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High-performance liquid chromatographic assay for the determination of a semisynthetic avermectin analog (eprinomectin) in bovine milk at parts per billion levels—method development and validation

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Abstract

A sensitive and automated method has been developed and validated to determine marker residue eprinomectin B_{1a} in bovine milk. Extraction of eprinomectin B_{1a} from milk is accomplished with acetonitrile after the addition of an internal standard. The extract containing the analytes is evaporated to dryness and reconstituted in a solution containing 30% 1-*N*-methylimidazole in acetonitrile. Online derivatization is carried out with trifluoroacetic anhydride. Determination of eprinomectin B_{1a} and its internal standard is carried out by HPLC using a reversed-phase C_{18} column with a mobile phase consisting of methanol, acetonitrile, water, triethylamine and phosphoric acid. The overall extraction recovery of eprinomectin B_{1a} is 94% with milk supplemented between 2 and 50 ng/ml eprinomectin B_{1a}. Precision RSD averaged 3.0% in Laboratory 1 (n=25) compared to 4.3% in Laboratory 2 (n=35). The limit of quantitation is approximately 2 ng/ml eprinomectin B_{1a}, the limit of detection is approximately 0.25 ng/ml using this method.

Keywords: Avermectin analogue; Eprinomectin

1. Introduction

IVOMEC EPRINEX® Pour-On[1] for beef and dairy cattle has recently been approved as a topical endectocide. The active ingredient is eprinomectin, a mixture of two analog compounds also known as 4″-epiacetylamino-4″-deoxyavermectin (B_{1a}≥90% (Fig. 1) and B_{1b}≤10%). Its precursor (avermectin= AVM) is obtained by fermentation from *Streptomyces avermitilis.* AVM is chemically modified to obtain eprinomectin according to Mrozik et al. [1]. There are a number of reports on the analysis of AVMs in milk and other matrices using different extraction and determination methods. Schenck and Lagman [2] used a series of liquid to liquid partitions and derivatization before fluorescence detection with

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[1]IVOMEC EPRINEX® Pour-on/EPRINEX® Pour-on, Merial Limited, a Merck and Aventis Company.

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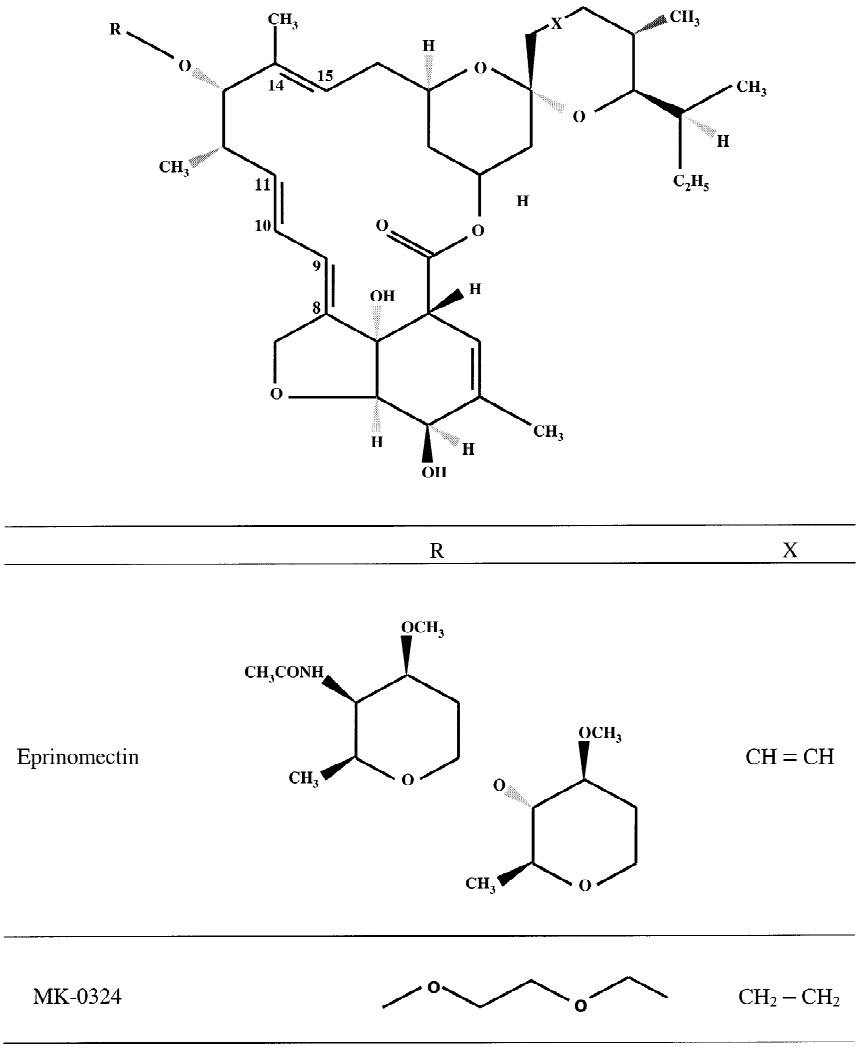

