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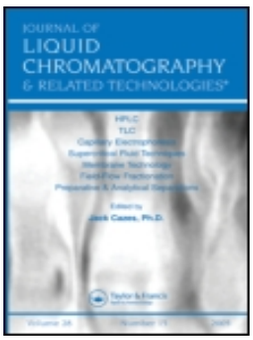

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Determining Terbinafine in Plasma and Saline Using HPLC

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A simple, sensitive, and accurate high-performance liquid chromatography (HPLC) method for the determination of terbinafine concentrations in small-volume plasma and saline has been developed. Following a liquid extraction using hexane, samples were separated by reversed-phase HPLC on a Symmetry Shield RP_{18} (5 μm) 4.6 mm × 100 mm column and quantified using ultraviolet detection at 224 nm. The mobile phase was a mixture of water, phosphoric acid, and triethylamine (pH 3.0), with acetonitrile (65:35, v/v), at a flow rate of 1.1 mL/min. The standard curve ranged from 5 to 1500 ng/mL for parrot plasma and 1 to 25 μg/mL for 0.9% saline. Intra- and inter-assay variability for terbinafine was less than 10% for both matrices, and the average recovery was greater than 90%. This method has been developed in parrot plasma and should be applicable to other species, making it useful to those investigators dealing with small sample volumes, particularly when conducting pharmacokinetic studies that require multiple sampling from the same animal. This method would also be valuable to aquariums that need to determine terbinafine concentrations in salt water.

Keywords: extraction, HPLC, plasma, saline, terbinafine, UV detection

Introduction

Terbinafine, (E)-N-(6,6-dimethyl-2-hepten-4-ynyl)-N-methyl-1-naphthalenemethanamine, is an allylamine with a broad range of *in vitro* antifungal activity.[1] Terbinafine inhibits squalene epoxidase, which is an enzyme involved in the synthesis of ergosterol, which gives terbinafine both fungicidal and fungistatic activity.[1] Historically, terbinafine has been used in humans to treat dermatophytosis. It has recently been found to be useful for the treatment of refractory and systemic fungal infections, particularly those caused by *Aspergillosis* sp.,[1] and may also be useful in the treatment of other systemic fungal diseases of animals.

Terbinafine concentrations have been determined using high-performance liquid chromatography (HPLC) methods with UV detection[1–15] and mass spectrometry (MS)[16] in plasma, tissue, urine, pharmaceutical products, and hair. MS can produce results with a lower limit of detection (LOD), but it may not be readily available in all laboratories due to the cost. Many extraction methods have been tried including solid-phase extraction (SPE),[2,3,11,16] multistep extractions, and hydrolysis reactions.[1,4,6–10,14,15] The existing SPE methods all require much larger sample sizes, and one method[11] is validated for urine and not plasma. The Baranowska et al.[3] method requires sample pretreatment with methanol, acetonitrile, and phosphate buffer prior to extraction, while the Gurule et al. method[16] requires sample pretreatment with phosphoric acid and has a recovery range of 78–87%. The aim of this article is to describe a simple, sensitive, and accurate method for extracting terbinafine from plasma and saline samples that eliminates the need for solid-phase cartridges and time-consuming hydrolysis reactions. To our knowledge, no method for the analysis of terbinafine has been reported for saline samples which could be valuable to aquariums that need to determine terbinafine concentrations in salt water during or after treatment of aquatic animals.

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Experimental

Instrumentation

The chromatography system consisted of a Model 2695 separation module and a Model 2487 ultraviolet detector (Waters, Milford, MA, USA). Separation was achieved on a Waters Symmetry Shield RP_{18} (4.6 mm × 100 mm, 5 μm) column preceded by a 5 μm Symmetry Shield RP_{18} (3.9 mm × 20 mm) guard column. The mobile phase was an isocratic mixture of A (1.5 mL 85% phosphoric acid, 1 mL trimethylamine in water, adjusted to pH 3.0 with 1 N sodium hydroxide) and B (acetonitrile (65:35)). All solutions were filtered through a 0.22 μm filter and degassed before use. The water was replaced on a daily basis. The flow rate was 1.1 mL/min, and the ultraviolet detector was set at a wavelength of 224 nm. The column and autosampler temperature were ambient which was 23°C.

