Detection of Halofuginone Residues in Chicken Liver Tissue by HPLC and a Monoclonal-Based Immunoassay

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The quinazolinone halofuginone (Hal) is a feed additive used worldwide to prevent coccidiosis in commercial poultry production. The current regulatory method for determining the action level of Hal residues in poultry involves measuring parent Hal in liver tissue by HPLC. That procedure is not amenable to high sample throughput due to a complex and tedious sample preparation scheme. A competitive enzyme-linked immunosorbent assay (cELISA) that can be used as a screening tool for determining Hal in chicken liver tissue is described. The cELISA method was evaluated using standard curves made in both assay buffer and chicken liver extract. The results demonstrated that standard curves made in assay buffer could be used for the cELISA. HPLC vs cELISA results were obtained during two studies; the first study used spiked chicken liver tissue, and the second study used both spiked chicken liver tissue and incurred levels of Hal in chicken liver tissue. There was good agreement in the results obtained by HPLC and cELISA. However, in most cases the recovery was higher using the cELISA method than with the HPLC method. In addition, the cELISA method does not require the use of organic solvents. These data clearly demonstrate that the cELISA method could be used as a screening method for the analysis of Hal in chicken liver tissue.

Keywords: *Broilers; chicken liver tissue; coccidiostat; ELISA; halofuginone; HPLC; immunoassay*

INTRODUCTION

Halofuginone [55837-2-2] (Hal, Figure 1) is used worldwide as an antiprotozoal drug to prevent coccidiosis in commercial poultry production (McDougald, 1990). The drug is used as the hydrobromide (HBr) salt [64924-67-0] (Shepard et al., 1996). Hal is a halogenated analogue of the naturally occurring quinazolinone alkaloid febrifugine (Openshaw, 1953; Cheng, 1976) and was among the top six coccidiostats used in poultry production in the U.S. from 1985 to 1990 (Anon., 1995). The U.S. poultry coccidiostat market increased from 78.7 million dollars in 1985 to 87.5 million dollars in 1990. The U.S. market was estimated to be 100 million dollars in 1993 (Anon., 1995). Hal was again estimated at being one of the top used coccidiostats in 1995−6, having an approximate U.S. market share of 5−10% (Anon., 1997a). The worldwide total coccidiostat market was estimated to be 500 million in 1995−6 (Anon., 1997b). Hal−HBr is used at 3 ppm in feed for the prevention of coccidiosis in growing turkeys and in broiler chickens (Sundlof et al., 1992). A residue tolerance of 160 ppb has been established for chicken liver tissue, and a 4-day withdrawal time is required for chickens before slaughter (Anon., 1985, 1991a).

The current regulatory method for determination of Hal−HBr residues in poultry involves the measurement of parent Hal in chicken liver tissue by HPLC. It was hoped that developing a simple chicken serum immunoassay for the screening of Hal (Beier et al., 1996) could greatly increase the rate of sample throughput. However, the lack of residues in serum of dosed birds after 24 h made the serum assay inapplicable.

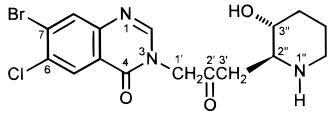

Figure 1. Structure of halofuginone (Hal).

Hal has been selected through the FSIS National Residue Program (NRP) as a compound included in their residue monitoring program. In the FSIS Compound Evaluation System (CES), Hal has a ranking of A-1. A compound with a ranking of A is considered a potential high hazard, and 1 indicates that exposure to this compound is likely to occur (Brown, 1995). Therefore, Hal was among the compounds in both the 1996 and 1997 Residue Monitoring Program. The HPLC method used by FSIS in this paper is the current method used by FSIS in their residue monitoring for halofuginone in chicken tissue.

A hapten containing Hal was synthesized (Rowe et al., 1993) and linked at the 1″ *N*-position of Hal via succinate to KLH, and a series of monoclonal antibodies (Mabs) specific for Hal were produced using this conjugate (Rowe et al., 1994). One of the Mabs, Hal-37, was used successfully in a preliminary study to evaluate levels of Hal in fortified chicken sera. The antibody cross-reactivity was determined for 12 structurally similar compounds, and in no case was the inhibition sufficient to calculate an IC_{50} (Rowe et al., 1994).