Fig. 1. Eprinomectin B_{1a} and MK-0324 B_{1a} structure.

recoveries >80% for abamectin (ABA), doramectin (DOR), ivermectin (IVM) and moxidectin (MOX). Heller and Schenck [3] applied particle beam liquid chromatography–mass spectrometry (LC–MS) using negative ion chemical ionization for the analysis of IVM residue in bovine milk and liver. Samples were prepared by liquid/liquid extraction followed by alumina B solid-phase extraction (SPE) clean-up but still gave co-eluting matrix compounds that enhanced the response and altered the abundance pattern of IVM. Turnipseed et al. [4] developed a LC–MS method to confirm AVM drug residues in several food matrices. IVM, DOR, eprinomectin and MOX were confirmed using atmospheric pressure chemical ionization (APCI) with negative ion detection and selected ion monitoring of three to four ions for each compound. Milk samples were prepared by liquid/liquid extraction followed by double SPE clean-up. Residues of DOR, IVM, and eprinomectin were confirmed in fortified milk at the 20 ng/ml level. Ali et al. simultaneously determined eprinomectin, MOX, ABA, DOR, and IVM in beef liver by LC

with fluorescence detection [5] and successfully confirmed this method at ≥25 ng/g levels [6]. Sutra et al. [7] published a report of successful validation of a high-performance liquid chromatography method with automated solid-phase extraction and fluorescence detection that was used to study the pharmacokinetics in plasma and milk in dairy cattle topically treated with eprinomectin [8].

There was a need to determine eprinomectin in milk in support of residue depletion studies of the drug in incurred samples. A simple and sensitive analytical method was required. A review of methods used to determine avermectin and ivermectin (hydrogenated avermectin) in biological samples revealed that the addition of acetic anhydride in pyridine promoted the formation of a fluorescent derivative [9]. Recent improvements led to the use by Tway et al. [10] of the more efficient nucleophile 1-*N*-methylimidazole (1-*N*-MIM) and of acylation reagent trifluoracetic anhydride (TFAA) for acetic anhydride by DeMontigny et al. [11]. Payne et al. [12] developed and validated a method to determine eprinomectin in bovine tissue and Antonian et al. [13] proposed an automated method for the determination of eprinomectin in bovine plasma. The latter methods used pre-column derivatization of eprinomectin with a fluorogenic reagent system which included 1-*N*-MIM and TFAA.

This paper describes the development and validation of a new analytical method to determine eprinomectin B_{1a} in bovine milk by HPLC with on-line derivatization to obtain the fluorescent derivative of eprinomectin B_{1a}.

2. Experimental

2.1. Participating laboratories

The development and validation of the method were accomplished in two laboratories. Laboratory 1 carried out the method development and was based in Merck Research Laboratories, West Point, PA, USA. Laboratory 2 fully validated the method and was based in Merial GmbH, Kathrinenhof Research Center, Rohrdorf, Germany. The following experimental sections refer to the method validation unless stated otherwise.

