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Sherry Cox
University of Tennessee, Knoxville, scox6@utk.edu

Marcy Souza
University of Tennessee, Knoxville, msouza@utk.edu

Joan Bailey
University of Tennessee, Knoxville, jbaile15@utk.edu

Molly White
University of Tennessee, Knoxville, molswhit@utk.edu

Krissten Gordon
University of Tennessee, Knoxville, csf916@vols.utk.edu

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Determination of Meloxicam in Egg Whites and Yolks Using Reverse Phase Chromatography

Sherry Cox*, Joan Bailey, Molly White, Kristen Gordon, and Marcy Souza

Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA

*Author to whom correspondence should be addressed. Email: scox6@utk.edu

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Abstract

A new method of analysis has been developed and validated for the determination of meloxicam in egg whites and yolks. Following a liquid extraction for the whites and a solid phase extraction for the yolks, samples were separated on an XBridge C_{18} column and quantified using ultraviolet detection at 360 nm. The mobile phase was a mixture of water with glacial acetic acid and acetonitrile, with a flow rate of 1 mL/min. The procedure produced a linear graph over the concentration range 5–1500 ng/mL with a lower limit of quantification of 5 ng/mL. Intra- and inter-assay variability was 10% or less for both the whites and yolks. The average recovery for whites was 96% and the average recovery in yolks was 97%.

Introduction

With the increasing popularity of backyard poultry ownership, veterinarians are faced with how to treat these animals with regard to drug withdrawal times for egg consumption. Veterinary drugs and feed additives can be absorbed by the digestive tract of laying hens and transferred to the egg. Physicochemical characteristics of these compounds determine their pharmacokinetic behavior and distribution to and within eggs. A recent review describes allowable medications for use in egg-laying poultry; however, few drugs have recommended doses or withdrawal times for use in laying hens (1).

Meloxicam (4-hydroxy-2-methyl-*N*-(5-methyl-1, 3-thiazol-2-yl)-1, 1-dioxo-1λ^6, 2-benzothiazine-3-carboxamide) is a non-steroidal anti-inflammatory drug (NSAID) that is commonly used in avian medicine. NSAIDs are generally used to treat painful conditions such as injuries, cancer surgery and dental infections. Meloxicam preferentially inhibits the cyclooxygenase-2 (COX-2) enzyme over COX-1. The ability to only inhibit the inflammatory COX-2 proved to be revolutionary for pain management. The introduction of COX-2 preferential NSAIDs has reduced stomach and intestinal side effects. A commercially liquid form is readily available and is easily administered to birds. A recent publication from Marmulak *et al.* (1) reported that the Food Animal Residue Avoidance Databank received more requests for egg withdrawal intervals for hens following meloxicam administration than for any other drug (1). Although one study examined meloxicam in chickens following intravenous administration (2), no published pharmacokinetic studies are available to aid in recommendations regarding dosing or withdrawal times for oral meloxicam administration.

Meloxicam levels in plasma have been determined using a number of analytical methods (2–17). Although mass spectrometry (3, 4, 12, 16) can produce the highest detection sensitivity, it may not be readily available in all laboratories and is expensive. The most economical method is high performance liquid chromatography with ultraviolet detection (HPLC–UV). In order to obtain a suitable limit of quantification (LOQ) for the determination of meloxicam in biological samples, several different processes have been used. Some of the methods employed include liquid–liquid extractions using diethyl ether (2, 5, 7, 9, 14), protein precipitation (6, 8, 15), pre-column enrichment (10, 11, 17) and solid phase extraction (13). To the authors' knowledge, there are no methods for the determination of meloxicam in eggs. Therefore, the aim of this paper was to describe a sensitive, specific, and accurate method for determining meloxicam in both egg whites and yolks using HPLC.

