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Rapid high-performance liquid chromatographic method for the determination of propranolol levels in canine and feline plasma

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ABSTRACT

A sensitive high-performance liquid chromatographic method that does not require organic extraction has been developed for the determination of propranolol levels in canine and feline plasma. Equal volumes of plasma and a mixture of methanol–acetonitrile–0.1 *M* sodium hydroxide (3:3:4, v/v/v) were added to a microseparation unit with a 10 000 molecular mass cut-off filter. The ultrafiltrate was analyzed by reversed-phase liquid chromatography with fluorimetric detection. The consistency of the recoveries obtained eliminated the need for an internal standard (coefficients of variation < 4%). Linear regressions for the standard curves (2.5–100 ng/ml) gave correlation coefficients above 0.9955. The detection limit was 1 ng/ml. The assay retains high sensitivity while eliminating laborious sample preparation.

INTRODUCTION

Although the β-blocking activity of propranolol makes it useful in the treatment of such cardiac disorders as arrhythmia and hypertension, high plasma levels of the drug can cause severe respiratory problems and heart failure [1]. Plasma concentrations of propranolol can indicate dangerous levels of the drug and give further insight into clinically effective levels [2]. High-performance liquid chromatography (HPLC) using fluorimetric detection provides the selectivity and sensitivity necessary for routine analysis of plasma propranolol concentrations.

Many HPLC methods using fluorimetric detection have been reported in the literature for the determination of propranolol in plasma. These methods all

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involve time-consuming sample preparation, which complicates routine analysis. The majority require labor-intensive organic extraction, often followed by back-extraction into aqueous acid [3–18]. Methods involving solid-phase extraction [19] and acetonitrile precipitation [20] do not significantly reduce sample preparation time.

This paper describes a one-step sample preparation that simplifies the analysis of plasma propranolol levels. The plasma is treated with a solution of methanol and acetonitrile in dilute sodium hydroxide and then is centrifuged through a 10 000 molecular mass (M_r) cut-off filter. Consistent recoveries of propranolol and the lack of volume transfers eliminate the need for an internal standard. These advantages allow investigators to study large sample sets in a reasonable time frame.

EXPERIMENTAL

Materials and equipment

The HPLC system consisted of a Perkin Elmer (Norwalk, CT, USA) 1020 integrator, a Waters (Milford, MA, USA) 715 WISP autosampler, a Perkin Elmer 250 isocratic pump, and a McPhearson (Acton, MA, USA) FL-750 spectrofluorimeter with high-sensitivity attachment (no filter). The FL-750 was set at an excitation wavelength of 222 nm and an emission wavelength of 340 nm with a sensitivity of 0.1, a time constant of 1.0 s, and a gain of 6.5.

The HPLC column (Zorbax R$_x$ C$_8$, 5 μm, 250 mm × 4.6 mm I.D.) was from MacMod Analytical (Chadds Ford, PA, USA). An IEC Model CS centrifuge with a 16-shield fixed-angle rotor (International Equipment, Needham, MA, USA) was used. The microseparation units (Centricon-10) with a 10 000 M_r cut-off filter were obtained from Amicon (Danvers, MA, USA).

Male Beagle dogs were obtained from Marshall Farms (North Rose, NY, USA). Lavender top Vacutainer tubes (10 ml) for plasma collection (0.1 ml of 15% EDTA) were obtained from Fisher Scientific (Raleigh, NC, USA).

Reagents

HPLC-grade acetonitrile and methanol were obtained from Baxter Healthcare (Charlotte, NC, USA). HPLC-grade triethylamine (TEA), HPLC-grade ammonium acetate, 0.1 *M* sodium hydroxide, and analytical-grade glacial acetic acid were obtained from Fisher Scientific. 1-Octanesulfonic acid (sodium salt) and DL-propranolol (HCl) were obtained from Sigma (St. Louis, MO, USA), and 1-dodecanesulfonic acid (sodium salt) was from Regis (Morton Grove, IL, USA). The HPLC-grade water came from a Milli-Q UF PLUS water purification system (Millipore, Bedford, MA, USA).