2.2. Avermectins

Eprinomectin and MK-324 (internal standard= I.S., Fig. 1) were obtained from Merck Research Laboratories (Rahway, NJ, USA). Analytical standards of eprinomectin and MK-324 were 95.3% (90.4% B_{1a} and 4.9% B_{1b}) and 89.5% (85.7% B_{1a} and 3.8% B_{1b}) pure, respectively.

2.3. Chemicals

Acetonitrile (ACN), methanol and water of HPLC-grade and *o*-phosphoric acid (85%), triethylamine (TEA) were obtained from Merck (Darmstadt, Germany); 1-*N*-MIM (99%+) and TFAA (99%+) were obtained from Sigma Aldrich (Taufkirchen, Germany).

2.4. Instrumentation

The HPLC system consisted of Hitachi instruments. A L-6200 intelligent pump, a AS-4000 programmable auto sampler, a T-6300 column thermostat, a F-1080 fluorescence detector and a D-2500 chromatointegrator were used. A Zorbax RX-C_{18} analytical column (25 cm×4.6 mm I.D., particle size 5 μm) was used. For sample preparation, an IKA vortex mixer, a Heraeus Labofuge A centrifuge and a aluminum heat block were used. The mobile phase was degassed with a Branson sonicator.

2.5. Chromatographic conditions

The composition of the isocratic mobile phase was methanol, ACN, water, TEA and *o*-phosphoric acid (25:68:7:0.2:0.2, v/v), premixed and degassed for 20 min by sonication. The flow-rate was 1.0 ml/min. The detector settings were at an excitation wavelength of 365 nm and an emission wavelength of 475 nm. The column was maintained at 35 °C.

2.6. Preparation of standards

Stock solutions of eprinomectin B_{1a} and I.S. were prepared at 0.45 and 0.26 mg/ml, respectively. Both stocks and all subsequent dilutions were made using ACN. The working solutions for eprinomectin B_{1a} were prepared at 180 and 9.2 ng/ml and the I.S. was

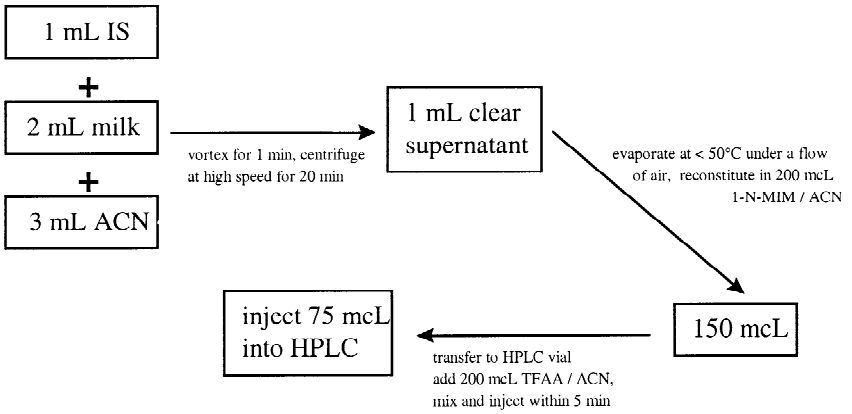

Fig. 2. Flow chart of sample preparation procedure.

prepared at 43 ng/ml. Eprinomectin B_{1a} working solutions were used to spike milk samples at 2.3, 4.6, 9.2, 23 and 46 ng/ml.

2.7. Sample preparation

A 2-ml milk sample and 1 ml of working standard solution of I.S. were combined and the sample was extracted with 3 ml of ACN and vortexed vigorously for 1 min. The sample was centrifuged for 20 min; 1 ml of the clear supernatant was transferred to a new tube and evaporated to complete dryness at <50 °C under a flow of air. The residue was reconstituted in 200 μl of 30% 1-*N*-MIM in ACN. A known amount of the sample (150 μl) was transferred to a vial (Fig. 2).