Experimental

Instrumentation and reagents

The chromatography system consisted of a 2695 separation module and a 2487 ultraviolet detector (Waters, Milford, MA). Separation was achieved on a Waters XBridge C_{18} column (4.6 × 250 mm, 5 µm) preceded by a 5 µm XBridge C_{18} guard column (3.9 × 20 mm). The mobile phase was an isocratic mixture of A: 990 mL water with 10 mL 85% glacial acetic acid, pH 3.0 with 1 M sodium hydroxide and B: acetonitrile. This was mixed at a ratio of 50% A and 50% B throughout the entire run. All solutions were filtered through a 0.22 µm filter and degassed before their use. The water was replaced on a daily basis. The flow rate was 1.0 mL/min, and the column and autosampler temperature were ambient which was 23°C. The ultraviolet detector was set at a wavelength of 360 nm.

Meloxicam (Figure 1) was purchased from Toronto Research Chemicals (Ontario, Canada) and was 99% pure. Piroxicam (Figure 1), which was the internal standard (IS) (99% purity), was purchased from US Pharmacopeia (Rockville, MD, USA). All reagent grade chemicals and solvents were purchased from Fisher Scientific (Pittsburg, PA, USA). Water (18.2 MΩ) was obtained from a Barnstead Nanopure Infinity (Dubuque, IA, USA) ultrapure water system.

Preparation of calibration standards

Five milligrams each of meloxicam or piroxicam were weighed using a Sartorius Microbalance (Elk Grove, IL, USA) and dissolved in methanol to produce stock concentrations of 100 µg/mL. Dilutions of the meloxicam and piroxicam stock standards were prepared in methanol to produce 0.1, 1, 5 and 10 µg/mL working stock solutions. Standards were aliquoted into 2-mL vials to prevent evaporation and cross contamination. All solutions were protected from light in bottles wrapped in aluminum foil and stored at −20°C. By comparing standard areas over time, it was determined that solutions were stable for a minimum of 6 months.

For preparation of calibration standards and quality control samples, appropriate volumes of stock solutions were placed in screw top tubes and evaporated with nitrogen gas then untreated egg white or yolk was added.

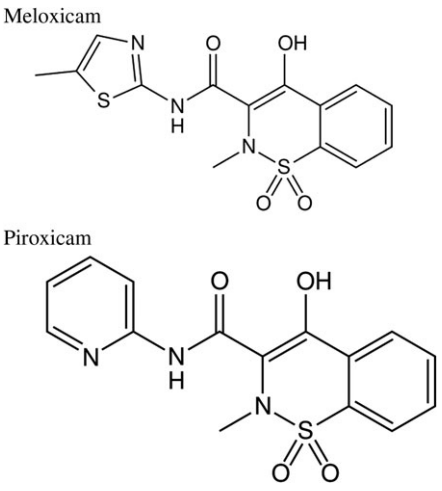

Figure 1. Structures of meloxicam and piroxicam.

Sample preparation

Meloxicam was extracted from egg whites using a modified version of the plasma method developed by Cox *et al.* (18). The white and yolk were separated then 1 mL of white was placed in a 15 mL screw cap tube. An aliquot of 75 µL of piroxicam (5 µg/mL) was added followed by 1 mL of 1 M hydrochloric acid (HCl) and 5 mL of chloroform. The tubes were vortex-mixed at high speed for 60 s followed by centrifugation for 20 min at 1000 × *g*. The supernatant was removed and placed in a 16 × 100 mm glass test tube (15 mL capacity) and evaporated to dryness with nitrogen. Samples were reconstituted in 300 µL of mobile phase, vortex-mixed and the supernatant was placed in total recovery chromatographic vials then 100 µL injected into the system.

After separation from the white, 1 mL of yolk was placed in a 15 mL screw top tube. An aliquot of 75 µL of piroxicam (5 µg/mL) was added followed by 200 µL of 1 M HCl and 2 mL of acetonitrile. The tubes were vortexed for 30 s followed by centrifugation for 20 min at 1000 × *g*. The supernatant was removed and placed in a 16 × 100 glass test tube. The extraction process on the yolk protein pellet was repeated and the supernatants were combined, and then evaporated until roughly 100 µL remain. Methanol (100 µL) and water (900 µL) were added to the tube and vortexed for 10 s. The tubes were centrifuged for 20 min at 1000 × *g*. The supernatant was removed and added to a prewet (1 mL methanol, 1 mL water) Oasis HLB 3 cc extraction cartridge (Waters, Milford, MA, USA). The cartridge was washed with 5% methanol in water and the sample was eluted with 2 mL of acetonitrile water (90:10, v/v). The eluent was evaporated to dryness with nitrogen and reconstituted in 300 µL of mobile phase, vortex-mixed and the supernatant was placed in total recovery chromatographic vials, then 100 µL injected into the system.

