Determination of ivermectin in bovine liver by optical immunobiosensor

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Abstract

A rapid and sensitive biosensor immunoassay was developed for residues of the antiparasitic agent ivermectin in bovine liver. A detection limit of 19.1 ng g⁻¹ was achieved. The sensor employed was a Biacore optical instrument based on surface plasmon resonance. 5-O-succinoylivermectin-apo-transferrin conjugate was used to produce monoclonal antibody while a second derivative, ivermectin-oxime, was immobilised onto the surface of a sensor chip. A range of assay parameters (flow rate, injection time, temperature) and extraction techniques were investigated. In the final assay procedure, ivermectin was extracted with acetonitrile followed by C₈ SPE clean-up. Matrix effect was minimised by increasing the flow rate to 25 μl min⁻¹ and reducing the sample injection time to 2 min. The average value for liver samples spiked at 100 ng g⁻¹ (the MRL for the drug) and 50 ng g⁻¹ concentrations were 93.7 and 43.2 ng g⁻¹, respectively. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Ivermectin; Avermectins; Biosensor; Bovine liver

1. Introduction

Ivermectin is a semi-synthetic 22,23-dihydro derivative of avermectin B₁ (abamectin) which is the major and most important product of the fermentation of Streptomyces avermitilis (Campbell, 1989). Ivermectin consists of a mixture of two homologous compounds (‘H₂B₁a’ and ‘H₂B₁b’) in an average ratio of 80:20 (Table 1). These compounds differ only by one methylene group at the C-25 position. Since the drug’s introduction to the market in 1981 it has found wide usage as an antiparasitic agent against endo- and ectoparasites of animals (cattle, sheep, swine, horses, dogs). It is also used as a very effective treatment for human filarial worm infections (Onchocerca volvulus, River Blindness).

Ivermectin is used at substantially lower doses than other veterinary medicines (up to 300 μg kg⁻¹; Campbell, 1989). This makes the detection of residues in animal tissue, which have resulted from treatment, very challenging. To enable residue detection very sensitive analytical assays are required. During the last 15 years a number of chromatographic methods (LC, HPLC) were developed for determination of ivermectin in different matrices such as liver, muscle (Abjean and Gaugain, 1995; Nordlander and Johnsson, 1990; Reuvers et al., 1993; Scott and McKellar, 1992), milk, serum, plasma (Chiou et al., 1987; De Montigny et al., 1990; Dickinson, 1990), salmon (Kennedy et al., 1993) and others. While sensitive these methods can be described as lengthy and tedious to perform. As routine screening methods they accomplish the analytical need but take much longer to perform than is desired.

Immunoassay, as a screening technique for residue analysis, has proven to be a promising alternative to conventional physicochemical methods. Both monoclonal (Schmitt et al., 1990) and polyclonal (Mitsui et al., 1996; Crooks et al., 1998) anti-ivermectin antibodies have been produced and successfully applied to ELISA based techniques. It is a logical progression to try to further develop ivermectin residue tests using immunosensor-
Table 1
Chemical structure of avermectins

<table>
<thead>
<tr>
<th>Substance</th>
<th>X(C22–C23)</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ivermectin</td>
<td>–CH₃–CH₂–</td>
<td>OH</td>
<td>CH(CH₃)R⁴</td>
</tr>
<tr>
<td>Abamectin</td>
<td>–CH=CH⁻</td>
<td>OH</td>
<td>CH(CH₃)R⁴</td>
</tr>
<tr>
<td>Eprinomectin</td>
<td>–CH=CH⁻</td>
<td>NHCH₃</td>
<td>CH(CH₃)R⁴</td>
</tr>
<tr>
<td>Emamectin</td>
<td>–CH=CH⁻</td>
<td>OH</td>
<td>C₂₁H₃</td>
</tr>
<tr>
<td>Doramectin</td>
<td>–CH=CH⁻</td>
<td>OH</td>
<td>C₂₁H₃</td>
</tr>
</tbody>
</table>

* Component B₁ₐ–R=C₂₁H₅, component B₁₉–R=CH₃.

was obtained from Pfizer (Louvain-la-Neuve, Belgium) and moxidectin was kindly supplied by Cyanamid (Gosport, Hampshire, UK). Ready-to-use HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4), Pioneer sensor chips F1, Amine Coupling Kit were obtained from Biacore AB (Uppsala, Sweden). Isolute C₈ SPE columns (500 mg/3 ml) were supplied by Jones Chromatography (Hengoed, UK). All other chemicals and reagents were purchased from Sigma (Poole, Dorset, UK).

