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Development and validation of an automated extraction method (accelerated solvent extraction®) and a reverse-phase HPLC analysis method for assay of ivermectin in a meat-based chewable formulation

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Abstract

A new method for monitoring ivermectin content in HEARTGARD CHEWABLES® has been developed and validated. The method consists of the automated extraction of ivermectin from the meat-based formulation under conditions of elevated temperature and pressure (accelerated solvent extraction, ASE®), and determination of the active by reverse-phase high performance liquid chromatography (HPLC). The method resolves both active species of ivermectin (components H_2B_{1a} and H_2B_{1b}) from the formulation matrix.

Keywords: Extraction; Accelerated solvent extraction; Ivermectin; HEARTGARD CHEWABLES; Reversed-phase HPLC

1. Introduction

Isolation of vitamins, hormones, preservatives, colorants, dyes, and other desired or undesired ingredients from foods, or pesticides from contaminated soil, has always presented a difficult challenge for an analytical chemist working in a quality control or environmental analysis laboratory. Traditionally, these problems were solved by employing extractions with mechanical shaking, ultra-sonication, micro-wave, or Soxhlet extractions. Typically in pharmaceutical formulations, extraction of the active ingredient is not a significant issue. For example, most oral formulations are designed so that the active will be readily released under appropriate conditions. Some drugs, however, are formulated to release the active ingredient slowly; consequently the active may also not readily be extracted into a solvent. Other drug products, such as veterinary dosage

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forms, may consist of complex matrices of drug and food rendering the isolation of active drug difficult.

The HEARTGARD CHEWABLES® product line is a series of beef-based formulations designed to dose household dogs and cats with ivermectin, an anthelmintic used in the treatment of common parasitic diseases such as heartworm [1,2]. The structure of ivermectin is shown in Fig. 1. Ivermectin consists of two active species, H_2B_{1a} and H_2B_{1b}, which differ structurally by the nature of the side chain at carbon 25 (*sec*-butyl and isopropyl, respectively). Due to the potency of ivermectin, it is present in very low doses (55–272 mcg in a multigram meat tablet). Also due to the nature of meat, which dries as it ages, efficient extraction of the active from the formulation over shelf life can be a challenge using the common extraction procedures described above. Conventional extraction methods require significant time to achieve efficient and reproducible recovery. Supercritical fluid extraction (SFE) has been explored in the analysis of ivermectin in liver samples [3]; this methodology was not applied to HEARTGARD CHEWABLES® products.

In order to streamline testing of this product, we have investigated the applicability of new automated extraction systems for analysis [4]. These systems fundamentally enhance the efficacy of extraction by optimizing solvation of the analyte with applied heat and pressure. The benefits of this technology for isolation for solid environmental samples [5], and for solid pharmaceutical dosage forms [6] has been reported.

We describe, herein the application of automated solvent extraction technology for the analysis of ivermectin in the HEARTGARD CHEWABLES® products. The selected conditions are described in Table 1. In addition, a new reverse-phase HPLC method is described (see Table 2 for details), which was designed to effect resolution of the active from the various excipient components extracted from the matrix under the modified conditions. Development of all appropriate conditions and validation of the method are described.

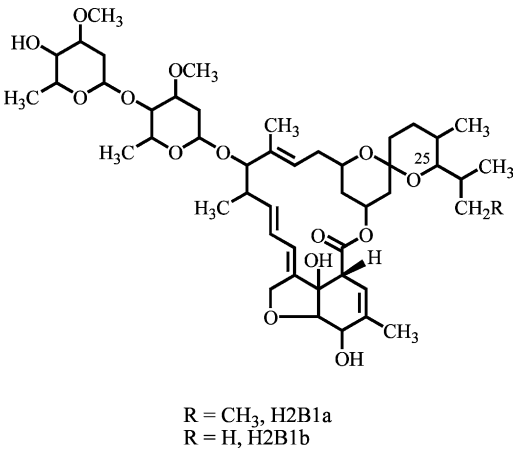

Fig. 1. Structure of Ivermectin (H_2B_{1a} and H_2B_{1b}).

2. Experimental

2.1. Materials and reagents

A laboratory scale formulated placebo of HEARTGARD CHEWABLES® was supplied by Merial Ltd., department of Animal Formulation Development (New Brunswick, NJ, USA).

Bulk ivermectin, and all product samples were obtained from the Merck manufacturing site (Barceloneta, Puerto Rico). The analytical standard for ivermectin was obtained as a solution in glycerol formal (1.49% by weight) from the Merck Reference Standard department.