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We have developed an immunoassay method suitable for the analysis of Hal in chicken liver tissue. Hal spiked tissue and incurred levels of Hal in chicken liver tissue were evaluated by both the competitive enzyme-linked immunosorbent assay (cELISA) method produced by the USDA Agricultural Research Service (ARS) Laboratory, College Station, TX, and also the HPLC method routinely used by the USDA Food Safety Inspection Service (FSIS) Laboratory, St. Louis, MO. The results of the combined analyses are presented in this paper.

MATERIALS AND METHODS

Chemicals Used for Competitive Inhibition ELISA (cELISA). Ammonium acetate was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Dimethyldichlorosilane (Cat. No. 83410) was obtained from Pierce Chemical Co. (Rockford, IL). Polyoxyethylene−sorbitan monolaurate (Tween 20), sodium carbonate, sodium bicarbonate, magnesium chloride, NaCl, Na_2HPO_4, TRIZMA hydrochloride, TRIZMA base, and goat anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma A-5278) were obtained from Sigma Chemical Co. (St. Louis, MO). Halofuginone−HBr (Hal−HBr) (99.4%, lot 0A3081B) and Stenorol Premix (2.72 g of Hal/lb, lot 42307062) were provided by Hoechst-Roussel Agri-Vet Co. (Somerville, NJ). The Hal−BSA conjugate used to coat microtiter plates and Hal 37/1 antibody were previously produced in our laboratory (Rowe et al., 1994). K-Blue substrate was obtained from Elisa Technologies (Lexington, KY). Nonfat dry milk (NFDM) Janet Lee instant nonfat dry milk fortified with vitamins A and D_3 contained 35.5% protein (Albertson's Inc., Boise, ID; obtained from a local grocery store). Reverse osmosis water, pyrogen free (RO H_2O) was produced on site by a reverse osmosis system obtained from Millipore Corp. (Bedford, MA) and used for the cELISA experiments.

Chemicals, Materials, and Instrumentation for HPLC. Glacial acetic acid was obtained from J. T. Baker (Phillipsburg, NJ). Acetonitrile was Baxter HPLC grade (Muskegon, MI). Ammonium acetate was obtained from EM Science (Gibbstown, NJ), and HPLC grade water was obtained from Millipore (Marlborough, MA). Bond Elut C_{18} bonded phase columns (6 cm^3/500 mg) were obtained from Varian (Harbor City, CA). HP 1050 Series Autosampler, HPLC pump, and variable wavelength detector and an HP 3396A Integrator were obtained from Hewlett-Packard (Palo Alto, CA). HPLC separation was accomplished on a 4.0 mm i.d. × 25 cm × 5 μm Hibar LiChrosorb RP 18 column (E. Merck, Darmstadt, Germany).

cELISA Materials and Instrumentation. Polytron Homogenizer, Type PT 10, 20, 350D, was used during extraction; the probe used was a PT 10 (Brinkmann Instruments, Westbury, NY). Polypropylene conical tubes were used for sample dilution, 15 mL (CEL Associates Inc., Houston, TX). Samples were weighed out and ground in silanized KIMAX culture tubes (25 × 150 mm, 24-410 thread with Teflon lined caps, Kimble No. 45066-25150) obtained from Fisher Scientific (Pittsburgh, PA). The method for silanizing the KIMAX culture tubes was previously reported by Beier et al. (1996). Sample dilutions were made with a portable pipet-aid, electrically actuated (Drummond Scientific Co., Broomall, PA). A multichannel pipettor was used to conduct the cELISA (Finnpipette, digital multichannel 50−300 μL pipet (LSI North America, Needham Heights, MA). Nunc-immuno plates F96 MaxiSorp and Nunc-lids used for immunoassays were obtained from PGC Scientifics Corp. (Frederick, MD). Optical densities of developed assays were read with a 96-well Microplate Reader, Bio-Rad model 3550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA). The Bio-Rad Reader Driver Software version 1.0 (for Power Macintosh 6100/60) was used at the following settings: 8 × 12 format, automix (3 min), and measurement wavelength of 655 nm.