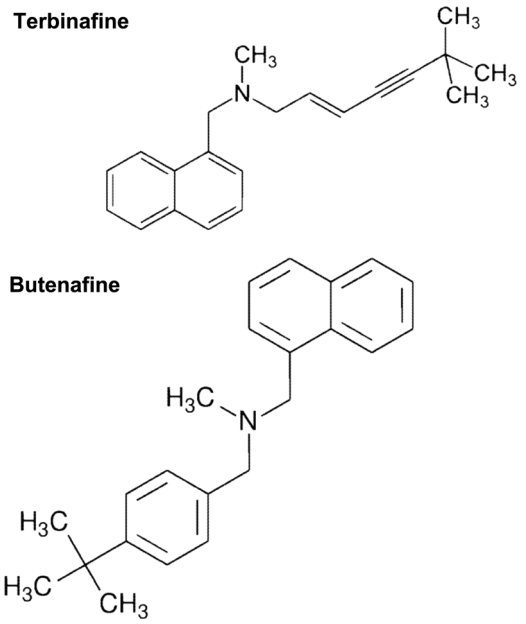

Fig. 1. Structures of terbinafine and butenafine.

Reagents

Terbinafine HCl (Figure 1) was purchased from US Pharmacopeia (Rockville, MD, USA) and was 99% pure. Butenafine HCl (Figure 1), which was used as the internal standard (99% purity), was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Triethylamine was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagent grade chemicals and solvents were purchased from Fisher Scientific (Pittsburg, PA, USA). Water (18.2 megaohm) was obtained from a Barnstead Nanopure Infinity (Dubuque, IA, USA) ultrapure water system.

Preparation of Calibration Standards

Five milligrams each of terbinafine and butenafine (internal standard) were weighed and dissolved in methanol to produce stock concentrations of 100 μg/mL. Dilutions of the terbinafine stock solution were prepared in methanol to produce 0.1, 1, and 10 μg/mL working stock solutions, while dilutions of the butenafine stock solution were made to produce a 1 μg/mL working stock solution. 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 4°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 and then untreated parrot plasma, or 0.9% saline was added. The final concentrations for the plasma calibration standard curve were 5, 10, 25, 50, 100, 250, 500, 800, 1000, and 1500 ng/mL with quality control standards of 15, 350, and 1200 ng/mL. The final concentrations for the saline standard curve were 1, 2.5, 5, 7.5, 10, 15, and 25 μg/mL with quality control standards of 3, 12, and 20 μg/mL. Calibration standards and control samples were treated the same as test samples. The calibration curve was constructed by using the ratio of the peak area of the analyte divided by the peak area of the internal standard versus the concentration. Linearity was assessed by unweighted linear regression analysis. The calibration curve had a correlation coefficient of 0.99 or better. The acceptance criterion for each back-calculated standard and quality control concentration was 15% deviation from the nominal value except lower limit of quantification (LLOQ), which was set at 20%.

Sample Extraction

Terbinafine was extracted from parrot plasma and saline using a liquid extraction. Previously frozen plasma or saline samples were thawed, and 100 μL was placed into a 7 mL screw cap tube. Seventy-five microliters of butenafine (internal standard, 1 μg/mL) for plasma or 10 μL of butenafine (internal standard, 100 μg/mL) for saline was added. The tubes were vortex mixed at a high speed for 5 s to mix solutions, and then 3 mL of hexane was added. Tubes were then capped and rocked for 20 min before centrifugation at 1000*g* for 15 min. The supernatant was removed and placed in a clean 16 × 100 glass test tube and then evaporated with nitrogen. After evaporation, they were redissolved in 250 μL of the mobile phase, vortex mixed for 5 s at high speed, and centrifuged for another 5 min at 1000*g*. The supernatant was placed in total recovery chromatographic vials (Waters) and 100 μL injected into the system.

