Production and Characterization of Monoclonal Antibodies against the Poultry Coccidiostat Halofuginone

Loyd D. Rowe, Ross C. Beier,* Marcel H. Elissalde, and Larry H. Stanker

Food Animal Protection Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Route 5, Box 810, College Station, Texas 77845-9594

A succinyl derivative of halofuginone (Hal) was covalently linked to keyhole limpet hemocyanin to provide an immunogen for production of anti-Hal antibodies. This resulted in six hybridoma cell lines producing Hal-specific monoclonal antibodies (Mabs) of moderate to high affinity. All 6 Mabs were highly specific for Hal relative to 12 compounds with similar structural components. Fifty percent inhibition of control values (IC_{50}) for the Mabs ranged from 0.19 to 12.0 ng of Hal hydrobromide/well. One Mab (IC_{50} = 0.19 ng/well) was used to develop a competitive enzyme-linked immunosorbent assay (cELISA) for Hal in chicken serum. Interference due to serum matrix effects was effectively eliminated by 4-fold dilution of the sample with assay buffer. The minimum detectable amount of Hal was 0.05 ng/well. Analysis of Hal-fortified serum (6.25–50 ppb) showed excellent agreement (92–100%) with the amount of Hal added.

INTRODUCTION

Halofuginone (Hal, 1, Figure 1) is an antiprotozoal drug used worldwide to prevent coccidiosis in commercial poultry production (McDougald, 1990). The drug is a halogenated analog of a quinazoline alkaloid, febrifugine, originally isolated from the ancient Chinese antimalarial drug Ch'ang Shan (roots of the shrub *Dichroa febrifuga*, Lour.) (Openshaw, 1953; Cheng, 1976). In the United States, Hal hydrobromide (Stenorol, Hoechst-Roussel Agri-Vet Co., Somerville, NJ) is approved for use in feed at 3 ppm for the prevention of coccidiosis in broiler chickens and growing turkeys (Sundlof *et al.*, 1992). Halofuginone is also effective in the treatment of protozoal infections in cattle caused by *Theileria parva parva* (east coast fever) and *Theileria annulata* (Mediterranean coast fever) (Kinabo *et al.*, 1989). The current regulatory method for the determination of Hal residues in poultry tissues (USDA, 1991; Brown, 1992) is similar to previously published methods (Anderson *et al.*, 1981; Analytical Methods Committee, 1984) and involves the measurement of parent Hal in liver by HPLC. This procedure is not amenable to high sample throughput primarily due to a complex sample preparation scheme. Kinabo *et al.* (1989) developed a sensitive method for the determination of Hal in bovine plasma by competing-ion HPLC after solid-phase extraction; however, application of the method to plasma from small avian species appears limited due to a large (4 mL) sample size requirement. Because antibody-based assays are established as capable quantitative analytical procedures for food contaminants (*e.g.*, mycotoxins and drugs) and have reduced requirements for sample preparation, sample size, equipment, and use of organic solvents relative to physicochemical procedures (Haagsma and van der Water, 1992; Lee *et al.*, 1992; Gazzaz *et al.*, 1992; Nugent, 1992), development of an immunochemical method to measure Hal residues in poultry tissues appears appropriate. In this study, an attempt was made to produce Hal-specific antibodies useful for this purpose. Our approach involved the production of monoclonal antibodies (Mabs) to provide a continuous, expandable source of standardized reagent. Six hybridoma cell lines eliciting high- to moderate-affinity Mabs were obtained. Details of production and characterization of these antibodies are reported in this paper. Results obtained with fortified chicken serum indicate that a competitive enzyme-linked immunosorbent assay (cELISA) based on one of these Mabs is capable of detecting and quantifying Hal at the low parts per billion level in a relevant biological matrix.

