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Simultaneous determination of phenolphthalein and phenolphthalein glucuronide from dog serum, urine and bile by high-performance liquid chromatography

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ABSTRACT

A procedure is described to simultaneously quantitate phenolphthalein and its glucuronide metabolite from dog serum, urine and bile using high-performance liquid chromatography. The major advantages of this over pre-existing methods include direct analysis of the parent compound and glucuronide metabolite without enzymatic hydrolysis, increased sensitivity and the potential for automation of a large number of samples. Analytes were extracted from serum and urine using a combination of liquid- and solid-phase extraction methodology. Bile samples were analyzed directly after a twenty-fold dilution with mobile phase. The components plus internal standard were separated by reversed-phase high-performance liquid chromatography using step gradient elution and quantitated by the absorbance of ultraviolet light at 230 nm. Limits of detection from 1 ml of serum, 0.1 ml of urine and 0.05 ml of bile were 0.1, 0.5 and 10 μg/ml for phenolphthalein and 0.1, 10 and 50 μg/ml for phenolphthalein glucuronide, respectively.

INTRODUCTION

Phenolphthalein, 3,3-bis(4-hydroxyphenyl)-1-(3H)-isobenzofuranone (PT), is a compound indicated for human use for the short-term treatment of constipation. In rats, it is metabolized exclusively to a glucuronide conjugate (phenolphthalein glucuronide, PTG) and has therefore been used as a model compound for enterohepatic circulation studies [1,2]. While PT's disposition is well documented in rats, there have been no published reports regarding its behavior in the dog. Since the dog is a commonly used laboratory animal for assessing the *in vivo* disposition of pharmaceuticals, we have chosen this species to investigate the extent of enterohepatic circulation after parenteral administration of PT and PTG. To do so, a sensitive analytical method is needed to separately quantitate PT and PTG levels from various biological matrices (serum, urine and bile).

Of the available assays for PT, they were either qualitative [3–5], did not involve quantitation of analytes from biological fluids [6–10] or required an enzymatic or chemical hydrolytic conversion of PTG prior to analysis of PT [5,11,12]. None of the above assays quantitated PT or PTG from bile. The only assay describing quantitation of

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PT and PTG from several biological matrices [13] involved pretreatment of serum, urine or intestinal perfusate with acidified aqueous acetone followed by addition of a pH 10.4 glycine buffer. Quantitation of PT was then made spectrophotometrically at 550 nm after development of a pink color. For PTG, the metabolite was first hydrolyzed to PT enzymatically using β-glucuronidase/arylsulfatase and then analyzed as previously described for PT. This method of analysis for PT and PTG suffers from several disadvantages. First, visible monitoring of PT at 550 nm results in a limit of detection from serum and urine of only ≈ 10 μg/ml, whereas the limit of detection for PTG from urine was ≈ 75 μg/ml; detectability for both compounds would likely be inadequate to support most pharmacokinetic studies. Secondly, the hydrolysis reaction of PTG with β-glucuronidase/arylsulfatase is somewhat variable (reactions are typically left for 24 h before analysis, thus creating the potential for compound instability to play an important factor in the accuracy of the analysis) and non-specific (commercially available β-glucuronidase contains both glucuronidase and sulfatase activities). Finally, the method is not amendable to automation of a large number of samples typically produced during a pharmacokinetic study.

In response to these shortcomings, we have developed a reversed-phase high-performance liquid chromatographic (HPLC) assay employing step gradient elution and quantitation of PT and PTG from serum and urine by UV absorption. Sample preparation consists of a combination of liquid- and solid-phase extraction methodology. In addition, this method was applied to quantitate PT and PTG from bile following direct dilution with the HPLC mobile phase. The internal standard (I.S.) used during the extraction procedure is a structurally similar compound, bromocresol purple. The method described herein was used to analyze actual serum and urine samples obtained during a pilot pharmacokinetic study. The chemical structures of PT, PTG and the I.S. are shown in Fig. 1.