2.8. Reactions

Derivatization was accomplished with the on-line addition of base catalyst 1-*N*-MIM and acylation reagent TFAA. Eprinomectin contains a tertiary hydroxyl group at C7 and a secondary hydroxyl group at C5. When eprinomectin reacts with TFAA in the presence of 1-*N*-MIM, these hydroxyl groups are thought to be acylated with subsequent deacetylation of the intermediate. The fluorescent derivatization product contains an aromatic ring which is conjugated with the butadiene system in the macrocyclic ring. The fluorescence excitation and emission maxima for the derivative were at 365 and 475 nm, respectively. Eprinomectin and I.S. have the same fluorescence characteristics (Fig. 3). The autoinjector system was programmed to automatically complete

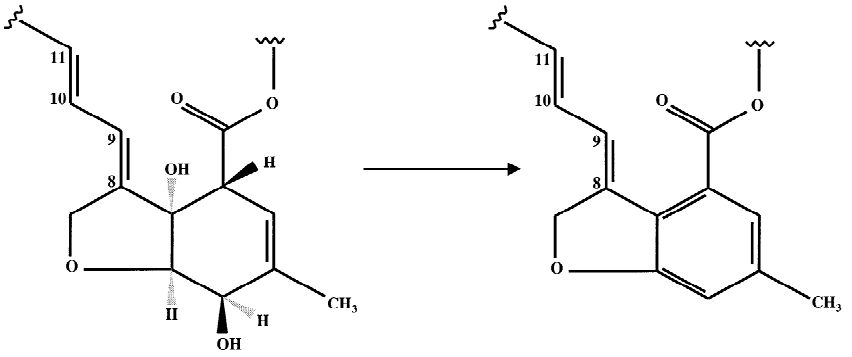

Fig. 3. Fluorescent derivatives of MK-0324 (I.S.) and eprinomectin.

the derivatization reaction by adding 200 μl 30% TFAA in ACN to the sample vial. Derivatization of eprinomectin was complete in less than 30 s at room temperature. Seventy-five microliters of the mixture were injected onto the HPLC system within no more than 5 min of the addition of the TFAA solution.

2.9. Calculations

All results were expressed in ng/ml. Peak height ratio (PHR) measurements were obtained by determining the peak heights of eprinomectin B_{1a} and the internal standard B_{1a} peak in the sample for comparison with the average of at least four (4) standards injections (PHR standard). The residue marker concentration (eprinomectin B_{1a}) in the sample was determined using the following equation:

$$\text{eprinomectin } B_{1a}\left(\frac{\text{ng}}{\text{ml}}\right) = \frac{\text{PHR sample}}{\text{PHR standard}} \times C_{\text{std}}\left(\frac{\text{ng}}{\text{ml}}\right)$$

where PHR = peak height ratio of eprinomectin B_{1a}/MK-324 B_{1a} and C_{std} = concentration of eprinomectin B_{1a} standard.

2.10. Notes on procedure

Injection of the samples must be made at a predetermined reaction time for all samples following mixing (within 5 min of reagent addition). If all system suitability criteria are met, two standard solutions should be injected followed by the injection of approximately 10 sample extracts and then injections of both standard solutions. Repeat the injections of two more standard solutions after the injection of approximately 10 more sample extracts as necessary. The standard solutions and sample extracts are stable for at least 24 h.

2.11. Precision, linearity, accuracy, sensitivity, specificity and HPLC system suitability

Method parameters were assessed in both laboratories. The precision of the method was assessed with at least five milk samples at five different concentrations ranging between 2 and 50 ng/ml used to construct the calibration curve. Linearity was confirmed by regression analysis of the average peak height ratio versus the concentration of eprinomectin B_{1a}. The accuracy of the method was established by calculating sample concentrations (mean observed concentration/spiked concentration × 100%). The limit of quantification (LOQ) was defined as the lowest concentration that could be assayed with acceptable accuracy (−30 to +10% of the nominal values) and precision (calculated as the relative standard deviation, %RSD ≤ 10%). Assay specificity was determined by injection of blank control and pre-dose milk samples from different cattle. The suitability of the HPLC system was verified with the injection of five replicate standard solutions. The acceptance criteria required that the %RSD for the peak height ratio measurements was less than 3% and that the resolution between B_{1a} and B_{1b} was greater than 1.2.