Results

Plasma

For specificity testing, untreated plasma was prepared in the same manner as study samples, and no endogenous plasma components interfered with the elution of the compounds of interest. Five different blank plasma samples from adult male and female parrots were used in the pre-validation process, and a blank sample from each animal was included in the analysis. Figure 2 illustrates chromatograms of a (A) blank plasma sample, (B) a 800 ng/mL spiked plasma standard, and (C) a plasma sample from a parrot after oral administration of a 60 mg/kg dose of terbinafine. The retention times in plasma were 6.30 and 8.88 min for terbinafine and butenafine. The method for plasma analysis produced a linear curve for the concentration range used (5–1500 ng/mL) with a correlation coefficient of 0.997. The mean slope, intercept, and r^2 values are reported in Table 1. Intra- and inter-day assay relative standard deviation (RSD) for plasma spiked with specific concentrations of terbinafine was used to determine the accuracy and precision which ranged from 2.2% to 9.1% (Tables 1 and 2). The recovery of terbinafine from spiked plasma was compared to directly injected analytes at concentrations of 15, 350, and 1200 ng/mL dissolved in mobile phase without undergoing extraction. The average recovery ranged from 85% to 90% for terbinafine (Table 2). The recovery of the internal standard was 81%. The LOD

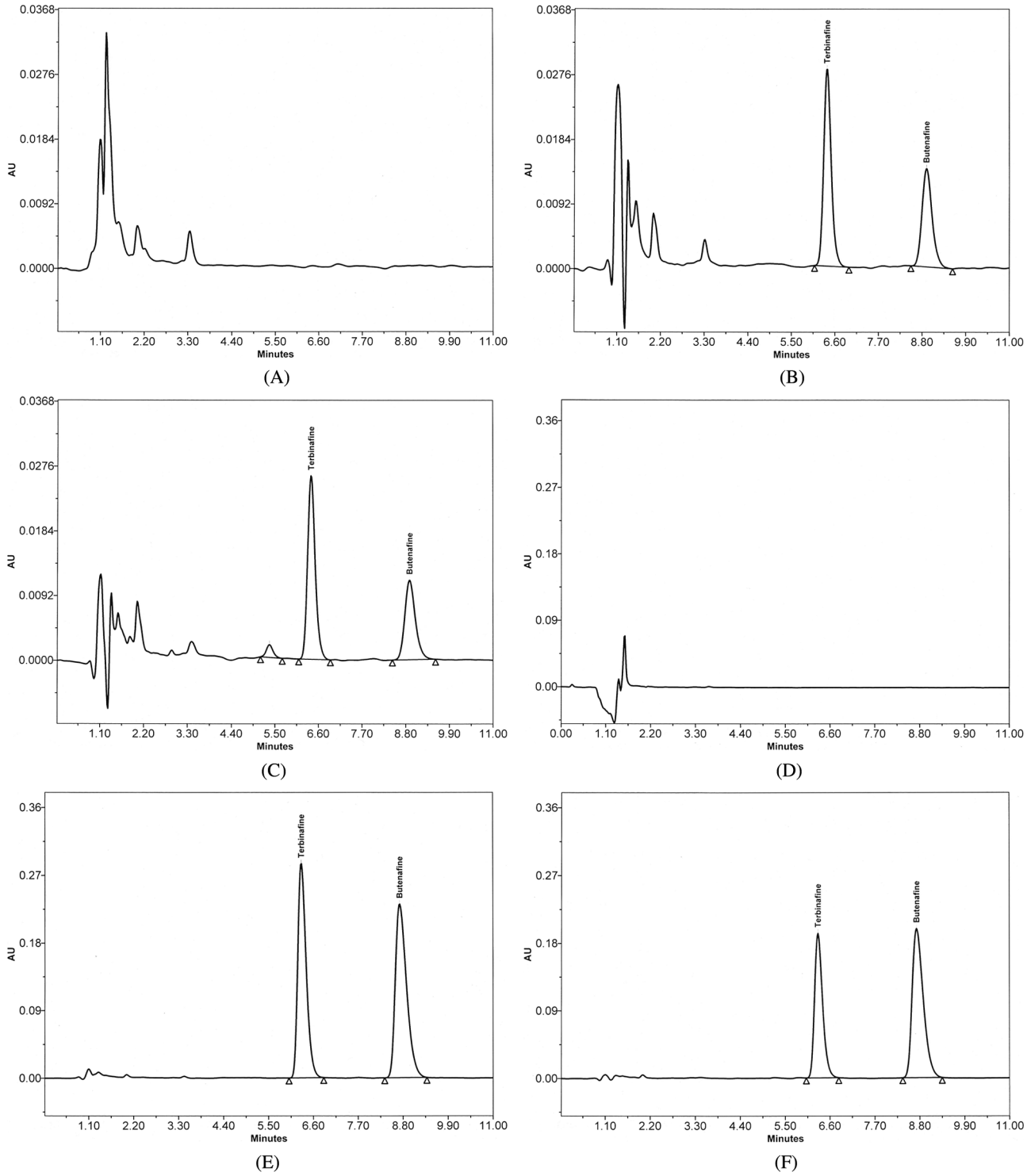

Fig. 2. Chromatograms for terbinafine. (A) Blank parrot plasma, (B) 800 ng/mL spiked plasma standard, (C) a parrot plasma sample 30 min after a 60 mg/kg oral dose of terbinafine was administered, (D) blank saline, (E) 5 μg/mL spiked saline standard, and (F) a saline sample from a 37°C incubator.