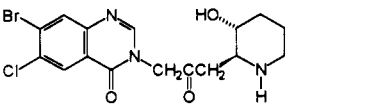


Figure 1. Structures of halofuginone (1) and the succinyl derivative Hal-suc (2) used as the immunizing hapten.

MATERIALS AND METHODS

A. Materials. Bovine serum albumin (BSA, RIA grade), keyhole limpet hemocyanin (KLH), goat anti-mouse IgG (whole molecule), horesradish peroxidase conjugate (A-5278, lots 40H8850 and 22H8880), polyoxyethylene sorbitan monolaurate (Tween 20), hypoxanthine–aminopterin–thymidine media supplement (HAT), hypoxanthine–thymidine media supplement (HT), sterile filtered dimethyl sulfoxide (DMSO), protein G affinity gel (P-3296), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), Tris-HCl, Trizma Base, amodiaquin dihydrochloride, chloroquine diphosphate, emetine dihydrochloride, primaquine diphosphate, quinacrine dihydrochloride, and quinine were purchased from Sigma Chemical Co. (St. Louis, MO). A solution containing penicillin G, 10 000 units/mL, and streptomycin sulfate, 10 000 μg/mL, was purchased from Life Technologies, Inc. (Grand Island, NY). *N,N*-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), and silanizing fluid (AquaSil) were obtained from Pierce Chemical Co. (Rockford, IL). Polyethylene glycol 4000 was a product of Fluka Chemie AG (Buchs, Switzerland). Dialysis membrane tubing (1200–1400 molecular weight cutoff) was obtained from Scientific Products (McGaw Park, IL). Quinazoline, 2-mercapto-4(3*H*)-quinazolinone, 2,3-dihydro-2-[2-(2-pyridyl)ethyl]-1,4-phthalazinedione,

* Author to whom correspondence should be addressed [(409) 260-9411].

6-chloro-2,4(1*H*,3*H*)-quinazolinedione, 4,6-dichloroquinazoline, 3-[2-(4-pyridyl)ethyl]-4(3*H*)-quinazolinone, 3-phenyl-4(3*H*)-quinazolinone, and 2,6,10,14-tetramethylpentadecane (pristane) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). *N,N*-Dimethylformamide (DMF, chromatography grade) was purchased from EM Industries, Inc. (Gibbstown, NJ). Powdered milk (35.3% protein) was a product of Albertson's Inc. (Boise, ID). Deionized water was produced using a Milli-RO4 water purification system (Millipore Corp., Bedford, MA). Halofuginone hydrobromide analytical standard (Roussel-Uclaf, lot 6L1321) was provided by Hoechst-Roussel Agri-Vet Co. (Somerville, NJ) and determined to be 100.8% pure by elemental analysis determinations. All other chemicals and organic solvents used were of reagent grade or better. Sterile plastic plates and culture flasks used for hybridoma cell culture were manufactured by Becton Dickinson & Co. (Lincoln Park, NJ). Microtiter plates used for immunoassays were obtained from CoStar Corp. (Cambridge, MA).

B. Preparation of Immunogen. A Hal derivative having a succinyl linker was synthesized from Hal hydrobromide as described by Rowe *et al.* (1993). Briefly, *N*-(trimethylsilyl)-imidazole was used to protect the Hal hydroxyl group during reaction of the piperidyl nitrogen with succinic anhydride. Removal of the trimethylsilyl moiety and regeneration of the hydroxyl was accomplished by acid hydrolysis to afford the required derivative, 7-bromo-6-chloro-3-[3-(1-succinyl-3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3*H*)-quinazolinone [144902-96-5] (Hal-suc, **2**, Figure 1). The succinyl derivative (**2**) was conjugated to KLH and BSA *via* an NHS-enhanced, carbodiimide-mediated coupling reaction described by Staros *et al.* (1986). The derivative **2** (51.2 mg, 0.0997 mmol), NHS (22.5 mg, 0.195 mmol), and DCC (39.2 mg, 0.19 mmol) were added to 500 μL of DMF and stirred for 1.5 h at room temperature followed by stirring for 18 h at 4 °C. The reaction mixture was filtered through glass wool, and the filtrate (200 μL) was added to a solution of protein (BSA or KLH, 25.0 mg) initially dissolved in 500 μL of distilled water (adjusted to pH 9 with NaOH) followed by the addition of 4.5 mL of DMF. The resulting mixture was stirred at room temperature for 2 h and then at 4 °C for 18 h. The mixture was then dialyzed at 4 °C against decreasing concentrations of DMF in deionized water (initial DMF concentration 60%) followed by three times against deionized water. All dialysis solutions were adjusted to pH 8.5. Conjugates were frozen at −70 °C in 0.5-mL aliquots.