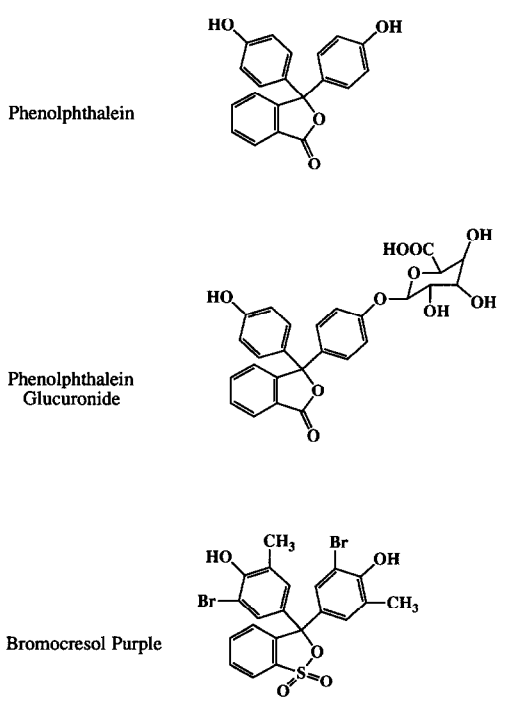

Fig. 1. Chemical structures for phenolphthalein, phenolphthalein glucuronide and the internal standard, bromocresol purple.

EXPERIMENTAL

Materials and reagents

PT and the sodium salt of PTG were obtained from Sigma (St. Louis, MO, USA). Bromocresol purple [0.04% (w/w) solution in water], sodium chloride, monobasic monohydrate sodium phosphate and dibasic anhydrous sodium phosphate were obtained from Aldrich (Milwaukee, WI, USA). All solvents were of HPLC grade (Fisher Scientific, Fair Lawn, NJ, USA).

Instrumentation and chromatographic conditions

The HPLC system consisted of two Kratos ABI Spectroflow 400 pumps (Ramsey, NJ, USA), and a Spectra Physics SP8780 automatic sampler (San Jose, CA, USA) attached to a Rheodyne injection valve (100-μl sample loop) which was coupled to a Supelco LC-18DB, 150 mm × 4.6 mm I.D., 5 μm particle size column (Bellefonte, PA, USA). Preceding the analytical column was a Waters μBondapak C_{18} guard-pak precolumn cartridge, 0.53 cm × 0.4 cm I.D., 10

μm particle size (Milford, MA, USA). All samples were run at ambient temperature.

The mobile phase in reservoir A consisted of acetonitrile–buffer (10:90, v/v) while that in the B reservoir consisted of acetonitrile–buffer (60:40, v/v). The buffer used in mobile phase preparation, 0.05 *M* monobasic sodium phosphate, was used as prepared (22.6 g in 4 l of water, pH ≈ 4.3). Prior to use, the mobile phase was filtered through a 0.45-μm Nylon 66 membrane (Fisher Scientific, Fair Lawn, NJ, USA). A flow-rate of 1.0 ml/min was maintained throughout the chromatographic run. A step gradient was employed for optimum peak separation and consisted of an initial 5-min isocratic segment of 65% A–35% B, followed by a 0.1-min step to 40% A–60% B which was held isocratically for 8 min before returning to the original 65% A–35% B composition (total run time = 13 min). Injections were made 18 min apart to allow for complete column re-equilibration.

Detection of the analytes and I.S. was accomplished using a Kratos ABI Spectroflow 783 variable-wavelength UV detector that was set at a fixed wavelength of 230 nm. Data were recorded on a Perkin Elmer 3212 LAS/LIMS (Laboratory Information Management System) information system (Norwalk, CT, USA).