Hydromatrix was purchased from Varian (Varian Instruments, 2700 Mitchell Dr., Walnut Creek, CA 94598). Neutral Alumina, Brockman Activity

Table 1
Conditions for extraction of ivermectin using the ASE®

Parameter	Setting
Pressure	1500 Psi
Preheat	0 min
Heat	6 min
Temperature	120 °C
Static time	10 min
Flush	30% volume
Purge	60 s
Cycles	1
Solvent	95% methanol, 5% Water

Table 2
Chromatographic conditions for analysis of ivermectin

Parameter	Setting
Column	Waters symmetry C8, 250 × 4.6 mm, 5 μm particle size
Mobile phase	72.5% acetonitrile, 27.5% water, 0.1% trifluoroacetic acid
Flow rate	1.2 ml/min
Injection volume	100 μl
Column temperature	40 °C
Detector	UV at 254 nm
Run Time	30 min

I, was purchased from Fisher Scientific (Fisher Scientific Worldwide, One Liberty Lane, Hampton, NH 03842), and was subsequently deactivated by addition of 6% water (w/w). Deionized water was further filtered using a Barnstead Nanopure Ultrapure Water System (Barnstead International, 2555 Kerper Boulevard, Dubuque, Iowa USA 52001).

Ottawa Sand, and all solvents and liquid reagents used were purchased from Fisher Scientific, and were HPLC grade or higher.

A Tyler 10 sieve was purchased from Fisher Scientific. Disposable polypropylene columns were purchased from BioRad (Life Science, Life Science Research, 2000 Alfred Nobel Drive, Hercules, California, USA 94547). Acrodisc 0.2 μm filters were purchased from Gelman (Pall Corporation, Life Sciences-BioPharmaceuticals Group, 2200 Northern Boulevard, East Hills, NY 11548).

2.2. Apparatus

A ToastMaster MiniChopper Model 1118 or Krups Type 203 Coffee Grinder was used for grinding the samples. A commercially available Dionex accelerated solvent extraction (ASE®) 200 Solvent Extractor was used. All HPLC results were generated on Thermal Separations Products (TSP) instruments (Degasser Model SCM-400, Pump Model P-4000, Autosampler Model AS-3000, UV-visible Model UV-2000).

2.3. Analysis of pharmaceutical formulations

To determine the content of ivermectin in formulated product, samples were prepared as follows. The tablet was ground using either a food chopper or coffee grinder, to a powder which can pass a Tyler 10 sieve. Approximately, 0.5–1.5 g (depending on the dose of the chewable) was transferred to a mortar and blended with approximately 1.5 g of Hydromatrix using a pestle. The mixture was transferred to an extraction cell and Ottawa sand was added to fill the cell. The cell was then extracted, using methanol/water (95/5% v/v) as the solvent, according to the parameters described in Table 1. The resultant solution was diluted with appropriate volumes (depending on the dose of the chewable) of methanol and water to produce a 0.9 μg/ml solution in methanol/water (approximately 80/20% v/v). The resultant solution was passed over a 5 ml alumina cartridge (the first 1 ml of eluent was discarded), and was filtered through a 0.2 μm PFTE filter into an HPLC vial.

2.4. Method validation

The method was developed with a target standard and sample concentration of 0.90 μg/ml, in order to accommodate appropriate dilution of all product strengths. The selectivity of the method was determined by injecting the working standard solution, a placebo sample preparation, and methanol (the primary component of the diluent). Chromatograms of a sample solution and placebo are shown in Figs. 2 and 3.

2.4.1. Accuracy and linearity

Spiked placebo preparations were prepared by adding Ivermectin of known purity to a fixed amount of ground placebo, in order to obtain extracts containing Ivermectin in the range of 50–150% of the target concentration (0.9 μg/ml). Each level was evaluated in duplicate on 2 different days. The data are presented in Table 3, and a plot of the linearity data is shown in Fig. 4.