Dog studies

Two fasted male beagle dogs were given an oral dose of 4 mg/kg propranolol

(in gelatin capsules). Blood samples were collected via the jugular vein at 15, 30, 45, 60, 90, 120, 300, and 420 min post dosing. The Vacutainer tubes were centrifuged for 20 min at 2500 *g*. The plasma was drawn off and stored at −80°C.

Sample preparation

Equal volumes (0.5 ml) of plasma and drug-releasing agent (DRA) were added to a microseparation unit (MSU). The DRA consisted of methanol–acetonitrile–0.1 *M* sodium hydroxide (3:3:4, v/v/v, pH 10). The MSU was vortex-mixed for about 10 s and centrifuged for 20 min at 2500 *g*. The colorless ultrafiltrate was transferred to HPLC vials for analysis.

Standard curves

The 1 mg/ml stock propranolol solution was made daily in methanol. This solution was diluted to 1000 ng/ml with DRA. Further serial dilutions in DRA gave concentrations of 200, 160, 120, 80, 40, 20, 10 and 5 ng/ml. These were used to produce the extracted (1:1 with plasma in the MSU) and the unextracted (1:1 with HPLC water in the MSU) standard curves. The MSUs were mixed and centrifuged as outlined above to give final concentrations in the ultrafiltrates of 100, 80, 60, 40, 20, 10, 5, and 2.5 ng/ml.

Reproducibility studies

Extracted (canine or feline plasma) and unextracted standards at two propranolol concentrations (100 and 10 ng/ml) were used to calculate recoveries. The MSUs were prepared as outlined above using the 200 and 20 ng/ml DRA standards 1:1 with plasma (extracted) or HPLC water (unextracted). Ten to eleven plasma replicates were used to evaluate the reproducibility of the extraction. Plasma blanks were also prepared in MSUs (1:1 DRA–plasma).

Chromatography

The mobile phase consisted of acetonitrile–methanol–0.05 *M* ammonium acetate (30:20:50) containing 0.002 *M* docecanesulfonic acid, 0.003 *M* octanesulfonic acid, and 0.2% TEA. The pH was adjusted to 5.2 with glacial acetic acid prior to adding the organic solvents. A flow-rate of 1.0 ml/min was used, giving a run time of 14 min. Ultrafiltrate volumes of 150 μl (extracted standards) and 500 μl (unextracted standards) were recovered from the MSUs. An injection volume of 30 μl was sufficient for the range of standards used. All calculations were based on peak height as determined by the Perkin Elmer 1020 integrator.

RESULTS AND DISCUSSION

Table I summarizes the statistical information obtained from spiking canine and feline plasma with known quantities of propranolol (10 and 100 ng/ml). Recoveries were based on the ratio of the extracted peak height to the unextract-

TABLE I

STATISTICAL SUMMARY OF PROPRANOLOL ANALYSIS FROM CANINE AND FELINE PLASMA

Concentration spiked (ng/ml)	*n*	Recovery (%)		C.V. (%)	Correlation coefficient	
		Range	Mean ± S.D.		Extracted standards[a]	Unextracted standards[a]
Canine plasma						
10	11	70.6–79.3	73.4 ± 2.5	3.3	0.9989	0.9999
100	11	67.5–74.7	72.0 ± 2.2	3.1		
Feline plasma						
10	10	69.5–79.5	75.2 ± 2.9	3.8	0.9956	0.9956
100	11	73.0–78.8	75.8 ± 2.0	2.7		

[a] Eight concentrations, $n = 3$.

ed peak height. No significant differences were noted between the canine and feline plasma extractions. A high level of precision was indicated by the low coefficients of variation (all < 4%), which also reflected a consistent level of drug recovery. These predictible recoveries and the lack of volume transfer in preparing the samples make the use of an internal standard unnecessary. The correlation coefficients for the extracted and unextracted standard curves (2.5–100 ng/ml range) are indicated in Table I. An average accuracy [21] of 96% was calculated for the eight standards (run in triplicate) extracted from canine plasma and was not significantly different from that obtained for the feline extracted standards.