cELISA Solutions. Detergent wash buffer was made by adding 0.05% Tween 20 to RO H_2O. Carbonate buffer consisted of Na_2CO_3 (0.015 M), $NaHCO_3$ (0.035 M), and $MgCl_2$ (0.002 M) in RO H_2O, pH 9.6. Phosphate-buffered saline (PBS-9) contained Na_2HPO_4 (0.01 M) and NaCl (0.15 M) in RO H_2O, pH 9. Blocking buffer consisted of NFDM (3%) in PBS-9. Assay buffer consisted of TRIZMA Hydrochloride (0.0726 M), TRIZMA Base (0.0275 M), NaCl (0.15 M), Tween 20 (0.0025%), and NFDM (0.005%) in RO H_2O, pH 7.75. Ab2 buffer consisted of 2% NFDM in assay buffer.

HPLC and cELISA Standards. An HPLC stock standard of Hal−HBr was prepared in 0.25 M ammonium acetate, pH 4.3. HPLC standards were prepared from the stock standard by adding HPLC mobile phase. HPLC fortification standards were prepared from the HPLC stock standard by adding HPLC grade water. A cELISA stock standard of Hal−HBr was prepared in 0.35 M ammonium acetate, pH 4.3. cELISA standards were prepared by using a 1:20 dilution of the stock standard with 0.35 M ammonium acetate buffer followed by an appropriate dilution with assay buffer to obtain a final Hal−HBr concentration of 2 ng/100 μL. cELISA fortification standards were prepared from the cELISA stock standard by diluting with RO H_2O.

Chicken Liver Extraction Method for cELISA. Chicken liver (5 g) was weighed out into a silanized KIMAX culture tube. Assay buffer (10 mL) was added to the tube and the liver homogenized using a Polytron for 1 min. The mixture was centrifuged for 10 min (g-force = 180) using a Clay Adams Dynac centrifuge (Thomas Scientific, Swedesboro, NJ). The supernatant was decanted into a 50 mL polypropylene conical tube. A second aliquot of assay buffer (10 mL) was added to the pellet and resuspended with the Polytron for 30 s. After a second centrifugation for 10 min (g-force = 180), the supernatant was combined with the first supernatant. The combined supernatant was centrifuged using a 15 mL polypropylene conical tube in a GT-2, MSE MINOR centrifuge (Sanyo Gallenkamp, Tasca, IL) for 15 min (g-force = 4750) to remove more solid material. This supernatant was diluted 1:20 with assay buffer (0.5 mL extract + 9.5 mL assay buffer) and used directly in the cELISA.

cELISA. The cELISA methods have been previously described (Rowe et al., 1994). Briefly, 96-well microtiter plates were washed with 0.05% Tween-20 in RO H_2O and rinsed with RO H_2O. The initial washing step was used because it increased the uniformity of the results. The plates were then coated by the addition of a Hal−BSA conjugate (120 ng conjugate in 100 μL carbonate buffer/well and stored overnight at 4 °C). Following the overnight incubation the plates were washed with 0.05% Tween-20 and rinsed with RO H_2O, and the nonreacted binding sites on the plates were blocked for 60 min at room temperature with blocking buffer (300 μL/well). The blocking buffer was removed from the wells and briefly washed with 0.05% Tween-20, rinsed with RO H_2O, and then incubated for 1 h at 37 °C with 100 μL samples of diluted chicken liver extract (a 1:20 dilution with assay buffer). Other wells on the same plate contained 100 μL diluted control chicken liver extract (extract of chicken liver that did not contain Hal) that served as the no competitor controls (100%) in assay buffer. Two standard curves of Hal−HBr in assay buffer starting at 2 ng/well (2-fold dilutions of Hal−HBr were made across the plate) and the appropriate negative control (background) were run on the same plate. Hal 37/1 antibody in assay buffer (22.5 ng/100 μL) was placed in all wells except for the negative controls. After the 1 h incubation, the plates were washed with 0.05% Tween-20 and rinsed with RO H_2O, and 100 μL of goat anti-mouse peroxidase conjugate (1:500 in Ab2 buffer) was added to each well. Following a second 1 h incubation at 37 °C, the plates were washed with 0.05% Tween-20 and rinsed with RO H_2O. K-Blue substrate (100 μL/well) was added to each well and incubated at room temperature for 20 min. The developed blue color was read without stopping at 655 nm.

