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Determination of ivermectin B_{1a} in animal plasma by liquid chromatography combined with electrospray ionization mass spectrometry

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A novel, sensitive and specific method for the quantitative determination of ivermectin B_{1a} in animal plasma using liquid chromatography combined with positive electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) is presented. Abamectin was used as the internal standard. Extraction of the samples was performed with a deproteinization step using acetonitrile. Chromatographic separation was achieved on a Nucleosil ODS 5 μm column, using gradient elution with 0.2% (v/v) acetic acid in water and 0.2% (v/v) acetic acid in acetonitrile. The method was validated according to the requirements defined by the European Community. Calibration curves using plasma fortified between 1 and 100 ng ml^{-1} showed a good linear correlation ($r \geq 0.9989$, goodness-of-fit coefficient $\leq 8.1\%$). The trueness at 2 and 25 ng ml^{-1} ($n = 6$) was +4.2 and −17.1%, respectively. The trueness and between-run precision for the analysis of quality control samples at 25 ng ml^{-1} was −4.0 and 11.0%, respectively ($n = 16$). The limit of quantification of the method was 1.0 ng ml^{-1}, for which the trueness and precision also fell within acceptable limits. Using a signal-to-noise ratio of 3 : 1, the limit of detection was calculated to be 0.2 ng ml^{-1}. The specificity was demonstrated with respect to ivermectin B_{1b}.

The method was successfully used for the quantitative determination of ivermectin B_{1a} in plasma samples from treated bovines, demonstrating the usefulness of the developed method for application in the field of pharmacokinetics. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: ivermectin; liquid chromatography/electrospray ionization tandem mass spectrometry; validation; plasma; quantification

INTRODUCTION

Ivermectin belongs to the macrocyclic lactone class of avermectins and consists of a mixture of two homologous compounds, 22,23-dihydroavermectin B_{1a} (H_2B_{1a}, not less than 80%) and 22,23-dihydroavermectin B_{1b} (H_2B_{1b}, not more than 20%). The B_{1a} component differs from B_{1b} by a single methylene group at the C-25 position of the molecule, i.e. B_{1a} contains a secondary butyl substituent and B_{1b} an isopropyl substituent (Fig. 1). Avermectins are potent anthelmintic, insecticidal and acaricidal compounds, which, by increasing the membrane permeability to chlorine ions, mediate the paralysis of nematodes and certain classes of ectoparasites.

Because of the high potency of ivermectin, efficacious doses given to cattle and sheep are 0.1 mg kg^{-1} and lower, resulting in the need for sensitive assays. The European Agency for the Evaluation of Medicinal Products (EMEA) has set maximum residue limits (MRLs) for food products of bovine, porcine, ovine, equidae species and deer (including reindeer).[1] The parent H_2B_{1a} component represents the major fraction of residue in all animal species. The marker residue in all species is therefore 22,23-dihydroavermectin B_{1a} and should be quantified in food monitoring programmes.

To evaluate the bioavailability and pharmacokinetics in different animal species, there is a need for a quantitative method capable of detecting only the marker H_2B_{1a} at concentrations in plasma as low as 1 ng ml^{-1}.

Several methods have been described for determining residues of macrocyclic lactones in plasma, tissues and crops. Both liquid chromatographic methods using UV detection and fluorescence detection have been reported. The evolution of the fluorogenic derivatization of ivermectin carried out with trifluoroacetic anhydride and 1-methylimidazole, which is most commonly used, was extensively reviewed by Fink *et al*.[2] The latest high-performance liquid chromatographic (HPLC) fluorescence assay reported resulted in a reaction time for the derivatization of less than 30 s and detection limits as low as 10 pg ml^{-1} in a 1.0 ml plasma sample.[3] However, De Montigny *et al*.[4] demonstrated that ivermectin degraded by 15% over a period of 18 h in the derivatization matrix. Unless one carries out derivatization just prior to injection by automation, there are uncertainties