Stock solutions of avermectins (1 mg ml⁻¹) were prepared in methanol and stored at −20 °C. Standard solutions in concentration range 0–10 µg ml⁻¹ were prepared in HBS-EP buffer and stored at 4 °C.

An anti-ivermectin monoclonal antibody against 5-O-succinoylivermectin-apo-transferrin conjugate (Crooks et al., 1998) was produced as described by Crooks et al. (2000). Ivermectin-oxime was synthesised according to the method described by Mitsui et al. (1996). A general scheme of the synthesis is presented in Fig. 1.

An optical biosensor BIACORE 1000 was obtained from Biacore AB (Uppsala, Sweden) and operated by BIACORE 1000 control software.

2.2. Immobilisation of ivermectin-oxime onto a sensor chip surface

A Pioneer sensor F1 chip was equilibrated to room temperature. N-hydroxysuccinimide (NHS) 0.05 M and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Amine Coupling Kit) were mixed in ratio 1:1. Of the mixture 50 µl was incubated on the chip surface for 30 min. The solution was removed then 50 µl of 1 M aqueous ethylenediamine (pH 8.5) added and incubated for 1 h. The chip was washed with HBS-EP buffer (3 × 50 µl). Free carboxyl groups were blocked with 50 µl 1 M ethanolamine hydrochloride (pH 8.5) for 20 min. The chip was washed with 50 µl of HBS-EP buffer. A solution of 5 mg EDC and 2 mg of NHS in 300 µl 10 mM sodium acetate (pH 4.5) was added dropwise to the solution of 2 mg of ivermectin-oxime in 300 µl dimethylformamide (DMF). An additional 300 µl DMF was then added to prevent sedimentation. The mixture (50 µl) was applied onto chip surface and incubated for 2 h. After incubation the solution was removed, the chip was washed with HBS buffer followed by 50 mM NaOH (6 × 50 µl) and dried under a stream of nitrogen. When not in use the chips were stored at 4 °C in plastic tubes containing silica gel desiccant.

2.3. Extraction of ivermectin from bovine liver

Liver samples were homogenised (Silverson, Chesham, UK) and weighed (2.0 ± 0.1) g into glass universals. Negative liver samples were spiked with 25 µl of the ivermectin solutions in HBS-EP buffer to provide 0;
10; 33; 100; 333; 1000 ng g\(^{-1}\) final concentrations. After 5 min incubation, 4 ml of acetonitrile was added to each sample and the universals shaken vigorously by hand for 10 s. The universals were mixed on a roller mixer (Denley Instruments Ltd, UK) for 15 min followed by centrifugation (International equipment company, Needham HTS, USA) at 3000 \(g\) for 10 min at 10 \(^\circ C\).

To 3 ml of supernatant was added 7 ml of distilled water and 20 ml triethanolamine. Gently vortex mixed solutions were applied onto C8 SPE columns (Isolute, 3 ml/500 mg) previously preconditioned by 3 ml of acetonitrile followed by 3 ml of acetonitrile–water solution (30:70, v/v) containing 0.1% triethanolamine. Ivermectin was eluted using 3 ml of acetonitrile. The eluates were evaporated to dryness under a stream of nitrogen on a dri-block sample concentrator (Techne Ltd, Cambridge, UK) at 60 \(^\circ C\).