cELISA Calculations. The results obtained from each plate were stored and processed on a computer with Microsoft Excel (Microsoft Corp., Redmond, WA) followed by final analysis with a four parameter curve fit using the software PeakFit (Jandel Scientific, San Rafael, CA). The standard

curves on each plate were entered into the PeakFit program as percent inhibition vs Hal−HBr per well, and the interpolated results were reported as nanogram of Hal−HBr per well. The "working range" of the standard curves was defined with a lower and upper limit of quantitation as 20 and 70% inhibition, respectively (Karu et al., 1991), and values outside that limit were not used. The final result was calculated using the following equation:

$$V(\text{ng}/100\ \mu\text{L})(20/1)(22.4\ \text{mL})(1000\ \mu\text{L}/1\ \text{mL})/(5\ \text{g}) = 896V\ \text{ppb} \quad (1)$$

where V was the value obtained from the program PeakFit in nanograms per well; 100 μL was the amount of sample placed in the well; 20:1 was the dilution of the extract, 22.4 mL was the average total volume of a 5 g chicken liver sample extracted with 20 mL of assay buffer, and 5 g was the total chicken liver sample that was extracted.

HPLC Solutions and Conditions. The mobile phase consisted of acetonitrile:0.25 M ammonium acetate (pH 4.3): H_2O (5:3:12 (v/v/v)) at pH 4.3 ± 0.05 (pH was adjusted with glacial acetic acid). The HPLC system flow rate was 1.2 mL/min. The column temperature was 40 °C, and the injection volume was 25 μL. The variable wavelength detector was set at 243 nm.

Extraction Method for HPLC. The extraction procedure used was the official FSIS method for halofuginone, and it and the required materials are found in-total in the FSIS halofuginone determinative method (Anon., 1991b). Briefly, food processor homogenized chicken liver (20 g) was placed into a 200 mL centrifuge bottle. Simultaneously, a 100 ppb recovery was performed by the addition of a 1 mL fortification standard solution to a 20 g blank chicken liver tissue sample. H_2O (10 mL) and trypsin (500 mg) were added to each sample, and the pH was adjusted to between 8 and 8.5 by addition of a 10% sodium carbonate solution. The samples were incubated in a 40 °C water bath for 3 h and cooled to room temperature. A 10% sodium carbonate solution (10 mL) was added to the samples and mixed. Ethyl acetate (100 mL) was added, and the samples were macerated for 3 min with a tissuemizer. They were centrifuged for 2 min at 10 °C and 2000 rpm. The ethyl acetate was decanted into a 500 mL separatory funnel via a vacuum suction apparatus. The steps starting with the addition of the ethyl acetate were repeated, and the second volume of ethyl acetate was added to the first extract. Salt-saturated sodium carbonate solution (5% (w/v), 50 mL) was added, and the mixture was shaken vigorously for 1 min. The aqueous layer was discarded. Ammonium acetate buffer (0.125 M, 50 mL) was added to the ethyl acetate extracts, and they were shaken for 1 min. The ammonium acetate layer (lower layer) was transferred into a 250 mL separatory funnel. The ammonium acetate buffer step was repeated, and the second ammonium acetate extract was added to the first extract. The ethyl acetate layer was discarded. Ethyl acetate (10 mL) was added to the ammonium acetate buffer and washed gently for 10 s. The ammonium acetate layer (lower layer) was transferred into a 250 mL round-bottom flask and evaporated to remove all remaining ethyl acetate from the buffer solution. The remaining ammonium acetate was transferred into a volumetric flask (100 mL) and brought up to volume with 0.125 M ammonium acetate buffer solution. The solution was filtered through Whatman GF/F filter paper, and the first few milliliters of the filtrate was discarded. Some of the filtrate (20 mL) was passed through a prewashed Bond Elute C_{18} cartridge and washed with water (3 mL). Hal was eluted from the cartridge with MeOH (10 mL) into a conical 15 mL polypropylene centrifuge tube. With a gentle stream of nitrogen, the sample was evaporated to 1−2 mL and passed through a Millex HV 0.45 filter unit. The unit was rinsed with a small amount of methanol, and the sample was evaporated to dryness. The residue was dissolved with 400 μL of mobile phase and was ready for HPLC analysis.