TABLE II

TIME COURSE PLASMA LEVELS OF PROPRANOLOL FROM DOGS DOSED AT 4 mg/kg ORALLY

Time post dose (min)	Propranolol concentration (ng/ml)	
	Dog 1	Dog 2
15	281	<2
30	227	<2
45	220	8
60	198	25
90	146	53
120	144	51
180	98	37
300	49	20
420	26	10

The linear regression equation ($y = 4.68x + 6.16$) for the standards extracted from canine plasma was used to calculate plasma concentrations for the oral time course study in dogs (Table II). The dog study (4 mg/kg oral propranolol) showed significant differences in peak plasma concentration (281 *versus* 53 ng/ml) and the time this concentration was reached (15 *versus* 90 min). The variability of these data reinforce the need for therapeutic monitoring of plasma propranolol concentrations to ensure that efficacious levels are maintained.

Propranolol has a high degree of lipophilicity with two potentially ionizable groups (R-OH, R-NH-R). It was necessary to combine organic solvents (60%) with a base (0.1 *M* sodium hydroxide) to denature the strong affinity propranolol has for plasma proteins. Attempts to increase either component to achieve higher recoveries of the drug endangered the integrity of the membrane in the MSU. The reproducibility of the recoveries, however, makes quantitative analysis possible and is the basis for the simplicity of this method.

Fig. 1 compares the chromatograms obtained from 30-μl injections of blank canine plasma and canine plasma spiked with 100 ng/ml progranolol. The baseline-resolved propranolol peak (A) elutes between 11 and 12 min. Three peaks that appear to be endogenous to canine plasma are actually extracted from the

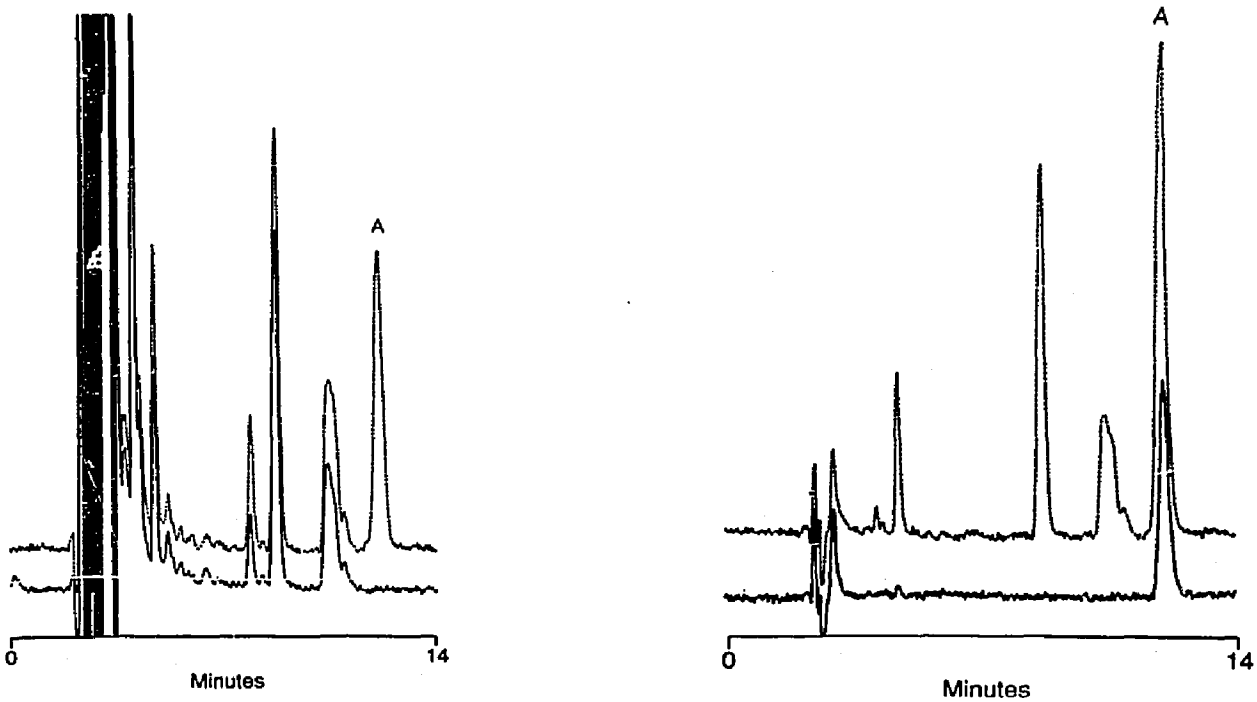

Fig. 1. Typical chromatograms for canine plasma blank (lower trace) and canine plasma spiked with 100 ng/ml propranolol (upper trace). The propranolol peak (A) is indicated.