C. Screening ELISA. Anti-hapten activity in mouse serum and hybridoma supernatants was monitored by a screening ELISA. Assay buffer (pH 7.75) used in the screening ELISA consisted of 0.1 M Tris, 0.15 M NaCl, 1% BSA, and 0.05% Tween 20 in deionized water. In general, 96-well plastic microtiter plates were coated by the addition of Hal-suc–BSA (0.1 μg in 100 μL of distilled water adjusted to pH 8.5) to each well followed by drying at 38 °C in a food dehydrator (Excalibur, Sacramento, CA) for 18 h. Following drying the nonreacted binding sites on the plates were blocked for 30 min at room temperature with a 3% solution of egg albumin in deionized water (pH 9, 300 μL/well). The blocking solution was removed from the wells (plates were not washed) and then incubated for 1 h at 37 °C with 50 μL/well mouse serum (diluted 10-fold with assay buffer) or hybridoma culture supernatant. The plates were then washed with a solution of 0.05% Tween 20 in deionized water, and 50 μL of goat anti-mouse peroxidase conjugate (1:500 in assay buffer) was added to each well. Following a second 1-h incubation at room temperature, the plates were washed, and 100 μL of 0.1 M citrate buffer (pH 5.5) containing ABTS (0.8 mM) and hydrogen peroxide (0.001%) was added to each well. After incubation for 60 min at room temperature, the absorbance (405 nm) of each well was measured (Bio-Tec Model EL309, Bio-Tec Instruments, Winooski, VT).

D. Production of Antibodies. *1. Immunization.* The immunogen was prepared for injection by emulsification of 1 mg of Hal-suc–KLH and 2 mL of sterile isotonic saline with the contents of one vial of RIBI adjuvant system (RIBI Immunochem Research Inc., Hamilton, MO). Five, 1-month-old, female BALB/c mice (Harlan Sprague-Dawley, Indianapolis, IN) were given Hal-suc–KLH by intraperitoneal injection (100 μg, 200 μL) at 111, 91, and 68 days prior to fusion and both intraperitoneal (67 μg, 134 μL) and intramuscular injections (33 μg , 66 μL) at 50 and 4 days prior to fusion. Fifty-six days before fusion, each mouse was tranquilized (4 μg of fentanyl and 200 μg of droperidol by subcutaneous injection) and bled from the orbital plexus using heparinized microhematocrit tubes. Plasma was obtained by centrifugation of the microhematocrit tubes at 13400*g* for 5 min. Anti-hapten titers were determined using the screening ELISA described above.

2. Culture Media. Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing $NaHCO_3$ (35 mM), glutamine (2.9 mg/mL), 2–4% fetal calf serum (Hazelton Biologics, Inc., Lenexa, KS), penicillin G (20 units/mL), and streptomycin sulfate (20 μg/mL) was used for culture of SP2/0 myeloma cells prior to fusion and for growth of hybridomas during Mab production. This medium, with the addition of HAT (hypoxanthine, 200 μM; aminopterin, 0.8 μM; thymidine, 32 μM) was used for hybridoma cultures immediately after fusion; thereafter, HT (hypoxanthine, 100 μM; thymidine, 16 μM) was added as needed through cloning by limiting dilution. Abdominal macrophages from pristane-treated BALB/c mice were added to the medium (2×10^5 cells/mL) to support hybridoma growth from fusion through cloning.