Serum extraction

Aliquots (1 ml) of serum were spiked with 25 μl of 0.4 mg/ml I.S. solution. To dog serum containing PT, PTG and the I.S., 3 ml of aqueous acidified acetone (880 ml of acetone, 0.5 ml of glacial acetic acid and water added to a 1-l final volume) were added to precipitate proteins. After vortex-mixing for 5 min, the serum proteins were pelleted by centrifugation at 1200 g for 10 min. A 3-ml volume of supernatant was transferred to clean test tubes and evaporated to dryness *in vacuo* (Savant Instruments vacuum evaporator, Farmingdale, NY, USA). The residue was resuspended in 1 ml of 0.1 *M* Na_2HPO_4–NaH_2PO_4–0.9% NaCl, pH 7.4 buffer and sonicated for 10 min. The resulting solution was further processed by solid-phase extraction using 1-ml C_{18} Bond-Elut columns (Analytichem, Harbor City, CA, USA) and a Speed-Wiz automated solid-phase extraction system (Applied Separations, Bethlehem, PA, USA). Conditioning of the columns occurred with two column volumes of methanol followed by two column volumes of water, respectively (keeping the sorbent bed wet after the final water wash). The buffer solutions containing the analytes were added to and then aspirated through the columns. Removal of endogenous material was accomplished with two column volume rinses of water. After the final water rinse, the columns were allowed to dry. Elution of the analytes into clean test tubes was accomplished using two 0.8-ml rinses of methanol. After evaporating the methanolic eluate to dryness *in vacuo*, the final residue was resuspended in 1 ml of HPLC mobile phase A and centrifuged at 1200 *g* for 10 min to remove particulates. A 100-μl aliquot was injected onto the HPLC column.

Urine extraction

Urine samples (0.1 ml) were spiked with 25 μl of 0.4 mg/ml I.S. solution and then brought to a final 1-ml volume with 0.875 ml of 0.1 *M* Na_2HPO_4–NaH_2PO_4–0.9% NaCl, pH 7.4 buffer. A 3-ml volume of non-acidified aqueous acetone (880 ml of acetone and water added to a 1-l final volume) was added and the solution vortex-mixed for 5 min. The urine samples were then processed as previously described for serum, with a 100-μl aliquot of the final supernatant injected onto the HPLC column.

Bile extraction

To a 50-μl bile sample, 950 μl of mobile phase A were added. Following centrifugation for 10 min at 1200 *g*, a 50-μl aliquot of the supernatant was injected directly onto the HPLC column. Since the preparation of bile samples involved only a dilution (and no extraction) step, the I.S. was not added to these samples.

Precision

Variability was determined by within- and between-day coefficients of variation (C.V.). Variability due to the HPLC system was determined from six replicate injections of a mobile phase

standard containing 10 μg/ml PT, 10 μg/ml PTG and 20 μg/ml I.S. Variability of the extraction method was determined from six separate matrix samples spiked with identical concentrations of PT, PTG and the I.S. and then carried through the extraction procedures described above.

Linearity and recovery

Linearity and recovery were evaluated by adding known amounts of PT and PTG to the appropriate biological matrices and extracting as described above. Results of regression analysis and recovery values were compared for standard curves generated on two different days. Absolute recovery of extracted standards was based upon comparison to detector responses of unextracted standards, correcting for volume differences after the initial liquid extraction step.

Accuracy

The accuracy of the assay was assessed by examining how well the back-calculated concentrations compared to the true concentrations within each standard curve. This was accomplished by calculating a percent error at each of the six concentrations comprising a standard curve; the mean percent error for each curve was reported.

Stability

Stabilities of PT and PTG were measured in the various matrices after storage at −20°C throughout a 35-day period (intervals of 0, 3, 7, 14 and 35 days). Triplicate stored samples containing PT and PTG at concentrations of 10 μg/ml (serum), 50 μg/ml (urine) or 250 μg/ml (bile) were analyzed and compared to freshly prepared standards on each day of analysis. In addition, matrix extracts and standards prepared in mobile phase were stored at room temperature and analyzed on days 0, 3, 7 and 14. Linear regression analysis of percent analyte found *versus* storage time was performed. Those matrices stored at −20°C having a slope steeper than −0.33 (*i.e.* > 10% degradation in thirty days) or room temper-