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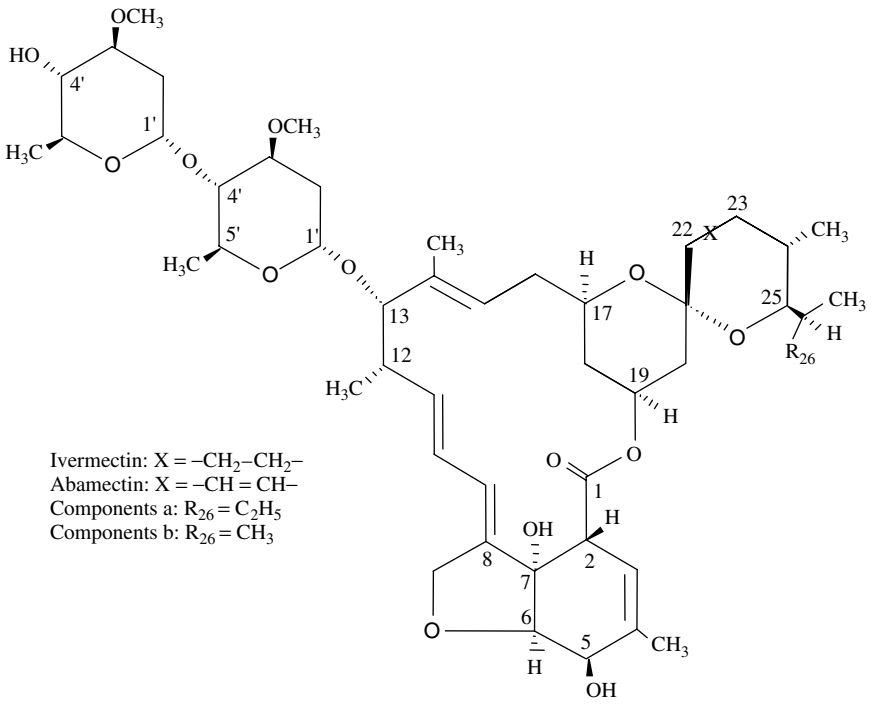

Figure 1. Structures of ivermectin and abamectin.

regarding degradation and/or relative rates of degradation of ivermectin and analogues during the period before injection. Therefore, we preferred to develop a method without the use of derivatization.

With the development of liquid chromatography/mass spectrometry (LC/MS), several groups of researchers have reported successful confirmations of avermectins using this technique. Previous LC/MS methods were developed to confirm avermectin residues in food matrices and included an MS method with negative ion chemical ionization for ivermectin in milk and bovine tissues,[5,6] a thermospray method for moxidectin in cattle fat,[7] an electrospray method for eprinomectin in bovine liver[8] and multi-residue methods in several food matrices,[9–14] including crops. However, none of the reported methods included the quantification of ivermectin in plasma.

Our objective in this work was to develop an electrospray ionization (ESI)-MS/MS method for ivermectin in animal plasma samples using a simple extraction procedure. The method presented was also validated according to EU requirements, in order to be suitable for regulatory purposes.

EXPERIMENTAL

Standards and chemicals

Ivermectin was a Chemical Reference Standard (CRS) from the European Pharmacopoeia (Strasbourg, France). Abamectin, used as the internal standard (IS), was a reference standard in a glycerol formal solution, donated by Merck (New York, USA). It was stored at 2–8 °C. The chemical structure of ivermectin and abamectin is presented in Fig. 1. Stock solutions of 1 mg ml^{-1} ivermectin B_{1a} and abamectin B_{1a} in acetonitrile were prepared and stored at ≤−15 °C. Working solutions of ivermectin B_{1a} at 1000, 750, 500, 250, 100, 50, 20 and 10 ng ml^{-1} were prepared by appropriate dilution of the stock solution with acetonitrile. The IS working solution concentration was 0.5 μg ml^{-1}. All working solutions were stored in a refrigerator (2–8 °C).

Glacial acetic acid used for the mobile phase was of analytical grade (Merck, Darmstadt, Germany). Water and acetonitrile were of HPLC grade and were purchased from Acros (Geel, Belgium).

Biological samples

Known ivermectin-free plasma samples were obtained from calves which had not received any ivermectin. Incurred plasma samples were obtained from calves which had been treated subcutaneously with an ivermectin formulation at a therapeutic dose. Blood was taken until 35 days after treatment. Plasma was removed by centrifugation and stored at ≤−15 °C until analysis.

Plasma extraction and chromatography

The method of standardization was based on the use of an internal standard, abamectin, which was added to the homogenized plasma samples just before analysis. All validation samples were prepared in drug-free plasma. Quality control (QC) samples, a blank sample and a calibration curve were analyzed together with each batch of incurred samples to check the extraction and LC/MS/MS procedure. QC samples were prepared from drug-free calf plasma spiked at a concentration of 25 ng ml^{-1} ivermectin B_{1a}.