Fig. 2. Chromatograms comparing standards in 1:1 drug-releasing agent–water injected directly onto the column (40 ng/ml, lower trace) and run through a microseparation unit (100 ng/ml, upper trace). The propranolol peak (A) is indicated.

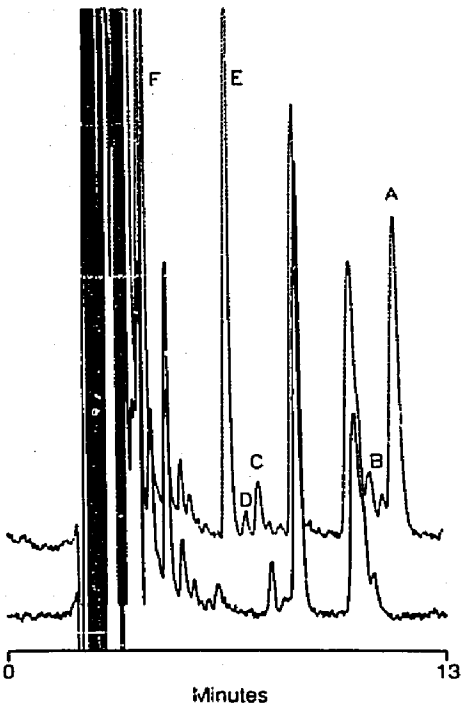

Fig. 3. Chromatograms comparing canine samples from an oral 4 mg/kg time course study. The lower trace represents the pre-dose plasma sample. The upper trace represents a plasma sample taken 60 min post dosing. The propranolol peak (A) and five potential metabolite peaks (B–F) are indicated.

membrane in the MSU. This is illustrated in Fig. 2, where a chromatogram of an unextracted standard (100 ng/ml in 1:1 DRA–water through an MSU) is compared to a standard that was not passed throught an MSU (40 ng/ml in 1:1 DRA–water). The feline plasma blank (not shown) had a profile similar to the canine plasma blank, indicating no endogenous interference with the propranolol standard. Plasma extracts from a dog dosed with 4 mg/kg oral propranolol (dog 1 from Table II) are shown in Fig. 3. Here a pre-dose plasma chromatogram is compared to a plasma chromatogram 60 min post dosing. In addition to the propranolol peak (A), five other peaks (B–F) are absent in the pre-dose sample indicating the presence of metabolites. We were unable to obtain metabolite standards to confirm the identity of these five peaks. However, the assay could be validated for the quantitation of these metabolites if the standards become available. The limit of detection (defined as a peak that is three times the baseline noise) for both canine and feline plasma was 1 ng/ml (60-μl injection volume). Injection volumes of 30–60 μl did not significantly reduce the life of the HPLC column, despite the high pH (10) of the ultrafiltrate.

Digoxin, quinidine, procainamide, furosemide, and phenobarbital, drugs commonly used in veterinary medicine, were evaluated for potential interference in the assay. Of the five, only quinidine had fluorescent character using the detector parameters in this method (excitation wavelength = 222 nm, emission wave-

length = 340 nm) and eluted at 16 min, causing no interference with propranolol or its metabolites.

Although this method was developed for a veterinary application, a human plasma blank was also analyzed. The HPLC trace (not shown) had fewer endogenous peaks than the canine plasma blank in Fig. 1. None of the endogenous peaks interfered with the analytical window for propranolol. This suggests that, after proper validation, the method could be adapted to therapeutic monitoring of plasma propranolol levels in human cardiac patients receiving the drug.

In conclusion, our method is as sensitive as other propranolol assays described in the literature and does not require the use of an internal standard. The simplified sample preparation is a distinct advantage in that large sample sets can be processed for analysis in a relatively short period of time.

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