HPLC Calculations. The HPLC derived values were compared to a Hal standard curve in micrograms per milliliter. The result from the standard curve was corrected for dilution with the following formula:

$$(V\ \mu\text{g/mL})(100\ \text{mL}/20\ \text{g})(400\ \mu\text{L}/20\ \text{mL})(1\ \text{mL}/1000\ \mu\text{L}) = (V) \times 1/10\ \text{ppm} \quad (2)$$

where V was the value obtained from the standard curve in micrograms per milliliter. For each 20 g of sample, the extract from that sample was taken to 100 mL total volume. A 20 mL aliquot of that sample was then processed down to 400 μL in mobile phase that was then ready for HPLC analysis. Since the standard curve was made with the same injection volume as was used during the sample analysis, the injection volume did not enter into the calculation.

Evaluation of the cELISA by Analyzing Hal-Spiked Chicken Liver Tissue. Hal free chicken liver tissue was spiked with cELISA fortification standards to produce samples at 47.8, 95.6, 191.2, 286.8, and 382.4 ppb Hal−HBr. These samples (in quadruplicate) were extracted with the chicken liver extraction method for cELISA and evaluated using the cELISA.

Study 1: Spiked Chicken Liver Tissue Analysis by HPLC and cELISA. Chicken liver was weighed out into 5 and 20 g samples. The 5 g samples were used for the cELISA methods, and the 20 g samples were used for the HPLC extraction method. Both sets of samples were spiked with cELISA fortification standards to provide each set with two samples at each level of Hal−HBr at 89.6, 149.4, and 209.1 ppb and two controls. All samples were frozen at −72 °C, and the FSIS 20 g samples were shipped overnight on dry ice for processing at the FSIS laboratory. Both sets of samples were processed starting on the same day.

Incurred Residues. Broilers (White Rock Cross), Hubbard × Peterson, were obtained at 1 day of age and fed unmedicated chicken feed for 3 weeks. They were then placed on feed consisting of unmedicated chicken feed to which Stenorol Premix had been added with mixing to provide a level of 3 ppm Hal−HBr. Liver tissue was taken from five groups of eight broilers each that were fed ad libitum for 10 days on unmedicated or Hal−HBr treated feed. Four groups were taken off Hal−HBr treated feed at 2, 6, 24, and 96 h prior to sacrifice. One group (controls) was fed only untreated feed and was taken off feed 2 h prior to sacrifice. The liver tissues were frozen (−72 °C) immediately after collection.

Study 2: Spiked Chicken Liver Tissue and Incurred Residue Analysis by HPLC and cELISA. *Part 1.* Chicken liver was weighed out into 5 and 20 g samples. The 5 g samples were used for the cELISA methods, and the 20 g samples were used for the HPLC extraction method. Both sets of samples were spiked with cELISA fortification standards to obtain levels of Hal−HBr at 100, 160, and 180 ppb. Some of the samples were not spiked and were used for controls.

Part 2. Chicken liver tissue from Hal incurred studies obtained at 2, 6, 24, and 96 h as well as chicken liver tissue from birds fed on Hal−HBr free feed for controls were thawed and immediately weighed out into 5 and 20 g samples. All samples used for the two methods of this study were frozen at −72 °C immediately after weighing, and the FSIS 20 g samples were shipped overnight on dry ice for processing at the FSIS laboratory. Both sets of samples were processed starting on the same day.