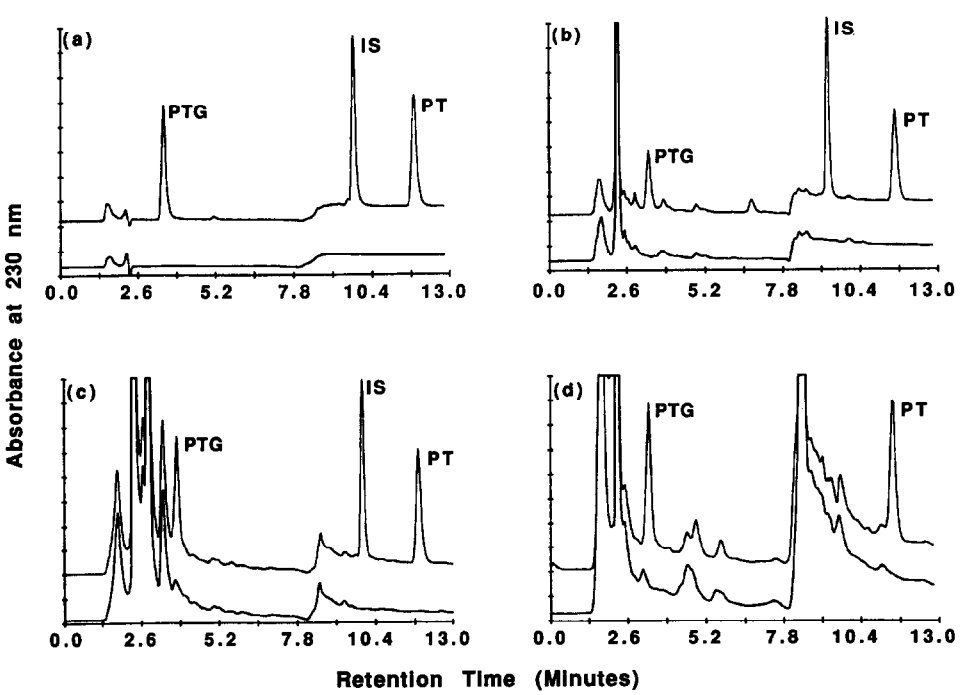

Fig. 2. Representative chromatograms of PT, PTG and I.S. in various matrices. (a) Mobile phase (2 μg/ml PT and PTG, 10 μg/ml I.S.); (b) serum (2 μg/ml PT and PTG, 10 μg/ml I.S.); (c) urine (20 μg/ml PT and PTG, 10 μg/ml I.S.); (d) bile (100 μg/ml PT and PTG, no I.S. added). Upper traces are analyte-spiked matrices, lower traces are blank matrices.

ature extracts having a slope steeper than −0.71 (*i.e.* > 10% degradation in fourteen days) were identified.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms of blank and analyte-spiked matrices containing PT, PTG and I.S. are shown in Fig. 2a–d. Elution of PTG, I.S. and PT from the HPLC column occurred at approximately 3.4, 9.5 and 11.4 min, respectively. Detection limits, based upon a 3:1 signal-to-noise ratio from 1 ml of serum, 0.1 ml of urine and 0.05 ml of bile, were 0.1, 0.5 and 10 μg/ml for PT and 0.1, 10 and 50 μg/ml for PTG, respectively.

Validation

Results for precision of the assay are represented in Table I. The percentage C.V. was based upon the response ratio of six replicates for each test. Response ratios were calculated as the quotient of analyte and I.S. peak areas or heights. The C.V. for the system ranged from 0.72 to 2.97% for samples analyzed on two different days. Similarly, the C.V. range for the extraction method of samples analyzed on two different days was between 0.25 and 4.92%.

Due to the large concentration range required for this assay, standard curves were constructed and weighted in order to adequately quantitate unknown samples. Reproducibility of the method was indicated by consistent slopes and intercepts obtained from curves generated on two different days (Table II). The accuracy of the method was considered acceptable by demonstrating typically ≤ 10% deviation of back-calculated concentrations from their true values (listed as "Error" in Table II). Mean recoveries shown in Table III for PT, PTG and I.S. demonstrated ≥

TABLE I

SUMMARY OF WITHIN- AND INTER-DAY PRECISION FOR PT AND PTG IN SEVERAL MATRICES (n = 6)

N.D. = Not determined.