Method validation

The method was validated according to the Guidelines for Bioanalytical Method Validation published by the Food and Drug Administration (19). Validation of the method was carried out using QC samples. All of the QC samples and calibration curves were prepared in egg white and yolk matrices. The validation process looked at accuracy, precision, selectivity, sensitivity, reproducibility and stability.

Selectivity

Selectivity was determined by injecting blank egg whites and yolks from six different chickens to confirm no interfering peaks around the retention time of both meloxicam and piroxicam, the IS.

Calibration curve, linearity and quality control samples

The final concentrations for the calibration standard curve were 5, 10, 25, 50, 100, 250, 500, 800, 1000 and 1500 ng/mL. The calibration curve was constructed by using the ratio of the peak area of the analyte divided by the peak area of the IS versus the concentration and obtained on five different days. Linearity was assessed by linear regression analysis and expressed as the coefficient of determination (r^2). The standard deviations of the slope, intercepts and regression coefficient were calculated. The QC samples were prepared in a similar manner as the calibration standards at four different levels, 15, 75, 350 and 1200 ng/mL. The acceptance criterion for each back-calculated standard was 15% deviation from the nominal value except lower (LLOQ), which was set at 20%.

Accuracy, precision and recovery

The precision and accuracy of the assay were determined using QC samples of known meloxicam concentrations (15, 75, 350 and 1200 ng/mL), which were processed freshly each validation day. Five replicates of each QC were analyzed during the same day and on five different days, and the intra- and inter-assay means, standard deviation (SD) and coefficient of variation (CV) were calculated. Recoveries were calculated as the measured concentrations divided by the expected concentrations and expressed as a percentage (19). Equations for accuracy, precision and recovery are listed below.

Accuracy equation: (Measured concentration/added concentration) × 100%

Precision equation: Absolute standard deviation (SD):

$$SD = \frac{\sqrt{\sum(x-\bar{x})^2}}{(n-1)},$$

relative standard deviation (RSD) or coefficient of variation (CV):

$$RSD\ (\%) = (SD/\bar{x})100$$

Recovery equation: $(X/\mu) \times 100\%$,

where, X = measured concentration and μ = added concentration.

Results

Selectivity

Endogenous components from either the white or yolk did not interfere with the elution of the compounds of interest. Six different blank whites and yolks were used in the pre-validation process. Figure 2 shows chromatograms of a (A) blank white, (B) a 75 ng/mL spiked white standard, (C) a white sample from a chicken 1 day after 1 mg/kg oral meloxicam administration, (D) a blank yolk, (E) a 350 ng/mL spiked yolk standard and (F) a yolk sample 3 days after 1 mg/kg oral meloxicam administration. Retention times were 7.7 min for meloxicam and 6.0 min for piroxicam.

Calibration curves, precision, accuracy and linearity

The egg white and yolk peak area ratio (area of meloxicam divided by IS area) versus concentration was plotted which produced a linear curve for the concentration range used (5–1500 ng/mL) with the correlation coefficients ranging from 0.9993 to 0.9996. The mean slopes, intercepts and r^2 values are reported in Tables I and III. A typical linear equation for the egg white calibration curve was y = 0.0022x −0.0071 while a typical curve for the egg yolk calibration curve was y = 0.0023x + 0.0050 where y represents the peak area ratios of meloxicam to IS and x represents the concentration of meloxicam in nanograms per milliliter.