was 2.5 ng/mL, which represents a peak approximately three times the baseline noise, and the LLOQ was 5 ng/mL, which represents a peak approximately five times the baseline noise. Testing of the short-term stability of the quality control standards indicated there was less than an 18% loss of drug after 24 hr in the auto sampler and a 12% loss after 24 hr in the refrigerator at 4°C.

Figure 3 is a representative concentration–time profile from a terbinafine pharmacokinetic study conducted in Amazon parrots after receiving a 60 mg/kg oral dose of terbinafine.

Table 1. Intra-assay accuracy, precision, and assay linearity for terbinafine in plasma

Intra-assay variability ($n = 5$)		
Concentration added (ng/mL)	Concentration measured (ng/mL) (mean ± SD)	RSD (%)
15	14 ± 1	9.2
350	349 ± 8	2.2
1200	1226 ± 83	6.8
	Assay linearity ($n = 5$)	
	Mean ± SD	RSD (%)
Y-intercept	−0.0168 ± 0.0160	5.6
Slope	0.00191 ± 0.00009	4.7
r^2	0.9972 ± 0.0005	0.05

SD: standard deviation; *n*: number of samples; RSD: relative standard deviation.

Table 2. Inter-assay variability and recovery for terbinafine in plasma and ($n = 5$)

Concentration added (ng/mL)	Concentration measured (ng/mL) (mean ± SD)	RSD (%)	Recovery (%)
15	15 ± 1	8.6	98
350	357 ± 14	4.0	87
1200	1243 ± 34	2.7	85

SD: standard deviation; *n*: number of days; RSD: relative standard deviation.

Table 3. Intra-assay accuracy, precision, and assay linearity for terbinafine in saline

Intra-assay variability ($n = 5$)		
Concentration added (μg/mL)	Concentration measured (μg/mL) (mean ± SD)	RSD (%)
3	3 ± 0.19	5.9
12	12 ± 0.79	6.8
20	21 ± 0.83	4.0
	Assay linearity ($n = 5$)	
	Mean ± SD	RSD (%)
Y-intercept	0.0393 ± 0.1061	2.3
Slope	0.121 ± 0.014	11.5
r^2	0.992 ± 0.008	0.8

SD: standard deviation; *n*: number of samples; RSD: relative standard deviation.