Matrix	Day	Compound	System			Method		
			Ratio	S.D.	C.V. (%)	Ratio	S.D.	C.V. (%)
Serum	1	PTG	1.52	0.02	1.32	1.42	0.03	2.12
		PT	1.81	0.03	1.47	2.18	0.11	4.92
	2	PTG	1.54	0.02	1.14	1.43	0.06	4.30
		PT	1.85	0.04	1.91	2.42	0.10	4.21
Urine	1	PTG	3.74	0.04	1.09	3.36	0.03	1.02
		PT	4.94	0.06	1.22	4.90	0.06	1.17
	2	PTG	3.91	0.03	0.72	3.45	0.09	2.65
		PT	4.79	0.04	0.81	4.97	0.08	1.56
Bile	1	PTG	N.D.	–	–	41.7[a]	0.31[a]	0.73
		PT	N.D.	–	–	57.3[a]	1.18[a]	2.06
	2	PTG	N.D.	–	–	42.6[a]	0.11[a]	0.25
		PT	N.D.	–	–	61.2[a]	0.45[a]	0.73
Mobile phase	1	PTG	1.76	0.04	2.38	N.D.	–	–
		PT	2.16	0.06	2.97	N.D.	–	–
	2	PTG	1.79	0.03	1.74	N.D.	–	–
		PT	2.19	0.04	1.69	N.D.	–	–
Mean					1.54			2.14

[a] Peak heights × 10^5 due to no internal standard used.

TABLE II

REPRODUCIBILITY OF PT AND PTG STANDARD CURVES

Matrix	Day	Range (μg/ml)	Slope	y-Intercept	Weight	r^2	Average error (%)
PTG							
Serum	1	0.1–50	0.2307	−0.0116	$1/C$	0.998	4.6
	2	0.1–50	0.2587	−0.0061	$1/C$	0.999	5.3
Urine	1	2–100	0.3370	0.0854	$1/C$	0.999	1.9
	2	2–100	0.3291	0.0814	$1/C$	0.999	7.2
Bile	1	50–800	6965	192073	$1/C$	0.999	3.2
	2	50–800	7448	147637	$1/C$	0.999	2.7
PT							
Serum	1	0.1–50	0.3981	0.0014	$1/C$	0.997	9.3
	2	0.1–50	0.6229	0.0019	$1/C$	0.998	4.2
Urine	1	0.5–100	0.4509	−0.1171	$1/C$	0.996	9.2
	2	0.5–100	0.4596	−0.0886	$1/C$	0.996	9.3
Bile	1	10–800	9955	69318	$1/C$	0.995	12.0
	2	10–800	10405	31932	$1/C$	0.997	7.2

77% recovery from serum and urine (bile extraction was assumed to be 100% since only a dilution took place).

PT and PTG appear to be stable in serum, urine and bile upon storage at −20°C for up to thirty days (Table IV). Examination of PT and PTG stability in mobile phase following extraction indicated a relative instability of the samples within fourteen days. Therefore, to ensure sample integrity, extraction should begin within thirty days of collection; HPLC analysis should be finalized within three to seven days of extraction.

TABLE III

SERUM AND URINE RECOVERIES OF PT, PTG AND I.S.

Bile recovery was assumed to be 100% since no extraction was performed.

Compound	Recovery (%)	
	Day 1	Day 2
Serum		
PTG	86	81
I.S.	92	87
PT	111	114
Urine		
PTG	79	77
I.S.	89	86
PT	93	86

Application to biological samples

The method has successfully been applied to a pilot pharmacokinetic study in dogs. Fig. 3 represents the serum PT and PTG concentration *versus* time profile following a 10-h constant rate intravenous infusion of phenolphthalein at 177 μg/min/kg to an 11-kg male beagle dog. Food was introduced at 2, 4, 6 and 8 h during the study to stimulate gallbladder contraction. Average steady-state serum levels were 7 μg/ml for PT and 30 μg/ml for PTG. Significant secondary peaks for both PT and PTG were seen; an observation consistent with a compound undergoing enterohepatic recirculation. The serum clearance of PT, calculated as the quotient of infusion rate and steady-state serum concentration, was 25.3 ml/min/kg.

