased from a local supplier and randomly divided into four groups of two each. Group A (controls) received non-medicated water throughout the duration of the trial. Groups B, C and D received target doses of DMZ (20 $mg\,kg^{-1}$), MNZ (20 $mg\,kg^{-1}$) and RNZ (10 $mg\,kg^{-1}$ bodyweight) respectively. These were added to communal drinking water and offered to the birds each morning for a period of 10 days. At the end of this time all groups were returned to non-medicated water and 24 h post-medication all birds were

Table 1 – Cross-reactivity profile of the MNZ sheep polyclonal antibody (S229)

Analyte	Buffer (%)	Porcine kidney (%)	Bovine kidney (%)	Ovine kidney (%)	Avian liver (%)	Avian egg (%)	Avian serum (%)	Bovine milk (%)
Dimetridazole	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Metronidazole	93.4	>100.0	>100.0	>100.0	80.5	>100.0	>100.0	>100.0
Ronidazole	>100.0	>100.0	>100.0	>100.0	>100.0	>100.0	>100.0	>100.0
Ipronidazole	>100.0	>100.0	>100.0	>100.0	>100.0	>100.0	>100.0	>100.0
Hydroxydimetridazole	51.3	79.9	>100.0	>100.0	67.5	>100.0	>100.0	71.0
Hydroxymetronidazole	>100.0	>100.0	85.6	>100.0	>100.0	>100.0	>100.0	>100.0
Hydroxyipronidazole	66.1	52.2	54.7	86.5	45.6	59.6	>100.0	52.3

sacrificed and experimental samples taken. Blood samples were centrifuged at 3000 rpm for 10 min, the sera was removed and immediately stored along with liver samples at −20 °C until analysed.

3. Results and discussion

3.1. Antibody characterisation

The sheep polyclonal antibody produced was assessed for cross-reactivity, in assay buffer and matrix, to a range of nitroimidazoles for each of the various species and sample types described. Significant cross-reactivity was found with all the major parent and metabolite nitroimidazoles tested (Table 1).

3.2. Biosensor assay validation

The developed immunobiosensor assay was validated in accordance with Commission Decision 2002/657/EC [19].

Nitroimidazole free samples ($n = 20$) for each species and matrix described were extracted and analysed as has been previously detailed. To determine the detection capability (CCβ) of DMZ for each matrix, the 20 blank samples were fortified at 1.0 μg kg^{-1} (or μg L^{-1}) DMZ. Results obtained showed the method to have a CCβ for DMZ of < 1.0 μg kg^{-1} (or μg L^{-1}) for all of the species and matrices tested.

To give increased confidence of detecting the other parent members and their respective metabolites within the nitroimidazole group, a reduced number of samples ($n = 5$) were fortified at various levels and evaluated against 0 and 1 μg kg^{-1} (or μg L^{-1}) DMZ calibration standards extracted from each matrix (Table 2). From the results obtained it was shown that the method will also detect the parent drugs RNZ and IPZ at <1 μg kg^{-1} (or μg L^{-1}) and MNZ and the metabolite MNZOH at <2.0 μg kg^{-1} (or μg L^{-1}). Both the metabolites DMZOH and IPZOH could be detected at <3.0 μg kg^{-1} (or μg L^{-1}). The assay is qualitative as it cannot distinguish between the various parent nitroimidazoles or their hydroxy metabolites but indicates that one or more is present. Any nitroimidazole concentration value obtained following biosensor analysis will represent the total concentration of DMZ-like immunoreactivity in the sample (which may contain one or more of the parent drugs and/or their metabolites).

