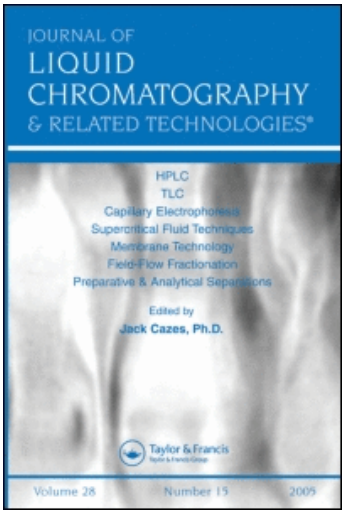

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EXTRACTION AND HPLC ANALYSIS OF HALOFUGINONE IN CHICKEN SERUM*

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ABSTRACT

Extraction and HPLC methods are described for the analysis of the anticoccidial quinazolinone drug, halofuginone (Hal), in chicken serum. Serum samples were diluted with acetic acid and Hal was adsorbed onto a Bond Elut® C8 column followed by elution and HPLC analysis. Recoveries of Hal from chicken serum spiked with 97 ng/ml Hal after 18 and 48 hours incubation were 97.6 and 96.2%, respectively, and recoveries of a 10 ng/ml spike after 18 and 48 hours incubation were 99.7 and 96.7%, respectively, with a detection limit of 1.5 ng/ml. Hal-HBr fed to chickens at 3 ppm for 10 days resulted in 3.75 ng/ml Hal in the serum.

*Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

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INTRODUCTION

Halofuginone [55837–2–2] (Hal, Fig. 1) is used worldwide as an antiprotozoal drug to prevent coccidiosis in commercial poultry production [1]. The drug is the hydrobromide (HBr) salt [64924–67–0] [2] of a halogenated analog of the naturally-occurring quinazolinone alkaloid, febrifugine [3], and was among the top five coccidiostats used in poultry production in the U.S. during 1991 [4]. Hal-HBr is an FDA approved feed additive at 3 ppm for the prevention of coccidiosis in broiler chickens, and has a tolerance in chicken liver of 0.16 ppm and a required withdrawal of 4 days [5,6,7].

Both GLC and HPLC methods for determination of Hal in chicken feed were investigated for the purpose of registering the drug [8]. It was shown that Hal decomposed when chromatographed by GLC, and that the use of HPLC resulted in a 95.6% recovery of Hal from feed. It was also demonstrated that Hal degrades in alkaline solution [8]. Hal appears to be susceptible to methanolysis; during modifications of the HPLC method [8] used for analysis of Hal in feed, an Analytical Methods Committee [9] found that recoveries ranged from 58–91% when Hal was chromatographed with methanolic solutions, and that increasing the solvent flow rate improved recoveries. When acetonitrile was substituted for methanol in the mobile phase, recoveries of 93.4–97.4% were obtained. We have noted while synthetically converting Hal to various derivatives that the use of methanol in the reactions results in degradation of Hal to a number of decomposition products [10].

An extraction and HPLC method was published for the analysis of Hal in feeds and chicken tissues, including liver, kidney, muscle, skin, and fat [11]. The recovery obtained from feed was 92.7%, while the recoveries from chicken tissues

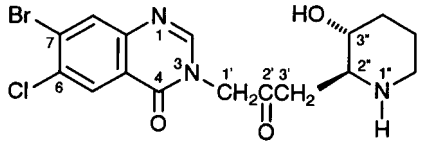

Figure 1. The chemical structure of halofuginone.

varied from 68–117%. Analytical methods accepted by the Food and Drug Administration (FDA) must have recoveries of 60–110% [12]. The Analytical Methods Committee accepted, with minor changes, the published method [11] for determining Hal in chicken tissues [13]. The recovery of Hal in a collaborative study from different chicken tissues ranged from 36–93% [13]. Extracting Hal from chicken tissues with a methanol:acetate buffer (1:1) followed by HPLC analysis resulted in recoveries that varied from 64–87.4% [14].

The Food Safety Inspection Service (FSIS) currently monitors chicken livers for the presence of Hal. The official method [15] used is a composite of a number of methods described above, and is complex and tedious. FSIS accepts recoveries of 60–114% for this method.

We have begun a systematic study of the levels of Hal found in chicken liver versus the levels found in a more workable tissue, chicken serum. The use of an immunochemical method based on monoclonal antibodies to Hal [16] would provide good sensitivity and a high through-put of samples. To verify the accuracy of the monoclonal technology, comparisons of data obtained by a competition enzyme-linked immunosorbent assay (ELISA) with data obtained from a standard HPLC analysis method is required.

A simple method for the determination of Hal in bovine plasma by solid phase extraction and HPLC analysis resulted in an average recovery of 75% [17]. This method for analysis of Hal in bovine serum was very simple, straight forward, but lacked an acceptable recovery rate.

This paper presents an improved method of extraction of Hal from chicken serum followed by HPLC analysis which provides consistent, high recoveries. The serum dilution and acidification step used during the extraction process was extensively investigated to determine its impact on the potential loss of Hal. The extraction and HPLC analysis of Hal as described here was used to determine the concentration of Hal in Leghorn chickens fed a ration containing 3 ppm Hal-HBr.

