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Determination of mifepristone levels in wild canid serum using liquid chromatography

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Abstract

An HPLC method was developed to determine levels of mifepristone, in coyote (*Canis latrans*) serum where mifepristone will be used as an oral contragestive agent for nonlethal predator control. Serum samples were extracted using C_{18} solid-phase extraction cartridges. A synthetic analog of mifepristone, RTI-3021-003, was used as the internal standard. Separation of the compounds was achieved by using a C_{18} (150×4.6 mm) column. The mobile phase was 55% acetonitrile in water running at 1.0 ml/min with UV detection at 305 nm. The assay was linear in the range of 10 to 1000 ng/ml. Inter-day accuracies for 10, 200 and 1000 ng/ml were 95.9, 99.4 and 104.7%, respectively. Inter-day precisions measured by RSD were 19.8, 9.7 and 4.5%. Intra-day accuracies were 117, 106.9 and 99.4% for 10, 200 and 1000 ng/ml, respectively. Intra-day RSDs were 19.7, 3.7 and 9.3%, respectively. A simple, sensitive and validated HPLC analytical method was developed to quantitate mifepristone in canine serum.
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1. Introduction

In the early 1980s, researchers at the pharmaceutical company Rochelle Uclaf (Paris, France) published the initial paper describing the antiprogestin mifepristone [1]. Synthesis of this compound created a new field of interest in reproductive endocrinology and made possible new approaches in the study of reproductive physiology. Use of hormone antagonists for medical and scientific purposes has increased since the introduction of these synthetic progesterone anti-hormone compounds [2]. Since mifepristone was the first compound developed in the category of progesterone antagonists it has become the benchmark by which all other antiprogestins are evaluated [3,4]. Mifepristone has been used in a variety of species as a competitive inhibitor of progesterone, the principal hormone necessary for maintenance of pregnancy, thereby terminating pregnancy. After its introduction and clinical investigation, numerous papers detailing dose, clinical application, and corresponding levels of success following a single oral dose have been published [5]. When used in conjunction with other compounds, such as prostaglandins, the success rate for complete expulsion of the conceptus approaches 100% [5].

The overall focus of this project has been to

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develop a protocol using mifepristone in coyotes to terminate pregnancy, effectively controlling reproduction. Progesterone production is required throughout pregnancy in canines with the corpus luteum producing progesterone independent of higher support (leuteotropic hormones) until day 18 of pregnancy [6]. It was necessary to develop a method for determining quantitative levels of mifepristone in coyote serum, to determine the correlation between oral dose and the level required for biological effect. In preliminary tests it was determined that the compound was absorbed from the gastrointestinal tract and that it could be detected in serum following administration. When mifepristone was administered to domestic canines, 5 mg/kg/day in two divided doses at 12 h intervals for 4–5 days, termination of pregnancy occurred without changes in general health, appearance or behavior [7,8]. No details regarding method of analysis or the rate of metabolism, bioavailability, or pharmacokinetics in canine matrices for mifepristone was reported.

Previous publications indicate that both high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) methods have been used to analyze mifepristone in human serum. The RIA method mentioned by Wang et al. is nonspecific for mifepristone and its metabolites [9]. The antiserum for mifepristone highly cross-reacted with at least two of its metabolites. Also, RIA requires the use of radioactive materials and the complications of handling, storage cost and waste disposal arises. There is also the disadvantage of long incubations and costly preparation of immunoglobulins.

The references available on HPLC methods [7,12] analyzing mifepristone both used human serum as the matrix for analysis. The HPLC method described by Heikinheimo et al. is a cumbersome method using Chromosorb column chromatography followed by HPLC separation [7]. The method was not validated and the sensitivity of the assay was poor (limit of detection 40 ng/ml and limit of quantification 250 ng/ml). The other HPLC method developed by Chang-hai et al. [12] was a liquid–liquid extraction procedure with UV detection. The sensitivity of the method was also poor (limit of detection 36 ng/ml and limit of quantification 240 ng/ml).

No specific plasma or serum data relating to mifepristone for canine species have been published. Since our objective is to use mifepristone as a reproductive control compound in coyotes we wanted to develop a simpler, sensitive HPLC analytical method to quantitate mifepristone specific to coyote serum.