3. Fusion and Cloning. On the day of fusion, the mouse having the highest polyclonal titer against Hal-suc–BSA was sacrificed by cervical dislocation and the spleen removed aseptically. Lymphocytes obtained from this spleen (1.2×10^8 cells) were fused with SP2/0 myeloma cells (1.6×10^8 cells) under conditions described by Stanker *et al.* (1986). Ten to 12 days after fusion, supernatants from the hybridoma cultures were screened for the presence of hapten-specific antibodies using the screening ELISA described above. Hybridoma cultures from wells showing the strongest competitive (Hal-specific) response were subcloned twice by limiting dilution. Cultures from each of the six monoclonal cell lines were preserved by freezing in liquid N_2 (Liddell and Cryer, 1991). All six cloned cell cultures were expanded to provide supernatants as a source of Mab. Prior to isolation of Mabs, supernatants were preserved with sodium azide (0.02%) and stored at 5 °C.

E. Purification of Antibodies. Monoclonal antibodies were isolated from cell culture supernatants by affinity chromatography using Sepharose-conjugated protein G at a flow rate of 1 mL/min. A protein G-containing column was preconditioned by 0.02 M phosphate buffer (pH 7) followed by passage of 100 mL of supernantant (pH 7). The column was then washed with 0.02 M phosphate buffer, and IgG was eluted using 0.1 M glycine hydrochloride buffer (pH 2.7). Detection and collection of the Mab-containing fraction was accomplished by on-line measurement of UV absorption (280 nm) of the column effluent. The resulting solutions of Mab were immediately brought to pH 7 with 2.0 M Tris base in deionized water and dialyzed against phosphate-buffered saline (PBS, pH 7.0) at 5 °C for 48 h. Protein concentration of the Mab solutions was estimated by measurement of UV absorption at 280 nm (Harlow and Lane, 1988). Sodium azide (0.02%) was added as a preservative, and the Mab solutions were stored in 0.5-mL aliquots at −70 °C.

F. Characterization of Antibodies. *1. Isotype and Affinity.* Antibody (IgG) isotypes were determined using reagents supplied in a commercially available kit (SBA Clonotyping System 1, lot L041-1071, Fisher-Biotec, Pittsburgh, PA) according to directions of the manufacturer. Relative affinity of the six Mabs for Hal was measured by determination of the 50% inhibition of control values (IC_{50}) for each at an antibody dilution producing 40% of plateau activity in the absence of competitor.

2. Antibody Specificity. Because Hal has no structural similarity to currently used coccidiostats, and a series of closely related analogs for use in this study was unavailable, a structural fragment-based computer search of available organic compounds (Aldrich Chemical Co., Inc., Milwaukee, WI) was performed in an attempt to identify compounds useful for study of cross-reactivity. As a result of this search, seven compounds were selected for study: quinazoline, 4,6-dichloroquinazoline, 2-mercapto-4(3*H*)-quinazolinone, 3-[2-(4-pyridyl)ethyl]-4(3*H*)-quinazolinone, 3-phenyl-4(3*H*)-quinazolinone, 6-chloro-2,4(1*H*, 3*H*)-quinazolinedione, and 2,3-dihydro-2-[2-(2-pyridyl)ethyl]-1,4-phthalazinedione. Chloroquine diphosphate and four related antimalarial agents, primaquine diphosphate, amodiaquin dihydrochloride, quinacrine dihydrochloride, and quinine, were

Table 1. Compounds Examined for Cross-Reactivity with Mabs Produced against Halofuginone[a]