RESULTS AND DISCUSSION

Chicken liver samples were spiked and then extracted as described under Materials and Methods. Table 1 presents recoveries of Hal obtained from the spiked chicken liver tissue after extracting it with assay buffer followed by cELISA analysis. The results were evaluated using both a Hal−HBr standard curve produced in assay buffer and a comparable curve generated in control chicken liver extract. The spike levels bracketed the tolerance level of Hal−HBr in chicken liver, which is at 160 ppb. The Hal−HBr standard curve made in chicken liver extract gave recovery results that were closer to the 100% values and, in general, had better

Table 1. Recovery of Hal after Extraction and Analysis by cELISA from Hal-Spiked Chicken Liver Tissue

	recovery[a] (%)	
spike (ppb)	buffer[b]	extract[c]
47.8	85.7 ± 8.1	95.3 ± 7.4
95.6	106.9 ± 11.6	92.8 ± 8.8
191.2	97.9 ± 8.0	101.7 ± 8.3
286.8	95.1 ± 4.9	99.8 ± 4.8
382.4	104.7 ± 0.6	92.6 ± 0.5

[a] Each data point is the mean of four samples. [b] Data obtained from a standard curve made in assay buffer. [c] Data obtained from a standard curve made in chicken liver extract (1:30 dilution).

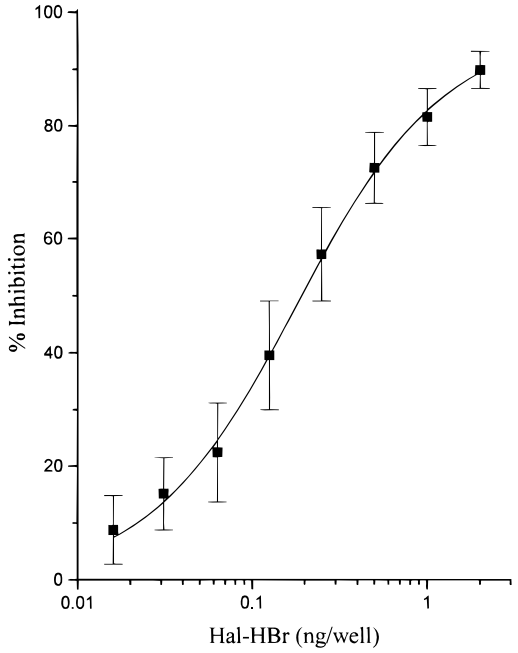

Figure 2. Average of 60 standard curves in assay buffer for the cELISA over a 12 day period.

standard deviations than the standard curve made in assay buffer. The line (not shown) generated from the data in Table 1 derived from the standard curve in assay buffer had an equation of (Hal recovered) = −0.00268 + 0.998 (Hal spike) with a coefficient of determination = 0.996. Therefore, the results using a Hal standard curve made in assay buffer are quite good and suggest that the assay using standards prepared in assay buffer can readily be used to quantify Hal−HBr levels. Due to the ease of use, the remaining experiments reported in this study used Hal−HBr standard curves made in assay buffer.

A series of Hal−HBr standard curves were run over a 12 day period, and an average of those curves is shown in Figure 2. This composite curve made from 60 standard curves shows that these curves were consistent from day to day and run to run. Due to the small variations in these curves, two standard curves were run on each 96-well plate during the analysis of samples, and the samples on that plate were compared to the average of those two standard curves.