2. Experimental

2.1. Chemicals and reagents

Mifepristone was purchased from Sigma–Aldrich (St. Louis, MO, USA). The internal standard (I.S.) is an analog of mifepristone, and was synthesized by the Research Triangle Institute (Research Triangle, NC, USA). The I.S. is 11β-4(*N*,*N*-dimethylamino-phenyl)-17α-methoxymethyl-17β-hydroxyestra-4,9-dien-3-one; RTI-3021-003. Purity of both compounds was reported as 98% and both were supplied in a fine crystalline dry powder. HPLC-grade acetonitrile (ACN) and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Extraction cartridges (Oasis HLB 1 ml, 30 mg), the HPLC separation column (Symmetry 150×4.6 mm, C_{18}) and the guard column (μBondapak C_{18}) were all purchased from Waters (Milford, MA, USA).

2.2. Instrumentation and operating parameters

The HPLC system was comprised of two Shimadzu LC 600 pumps, and a Waters 486 tunable absorbance detector. System management and hardware interface for data acquisition was done using the Millennium 3.1 computer software package from Waters. Samples were manually injected into the HPLC system using a Rheodyne injector (Cotati, CA, USA) fitted with a 50-μl injection loop. The mobile phase was ACN–water (55:45) pumped at 1.0 ml/min. Solutions were prepared fresh daily, filtered and degassed immediately prior to each analysis using a 0.2-μm poly(vinylidene fluoride) (PVDF) filter (Gelman Sciences, Ann Arbor, MI, USA). The column temperature was not regulated and ambient temperature for the system ranged from approximately 22 to 24 °C. Absorbance was measured by a tunable absorbance UV detector set at a wavelength of 305 nm. Data were collected, inte-

grated, and analyzed using Waters Millennium software.

2.3. Preparation of stock and working solutions

Stock solutions of both mifepristone and I.S. were prepared from dry chemicals weighed on an ultra balance using small foil strips which were then placed in cleaned 10-ml glass tubes. These were stored at −80 °C until they were prepared for use by adding the appropriate volume of acetonitrile to make a 100 μg/ml stock solution. Stock solutions were prepared new each week to eliminate changes in concentration due to evaporation of solvents, even though mifepristone in solution appeared to be very stable. Working solutions of 10 μg/ml were prepared from stock solutions fresh prior to each analysis, using water to prevent precipitation of proteins when added to the serum.

2.4. Preparation of standards in coyote serum matrix

Untreated serum was obtained from captive coyote blood collected via cephalic venepuncture into 10-ml red top vacuum tubes. Blood was allowed to clot approximately 4 h and centrifuged. Serum was harvested and stored at −80 °C until used. Preparation of the 1000 ng/ml serum standards of mifepristone were made by adding known amounts of mifepristone working solution to blank coyote serum. Serial dilutions of the 1000 ng/ml standard were made with blank serum to obtain additional standards of 10, 20, 50, 100, 200, and 500 ng/ml. Standard serum concentrations were analyzed in duplicate.

2.5. Sample preparation and extraction

Except for the blank, serum samples (1 ml) were spiked with 50 μl of I.S. working solution (10 μg/ml) and vortexed. All samples, (blanks, unknowns, and standards) were extracted using the disposable solid-phase extraction cartridges (Oasis HLB 1 ml, 30 mg). Each cartridge was preconditioned prior to use by washing with two 1-ml aliquots of ACN followed by two 1-ml aliquots of water. Samples were then passed through the cartridge under low-pressure (>5 mmHg) laboratory vacuum and washed with two 1-ml aliquots of water. The analytes were eluted with two 0.75-ml volumes of ACN and collected in clean glass tubes. The eluent was then dried under a stream of nitrogen gas and reconstituted in 100 μl of ACN prior to injection into the HPLC system. Samples (~70 μl) were manually injected to insure the 50-μl loop of the injector was totally full.

Extraction efficiencies were calculated from two sets of samples prepared by dissolving both mifepristone and I.S. together in 1 ml of either serum or ACN. The serum samples were subjected to extraction, drying and reconstitution procedures described above. The non-extracted ACN samples were dried, reconstituted and injected onto the column.