All values of accuracy and precision were within the recommended limits. Intra-assay precision ranged from 5.1 to 8.3% for egg whites and 4.9 to 9.0% for egg yolks. The inter-assay precision ranged from 3.2 to 6.4% for whites and 5.7 to 10.1 for yolks. These precision values are well below the set ±15% for all quality control samples as shown in Tables I through IV. The intra-assay accuracy ranged from 96 to 100% for whites and 100–106% for yolks. The inter-assay accuracy for whites ranged from 93 to 100% and for yolks 100 to 106%.

Recovery, LLOQ and stability

The recovery of meloxicam was determined by comparing the peak areas of extracted analytes with that of the directly injected standard solutions. The average recovery of meloxicam from the four egg white QC concentrations ranged from 95–98% while the average recovery from egg yolks ranged from 94 to 98%. The average recovery of piroxicam was 93 and 96% from egg whites and yolks, respectively.

The LLOQ was 5 ng/mL for both egg whites and yolks, which represents a peak approximately five times baseline noise.

Testing of short-term stability of the quality control standards for egg whites indicated that there was a 2% loss of drug after 24 h in the autosampler and no loss after 24 h in the refrigerator at 4°C. For egg yolks there was no loss of meloxicam after 24 h in the autosampler and a 5% loss after 24 h in the refrigerator. Samples in our studies were not frozen. The eggs were collected and then stored in the refrigerator until analysis which was within 10 days. On the day of analysis, the white and yolk were separated and analyzed on the same day, then both egg white and yolk samples were stored at −80°C. After one freeze–thaw cycle there was an average meloxicam loss of 30% in egg yolks and a 7% loss in egg whites.

Discussion

We have developed and validated the first method for quantification of meloxicam in both egg whites and yolks using reverse phase separation and UV detection. Various methods have been developed for the determination of meloxicam in plasma but to the authors' knowledge none were available for eggs.

Having previously developed and validated a method for meloxicam analysis in plasma (18), that method was applied to both egg whites and yolks with modifications. The application of the method to egg whites proved successful while the application to the yolks was not. The average recovery from whites was 96% while the recovery from yolks was approximately 10%. The method for egg whites was modified by increasing the sample size since sample quantity was not an issue with eggs. The 1:1 ratio for sample and hydrochloric acid produced the greatest recovery of meloxicam from plasma therefore the same ratio was applied to the egg white samples. Two different volumes of chloroform were initially tried; 8 and 5 mL. The recovery for the two volumes used were equal therefore 5 mL was used. Any volume <5 mL resulted in a decrease in the recovery.

Since the chloroform/hydrochloric acid extraction was not successful in extracting meloxicam from the yolks, other organic solvents and mixtures were used including acetonitrile, methanol, perchloric acid:chloroform, formic acid:acetonitrile, perchloric acid: acetonitrile and methanol:chloroform. All were found to produce either lower recoveries or poor peak resolution. Solid phase extraction was then applied to the yolk samples using Oasis PRiME HLB (Waters, Milford, MA, USA) cartridges; however, the recovery using these cartridges was less than 20% even with varied wash and elution volumes and solutions. Oasis HLB cartridges were then used and the hydrophilic–lipophilic balanced copolymer enabled high recoveries for both meloxicam and piroxicam from egg yolks. Once an appropriate cartridge was selected, we also looked at various wash solutions including 2.5, 5 and 10% methanol. The 10% solution caused a decrease in recovery and the 2.5% solution produced