2.4. Biosensor immunoassay procedure

A volume (175 \(\mu l\)) of each liver extract diluted 1/50 with HBS-EP buffer was mixed with 25 \(\mu l\) of anti-ivermectin monoclonal antibody diluted 1/700 with the same buffer. This mixture was injected over the chip surface at a flow rate of 25 \(\mu l\) min\(^{-1}\) for 2 min. Report points were taken on each sensogram produced before and after sample injection. Regeneration of the chip surface was achieved by injection of 15 \(\mu l\) 50 mM sodium hydroxide. A typical analytical cycle for each sample took about 5 min to complete. Each calibration point or sample was analysed in duplicate. BIACORE Evaluation 3.0 control software was used for data handling.

3. Results and discussion

3.1. The chip production and generation of ivermectin calibration curve

5-\(O\)-succinoylivermectin was used as the hapten in the immunogen to produce ivermectin specific monoclonal antibody. In order to obtain a heterologous biosensor assay format a second ivermectin derivative, ivermectin-oxime, was immobilised onto the surface of a sensor chip. A further reason for synthesising the oxime derivative was chemical stability of the 5-\(O\)-succinoylivermectin. This molecule contained an ester bond and was predicted to be unstable during the chemical regeneration of a chip surface when performed by either the injection of acid or alkali solutions.

The injection of an excess of antibody (1/10 dilution in HBS) at a flow rate of 10 \(\mu l\)/min for 5 min resulted in saturation curves with maximum responses in a range of 1800–2000 RU obtained. Chips were stored in HBS-EP buffer or dry with silica gel desiccant at 4 \(^\circ C\) over a period of 6 months. No appreciable difference was observed between these chips in terms of the surface stability and antibody binding.

Ivermectin is chemically unstable in solutions with high and low pH (Campbell, 1989). The acidic degradation results in hydrolysis of the two sugar rings to yield the monosaccharide and aglycone derivatives as the predominant products. In basic solution ivermectin can undergo isomerization at C-2 position. Previous experience with Biacore chips has shown that change of pH is the most convenient and effective way to regenerate chip surfaces (Bergstrom et al., 1999; Bjurling et al., 2000). Experiments were performed to determine the lowest concentration of alkali that would effectively regenerate the ivermectin surface without any detrimental effect on the chip stability. Sodium hydroxide solutions ranging from 180 to 25 mM were tested. It was found that a 50 mM solution of NaOH injection for 40 s could be used for regeneration of chips without significant loss of...
ivermectin activity on the chip surface. During the lifespan of a chip the baseline varied by about 40–50 RU (Fig. 2). There was evidence of a cyclical pattern corresponding to the concentration of drug present in injected sample. It was observed that between 80 and 100 injections per day resulted in loss of about 25% surface activity over a one week period (about 500 injections). Therefore a single ivermectin-oxime chip (4 lanes per chip) had the ability to be used for approximately 2000 injections.

The calibration curve with the highest sensitivity was obtained in a buffer system (ratio antibody: ivermectin standard solution 1:7; working antibody dilution 1/700). The curve had a midpoint of about 10 ng ml⁻¹ ivermectin and a working range of 1–100 ng ml⁻¹.

3.2. Assay optimisation

The influence of flow rate and injection time on assay sensitivity was investigated. These parameters were varied between 5–35 µl min⁻¹ and 1–6 min, respectively. Some examples of the dose–response curves obtained are given in Fig. 3. The lowest flow rate used (5 µl min⁻¹) across a spectrum of injection times resulted in the production of calibration curves with the highest background and lowest response (RU) at zero concentration (Fig. 3, curve 1). Calibration curves constructed from data obtained at higher flow rates (15, 25 and 35 µl min⁻¹) over a 2 min injection period improved the range (RU) of the curve, increased the maximum response and reduced the background. Longer injection times (up to 6 min) resulted in higher responses across the range of calibration points but did not improve the assay sensitivity. Increasing the dilution of antibody (up to 1/1200) used during the longer injection times did not result in lower detection limits (data not shown). The calibration curve produced using a flow rate of 15 µl min⁻¹ and injection time of 3 min was found to provide the highest sensitivity of the assay (Fig. 3, curve 2).