2.6. Validation procedure

According to Shah et al. [10] ''analytical method validation includes all the procedures required to demonstrate that a particular method for the quantitative determination of the concentration of an analyte (or series of analytes) in a particular biological matrix is reliable for the intended application''. Validation of the analytical procedure was performed over several days using data to evaluate the intra- and inter-day accuracy, intra- and inter-day precision as well as the specificity. Accuracy is expressed as the closeness of the standard samples to the actual known amount. Precision was the level of repeatability as reported between samples analyzed on the same day, intra-day, and samples run on three different days, inter-day. Specificity is the degree to which the procedure applies to a single analyte and is checked in each analysis by examining a blank matrix sample for any interfering peaks. Linearity of the analysis was determined from the correlation coefficient (r) of regression analysis of concentration versus peak area ratio of mifepristone and I.S. Standard concentrations were back calculated using the standard curve and the percent deviation was calculated. Calculation of r-values, processing of chromatograms and determination of slopes was completed using the Millenium[32] chromatography software.

2.7. Measurement of mifepristone in serum from treated coyotes

Blood samples from experiments on captive coyotes were taken for analysis after administration of 150 mg of mifepristone through cephalic vein. Blood samples were taken from either the opposite cephalic vein or a jugular vein using an evacuated glass tube with no anticoagulant (red top). Animals were manually restrained to avoid use of drugs such as sedatives and tranquilizers. After the blood was withdrawn the tubes were once again allowed to clot at room temperature for approximately 4 h, centrifuged for 20 min at 500 *g*, the serum was harvested and stored at −80 °C until analysis was performed.

3. Results and discussion

3.1. Measurement of mifepristone

Each HPLC standard curve included a blank containing neither analyte or I.S., zero blank containing only I.S., and duplicate standard samples. Peak area ratios of analyte to I.S. were reported as the response. The resulting standard curve from response versus concentration was evaluated for linearity and slope. Elution times for the internal standard and mifepristone were compared for repeatability and were approximately 8.0 and 9.6 min, respectively in each run. There are interfering peaks up to 6 min. Any attempt to reduce the run time resulted in poor separation between the two peaks. The columns were about 6 months old and had minimum 300 injections. The peak shape did not change with time. The guard column used was μBondapak C_{18}. Differences in packing materials in the guard column and the Symmetry C_{18} HPLC separation column may have contributed to some peak broadening.

3.2. Extraction efficiency

Extraction efficiency was determined by comparing the mean peak areas of three ($n=3$) samples extracted to the mean peak areas of three ($n=3$) non-extracted samples of the same concentration. Samples were prepared in serum and extracted using the method described and reconstituted to 100 μl. Non-extracted samples were prepared in ACN, dried and reconstituted along with the extracted samples. Extraction efficiency of mifepristone varied from 104.3% for the lowest concentration, 20 ng/ml, to 92.7% at the highest concentration, 1000 ng/ml and the internal standard extraction averaged 100.2% (Table 1). The extraction efficiency was calculated as (peak area found after extraction·100)/(peak area without extraction). Mifepristone showed very high extraction efficiency, i.e., very little was lost during the extraction process.

3.3. Specificity and linearity of the method

Chromatograms for the blank, have been included to illustrate that there were no interfering peaks coming at the elution times for either mifepristone or I.S. (Fig. 1A). Although there are characteristic peaks for the biological matrix and in some cases small peaks that may represent chemically degraded products of the principal compounds, these do not interfere with the peaks for the analyte or the I.S. Elution times for both the analyte and the I.S. were consistent and did not fluctuate significantly over the course of the validation. The lowest concentration for the standard curve was based on the limit of quantification. The standard curves were prepared using a weighting factor $1/x$. There was only slight

Table 1
Extraction efficiency for mifepristone and I.S. (mean±SD, $n=6$)

	Mifepristone (mean±SD)			I.S. (mean±SD), 500 ng/ml
	20 ng/ml	200 ng/ml	1000 ng/ml	
Non extracted peak area	36 077±1411	295 824±342	1 189 974±43 614	602 257±87 995
Extracted peak area	37 613±300	304 924±34 543	1 103 053±59 160	603 405±12 699
Extraction efficiency (%)	104.3	103.1	92.7	100.2