MATERIALS AND METHODS

Chemicals

Halofuginone-HBr (99.4%, Lot #0A3081B) and Stenorol® (2.72 g halofuginone/lb., Lot #42307062) were provided by Hoechst-Roussel Agri-Vet

Co., Somerville, NJ. Instrumental grade acetic acid [64–19–7] Tracepur® Plus, OmniSolv HPLC grade methanol [67–56–1] and acetonitrile [75–05–8] were obtained from EM Science, Gibbstown, NJ. HPLC grade ammonium acetate [631–61–8] was obtained from Fisher Scientific, Fair Lawn, NJ. Decylamine [2016–57–1] was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. Water was prepared on a Millipore RO system, Marlborough, MA.

Materials and Instrumentation

All surfaces that came in contact with Hal when preparing and storing standards or analyzing samples were either polypropylene or silylated glass. Glass active sites were exposed by cleaning the surfaces thoroughly with a solution of 5% potassium hydroxide in 95% ethanol followed by drying in an oven. The glass surfaces were then deactivated by reacting them in an atmosphere of dimethyldichlorosilane [75–78–5] (Pierce Chemical Co., Rockford, IL) overnight followed by rinsing with dry methanol.

Chicken serum (Sigma Cell Culture Reagents®) was used for Hal spiking experiments and methods development (Sigma Chemical Co., St. Louis, MO). Bond Elut® (1cc) C8 bonded phase columns, Lot #060956, were obtained from Varian, Harbor City, CA.

A Tracor model 951 LC Pump (Tracor, Inc., Austin, TX) delivered the solvent system through a Whatman guard column (Pierce Chemical Co., Rockford, IL) filled with CO:PELL ODS (octadecyl groups chemically bonded to 30–38 μm glass beads) (Whatman, Inc., Clifton, NJ), and then through a 4.6 mm ID x 25 cm 5 μm Supelcosil™ LC-18-DB column (Supelco, Inc., Bellefonte, PA).

Samples were introduced via a 50 μl loop injector, Model 7125 (Rheodyne, Inc., Cotati, CA). The column's effluent was monitored by a LDC/Milton Roy spectroMonitor® D, variable wavelength detector, at 243 nm and recorded with a HP 3390A integrator (Hewlett-Packard, Palo Alto, CA).

Solutions

Ammonium acetate buffer (AA buffer) was prepared at 0.25 M, pH 4.3. Elution solvent used with the C8 Bond Elut® columns consisted of 79.9:20:0.1

v/v/v water-acetonitrile-acetic acid plus 210.4 µl decylamine per 100 ml batch, pH approximately 4.3. The HPLC solvent system consisted of 22:15:63 v/v/v acetonitrile-AA buffer-water plus 210.4 µl decylamine per 1 liter batch, pH approximately 4.75. A stock standard solution of Hal-HBr was prepared by dissolving the compound in AA buffer. Fortification standards used for additions to serum were made by diluting the stock standard with water. HPLC standards were prepared by diluting the stock standard with the HPLC solvent system.

Extraction and HPLC Analysis

Extraction and HPLC analysis of chicken serum was similar to that reported for bovine serum [17]; however, a number of changes were required to improve recoveries of Hal. Hal eluted from HPLC with a uniform peak shape and consistently when both decylamine and a buffer system were used.

The C8 Bond Elut® columns were conditioned by passing methanol (2 ml) followed by acidified water (8 ml, adjusted to pH 4.3 with acetic acid) through the columns prior to use. Ten percent acetic acid (8 ml) was added to serum (4 ml) spiked with Hal-HBr and serum obtained from Hal-HBr treated chickens. The diluted acidified serum was immediately passed through a Bond Elut® column. The column was washed with acidified water (5 ml). The column was then washed with 35% methanol (1 ml, adjusted to pH 4.3 with acetic acid), and finally washed again with 1 ml of acidified water. Hal was then eluted from the column with the elution solvent (1 ml). The column eluent was injected (50 µl) on HPLC.

A standard curve was generated by the HPLC analysis of solutions that contained from 0.01 ng/µl to 0.6 ng/µl of Hal-HBr; the correlation coefficient for the standard curve was 0.9998.

Incurred Residues of Hal in Chicken Serum

Broilers (White Rock Cross), Hubbard x Pettersen, were obtained at the age of 1 day old and fed unmedicated chicken feed for 3 weeks at which time they were placed on feed consisting of unmedicated chicken feed to which Stenorol® had been added with mixing to provide a level of 3 ppm Hal-HBr. Blood was taken from 8 broilers that were fed *ad libitum* for 10 days on Hal-HBr treated feed; serum was collected after centrifugation following clot formation. The serum was pooled and frozen (-70°C) within two hours of collection.