2.4.2. Precision

In order to demonstrate HPLC injection reproducibility, six replicate injections of a working

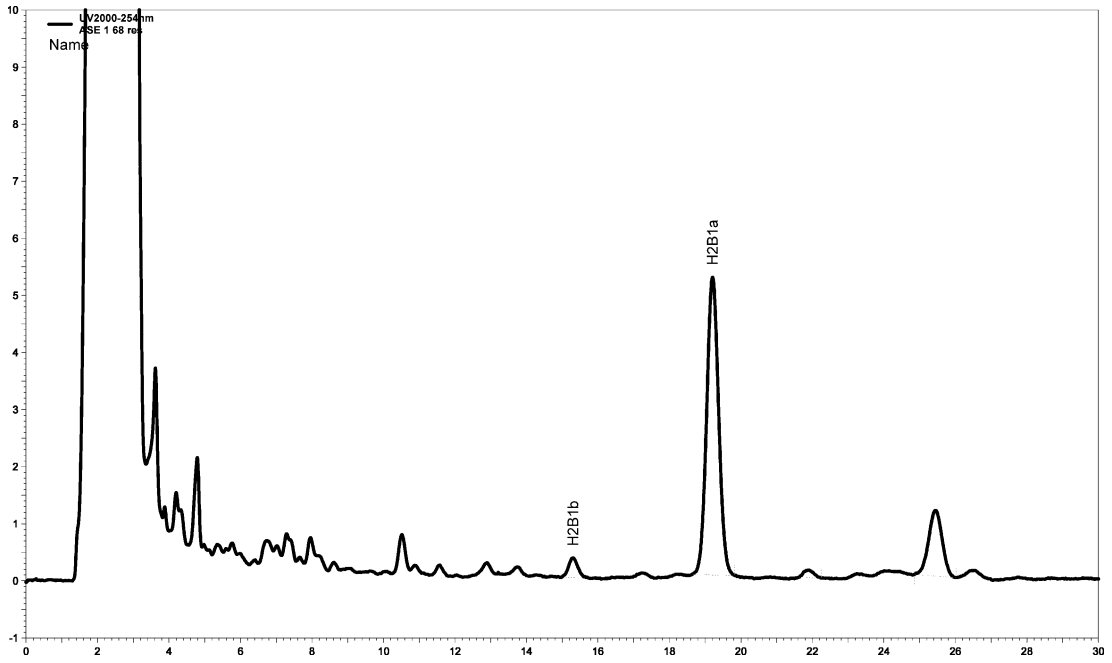

Fig. 2. Chromatogram of an extracted HEARTGARD CHEWABLE® sample.

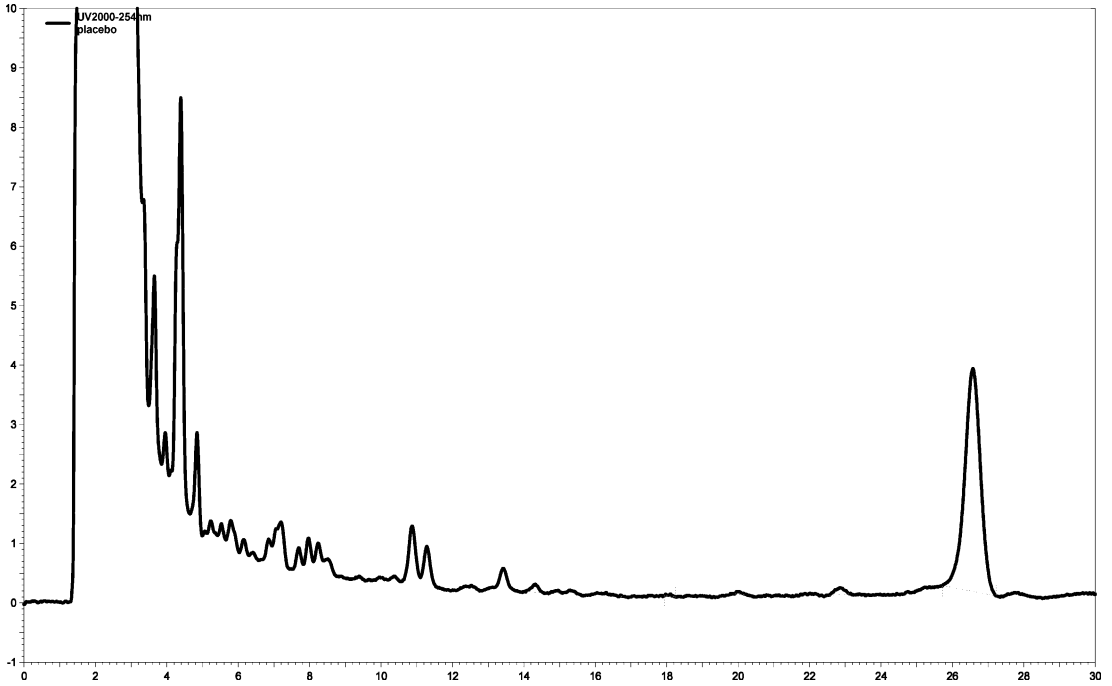

Fig. 3. Chromatogram of Placebo solution.