A 500 μl volume of plasma was transferred into a capped 1.5 ml microcentrifuge tube and spiked with 50 μl of the IS working solution of 0.5 μg ml^{-1}. After vortex mixing for 15 s, 450 μl of acetonitrile were added. The sample was again vortex mixed for 15 s. After centrifugation at 10 800 *g* at room temperature for 10 min, the supernatant was transferred into

an autosampler vial and a 100 µl aliquot was injected into the LC/MS instrument.

The HPLC system consisted of an Alliance Type 2690 separation pump and a column heater module, both from Waters (Milford, MA, USA). Chromatography was performed on a Nucleosil C_{18} column (100 × 3 mm i.d., d_p 5 µm) in combination with a guard column of the same type (10 × 2 mm i.d.) from Chrompack (Middelburg, The Netherlands). The mobile phase solvent A was a solution of 0.2% (v/v) glacial acetic acid in water and solvent B was 0.2% (v/v) glacial acetic acid in acetonitrile. The flow-rate was 0.6 ml min^{-1}. Gradient elution was performed to elute ivermectin and the IS and some late-eluting endogenous plasma compounds from the column (0 min, 20% A–80% B; 1.5–5.5 min, 100% B; 6–10 min, 20% A–80% B).

Mass spectrometry

The HPLC column effluent was split before it was pumped to the MS instrument, at such a rate that about one-third of the volume was sent to the detector. A Quattro Ultima triple-quadrupole instrument (Micromass, Manchester, UK) was used, equipped with an ESI z-spray source, which was operated in the positive ion MS/MS mode. The instrument was first calibrated with a solution of sodium iodide according to the manufacturer's instructions. Thereafter, tuning was performed for both ivermectin and the IS by direct infusion of a 10 µg ml^{-1} solution. The following tune parameters were used for both analytes: capillary, 3.5 kV; cone, 70 V; source temperature, 120 °C; desolvation temperature, 250 °C; cone gas flow-rate, ~30 l h^{-1}; desolvation gas flow-rate, ~500 l h^{-1}; resolution (LM1, HM1, LM2, HM2), 14.0; ion energy 1 and 2, 1.0; entrance and exit, 5.0; multiplier, 650 V; and Pirani pressure of the argon gas used for collision, 2.49×10^{-3} mbar. The optimum collision energy in the MS/MS mode for the protonated sodium adduct of the molecular ion of both ivermectin and abamectin (m/z 897.6 and 895.5, respectively) was 40 eV.

For our quantitative purposes, the instrument was operated in the multiple reaction monitoring (MRM) mode, using the product ions at m/z 753.5 and 751.5 for ivermectin and abamectin, respectively. The MRM transition m/z 895.5 → 751.5 for abamectin was monitored from 0 to 3.6 min, whereas the transition m/z 897.6 → 753.5 for ivermectin B_{1a} was only monitored from 3.4 to 10 min.

Method validation and criteria

The proposed method for the quantitative determination of ivermectin in animal plasma was validated by a set of parameters which are in compliance with the recommendations as defined by the European Community.[15–18]

The linearity of the method was evaluated using fortified blank plasma samples. The addition of 50 µl of the above-mentioned standard working solutions resulted in calibration curves with ivermectin B_{1a} concentrations of 1, 2, 5, 10, 25, 50, 75 and 100 ng ml^{-1} plasma. Peak area ratios between ivermectin B_{1a} and the IS were plotted against their concentration ratios and a linear regression was performed. The acceptance criterion for the correlation coefficient (r) was $r \geq 0.99$ and for the goodness-of-fit coefficient (g)[18] was $g \leq 10\%$.

The trueness and within-day precision (repeatability) was determined by analyzing, on the same day, blank plasma samples fortified at 2 ($n = 6$) and 25 ($n = 6$) ng ml^{-1}. The between-day precision or reproducibility was determined using blank plasma samples fortified at 25 ng ml^{-1} ($n = 6$), but analyzed on different days. The maximum allowable tolerances for the within-day imprecision (RSD_{max}) are two-thirds of the values calculated according to the Horwitz equation ($RSD = 2^{(1-0.5 \log C)}$, where C is the concentration at which plasma is fortified[15–17]). For the between-day imprecision, the maximum tolerances are equal to the values of the Horwitz equation.

The trueness was evaluated in the same experiment as the precision by comparing the mean measured concentration with the fortified concentration of the six plasma samples fortified at 2 and 25 ng ml^{-1}. The trueness should be in the range −50 to +20% for levels ≤1 ng ml^{-1}, −30 to +10% for levels >1–10 ng ml^{-1} and −20 to +10% for levels >10 ng ml^{-1}.[15–17]

The limit of quantification (LOQ) was determined by analyzing six blank plasma samples fortified at a concentration of 1 ng ml^{-1}. The trueness and precision at that level should fall within the recommended ranges. The LOQ was also established as the lowest point of the calibration curve.