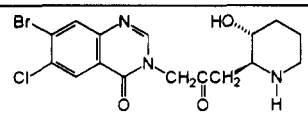
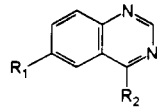
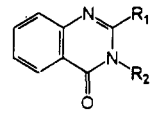
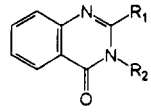
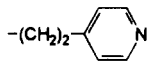
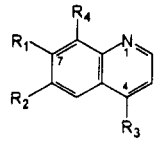
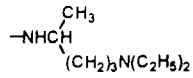
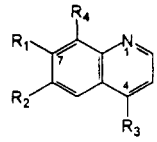
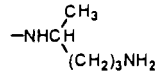
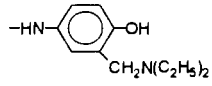
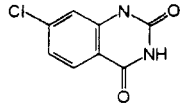
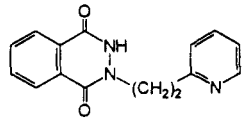
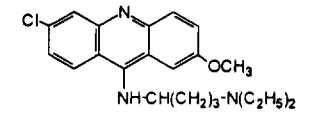
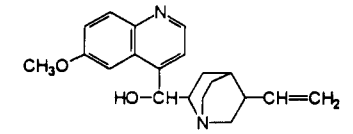
compound	max concn used (ng/well)	structure			
halofuginone	-----				
					
		R_1	**R_2**	**R_3**	**R_4**
quinazoline[b]	3442	–H	–H	------	------
4,6-dichloroquinazoline[b]	1000	–Cl	–Cl	------	------
					
		R_1	**R_2**	**R_3**	**R_4**
2-mercapto-4(3H)-quinazolinone[b]	1200	–SH	–H	------	------
					
		R_1	**R_2**	**R_3**	**R_4**
3-(2-(4-pyridyl)-ethyl-4(3H)-quinazolinone[b]	2855	–H		------	------
3-phenyl-4(3H)-quinazolinone[b]	2535	–H	$-C_6H_5$	------	------
					
		R_1	**R_2**	**R_3**	**R_4**
chloroquine	3160	–Cl	–H		–H
					
		R_1	**R_2**	**R_3**	**R_4**
primaquine	2765	–H	$-OCH_3$	–H	
amodiaquine	2965	–Cl	–H		–H
6-chloro-2,4(1H,3H)-quinazolinedione[b]	1250				
2,3-dihydro-2-(2-(2-pyridyl)-ethyl)-1,4-phthalazinedione[b]	2805				

Table 1 (Continued)

compound	max concn used (ng/well)	structure
quinacrine	3175	
quinine	2650	

[a] For all compounds other than Hal, inhibition in the cELISA was minimal or absent up to the maximum concentration used and in all cases insufficient to provide an IC_{50} value. [b] These compounds were selected for study by means of a structural fragment-based computer search based on halofuginone.

also included on the basis of the reported structural similarity of chloroquine with the naturally occurring Hal analog, febrifugine (Cheng, 1976). Compounds to be studied were dissolved in assay buffer and examined as potential competitors using the cELISA described below. Structural information regarding these compounds is presented in Table 1.