3.3. Incurred samples

Incurred liver and serum samples were analysed by the developed biosensor-based procedure and by LC–MS/MS [20] (Table 3). Of the six incurred liver samples only two gave screening results above the 3 μg kg^{-1} recommended concentration, one being confirmed above this level by LC–MS/MS. Concentrations of nitroimidazole residues found in the liver samples of each of the three medicated groups 24 h after slaughter were considerably lower than the serum samples taken from the same animals. Concentrations measured using the developed method in liver ranged from 1.44 μg kg^{-1} for DMZ/DMZOH to 6.42 μg kg^{-1} for MNZ/MNZOH while concentration in the serum of all three medicated groups was

Table 2 – Fortification level required for each nitroimidazole in various matrices to allow for the classification of five sample replicates as >1.0 μg kg^{-1} (or L^{-1}) DMZ

Analyte	Buffer (μg L^{-1})	Porcine kidney (μg kg^{-1})	Bovine kidney (μg kg^{-1})	Ovine kidney (μg kg^{-1})	Avian liver (μg kg^{-1})	Avian egg (μg kg^{-1})	Avian serum (μg L^{-1})	Bovine milk (μg L^{-1})
Metronidazole	1	1	2	2	2	1	1	2
Ronidazole	1	1	1	1	1	1	1	1
Ipronidazole	1	1	1	1	1	1	1	1
Hydroxydimetridazole	2	3	3	2	3	3	3	3
Hydroxymetronidazole	1	1	1	1	1	1	1	2
Hydroxyipronidazole	2	3	3	3	3	3	3	3

Table 3 – Results from the analysis of incurred liver and serum samples taken from broilers 24 h post-medication

Analyte	Liver ($\mu g\,kg^{-1}$)				Serum ($\mu g\,L^{-1}$)			
	Biosensor		LC–MS/MS		Biosensor		LC–MS/MS	
	Animal 1	Animal 2	Animal 1	Animal 2	Animal 1	Animal 2	Animal 1	Animal 2
Dimetridazole	1.44	1.67	ND	ND	>10.0	>10.0	1275.6	949.3
Metronidazole	2.76	6.42	0.36	1.31	>10.0	>10.0	482.2	527.3
Ronidazole	5.23	2.50	4.20	2.00	>10.0	>10.0	1356.8	1103.7
Controls	<0.01	<0.01	ND	ND	<0.01	0.03	ND	ND

>10.0 $\mu g\,L^{-1}$. The unmedicated birds tested negative in both matrices.

4. Conclusions

A simple and reliable screening procedure, based on optical biosensor technology, has been developed which has been shown to be capable of detecting low concentrations of a range of nitroimidazoles and their metabolites in various species and matrices. The cross-reactivity data obtained has shown that the antibody is suitable for the detection of at least seven of the major nitroimidazole parent drugs and their metabolites.

An assay employed to detect substances that are banned within the European Union, such as the nitroimidazoles, should be as sensitive as possible. Currently, a recommended concentration (setting the minimum standards that methods used in the EU should reach) has been set at 3 $\mu g\,kg^{-1}$ for DMZ, MNZ and RNZ and their metabolites. No recommendation has been proposed for IPZ or IPZOH to date. The CCβ values obtained in this study have demonstrated that the procedure is suitable for the analysis of these compounds in the <1.0–3.0 $\mu g\,kg^{-1}$ range and is therefore capable of meeting these minimum requirements.

Many methods for the detection of drug residues in biological matrices employ elaborate physicochemical procedures such as HPLC and LC–MS/MS. These can cause significant drawbacks to laboratories due to the cost and complexity of the analytical equipment involved and the time-consuming extraction procedures that are often associated with them. As a result, many laboratories adopt a two-step approach to drug residue analysis involving an initial screening programme involving the use of inexpensive and reliable techniques such as enzyme linked immunoassays (ELISAs) or immunobiosensor assays followed by confirmatory analysis of samples shown to be potentially non-compliant. An ELISA procedure has been reported for the determination of nitroimidazoles in eggs and chicken muscle [21], however, it offers negligible antibody cross-reactivity with the metabolites MNZOH and IPZOH and has a limited detection capability for many of the other major compounds. A biosensor method has previously been described for detection of nitroimidazoles in avian muscle [15] and while it employs a simple and inexpensive extraction procedure, it was not demonstrated as suitable for application to a broader range of species and sample types. By comparing with this study, the antibody employed previously [15] displayed limited cross-reactivity with the metabolites MNZOH and DMZOH while no data was presented for the parent drug IPZ and its metabolite IPZOH.