The limit of detection (LOD) was defined as the lowest concentration of ivermectin B_{1a} that could be recognized by the detector with a signal-to-noise (S/N) ratio of ≥3. The LOD was calculated using plasma samples spiked at 1 ng ml^{-1}.

The specificity of the method was investigated by analyzing blank plasma samples to exclude possible interference of endogenous plasma substances. Also, the specificity with respect to ivermectin B_{1b} was demonstrated.

RESULTS AND DISCUSSION

Plasma extraction and chromatography

None of the published LC/MS methods mention a procedure for the extraction of plasma samples. Because of the selective MS detector, the samples often need only very little preparation, if care is taken that the mobile phase is diverted to waste before the compounds of interest elute from the column. Also, one should take care that the HPLC column is rinsed sufficiently between sample injections to lengthen its lifetime. By applying a deproteinization with acetonitrile, a very rapid sample preparation procedure was established.

Ivermectin gave a good electrospray response in the positive ion mode when using the mobile phase suggested by Yoshii *et al*.[14] This was a combination of 0.2% (v/v) acetic acid in water (solvent A) and 0.2% (v/v) acetic acid in acetonitrile (solvent B). Gradient elution was performed to obtain good quality chromatograms for both ivermectin and the IS. Indeed, when applying isocratic elution with 20% A–80% B, retention times were 9.0 and 15.6 min for the IS and ivermectin, respectively. However, on increasing the level of organic phase B to 100% immediately after the start of the run, both compounds eluted as a sharp

peak with an improved S/N ratio at 8.5 and 11.9 min, respectively. This had the simultaneous advantage that the column was well rinsed between sample injections. These retention times were recorded at a flow-rate of 0.2 ml min^{-1}. However, our aim was to develop a rapid extraction and chromatographic procedure. Therefore, the flow-rate was raised to 0.6 ml min^{-1} in order to obtain retention times of only 3.0 and 3.9 min and to shorten the total chromatographic run time. However, in the ESI mode, this involved the use of a flow splitter operating at a rate about one-third of the eluent to be sent to the detector. This offered the additional advantage in ESI that the noise was lowered, resulting in an improved S/N ratio. Indeed, the LOQ of the method before the use of a flow splitter was 5 ng ml^{-1}, whereas with splitter the LOQ was determined as 1 ng ml^{-1} plasma.

Mass spectrometry

In the ESI positive ion MS mode, the $[M + Na]^+$ ions were mainly produced, which has also been described by other researchers. The negative ion mode was also tested, but neither ivermectin nor IS could be visualized as the molecular ion. Since electrospray is a 'soft' ionization technique that induces little initial fragmentation, we switched to tandem mass spectrometry (MS/MS).

Figures 2 and 3 show the full-scan tandem mass spectra of ivermectin and the IS after direct infusion of a standard solution, obtained in the positive ion mode and using the tune parameters mentioned above. Interestingly, a high cone voltage and collision energy were needed to obtain an acceptable response of the main product ion $[M - 144 + Na]^+$. A cone voltage of 70 V was superior to a value of 30 V. The collision energy was varied from 10 to 60 eV to find the maximum response of this product ion. A minimum of 20 eV was necessary to induce fragmentation, while the molecular ion was still present. Values higher than 55 eV resulted in the loss of typical structure-related ions. Therefore, a cone voltage of 70 V and a collision energy of 40 eV were chosen to obtain the maximum response of the product ion $[M - 144 + Na]^+$.

The fragmentations observed in the product ion spectra of $[M + Na]^+$ ions of ivermectin and the IS matched those reported by Gianelli *et al*.[19] ESI fragmentations produce ions A and D, due to the loss of one or two monosaccharide residues, respectively (Figs 2 and 3). This corresponds to the ion A = $[M - 144 + Na]^+$ at m/z 753.3 and ion D = $[M - 2 \times 144 + Na]^+$ at m/z 609.4 for ivermectin. For abamectin, the ions A and D are at m/z 751.4 and 607.4, respectively. Since the $[M - 144 + Na]^+$ ion was the most abundant product ion present, it was chosen for quantitation of both compounds.