The temperature at which the assays are performed can be tightly controlled in the BIACORE system. The above experiments were performed at 25 °C. To determine the effect of temperature on the sensitivity of the assay calibration curves were constructed at 20, 25, 30 and 37 °C (Fig. 4). It was found that the higher the temperature the assay was performed the higher the absolute response was obtained (Fig. 4). However, increasing or decreasing the temperature did not alter the midpoint value gained significantly. IC50 for 20, 25, 30 and 37 °C were 9.4, 12.8, 11.8 and 14.4 ng ml⁻¹, respectively.

![Fig. 3. Influence of assay parameters on 'dose–response' curves for ivermectin in buffer. For curves 1–4 assay parameters-flow rate (µl min⁻¹), injection volume (µl), injection volume of regeneration solution (µl)-are 5–10–5, 15–45–10, 15–30–10 and 25–50–15 correspondingly. Antibodies dilution is 1/700 for all curves. Ratio antibody–standard solution is 1:7.

![Fig. 4. Influence of temperature on biosensor ivermectin assay in buffer. Temperature for curves 1–4, is 20, 25, 30 and 37 °C correspondingly. Assay parameters-flow rate 15 µl min⁻¹, injection time volume 45 µl, injection volume of regeneration solution-10 µl. Antibodies dilution is 1/700 for all curves. Ratio antibody–standard solution is 1:7.

Table 2

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>100</td>
</tr>
<tr>
<td>Abamectin</td>
<td>178</td>
</tr>
<tr>
<td>Eprinomectin</td>
<td>112</td>
</tr>
<tr>
<td>Emamectin</td>
<td>70</td>
</tr>
<tr>
<td>Doramectin</td>
<td>20</td>
</tr>
<tr>
<td>Moxidectin</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>

ᵃ Liver samples were extracted then spiked with compound.
ᵇ Liver samples were spiked with compound then extracted.
3.3. Cross-reactivity of the ivermectin monoclonal antibody in the biosensor assay

The cross-reactivity of the monoclonal antibody was tested against a range of avermectin molecules (Table 2). Ivermectin, eprinomectin and emamectin had very similar cross-reactivity profiles whereas with abamectin and doramectin substantive differences were observed (178 and 20%, respectively). The low values obtained for doramectin may be related to the presence of a cyclohexyl moiety at the C-25 position. Negligible cross-reactivity was found against moxidectin, one of the milbemycins which differ structurally with the avermectins mainly due to the absence of a disaccharide group at position C-13. These data show the requirement for a disaccharide group on the molecule to allow antibody recognition to take place.

3.4. Determination of ivermectin residues in bovine liver: optimisation of an extraction procedure

Methods used for ivermectin extraction from biological samples can be divided into two groups; solvent–solvent extraction or solvent extraction followed by SPE clean-up. The main disadvantage of solvent–solvent extraction relates to the use of several organic solvents in large quantities. A second problem with such procedures is that they tend to be very time consuming. A method previously developed for the determination of ivermectin in bovine liver by ELISA combined solvent–solvent extraction and SPE clean-up. This reduced the need for some solvent but remained rather lengthy to perform (Crooks et al., 1998). This procedure was used to prepare liver extracts for the sensor based assay. Satisfactory results were obtained but the length of the method warranted further investigations to decrease the time required for sample preparation.

The scientific literature shows that for SPE based clean-up methods acetonitrile is the most commonly used as extractant. The next stage requires ivermectin to be eluted from a column with either acetonitrile (Reuers et al., 1993; Nordlander and Johnsson, 1990; Abjean and Gaugain, 1995), chloroform (Scott and McKellar, 1992; De Montigny et al., 1990), methyl tertbuthyl ether (Dickinson, 1990; Dusi et al., 1997) or methanol (Chiou et al., 1987). In some of these methods triethylamine was added to the column preconditioning solution or to the sample itself.