The results from study 1, analysis of spiked chicken liver tissue, are summarized in Table 2. The recovery is shown for both the HPLC method and cELISA method for Hal−HBr in these chicken liver tissues. The recoveries obtained by the cELISA method were consistently higher than those obtained by the HPLC method. To view the correlation between HPLC and cELISA results, Figure 3 shows a graphical comparison

Table 2. Recovery of Hal−HBr (Study 1) from Hal−HBr-Spiked Chicken Liver Tissue Analyzed by HPLC and cELISA

	HPLC		cELISA	
spike (ppb)	ppb	% recovery	ppb	% recovery
89.6	65.0	72.5	76.7 ± 18.1	85.6 ± 20.2
89.6	69.0	77.0	81.9 ± 35.9	91.4 ± 40.1
149.4	115.0	77.0	154.6 ± 35.5	103.5 ± 23.8
149.4	118.0	79.0	117.4 ± 23.2	78.6 ± 15.5
209.1	157.0	75.1	183.4 ± 13.7	87.7 ± 6.5
209.1	157.0	75.1	179.9 ± 15.5	86.0 ± 7.4
control			*a*	
control				

[a] These controls were used for the no competitor controls (100%) in the cELISA.

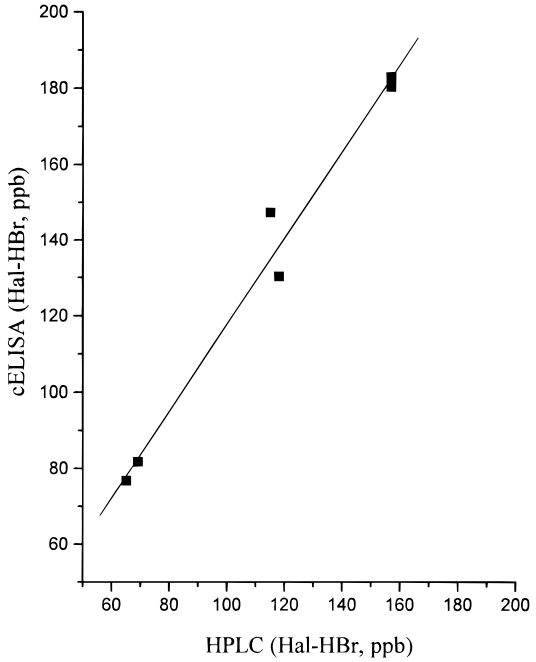

Figure 3. Correlation of HPLC vs cELISA results for Hal-spiked chicken liver tissue in study 1 (Table 2).

of the data from the two methods. Even though the recoveries were higher with the cELISA method, which differs greatly from the complex extraction method required for HPLC analysis, the results obtained from the two methods were consistent, as seen in Figure 3 having a linear equation of (Hal cELISA) = 4.281 + 1.136 (Hal HPLC) with a coefficient of determination = 0.989.

In a second set of experiments, study 2, Hal-spiked chicken liver tissue and tissues with incurred residues were analyzed by the HPLC method and by the cELISA method. These results are shown in Table 3. In study 2, a smaller range of Hal concentrations was used in order to bracket the tolerance level of 160 ppb. In two out of the three spike levels the cELISA again gave higher recoveries. However, the recoveries observed remained in general agreement for both methods.

The second part of study 2 was the analysis of incurred levels of Hal−HBr in chicken liver tissue by both the HPLC and cELISA methods. Table 4 shows the results of the analysis of chicken liver tissue after the birds were removed from Hal−HBr treated feed. There is good agreement in the results for all withdrawal times except the 24 h time period. For this time point the HPLC method gave a significantly higher value for the level of Hal−HBr than did the cELISA

Table 3. Recovery of Hal−HBr (Study 2, Part 1) from Hal−HBr-Spiked Chicken Liver Tissue Analyzed by HPLC and cELISA

	HPLC[a]		cELISA[a]	
spike (ppb)	ppb	% recovery	ppb	% recovery
100	82.2 ± 3.4	82.2 ± 3.4	90.9 ± 29.7	90.9 ± 29.7
160[b]	126.0 ± 6.5	78.8 ± 4.1	99.8 ± 12.0	62.4 ± 7.5
180	146.0 ± 7.0	81.1 ± 3.9	151.7 ± 20.7	84.4 ± 11.5
control	ND[c]		<38[d]	

[a] Each data point is the mean of four samples. [b] Tolerance level in chicken liver tissue. [c] ND = nondetected. [d] cELISA lower quantitation limit.