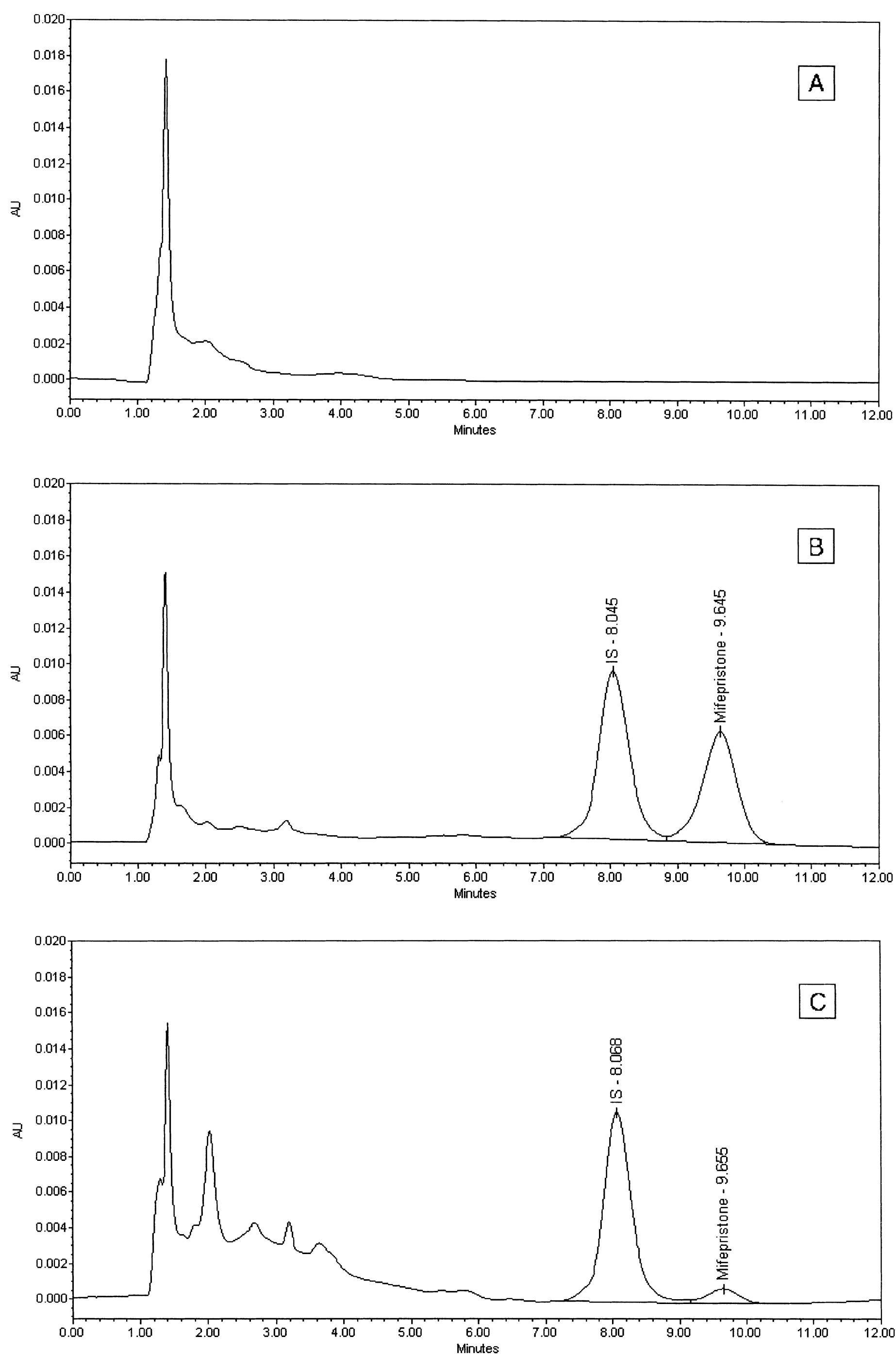

Fig. 1. Chromatograms of mifepristone and I.S. in coyote serum. (A) Blank coyote serum depicting no interfering peaks at the corresponding elution times of mifepristone and I.S. (B) Standard serum containing 200 ng/ml of mifepristone. (C) Coyote serum, drawn at 24 h after i.v. administration of 150 mg mifepristone (35 ng/ml).

Table 2
Inter-day analysis of mifepristone (mean±SD, n=6)

Injected concentration (ng/ml)	10	200	1000
Found concentration (ng/ml)	9.59±1.9	198.8±19.3	1047.3±46.9
Accuracy (%)	95.9	99.4	104.7
Precision (RSD, %)	19.8	9.7	4.5

variation in the slopes calculated on different days. Linearity of the curve was determined from the correlation coefficients and back calculated values from the standard curve. The HPLC method described here is linear in the range of 10 to 1000 ng/ml. Fig. 1C is a representative chromatogram from one of the coyotes treated with 150 mg intravenous mifepristone.