Table 3
Summary of recovery data for ivermectin (H_2B_{1a} and H_2B_{1b})

Target concentration (%)	% Recovery				Mean	% R.S.D.
	Set 1	Set 2	Set 3	Set 4		
50	98.4	103.9	97.3	100.0	99.9	2.9
75	99.2	102.0	98.7	98.7	99.7	1.6
100	102.3	99.9	100.4	100.6	100.8	1.0
125	103.3	101.2	103.3	100.8	102.1	1.3
150	100.9	98.2	99.6	101.8	100.1	1.6

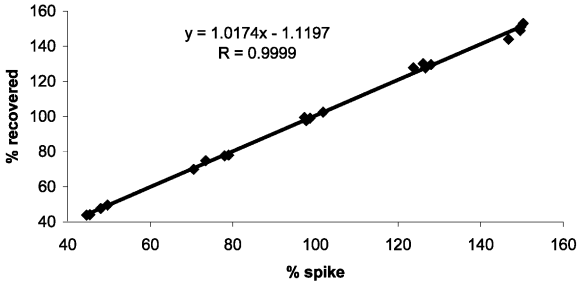

Fig. 4. Linearity of total Ivermectin ($H_2B_{1a}+H_2B_{1b}$). Over the range of 50–150% of the target sample concentration (0.9 μg/ml). In all four, spike and recovery experiments were performed on 2 days (two sets of experiments per day).

Table 4
Summary of measurement precision data for ivermectin (H_2B_{1a} and H_2B_{1b})

Injection	Area response ($H_2B_{1a}+H_2B_{1b}$)
1	118 477
2	119 085
3	119 166
4	118 663
5	119 683
6	120 084
Mean	119 193
R.S.D. (%)	0.51

standard solution (0.90 μg/ml) were analyzed, and the area responses were compared (see Table 4).

To demonstrate precision of the method, six individual preparations from one lot of HEARTGARD CHEWABLES® FOR CATS were analyzed (Table 5). The accuracy data from Section 2.4.1 were also analyzed across each individual level in order to evaluate reproducibility at each level across the validated range (see Table 3).

Table 5
Summary of method precision data

Preparation	Recovery (%)
1	101.3
2	103.5
3	101.6
4	102.4
5	101.6
6	103.2
Mean	102.3
R.S.D. (%)	0.90

Intermediate precision (variability from one analyst to another) was also evaluated. Three lots of HEARTGARD CHEWABLES® FOR CATS were assayed for percentage Ivermectin of the labeled amount. Two analysts each prepared two composites per lot and analyzed two samples per composite (total of four samples analyzed per lot per analyst) using different HPLC systems, different columns, independently prepared reagents and mobile phase on different days. The

Table 6
Summary of intermediate precision data

Lot	Recovery (%)		Absolute difference
	Chemist 1	Chemist 2	
1	102.3	101.4	0.9
2	101.0	101.9	0.9
3	103.0	102.8	0.2

means obtained for each lot by each analyst are compared in Table 6.

3. Results and discussion

3.1. Method development

The nature of the ASE® technology requires that the samples be ground and dispersed in alumina or sand prior to extraction. The solid support keeps the sample particles dispersed under conditions of high pressure, maximizing surface area and thus enabling the solvent system to rapidly permeate the sample matrix. Sample preparation is rather critical, it is essential to grind the samples to a fine and relatively uniform particle size in order to effect uniform extraction in the ASE®. Therefore, after milling with a mortar and pestle, we typically grind the samples further in a coffee mill or food processor. In order to separate larger particles remaining, we put the resultant mixture through a Tyler 10 sieve. Larger particles that do not pass the sieve screen are further ground and the material is again sieved. Aliquots from the fines are then used in the extraction. This procedure is applicable to the testing of single tablets as well as composite assay testing. An advantage to the approach of using aliquots from the ground sample is that the sample is not completely consumed in one extraction; thus when an atypical result is obtained, the same sample may be retested. The ground sample is then mixed with Hydromatrix dispersion agent and added to the ASE® extraction chamber of an appropriate size. The sample chamber is then filled to capacity with Ottawa Sand to maintain a relatively constant volume of extraction.