Table 4. Inter-assay variability and recovery for terbinafine in saline ($n = 5$)

Concentration added (μg/mL)	Concentration measured (μg/mL) (mean ± SD)	RSD (%)	Recovery (%)
3	3 ± 0.27	8.2	97
12	12 ± 0.34	2.9	101
20	20 ± 0.49	2.4	105

SD: standard deviation; *n*: number of days; RSD: relative standard deviation.

Saline

Blank saline was subjected to the extraction procedure to ensure the absence of any peaks at the retention times of the peaks of interest. Four different saline samples were used in the pre-validation process, and a blank sample from each batch was included in the analysis. Figure 2 illustrates chromatograms of a (D) blank saline, (E) 5 μg/mL spiked saline sample, and (F) saline from an implant sample placed in a 37°C incubator. The retention times of terbinafine and butenafine were 6.30 and 8.88 min. This method produced a linear curve for the concentration range used (1–25 μg/mL) with a correlation coefficient of 0.992. The mean slope, intercept, and r^2 values are reported in Table 3. Intra- and inter-day assay RSD for saline spiked with specific concentrations of terbinafine was used to determine the accuracy and precision, which ranged from 2.4% to 8.2% (Tables 3 and 4). The recovery of terbinafine from spiked saline was determined by comparing the areas of extracted saline samples to those of directly injected analytes at concentrations 3, 12, and 20 μg/mL without undergoing extraction. The average recovery ranged from 97% to 105% for terbinafine (Table 4). The recovery of the internal standard was 108%. The LOD was 0.0025 μg/mL, which represents a peak approximately three times the baseline noise, and the LLOQ was 0.005 μg/mL, which represents a peak approximately five times the baseline noise. Testing of the short-term stability of the standards at concentrations indicated there was less than an 18% loss of drug after 24 hr in the auto sampler, and a 6% loss after 24 hr in the refrigerator at 4°C.

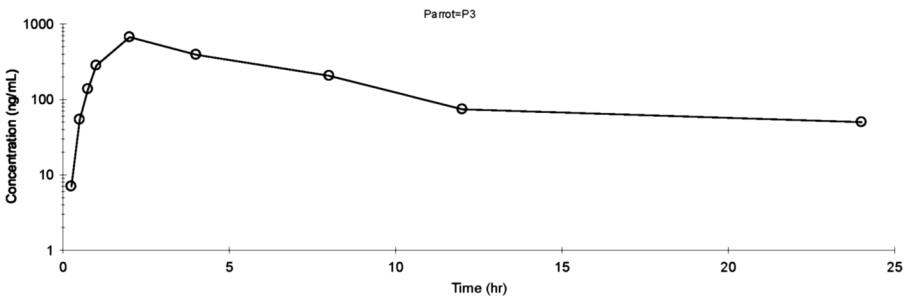

Fig. 3. Concentration time profile of terbinafine in a parrot after a 60 mg/kg oral dose.

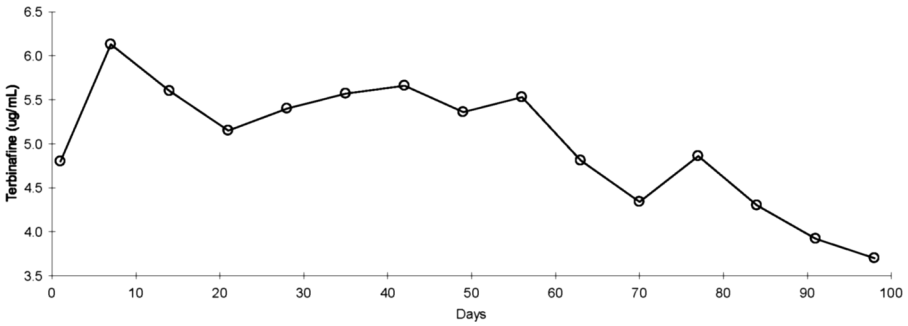

Fig. 4. Profile of terbinafine concentrations released from an implant in saline at 37°C.