G. Competitive ELISA. A competitive inhibition ELISA (cELISA) was developed to evaluate antibody specificity and to quantitate Hal in solution. The protocol was similar to the screening ELISA described above except that each microtiter well was coated with 0.07 μg of Hal-suc–BSA and a constant amount of antibody (50 μL of hybridoma culture supernatant or purified antibody diluted in assay buffer to 40% of plateau activity as determined in a ELISA without competitor) together with 50 μL of sample or Hal standard was added. Assay buffer used in the cELISA differed from that used in the screening ELISA in that 1% powdered milk was substituted for BSA. Powdered milk is an economical protein-rich product that equally substitutes for the more expensive BSA. Hal stock standard solution (200 μg/mL) was prepared in silanized glassware by dissolving an appropriate quantity of Hal hydrobromide in 0.25 M ammonium acetate buffer (pH 4.3) and stored at 5 °C. Fresh stock solutions of Hal were prepared every 6 weeks. A working solution for production of Hal standard curves in the cELISA was made on the day of assay by the dilution of an appropriate volume of Hal stock solution in assay buffer using silanized glassware. Working standards contained 0.1–0.6 μg of Hal hydrobromide/mL (5–30 ng/50 μL). Duplicate standard curve determinations for Hal were included on each microtiter plate used. Dosage–inhibition curves were obtained by plotting percent inhibition $[(1 - B/B_0) \times 100]$ *vs* the amount of competitor present (B_0 represents the absorbance measured without competitor added to the antibody and B the absorbance measured with various concentrations of competitor). Percent inhibition values ($I_{\%}$) and estimates of Hal in 50-μL samples were calculated from curves obtained by a fit of cELISA data from a series of known Hal concentrations to the logistic function model using a nonlinear curve fitting computer program (PeakFit, Jandel Scientific, San Rafael, CA).

H. Analysis of Hal-Fortified Serum. Blood was obtained via the jugular vein from 12 1-year-old white Leghorn hens maintained on custom-prepared feed to which no drugs were added since day 1 of age at our laboratory. Serum was harvested from the clotted blood by centrifugation and held at −20 °C. The possibility of a matrix effect due to the addition of chicken serum to the assay was investigated by inclusion of various dilutions of serum (without Hal) from each hen and from one commercial source (Sigma Chemical Co.) in the cELISA procedure.

Fresh fortification standard (2 μg of Hal/mL) was prepared every 14 days by dilution of Hal stock standard (see above) with deionized water in silanized glassware. Serum from nine hens was fortified by the addition of an appropriate volume of fortification standard to produce a Hal concentration of 50.0 ppb and was allowed to stand at room temperature for 1 h. Serum samples from each hen containing 25.0, 12.5, and 6.25 ppb were produced by serial dilution (1:1) of the 50.0 ppb sample with blank serum from the same hen and then diluted 4-fold with assay buffer to reduce serum matrix effects. The content of Hal in fortified samples (50 μL) was measured using the cELISA as described above; assay results on individual samples were the mean of 5 replicate wells.

RESULTS AND DISCUSSION

A. Preparation of Monoclonal Antibodies. Ten days after fusion, growing hybridomas were observed in 97% of the 1824 wells seeded. At this time, supernatant from 90% of the wells showed anti-hapten activity using the screening ELISA described above. Hybridoma cultures from the 96 wells with the highest OD readings were selected for further evaluation. Because the immunogen was Hal-suc–KLH, antibodies should not bind to BSA. Therefore, binding produced by supernatants in the screening ELISA (which utilized Hal-suc–BSA as the antigen) was assumed to involve an epitope associated with Hal-suc (2). Supernatant from 19 of the previously selected 96 hybridomas showed diminished binding to the Hal–BSA conjugate when free Hal hydrobromide was present, and the 9 hybridoma cell cultures producing the most competitive supernatants (lowest I_{50} values) were chosen for further development. Selection of clones from these cultures by limiting dilution led to six stable hybridoma cell lines. These monoclonal cultures and their corresponding Mabs were named Hal-16, Hal-18, Hal-37, Hal-52, Hal-53, and Hal-64.

B. Characterization of the Antibodies. *1. Isotype and Affinity.* Immunoglobulin isotypes of the anti-Hal Mabs were found to include $IgG1_\lambda$ (Hal-37), $IgG2a_\kappa$ (Hal-52, Hal-53, Hal-64), and $IgG3_\kappa$ (Hal-16, Hal-18). The concentrations of Hal hydrobromide resulting in 50% inhibition of control activity (*i.e.*, wells with no competitor present) were 0.19, 0.22, 1.0, 1.7, 2.4, and 12.0 ng/well for Mabs Hal-37, Hal-16, Hal-18, Hal-52, Hal-64, and Hal-53, respectively. Dosage–inhibition response curves in assay buffer for Mabs with the lowest (Hal-37) and highest (Hal-53) IC_{50} values are shown in Figure 2.