3.4. Validation

Validation of the method of analysis was done using comparisons of inter-day and intra-day variation of samples (Tables 2 and 3). Inter-day samples were comprised of high, medium and low concentrations of the compound in the serum matrix, analyzed in duplicate on three different days, for a

Table 3
Intra-day analysis of mifepristone (mean±SD, n=6)

Injected concentration (ng/ml)	10	200	1000
Found concentration (ng/ml)	11.7±2.3	213.8±8.0	994.1±92.3
Accuracy (%)	117.0	106.9	99.4
Precision (RSD, %)	19.7	3.7	9.3

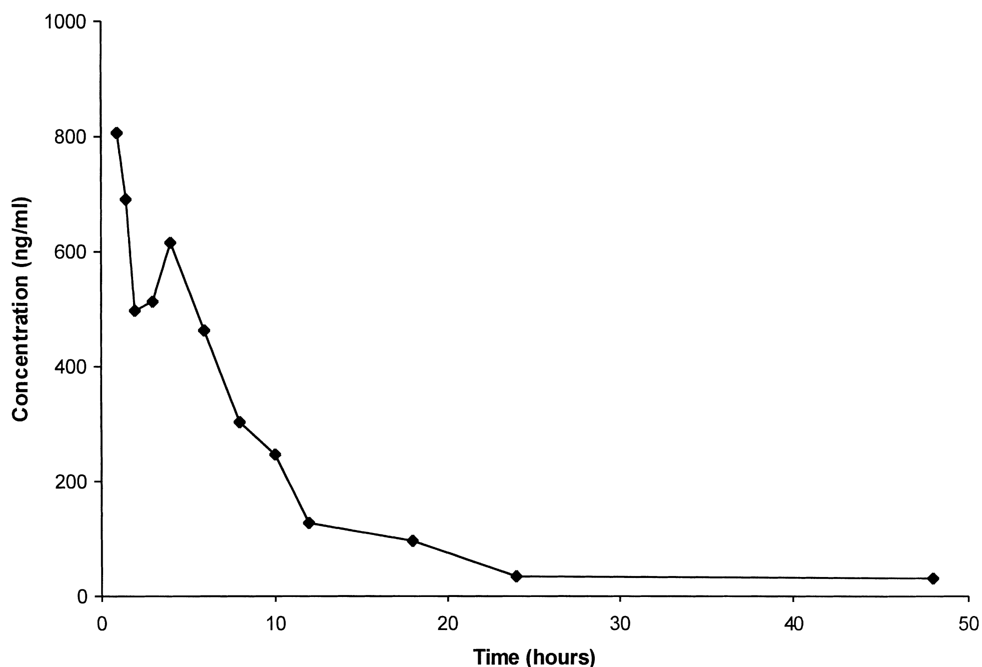

Fig. 2. Graph of serum concentrations analyzed over 48 h following i.v. administration of 150 mg of mifepristone.

total of six samples for each concentration. Intra-day samples were taken as a total of six samples for the same concentrations analyzed on the same day. Inter- and intra-day accuracies are within acceptable values [10]. Inter- and intra-day relative standard deviations (RSDs) (precisions) were less than 10% for 200 and 1000 ng/ml. Inter- and intra-day precisions are 19.8 and 19.7% for the lowest level, 10 ng/ml, respectively.

3.5. Measurement of mifepristone in serum samples from coyotes

Serum samples taken from captive coyotes used in the research projects were analyzed using this method. The time–concentration curve of mifepristone in serum samples collected from one coyote over 48 h after receiving a single 150 mg intravenous (i.v.) dose can be seen in Fig. 2.

Mifepristone levels decreased rapidly after administration in coyotes, however quantifiable amounts were found up to 48 h after treatment in the serum of this coyote. This HPLC method is currently being used to determine the least dose response, bioavailability and effectiveness of a bait delivery systems for mifepristone in coyotes.

4. Conclusion

We have developed and validated a simple, rapid, sensitive method for analysis of mifepristone in coyote serum. Using this method will allow accurate assessment of the serum levels of mifepristone in treated coyotes which is critical in our endeavor to develop new non-lethal strategies for the control of predators such as coyotes.

Mifepristone is appealing as a predator control drug due to the lack of toxicity and minimal impact on the environment or individuals other than targeted females [11]. Developing effective nonlethal methods will offer a humane alternative to previously used conventional methods and hopefully diminish some of the opposition to predator control.

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