3. Results

The method to determine eprinomectin B_{1a} and its internal standard MK-324 in milk was developed and validated with the analysis of drug-free milk samples supplemented with 2.3–51 ng/ml eprinomectin B_{1a}. Using this method in Laboratories 1 and 2, the overall mean extraction recovery of eprinomectin B_{1a} was 94.4% (range: 77.1–105%, $n = 60$, see Table 1) with a precision RSD ranging from 1.9 to 6.2%. The correlation coefficient (r) of the mean peak height ratio measurements obtained from samples with 2.3–51 ng/ml eprinomectin B_{1a} was 0.999. The typical equation describing the standard line was $Y = 2.89e-2\ X + 9.59e-3$ (Laboratory 1: $Y = 2.89e-2\ X + 2.24e-3$, $r = 1.000$; Laboratory 2: $Y = 2.89e-2\ X + 1.96e-2$, $r = 0.997$). Chromatograms are presented in Fig. 4a (drug-free milk), Fig. 4b (derivatized eprinomectin standard) and Fig. 4c (milk sample with 23 ng/ml eprinomectin B_{1a}). The internal standard eluted at ~18 min and eprinomectin B_{1a} at ~8 min. The LOQ for this assay was approximately 2 ng/ml and the limit of detection (LOD) for this assay was approximately 0.25 ng/ml (based on a signal-to-noise ratio of approximately 3:1). No interference was noted at the retention times of eprinomectin B_{1a} and the internal standard.

Table 1
Summary of eprinomectin B_{1a} method recoveries

Fortification level (ng/ml)	Mean recovery (%)	% RSD (*n*)	Source
2.5	100	3.2 (5)	Laboratory 1
5.1	95	2.2 (5)	
10	97	4.0 (5)	
25	95	2.4 (5)	
51	99	3.1 (5)	
Overall	97	≤4.0 (25)	
2.3	96	6.2 (7)	Laboratory 2
4.6	100	5.6 (7)	
9.2	92	2.1 (7)	
23	90	1.9 (7)	
46	84	5.9 (7)	
Overall	92	≤6.2 (35)	