2. Specificity. Cross-reactivity studies with all six antibodies yielded similar results. For all candidate cross-reacting compounds examined, inhibition of the cELISA response was absent or only weakly present at the maximum concentration used (Table 1); in no case was inhibition sufficient to calculate an IC_{50} value. Weak reaction at maximum concentration was observed for 4,6-dichloroquinazoline, 2-mercapto-4(3*H*)-quinazoline, and 6-chloro-2,4(1*H*,3*H*)-quinazolinedione with Hal-53, Hal-64, and Hal-52. Each of the three chemicals tested had

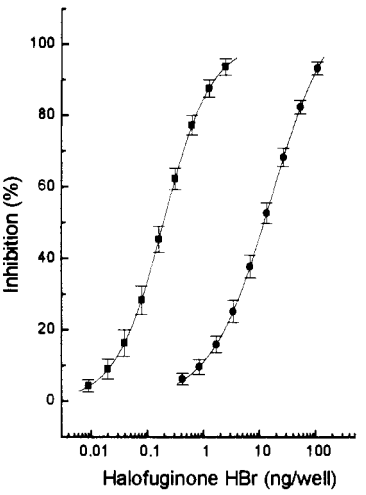


Figure 2. Inhibition of antibody binding by halofuginone hydrobromide in the cELISA with BSA–hapten-coated plates. Shown are dosage–inhibition curves for Hal-37 (■) (IC_{20}, IC_{50}, IC_{80} = 0.05, 0.19, and 0.70 ng/well, respectively) and Hal-53 (●) (IC_{20}, IC_{50}, IC_{80} = 2.4, 12.3, and 49.6 ng/well, respectively). Error bars represent ±1 SD of 24 assays for Hal-37 and 12 assays for Hal-53.

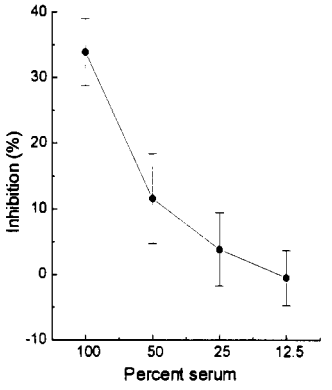


Figure 3. Inhibitory effect of chicken serum on the cELISA procedure (no Hal added) using Mab Hal-37. Serum was added neat or diluted in assay buffer. Solid circles (●) represent the mean, and error bars represent ±1 SD of sera from 12 drug-free hens and 1 commercial source.

equivalent cross-reactivities with the reacting antibodies, resulting in cross-reactivity estimates of <1.2%, <0.2%, and <0.2%, respectively, for each antibody. Cross-reactivity estimates for Hal-16, Hal-18, and Hal-37 were less than 0.08% for all compounds tested.

C. Analysis of Hal-Fortified Sera. Hal-37 was selected for development of a cELISA for analysis of Hal-fortified serum on the basis of its high affinity (low IC_{50} value), relative steepness of slope of its dosage–inhibition curve, growth rate, and hardiness of the monoclonal cell culture. The minimum detectable amount of Hal, defined here as the lower limit (20% inhibition of control or IC_{20}) of the linear portion of the curve, was 0.05 ng/well (Figure 2).