Whereas ivermectin is susceptible to heat-based degradation on prolonged exposure, excursions to high temperature are generally not of significant concern [1]. It was determined that extraction under the conditions listed in Table 1 resulted in complete recovery of ivermectin from spiked placebo samples, measured against an analytical standard prepared directly from bulk ivermectin without ASE® conditioning. Methanol containing 5% water was selected as the extraction solvent, in order to minimize heat of mixing in later dilution steps and with the partially aqueous mobile phase.

In order to determine the appropriate time of extraction, a composite sample (ten tablets) was prepared from a single lot. Several aliquots from this composite were extracted for varying time intervals, and were analyzed by the HPLC method described herein. Recovery reaches a plateau after 5 min of extraction under the conditions described in Table 1; an extraction time of 10 min was selected for testing.

Following extraction, the samples are somewhat cloudy due to the extraction of fats and other excipients from the matrix under the ASE® conditions. Each extract is diluted to a standard volume with methanol (to achieve a concentration of 0.9 μg/ml), and an aliquot of the final solution is passed through a short column of 6% deactivated alumina. The alumina removes insoluble components as well as some of the emulsified fat, rendering the solution suitable for HPLC analysis.

The principal challenge in development of the HPLC method was to minimize interference from the sample matrix. The HPLC conditions described in Table 2, were found to minimize interference from all excipient peaks, such that the two active species (H_2B_{1a} and H_2B_{1b}) could be quantified. See Figs. 2 and 3 for chromatograms of a sample solution and Placebo solutions.

3.2. Method validation

The isocratic HPLC method utilizes a mobile phase composed of 72.5% acetonitrile in water which is supplemented with 0.06% trifluoroacetic acid on a Waters Symmetry C8, 25 cm × 4.6 mm (5 micron particle size) column and UV detection at 254 nm. In Fig. 2, is shown a chromatogram of working sample solution (approx. 0.90 μg/ml). Fig. 3 shows the response for a placebo injection. The two active species H_2B_{1a} and H_2B_{1b} are labeled, and are clearly resolved from all matrix components.

The data shown in Tables 3–5 demonstrate excellent accuracy and precision of the assay method. The data shown in Fig. 4 demonstrate excellent linearity of the method and that no significant variation is observed from 1 day to

the next. The variability between analysts in analysis of actual product samples, Table 6, is within typical analytical variability reflected in the other precision data. These data demonstrate that the ASE® technology not only performs with adequate accuracy and reproducibility for pharmaceutical analysis, but it is also very rugged from one analyst to the next.

3.3. Method robustness

In order to demonstrate robustness of the ASE® conditions described in Table 1, extractions were performed under a variety of conditions. It was found that small changes, such as varying the static temperature by ±10 °C, the extraction time by ±5 min, or the organic content of the solvent by ±5%, did not have a significant impact on recovery.

Robustness of the HPLC method parameters described in Table 2 was demonstrated by variation of several method variables including flow rate (1.0–1.5 ml/min), detection wavelength (250–260 nm), temperature (30–50 °C), organic mobile phase composition (organic content varied by ± 5%), and column (columns from three different vendor lots). Robustness of each variable was determined by comparing duplicate injections of a sample solution. In all cases, acceptable chromatography was obtained with the small perturbations applied.

Whereas Ivermectin can be quantitatively extracted from fresh HEARTGARD CHEWABLES® using platform shaking and sonication, old aged samples (past expiry) are more challenging. Comparing the ivermectin recoveries from such samples using the ASE® technology versus platform shaking and sonication, we found that the ASE® consistently produced 1–5% higher recoveries (data not shown). Since the sample remains in the extraction cell, it was possible to demonstrate that the extraction using the ASE® was complete by repeating the extraction; negligible recovery was observed. In contrast, samples extracted by sonication/shaking are destroyed in the course of preparation, therefore, completion of the extraction cannot be demonstrated by reextraction.

4. Conclusion

The new assay method presented herein for assay of Ivermectin in the HEARTGARD CHEWABLES® product line is specific, accurate, reproducible and rugged. The method requires minimal effort on the part of the analyst and a minimal amount of solvent for analysis. The method constitutes a rare application of the automated ASE® extraction technology to the analysis of a pharmaceutical product. An attractive alternative to the sequentially operating ASE®, is the Pressurized Solvent extractor (PSE®) from Applied Separations, which allows extraction of six samples simultaneously.

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