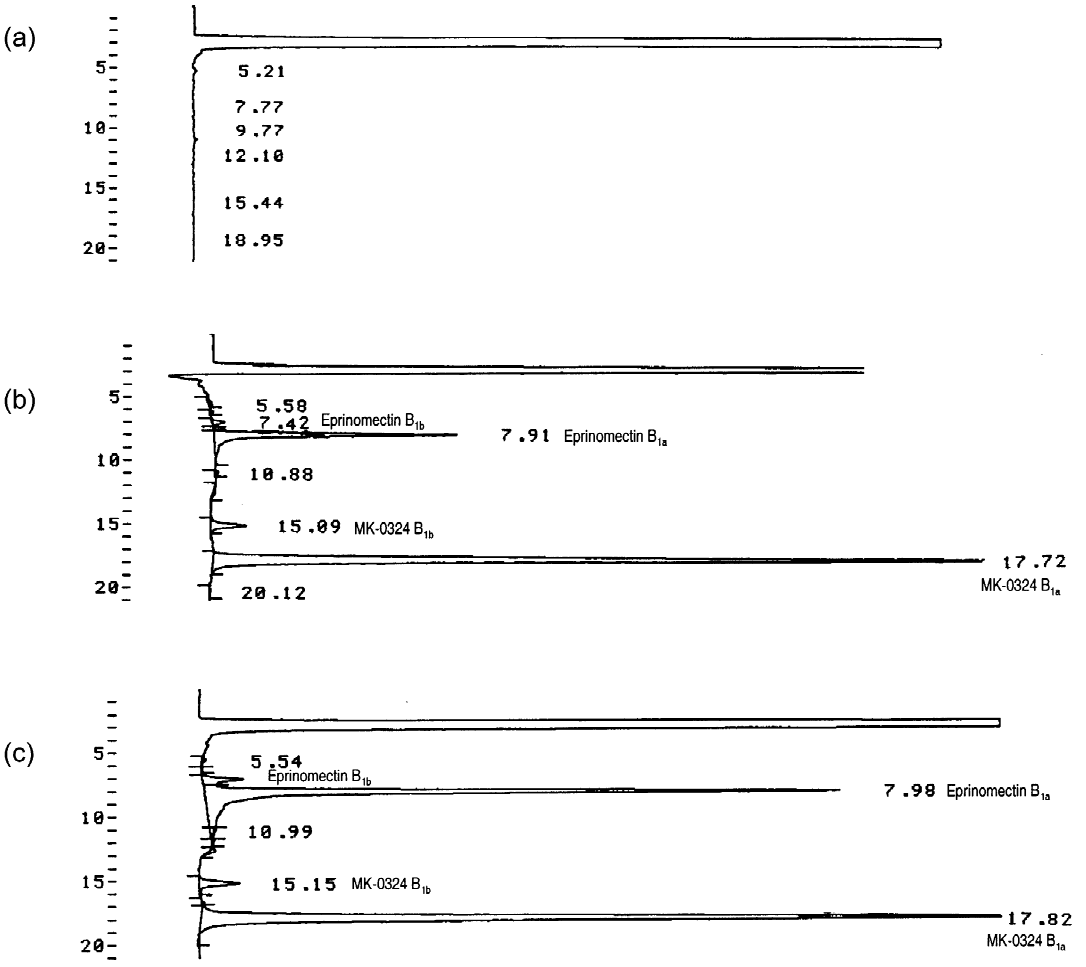

Fig. 4. (a) HPLC chromatogram of blank milk (no active); (b) HPLC chromatogram of derivatized eprinomectin standard; (c) HPLC chromatogram of fortified milk sample (23 ng/ml).

4. Discussion

A very simple and sensitive analytical method to determine eprinomectin in bovine milk was recently developed and validated in our laboratories. Sensitivity was accomplished by reacting eprinomectin and the internal standard MK-324 with catalyst *N*-methylimidazole and acylating reacting trifluoroacetic anhydride. Using this procedure, a strongly fluorescent derivative of each analyte was obtained. Because the reaction was found to proceed rapidly, the derivatization step was automated by programming the autosampler to add and mix the reagent with the analytes in the sample vial. Upon completion of the mixing of the derivatization mixture by the autosampler, the derivatized analytes were immediately injected into the HPLC for analysis.

The method was linear over the range of 2.3–51 ng/ml using a 2-ml aliquot of milk. A plot of eprinomectin B_{1a} concentration against the ratio of eprinomectin B_{1a} peak height to internal standard peak height confirmed the linearity. The overall correlation coefficient (r) was 0.99. The overall recovery of eprinomectin B_{1a} from milk was 94.4%; the relative standard deviation was $\leq$6.2% confirming reproducibility. The selectivity of the method was checked by processing blank milk samples. No significant interferences were observed at the retention times of eprinomectin and I.S. Controls were generally clean from interferences. HPLC system suitability criteria were met. All method parameters (precision, linearity, accuracy and sensitivity) were found to be similar between both laboratories. In both laboratories, it was possible to obtain a signal-to-noise ratio of approximately 10 at a concentration of 1 ng/ml. Nonetheless, the LOQ was established at 2 ng/ml because only at that concentration, accuracy and precision criteria were met in Laboratory 2.

In conclusion, a highly sensitive, automated HPLC method for the determination of eprinomectin in bovine milk, using a simple clean-up procedure and programmable autosampler for automated pre-column derivatization, has been developed. This method has been validated in the concentration range of 2.3–51 ng/ml. The linearity of the method in the concentration range has been demonstrated. The method is rugged, reliable and was used routinely for the analysis of milk samples [14]. The use of solvent partitioning and/or solid-phase extraction of bovine milk was deemed unnecessary to obtain a cleaner extract and improved recovery. On-line derivatization with TFAA and 1-*N*-MIM was found to be the most efficient and simple procedure to determine eprinomectin residues from incurred milk samples with less than 50 ng/ml eprinomectin.

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