In order to determine the extraction procedure most compatible with the present biosensor assay a range of different published and modified extraction methods were investigated (Table 3). For each method a four-point calibration curve was obtained. Each procedure was investigated by using different dilutions of liver extract to compare the level of matrix effect encountered. For many of the methods investigated low levels of ivermectin recovery were observed. The best results were obtained using the method developed by Nordlander and Johnsson (1990) for the analysis of drug in swine muscle. The method proved to be relatively simple and was successfully adapted by Kennedy et al. (1993) for ivermectin extraction from salmon tissues. The procedure involves extracting ivermectin into acetonitrile followed by SPE C8 clean-up. An important finding of Nordlander and Johnsson (1990) was that the addition of triethylamine to the preconditioning solution for the SPE column and to the sample reduced the irreversible absorption of ivermectin on the residual silanol groups on the C8 column material and led to an improved recovery.

Table 3
SPE based extraction procedures for ivermectin investigated for compatibility with biosensor immunoassay

<table>
<thead>
<tr>
<th>Method</th>
<th>Add to sample</th>
<th>Column and preconditioning</th>
<th>Wash</th>
<th>Eluent</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>Water, triethylamine</td>
<td>Isolute C8; acetonitrile, acetonitrile–water (30:70) with 0.1% triethylamine</td>
<td>Acetonitrile</td>
<td>Acetonitrile</td>
<td>Nordlander and Johnsson (1990)</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Water, triethylamine</td>
<td>Sep-Pak C18; acetonitrile, acetonitrile–water (30:70) with 0.1% triethylamine</td>
<td>Acetonitrile–water (30:70)</td>
<td>Acetonitrile</td>
<td>Abjean and Gaugain (1995)</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Water</td>
<td>Sep-Pak C18; acetonitrile, acetonitrile–water (30:70) with 0.1% triethylamine</td>
<td>Acetonitrile–water (30:70)</td>
<td>Acetonitrile</td>
<td>Reuers et al. (1993)</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Water</td>
<td>Sep-Pak C18; chloroform, acetonitrile, water (1:1)</td>
<td>Acetonitrile–water (1:2)</td>
<td>Chloroform</td>
<td>Scott and McKellar (1992)</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Water</td>
<td>Sep-Pak C18; methyl tertbuthyl ether, acetonitrile, acetonitrile–water (1:1)</td>
<td>Acetonitrile–water (1:1)</td>
<td>Methyl tertbuthyl ether</td>
<td>Dickinson (1990)</td>
</tr>
<tr>
<td>Methanol</td>
<td>Water</td>
<td>Sep-Pak C18; methanol, water</td>
<td>Acetonitrile–water (1:1)</td>
<td>Water</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Methanol</td>
<td>Water</td>
<td>Sep-Pak C18; methanol, water</td>
<td>Methanol–water</td>
<td>Methanol</td>
<td>Methanol</td>
</tr>
<tr>
<td>Methanol</td>
<td>Water</td>
<td>Bond-Elute C8; methanol, water</td>
<td>Methanol–water</td>
<td>Methanol</td>
<td>Methanol</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Water, triethylamine</td>
<td>Bond-Elute C8; acetonitrile, acetonitrile–water (30:70) with 0.1% triethylamine</td>
<td>Methanol–water</td>
<td>Acetonitrile</td>
<td>Chiou et al. (1987)</td>
</tr>
</tbody>
</table>
In the present study the method was optimised by including 0.2% triethylamine in the extracted sample. This had the result of reducing the sample matrix effect experienced. Studies with the resultant extract (data not included) showed that some matrix effect was still observed even when diluted (1/50) in buffer. Further effort to reduce this interference was performed by altering the extraction procedure and assay conditions (flow rate and injection times). The addition of a hexane wash to the sample extract prior to SPE achieved a reduction in matrix effect. However, a similar effect could be found by a reduction in the injection times from 3 to 2 min without affecting assay sensitivity. Benefit was also gained by using a flow rate of 25 µl min⁻¹ (Fig. 5). These modifications to the assay conditions aided the goal of keeping the sample extraction procedure as simple and fast as possible.