In ESI-MS/MS, some other pathways produce ions which have a low abundance but which are important for structure-related data or unambiguous identification of the compounds. Gianelli *et al*.[19] mentioned that ion D generates a fragment ion F via one of two possible McLafferty rearrangements involved at C-1 and C-19, followed by an allylic cleavage between C-12 and C-13 (Fig. 4). This fragment corresponds to m/z 327 for avermectin B_{1a} and to m/z 329 for ivermectin B_{1a}, which are also seen in the tandem mass spectra (Figs 2 and 3). The same authors found that for the ESI-MS/MS process, after the McLafferty rearrangement at C-1 and C-19, the double bond was formed in two different positions, in C-19–C-20 or C-18–C-19. These provide two different fragmentation pathways and two ions (ion C and B), shown in Fig. 4. Ion E is produced after further allylic cleavage between C-12 and C-13 of ion B (Fig. 4). These three ions C, B and E at m/z 641, 665 and 449 are seen in the tandem mass spectrum of ivermectin and in that of abamectin also, since these fragments result from the loss of that part of the molecule that differ from each other. All these product ions are shown in Figs 2 and 3.

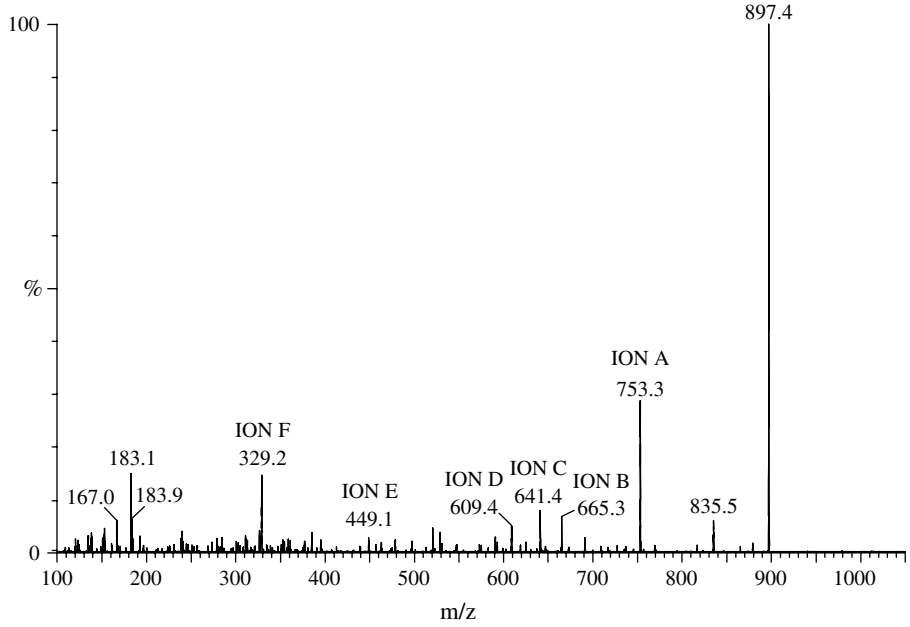

Figure 2. ESI tandem mass spectrum of ivermectin B_{1a}, obtained after direct infusion of a standard solution of 10 μg ml^{-1} (positive ion mode, collision energy in MS/MS = 40 eV).