The goal of the implant study was to investigate a terbinafine impregnated implant designed for subcutaneous placement over the dorsum of bats infected with *Geomyces pseudogymanoascus.* The first phase of the study was to place the implant in saline at two different temperatures, 4°C and 37°C, and determine the concentrations released over the course of approximately 6 months. Figure 4 is a representative profile of terbinafine from this study.[17]

Discussion

We wanted to develop a simple method that did not require SPE cartridges or lengthy hydrolysis reactions. During method development, several organic solutions and mixtures were investigated for analyte recovery including methylene chloride, ethyl acetate, acetonitrile, ethyl acetate and hexane, methanol, and chloroform. Ethyl acetate and hexane, methylene chloride, chloroform, and ethyl acetate were found to produce 68%, 55%, 30%, and 60% recoveries, while extractions with acetonitrile and methanol decreased the peak resolution and produced interfering peaks. Once hexane was selected as the extraction solvent, we wanted to determine the appropriate volume for the extraction process and found that 2 mL produced a recovery of roughly 80% and 4 mL produced the same recovery as the 3 mL which was selected. We also checked varying methods for mixing the samples and determined that rocking the samples produced a greater recovery than vortexing for 60 s.

This HPLC method quantifies terbinafine from plasma and saline by combining a liquid–liquid extraction procedure with ultraviolet detection. To our knowledge, this is the first reported method for the analysis of terbinafine in saline samples, which could be valuable to aquariums that have to measure drug concentrations in sea water. Some methods involved in terbinafine analysis in plasma use MS,[16,18] but MS equipment is expensive and may not be readily available to all laboratories. The method described here actually has the same LLOQ as both[16,18] of the previously reported MS methods. The Gurule et al.[16] method also required 0.5 mL of plasma to achieve their LLOQ versus the 0.1 mL sample of this method. The hexane extraction method has a LLOQ that is more sensitive than many previously described UV detection methods[1–3,10,11,15] while using a much smaller sample size than previous methods.[1–3,8,10,14–16] The two previous methods[8,14] that reported 2 ng/mL LLOQ used a larger sample volume than the present method to obtain that level of sensitivity. The present method eliminates the need for expensive SPE cartridges,[2,3,11,16] online solid phase equipment,[15] and the use of a robotic liquid handling workstation.[18] There is no need for sample hydrolysis or multiple extraction steps, thus eliminating the use of strong acids and bases, and the additional time needed to perform these steps.[1,8,10,14,15] The average terbinafine recovery of 90% for plasma is at least 25% better than most of the reported plasma methods.[1,4,8–10,14–16,18] The use of butenafine as an internal standard corrects for the intra- and inter-day assay variability in the extraction. The LOQ and recovery are more than adequate for use in pharmacokinetic studies.

Conclusion

In conclusion, this analytical procedure was authenticated in terms of recovery, linearity, LLOQ, precision, and accuracy. The results of this study indicate that this HPLC procedure is a reproducible method that provides consistent quantification of terbinafine in plasma and saline. It is a simple method that eliminates the need for SPE cartridges and hydrolysis reactions that allows numerous samples to be processed by a single technician.

This method has been used successfully to determine terbinafine concentrations in both plasma and saline samples at this institution. It has been used in the quantitation of terbinafine in other species and could be applicable to other groups. This method utilizes a small sample size of 100 μL, making it potentially useful for small dogs, cats, other small animals or to those investigators dealing with small sample volumes, particularly when conducting pharmacokinetic studies that require multiple sampling from the same animal. This method would also be valuable to aquariums that need to determine terbinafine levels in salt water.

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