In a separate dog infused with 177 μg/min/kg for 8 h, timed urine collections were performed throughout the study. After recording total vol-

TABLE IV

STABILITY OF PT AND PTG IN SEVERAL MATRICES OR EXTRACTS

Matrix	Percentage found				Slope (%/day)
	Day 3	Day 7	Day 14	Day 35	
PTG					
Serum	115	93	105	97	−0.31
Serum extract	86	83	71	N.D.[a]	−1.43[b]
Mobile phase	103	88	100	N.D.	0.01
Urine	110	104	105	101	−0.22
Urine extract	81	91	96	N.D.	1.23
Bile	111	99	101	98	−0.28
Bile extract	111	106	92	N.D.	−1.76[b]
PT					
Serum	104	86	84	89	−0.24
Serum extract	129	85	103	N.D.	−1.81[b]
Mobile phase	99	91	94	N.D.	−0.39
Urine	105	98	97	110	0.28
Urine extract	113	91	84	N.D.	−2.42[b]
Bile	107	103	97	95	−0.33
Bile extract	108	106	94	N.D.	−1.27[b]

[a] Not determined.

[b] Negative slope indicating > 10% degradation at day 14 (extracts or mobile phase standards at room temperature) or day 30 (matrices at −20°C).

umes, the urine was analyzed for PT and PTG as previously described. Table V displays the results of this study.

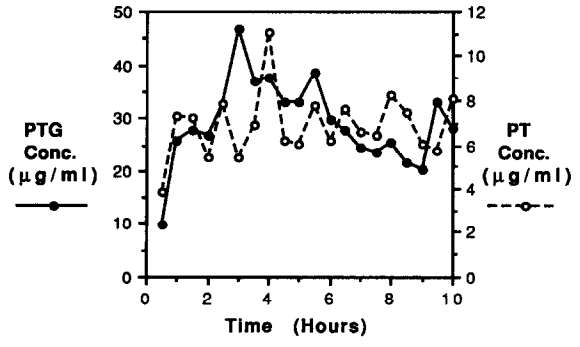

Fig. 3. Serum PT and PTG concentration *versus* time profile following an intravenous infusion of 177 µg/min/kg phenolphthalein to a dog. Food was introduced at 2, 4, 6 and 8 h to stimulate gallbladder contraction.

CONCLUSIONS

Complete pharmacokinetic characterization of a drug requires a sensitive and selective assay for both the parent drug and its major metabolite(s) in several biological fluids. We report here such an assay for the analysis of PT and PTG from dog serum and urine. By combining liquid- and solid-phase extraction techniques with reversed-phase HPLC, detection limits of appropriate sensitivity have been achieved. The major advantage of this method is the direct analysis of a glucuronide metabolite without the need for a separate hydrolysis step. The method has been succesfully applied by assaying serum and urine samples obtained from a pilot dog pharmacokinetic study. Coupling the expected significant biliary excretion of PT with the normal concentrating function of the gallbladder [14], we anticipate biliary concentrations of PT and PTG to exceed serum

TABLE V

URINE COLLECTION DATA AND CONCENTRATIONS OF PT AND PTG FOUND

Collection period (h)	Urine volume (ml)	PTG concentration (μg/ml)	PT concentration (μg/ml)
2	28	567	7.7
3	40	604	11.6
3	60	198	5.9

concentrations by at least 10-fold and probably closer to 100-fold. As such, we anticipate having sufficient analytical capability to quantitate levels of PT and PTG from this biological matrix.

During the development of this assay, several problems arose. The first was noted when we attempted to adapt this method to another HPLC instrument and found slight to moderate changes in retention times for the analytes. Any chromatography using a step gradient is dependent upon tubing length and diameter, solvent mixer, column length, etc. Therefore, slight modifications to the system or mobile phases may be needed to exactly reproduce retention times reported here when using another HPLC system. The second concern is with interferences seen in urine and bile samples which directly influenced the PTG detection limit of 10 and 50 μg/ml, respectively. Since these interferences may be subject to considerable dog-to-dog variability, a blank matrix sample should always be evaluated to determine the magnitude of these interfering peaks. Since the time of method validation, we have found through retrospective analysis of urine from many dogs that the detection limit reported here is on the conservative side (*i.e.* some urine samples afforded a detection limit in the 2–5 μg/ml range).

In summary, we have developed an assay for the quantitative analysis of PT and PTG from serum and urine (and likely bile) that has the advantages of speed and sensitivity sufficient to analyze samples obtained from a complete pharmacokinetic study of PT in dogs.

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