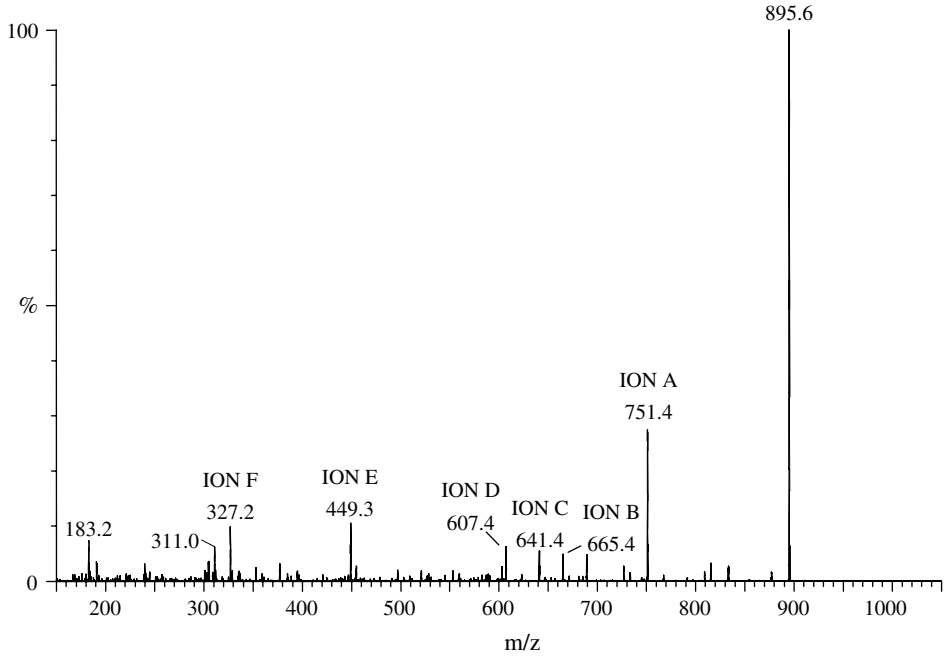

Figure 3. ESI tandem mass spectrum of abamectin (IS), obtained after direct infusion of a standard solution of 10 μg ml^{-1} (positive ion mode, collision energy in MS/MS = 40 eV).

Method validation

The results of the method validation are summarized in Table 1.

Linearity

The correlation coefficient (r) and the goodness-of-fit coefficient (g) for the concentration range 0–100 ng ml^{-1} plasma were >0.99 and <10%, respectively.

Trueness and precision

The within-day RSD values (n = 6) were 25.8 and 12.9% for 2 and 25 ng ml^{-1} and were below the RSD_{max} values of 27.2 and 18.6%, respectively. The between-day precision was evaluated using quality control samples spiked at 25 ng ml^{-1} (n = 16). The RSD was 11.0% and was below the RSD_{max} value of 27.9%.

The trueness was in all cases within the accepted ranges of −30 to +10% and −20 to +10% for 2 and 25 ng ml^{-1}, respectively.

Limit of quantification

The LOQ was established as the lowest point of the calibration curve, i.e. 1 ng ml^{-1}, and was also tested for the precision and trueness. Table 1 shows that both parameters were within the recommended ranges. In addition, a mean S/N ratio of 17.2 (n = 6) was determined for plasma fortified at 1 ng ml^{-1}. These S/N ratios were still above 10, which is

Table 1. Validation results for the determination of ivermectin B_{1a} in calf plasma by LC/ESI-MS/MS

	Concentration	Trueness (%)	Precision (RSD, %)
Calibration curve r = 0.9989 g = 8.1%	0 → 100 ng ml^{-1}	within −50 to +20%, depending on the concentration	
Trueness and precision:			
within-run	2 ng ml^{-1}	+4.2	25.8
within-run	25 ng ml^{-1}	−17.1	12.9
between-run	25 ng ml^{-1}	−4.0	11.0
Limit of quantification	1 ng ml^{-1}	+13.3	14.4
Limit of detection	0.2 ng ml^{-1}		
Specificity	no interference of endogenous compounds no interference of analogous compounds (ivermectin B_{1b})		

g = goodness-of-fit coefficient, which has to be ≤10% (Knecht *et al.*).[18]
RSD_{max} repeatability: 1 ng ml^{-1}: 30.2%, 2 ng ml^{-1}: 27.2%, 25 ng ml^{-1}: 18.6%; RSD_{max} reproducibility: 25 ng ml^{-1}: 27.9%.