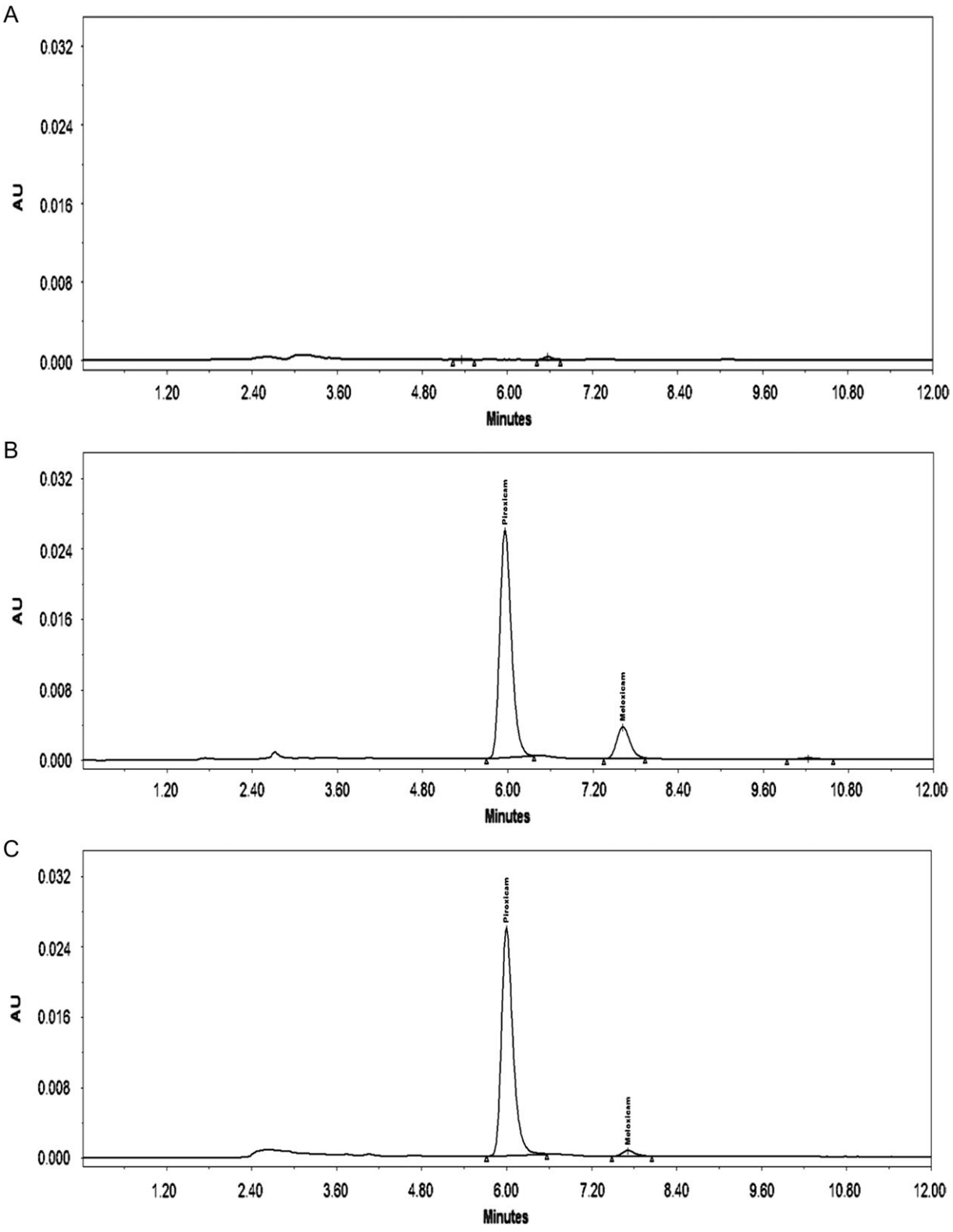

Figure 2. Chromatograms for a (A) blank egg white, (B) an egg white standard spiked with 75 ng/mL meloxicam and IS, (C) an egg white sample from a chicken 1 day after a 1 mg/kg oral meloxicam administration, (D) a blank egg yolk, (E) an egg yolk standard spiked with 350 ng/mL of meloxicam and IS and (F) an egg yolk sample 3 days after a 1 mg/kg oral meloxicam administration.

interfering peaks. The use of 2 mL of wash solution also decreased the recovery of meloxicam; therefore 1 mL of wash solution was used. The elution composition of 90:10 acetonitrile:methanol was part of a standard protocol recommended by the manufacturer which worked well for the yolk application and was not varied. The amount of hydrochloric acid and acetonitrile used in the