Specificity data obtained for liver extracts clearly indicated the matrix influence on cross-reactivity pattern (Table 2). Substantially reduced cross-reactivities were observed for emamectin and doramectin. Smaller reductions were also noted for abamectin and eprinomectin. Average recovery of ivermectin from spiked liver samples was found to be 82%. Further declines in apparent cross-reactivities were seen to occur when samples were spiked with compound and then extracted (Table 2). All compounds with the exception of doramectin showed reduced values of cross-reactivities. These decreases are more likely to be caused by reduced extraction efficiencies for these compounds than by changes in antibody binding affinities.

### 3.5. Assay validation

The assay developed was fully validated (Table 4). The detection limit of the assay (19.1 ng g⁻¹) was calculated as the concentration corresponding the average response mean for 20 negative bovine liver samples minus three times the standard deviation. Within run repeatability (CV, %) was determined to be between 18.3–21.3 and 13.5–16.4% for concentrations of 50 and 100 ng g⁻¹, respectively. The average value for liver samples spiked at 100 ng g⁻¹ (the MRL for the drug) and 50 ng g⁻¹ concentrations were 93.7 and 43.2 ng g⁻¹, respectively.

The method was not validated against the full spectrum of avermectins due to insufficient extraction efficiency recorded for many of these compounds. It is likely, however, that with some further modifications to the extraction procedure the assay could be used in a ‘multi-residue’ manner.

The data produced clearly shows that ivermectin, the most widely used avermectin, can be detected well below the MRL. Furthermore, the method developed has a quicker and simpler sample extraction procedure than published methods which allows results to be generated in a more timely manner, for instance the extraction and analysis of 20 liver samples could be performed within a single working day. Significant reductions in solvent usage compared to other published methods were also considered to be an advantage of the method developed.

### 4. Conclusions

During assay optimisation studies the effects of flow rate, injection time and assay temperature all had pronounced effects on sensitivity, robustness and sample extract interference. Ultimately a simple and straightforward extraction procedure for ivermectin residues from bovine liver, combined with a rapid biosensor based immunoassay, was developed. Such procedures are a welcome addition to laboratories with the function of performing routine control programmes within strict time limits.

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![Graph](image-url)  
**Fig. 5.** Influence of assay parameters on ivermectin biosensor assay for bovine liver. 1-dose-response curve for HBS buffer (ng x⁻¹ corresponds to ng ml⁻¹); 2-4-dose-response curves for bovine liver (ng x⁻¹ corresponds to ng g⁻¹). For curves 1-4 assay parameters-flow rate (µl min⁻¹), injection volume (µl), injection volume of regeneration solution (µl)-are 15–45–10, 15–45–10, 15–30–10 and 25–50–15 correspondingly. Liver extracts were diluted 1/50 with HBS-EP buffer. Antibodies dilution is 1/700 for all curves. Ratio antibody–standard solution is 1:7.

---

**Table 4**

<table>
<thead>
<tr>
<th>Level added (ng g⁻¹)</th>
<th>50 (ng g⁻¹) (0.5 MRL)</th>
<th>100 (ng g⁻¹) (MRL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level found (ng g⁻¹)</td>
<td><strong>Intra-assay</strong></td>
<td></td>
</tr>
<tr>
<td>(n = 20)</td>
<td>42.2–44.3</td>
<td>86.8–104.0</td>
</tr>
<tr>
<td>CV (%)</td>
<td>18.3–21.3</td>
<td>13.5–16.4</td>
</tr>
<tr>
<td>Level found (ng g⁻¹)</td>
<td><strong>Inter-assay</strong></td>
<td></td>
</tr>
<tr>
<td>(n = 3)</td>
<td>43.2</td>
<td>93.7</td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.4</td>
<td>9.7</td>
</tr>
</tbody>
</table>
Acknowledgements

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References