The current study has provided a fully validated screening method which has many advantages over other procedures in terms of one or more of the following criteria: cost, simplicity of extraction, range of parent nitroimidazoles and their metabolites detected and ease of application to a wide range of species and matrices. Employing the current procedure, 24 samples could be extracted and analysed within 15 h.

Furthermore, the developed method was applied to a small number of incurred samples and was shown to be capable of extracting the drugs from the liver and serum of the treated broilers. As was previously determined by the work of Polzer et al. [22] on medicated turkeys, the results obtained would appear to suggest that levels of nitroimidazoles are to be found in substantially higher concentrations in serum than in liver post-medication. It is therefore proposed that a much more extensive depletion study will be undertaken to ascertain which would be the most suitable matrix for the long-term detection of nitroimidazole abuse in broilers following drug withdrawal from the diet.

REFERENCES

[1] C.E. Voogd, Mutat. Res. 86 (1981) 243.
[2] Council Regulation 2377/90, Off. J. Eur. Commun. No. L224/1 (1990).
[3] Council Regulation 2205/2001, Off. J. Eur. Commun. No. L297/3-4 (2001).
[4] European Commission Staff Working Document on the Implementation of National Residue Monitoring Plans in the Member States in 2004, Sanco/3400/2005, Annex 21–22, 2005.
[5] A. Canavan, D.G. Kennedy, Analyst 122 (1997) 963.
[6] E. Daeseleire, H. De Ruyck, R. van Renterghem, Analyst 125 (2000) 1533.
[7] D. Hurtaud-Pessel, B. Delepine, M. Laurentie, J. Chromatogr. A 882 (2000) 89.
[8] J. Polzer, P. Gowik, J. Chromatogr. B 761 (2001) 47.
[9] D.R. Shankaran, K.V.A. Gobi, N. Miura, Sens. Actuator B: Chem. 121 (2007) 158.
[10] J.V. Samsonova, N.A. Uskova, A.N. Andresyuk, M. Franek, C.T. Elliott, Chemosphere 57 (2004) 975.
[11] V. Gaudin, P. Maris, Food Agric. Immunol. 13 (2001) 77.
[12] T. McGrath, G.A. Baxter, J. Ferguson, S.A. Haughey, P. Bjurling, Anal. Chim. Acta 529 (2005) 123.
[13] S.R.H. Crooks, B. McCarney, I.M. Traynor, C.S. Thompson, S. Floyd, C.T. Elliott, Anal. Chim. Acta 483 (2003) 181.
[14] C.S. Thompson, S.A. Haughey, I.M. Traynor, T.L. Fodey, C.T. Elliott, J.P. Antignac, B. Le Bizec, S.R.H. Crooks, Anal. Chim. Acta 608 (2008) 217.

[15] L. Connolly, C.S. Thompson, S.A. Haughey, I.M. Traynor, S. Tittlemeier, C.T. Elliott, Anal. Chim. Acta 598 (2007) 155.
[16] CRL Guidance Paper—CRLs view on state of the art analytical methods for the national residue plans for control of residues, available from the EU CRLs for veterinary drug residue analysis, 2007.
[17] C. Situ, A.R.G. Wylie, A. Douglas, C.T. Elliott, Talanta 76 (2008) 832.
[18] T.L. Fodey, L. Connolly, S.R.H. Crooks, P. Delahaut, C.T. Elliott, Anal. Chim. Acta 483 (2003) 193.
[19] Commission Decision 2002/657/EC, Off. J. Eur. Commun. L221/8 (2002).
[20] R. McCracken, D.G. Kennedy, in preparation.
[21] A.-C. Huet, L. Mortier, E. Daeseleire, T. Fodey, C. Elliott, P. Delahaut, Anal. Chim. Acta 534 (2005) 157.
[22] J. Polzer, C. Stachel, P. Gowik, Anal. Chim. Acta 521 (2004) 189.