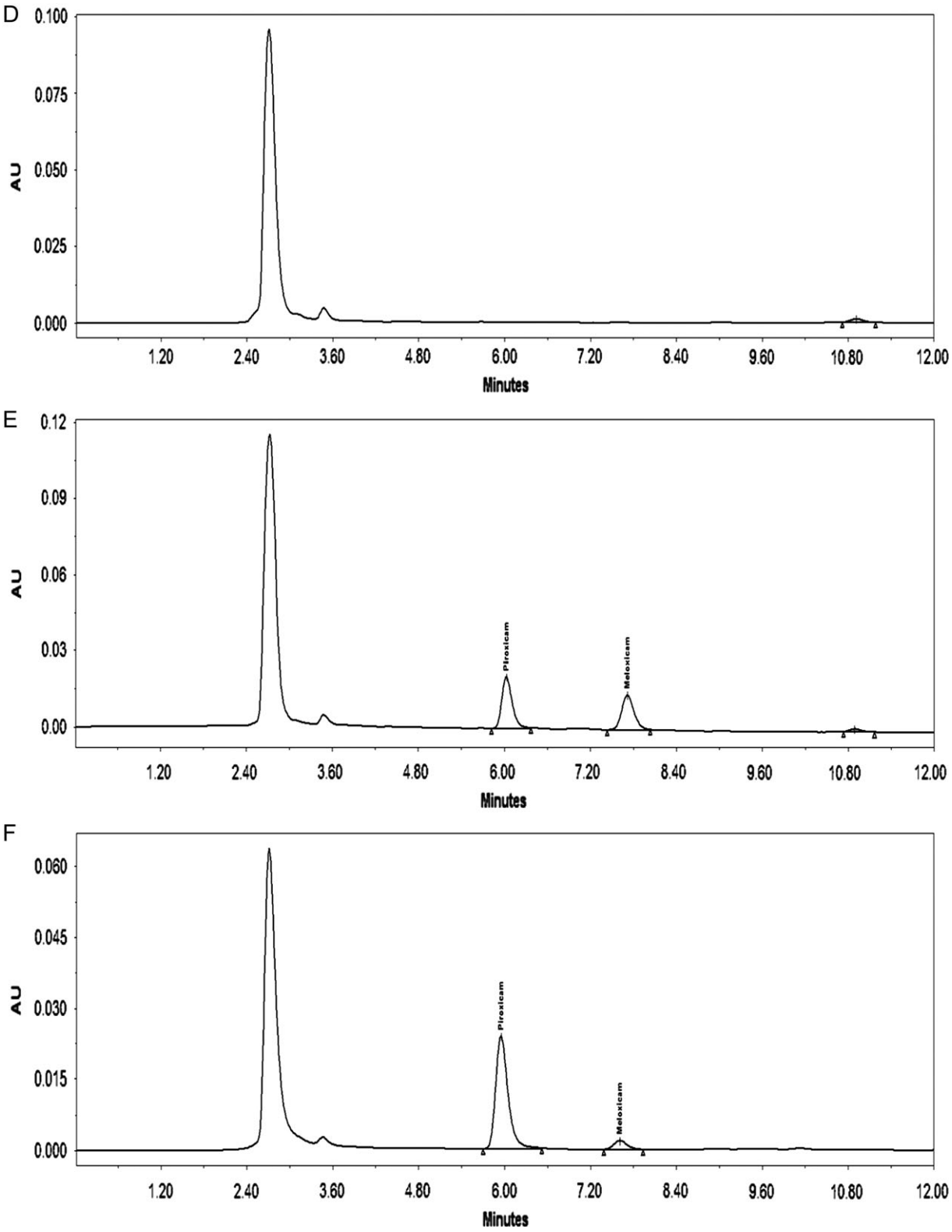

Figure 2. Continued

protein precipitation phase were also optimized. If no acid was used the meloxicam recovery was less than 10%. We looked at the addition of 200, 300 and 400 µL amounts of 1 M HCl and found that there was very little change in meloxicam recovery; therefore, we chose 200 µL. The amount of acetonitrile used in the precipitation phase was also varied and the amounts ranged from 2 to 4 mL. There was very little change in the recovery of meloxicam thus we chose 2 mL.