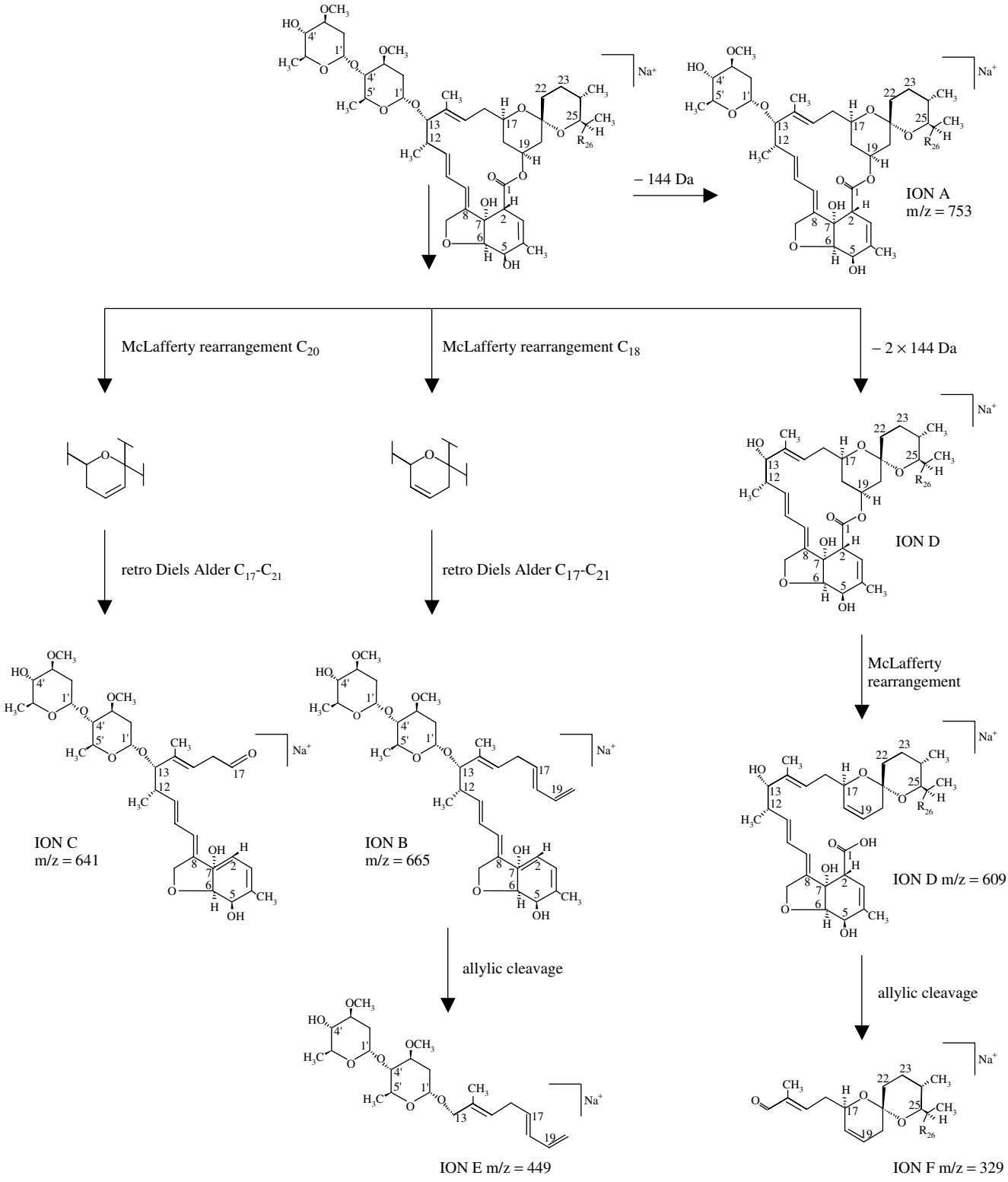

Figure 4. Proposed fragmentation pattern of ivermectin B_{1a} in the positive ionization MS/MS mode (Gianelli *et al*.[19]).

generally accepted as the minimum S/N ratio of the LOQ in the literature.

Limit of detection

From the S/N ratio of 17.2 for plasma fortified at 1 ng ml^{-1}, the concentration corresponding to an S/N ratio of 3 : 1 was 0.2 ng ml^{-1}.

Specificity

The described method proved to be specific with respect to the interference of endogenous compounds with the same retention time as ivermectin and the IS, as can be seen in the ion chromatogram of a blank plasma sample (Fig. 5(A)). The specificity with respect to ivermectin B_{1b} was also proved since ivermectin B_{1b} was only detected with the