A matrix effect resulting in inhibition of color development was found upon addition of high concentrations of Hal-free serum to the assay. Undiluted Hal-free sera from 12 hens and 1 commercial source (Sigma Chemical Co.) produced 34 ± 5% (mean ± SD) inhibition of the cELISA response relative to assay buffer. This effect was essentially eliminated by 8-fold dilution of the sera with assay buffer (Figure 3). Of the 13 sera studied, nonspecific inhibition was highest (44%) for undiluted serum from the commercial source. However, inhibition by this serum

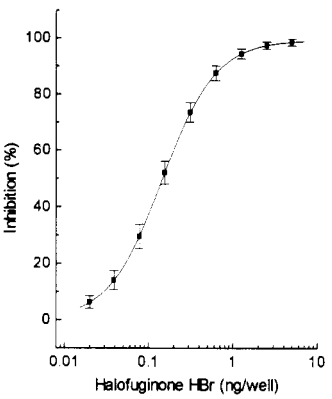


Figure 4. Inhibition of Mab Hal-37 binding by halofuginone hydrobromide in the cELISA using assay buffer containing 25% chicken serum (IC_{20}, IC_{50}, IC_{80} = 0.05, 0.15, and 0.41 ng/well, respectively). Solid squares (■) are means, and error bars represent ±1 SD of 24 assays.

Table 2. Determination of Halofuginone (Hal) in Fortified Chicken Sera (N = 9 Hens) by cELISA Using Mab Hal-37

	cELISA	
Hal-HBr fortification (ppb)	ppb[a] (%)[b]	SD (CV)[c]
6.25	6.16 (98.6)	0.32 (5.2)
12.5	12.2 (97.6)	0.32 (2.6)
25.0	25.0 (100.0)	1.76 (7.0)
50.0	46.2 (92.4)	6.08 (13.2)

[a] Calculated according to standards made of halofuginone hydrobromide in assay buffer containing 25% chicken serum. Result for serum of each hen is the average of five determinations. [b] The percentage of halofuginone found by cELISA is defined as [(ppb measured by cELISA after addition/ppb added) × 100]. [c] SD, standard deviation; CV, coefficient of variation (average of nine sera).

was within ±1 SD of the mean of all sera at all other dilutions. Because 4-fold dilution of sera was sufficient to allow ODs in the range 0.5–0.6 when no Hal was present, assessment of cELISA performance was conducted using Hal-fortified sera diluted 4- rather than 8-fold to limit loss of assay sensitivity.

In initial experiments with fortified sera, Hal standard curves were produced in both assay buffer and assay buffer containing 25% commercial chicken serum. Use of the former was abandoned because Hal standard curves using 25% serum gave improved agreement between fortification level and cELISA results. This was found to be due to an increase in the slope of the Hal standard curve when assay buffer was replaced by assay buffer containing 25% serum. This increase in slope was found to be statistically significant ($P < 0.001$) by application of the t-test for comparison of two slopes (Zar, 1984) to concurrently generated data from the linear portion of Hal dosage–inhibition curves obtained with assay buffer of each type (N = 24 per Hal level per curve). This change in slope was reflected by a lowering the IC_{80} and IC_{50} values from 0.70 and 0.19 to 0.41 and 0.15 ng of Hal/well, respectively. IC_{20} values for both curves remained at about 0.05 ng of Hal/well (Figures 2 and 4). The mechanisms involved in these serum-induced matrix effects are not understood, but it appears that one or more of the following may be possible: (1) the presence of serum (at least at the level of 25%) may increase binding of the first antibody with free Hal in a concentration-dependent manner, (2) serum proteins may block the first antibody in some way from binding the hapten–conjugate coating and, (3) levels of serum ranging from 12 to 100% may result in decreased binding of the second antibody with the first antibody.

Analysis of Hal-fortified sera from nine hens produced excellent agreement between fortification level (6.25–50.0

ppb) and cELISA results (Table 2). The average percentage of Hal found by cELISA [(ppb measured by cELISA/ppb added) × 100] over all levels of fortification ranged from 92.4 to 100%. Coefficients of variation (CV) for assay results between sera from individual birds ranged from 2.6 to 13.2% over all levels of fortification. These results indicate that this Mab-based cELISA is sensitive and selective and constitutes a valuable method for detection of Hal in chicken serum down to the low parts per billion level.

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