Table I. Intra-Assay Accuracy, Precision and Assay Linearity for Meloxicam in Egg Whites

Intra-assay variability ($n = 5$)			
Concentration added (ng/mL)	Concentration measured (ng/mL) (mean ± SD)	RSD (%)	Accuracy (%) (mean ± SD)
15	15 ± 1	6.6	100 ± 9
75	72 ± 6	8.3	96 ± 7
350	350 ± 18	5.1	100 ± 5
1,200	1201 ± 74	6.2	100 ± 5
Assay linearity ($n = 5$)			
	Mean ± SD	RSD (%)	
Y-intercept	−0.0071 ± 0.0010	14.5	
Slope	0.0022 ± 0.0000	0.5	
r^2	0.9993 ± 0.0002	0.02	

n: number of samples.

Table II. Inter-Assay Variability and Recovery for Meloxicam in Egg Whites ($n = 5$)

Concentration added (ng/mL)	Concentration measured (ng/mL) (mean ± SD)	RSD (%)	Accuracy (%) (mean ± SD)	Recovery (%)
15	14 ± 1	6.4	93 ± 5	95
75	77 ± 3	3.9	103 ± 3	96
350	351 ± 17	4.8	100 ± 4	98
1200	1201 ± 38	3.2	100 ± 3	97

n: number of days.

Table III. Intra-Assay Accuracy, Precision and Assay Linearity for Meloxicam in Egg Yolks

Intra-assay variability ($n = 5$)			
Concentration added (ng/mL)	Concentration measured (ng/mL) (mean ± SD)	RSD (%)	Accuracy (%) (mean ± SD)
15	16 ± 1	8.1	106 ± 8
75	77 ± 4	4.9	103 ± 5
350	350 ± 31	9.0	100 ± 9
1200	1210 ± 93	7.7	101 ± 7
Assay linearity ($n = 5$)			
	Mean ± SD	RSD (%)	
Y-Intercept	0.0050 ± 0.0006	12.4	
Slope	0.0023 ± 0.0000	1.11	
r^2	0.9996 ± 0.0003	0.03	

n: number of samples.

The recovery and LOQ are more than adequate for use in the determination of meloxicam in eggs. There is a 2% or less, sample loss in whites and 5% or less sample loss in yolks after storage in either the autosampler or refrigerator; therefore, if there were a power or equipment failure, samples could be reanalyzed. The addition of piroxicam as an IS allows for the correction of intra- and inter-assay variability.

Table IV. Inter-Assay Variability and Recovery for Meloxicam in Egg Yolks ($n = 5$)

Concentration added (ng/mL)	Concentration measured (ng/mL) (mean ± SD)	RSD (%)	Accuracy (%) (mean ± SD)	Recovery (%)
15	16 ± 1	10.1	106 ± 8	94
75	76 ± 6	7.9	101 ± 8	98
350	353 ± 21	5.9	101 ± 5	98
1200	1204 ± 68	5.7	100 ± 5	98

n: no. of days.

Conclusion

This is the first method developed for the analysis of meloxicam in egg whites and yolks. More people are choosing to keep live poultry as a way to locally produce food in a humane manner. Veterinarians are faced with how to treat these animals with regard to drug withdrawal times for egg consumption, thus the need for analytical methodology to aid in determining these times. This analytical procedure was validated in terms of recovery, linearity, LLOQ, precision and accuracy. Our results indicate the method is sensitive, accurate and reproducible, providing consistent quantification of meloxicam. This method has been used successfully to determine meloxicam in both egg whites and yolks in two studies conducted at this institution (20) (Souza *et al.*, submitted for publication). In the studies egg concentrations of meloxicam following a single oral dose and following oral dosing at 1 mg/kg every 12 h for a total of nine doses (5 days) were determined. Meloxicam was detected in egg whites up to 4 days and in egg yolks up to 8 days after single dosing. While drug was detected in egg whites up to 3 days and in egg yolks up to 8 days after multiple doses. Based on these results a 2-week withdrawal time should be adequate to avoid drug residues in eggs meant for consumption.

Conflict of Interest Statement

None declared.

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