Table 4. Analysis of Incurred Levels of Hal−HBr from Chicken Liver Tissue by HPLC and cELISA (Study 2, Part 2)

time (h)	HPLC[a] (ppb)	cELISA[a] (ppb)
2	698.5 ± 100.3	921.6 ± 196.3
6	1031.2 ± 114.0	1018.2 ± 36.6
24	333.0 ± 80.1	200.5 ± 24.6
96	<50[b](16) ± (4.0)	<38[c](30) ± (12.2)
control	ND[d]	<38

[a] Each data point is the mean of four samples. [b] The HPLC lower reporting limit. [c] cELISA lower quantitation limit. [d] ND = nondetected.

method. However, the standard deviations obtained from the cELISA were much better, at both the 6 and 24 h time periods, than those of the HPLC method. The 96 h sample result from the HPLC method is in parentheses because it is lower than the normal reporting limit (50 ppb) of the HPLC method, and the result from the cELISA is in parentheses because it is below the cELISA lower quantitation limit (38 ppb). The cELISA lower quantitation limit is defined as 20% inhibition (Karu et al., 1991), and a 20% inhibition is at 38 ppb Hal−HBr in the cELISA. Technically, the 96 h points for either method are a nondetect. To view the correlation between HPLC and cELISA results, the data from the spiked samples (study 2, part 1) were combined with the data from the incurred samples (study 2, part 2), and are shown in Figure 4 as cELISA vs HPLC data. Figure 4 shows a linear fit of the data of (Hal cELISA) = −16.486 + 1.08 (Hal HPLC) with a coefficient of determination = 0.970. The results from the two studies compare favorably, especially in the region close to the tolerance level. During monitoring for Hal by FSIS the typical levels of Hal detected in samples range from 50 to 100 ppb.

CONCLUSIONS

This study demonstrates that the cELISA method described here gives comparable results with the HPLC method. In addition, the cELISA method has a greatly simplified sample preparation. Higher recoveries were usually obtained using the cELISA method, which may reflect the more simplified sample preparation method used with the cELISA.

Even though we were comparing totally different extraction methodologies and detection schemes, we observed good correlation between both methods. However, the cELISA method is faster and a much simpler method and also requires less technical expertise. In addition, to perform the cELISA method does not require the use of organic solvents, and this is environmentally friendly. Finally, the cELISA method, because of its simple extraction method, has a high throughput

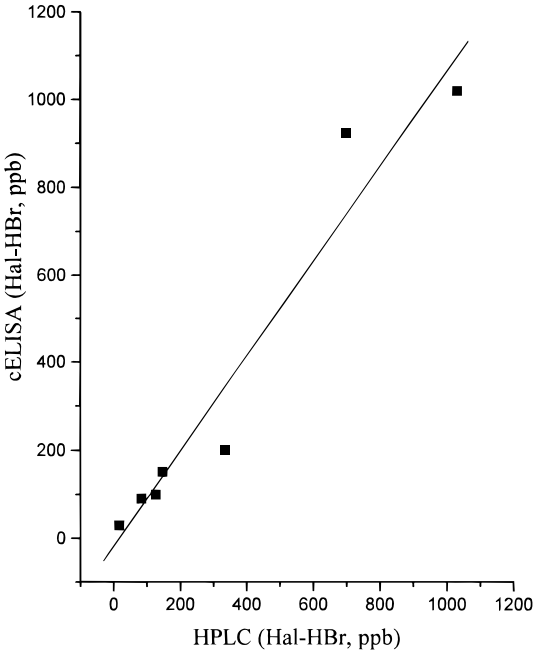

Figure 4. Correlation of HPLC vs cELISA recovery results for levels of spiked Hal−HBr residues (study 2, part 1) and levels of Hal−HBr incurred residues (study 2, part 2) in chicken liver tissue. Each data point is the mean of four samples (Tables 3 and 4).

of samples and could be used as a screening method for Hal in samples of chicken liver tissue. The work presented here clearly demonstrates that specific monoclonal antibodies can be developed that work effectively and the immunoassay can have a place in residue detection.

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