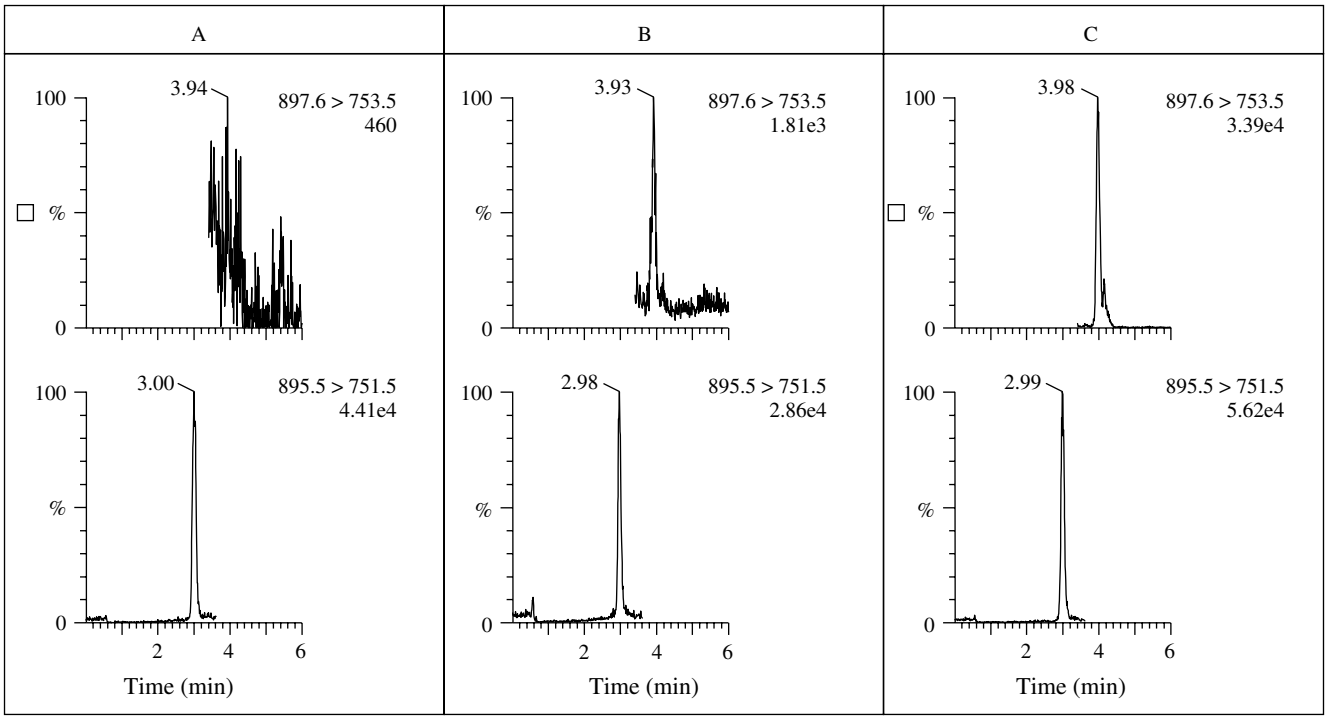

Figure 5. LC/ESI-MS/MS ion chromatogram of (A) a plasma sample taken before the treatment started, (B) a sample fortified at 1 ng ml^{-1} and (C) a sample of the same calf as presented in (A), but taken after treatment with a commercial formulation (representing 17.9 ng ml^{-1} of ivermectin B_{1a}). The upper trace represents ivermectin B_{1a} and the lower trace the IS.

MRM transition of m/z 883.8 → 739.7 at a retention time of 3.5 min and not with the transition m/z 897.6 → 753.5 of ivermectin B_{1a}.

Analysis of biological samples

To evaluate the applicability of the proposed method, plasma samples from calves which had been treated subcutaneously with ivermectin at a therapeutic concentration were analyzed. Quantification was necessary since the results were used for a pharmacokinetic study. A representative ion chromatogram of a blank plasma sample (A), a sample fortified at 1 ng ml^{-1} (B) and a sample of a calf that was treated with a commercial formulation (C) is presented in Fig. 5. A total of 200 samples were analyzed on the same HPLC column, and the guard column was replaced once during the study, demonstrating the practicability and applicability of the method.

Interestingly, the ion chromatograms of samples from treated calves and fortified samples showed the presence of an 'extra' peak that was tailing on the ivermectin B_{1a} peak (see Fig. 5(C)). This was seen to a larger extent for the samples from the treated calves than for the fortified samples. The origin is not well understood, but for the quantification the total peak area was taken into account. It is well known that UV radiation below 280 nm rapidly isomerizes the *E* (*trans*) 8,9 and 10,11 double bonds of avermectins to the 8,9- and 10,11-*Z* isomers.[20] These isomers have the same molecular mass, which can explain their detection with the MRM transition that we applied. Further investigation with analytical standards of the 8,9- and 10,11-*Z* isomers should be performed to elucidate this phenomenon.

CONCLUSIONS

The method proposed is, to our knowledge, the first quantitative and validated method available in the literature for the determination of ivermectin B_{1a} in animal plasma by LC/ESI-MS/MS. It has the advantages first that it includes a very rapid sample preparation and chromatographic procedure, which promotes the applicability and practicability of the method, and second it is a very sensitive method using only 500 µl of sample. We were able to quantify ivermectin B_{1a} at levels as low as 1 ng ml^{-1}. The LOD was calculated as 0.2 ng ml^{-1}, which is in the same range as in reported HPLC/fluorescence methods.

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