RESULTS AND DISCUSSION

Initial attempts at using the extraction and HPLC analysis method for Hal in bovine serum [17] for the detection of Hal in chicken serum gave recoveries that ranged from 65–85%. These recoveries were in good agreement with those published with the method [17]. However, these low and variable recoveries prompted further investigation aimed at improving the method. It was discovered that adjusting all solvents used for washing the Bond Elut® C8 column to pH 4.3 with acetic acid, and collecting 1 ml of the eluted Hal rather than 0.5 ml drastically improved the recoveries of the method. However, variability in recoveries of Hal remained unacceptable.

We subsequently observed that serum samples diluted with 10% acetic acid but not processed immediately had lower recoveries than others that were processed immediately. An experiment was carried out to evaluate the apparent loss of Hal in the acidified serum. Chicken serum samples were spiked with 97 ng/ml Hal-HBr and allowed to sit for 48 hours at 13°C. Samples were diluted with the 10% acetic acid and processed at different time intervals. Fig. 2 shows the recovery results of the timed study of Hal in acidified serum. A linear loss of

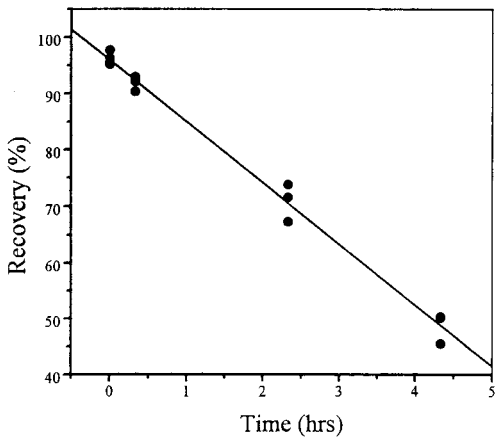

Figure 2. Recovery of Hal from serum at different time intervals after acidification, at room temperature.

available Hal at the rate of 11% per hour was observed. Upon dilution of the serum with 10% acetic acid, the resultant solution had a pH of ≈ 2.8. Since Hal is stable for considerable periods of time in acid solutions, FSIS makes their stock standard reagent of Hal-HBr in an ammonium acetate buffer at pH 4.3 [15]; therefore, it seems most likely that the diminished levels of Hal is the result of Hal binding to serum components under acid conditions.

The HPLC system containing both ammonium acetate buffer and decylamine gave very good peak shape for Hal as seen in Fig. 3. Frame A is a tracing of an

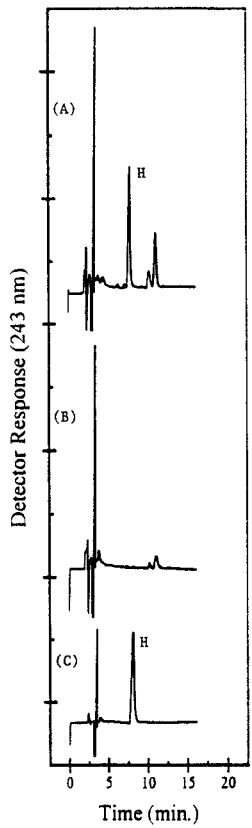

Figure 3. HPLC chromatograms of a 48 hour Hal spiked serum extract (A), of a serum control extract (B), and a Hal standard (C). The Hal peak is marked with an H.

Table 1

Observed Recoveries of Hal After Incubation in Chicken Serum at Different Time Intervals.

High Spike Level[1]			
Time (hours)[2]	Recovery	SD[3]	CV[4]
18	97.6%	± 1.75	1.79%
48	96.2%	± 1.12	1.16%
Low Spike Level[5]			
18	99.7%	± 3.30	3.31%
48	96.7%	± 3.16	3.26%

[1]97 ng/ml serum.
[2]Incubation period prior to acidification and imediate HPLC analysis.
[3]SD = standard deviation, n = 3 or 4.
[4]CV = coefficient of variation.
[5]10 ng/ml serum.

extract of a 48 hour spiked chicken serum sample. Frame B is a tracing of an extract of control chicken serum. Frame C is a tracing of a Hal standard. Not only was the peak shape for Hal symmetrical, but there also were no interfering peaks in chicken serum using this system. This method can quantitate as low as 1.5 ppb Hal in chicken serum.

The recoveries of Hal spiked at a high and low level from chicken serum after different times of incubation are shown in Table 1. After 18 or 48 hours of incubation, high and consistent recoveries were observed with both high and low spiked levels of Hal. The standard deviations bear out the results showing that the recoveries observed are consistent.

Finally, we determined the quantity of Hal that occurred in chicken serum after administration of Hal-HBr in the feed at 3 ppm for 10 days. The frozen pooled chicken serum was thawed and processed after reaching room temperature. The analysis showed Hal residues of 3.75 ng/ml (SD = ± 0.36, CV = 9.6), and the chromatography was as clean as that of the spiked chicken serum (Fig. 3, A).

The extraction and analysis method developed here for Hal in chicken serum provides an HPLC method that is moderately fast with consistently high recoveries. The confirmation that Hal decomposes readily in methanolic solutions,

and the knowledge that it binds to serum components under acid conditions will be successful work with Hal in future studies.

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