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DETERMINATION OF THIABENDAZOLE AND 5-HYDROXYTHIABENDAZOLE IN HUMAN SERUM BY FLUORESCENCE-DETECTED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We have developed a rapid, sensitive and precise high-performance liquid chromatographic method using fluorescence detection for the simultaneous determination of thiabendazole and unconjugated 5-hydroxythiabendazole in serum. Sample pretreatment consists only of protein precipitation with acetonitrile containing the internal standard, 2-methylindole. Detection limits were found to be 0.1 μg/ml serum for thiabendazole and 0.4 μg/ml serum for 5-hydroxythiabendazole. Between-day analytical precision coefficients of variation for serum-based controls were 7% and 11% for thiabendazole levels of 1 and 5 μg/ml, respectively; and 43% and 8% for 5-hydroxythiabendazole levels of 6 and 60 μg/ml, respectively. We also devised a microenzymatic method for the conversion of the glucuronide and sulfate esters of 5-hydroxythiabendazole using β-glucuronidase [EC 3.2.1.31] and sulfatase [EC 3.1.6.1]. Thus, quantitation of the separate metabolites was possible. We also utilized a special adaptation of the chromatographic procedure for the determination of the 5-hydroxythiabendazole metabolites in the sera of uremic patients, which can contain large amounts of interfering fluorescent substances. The method should be particularly useful for monitoring thiabendazole therapy in patients unable to eliminate the potentially toxic metabolites.

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INTRODUCTION

Thiabendazole, 2-(4-thiazoyl)-1H-benzimidazole (TBZ) is a broad-spectrum anthelmintic agent that is also extensively used as a fungicide for the post harvest protection of citrus fruits and bananas. Several high-performance liquid chromatographic (HPLC) methods for the quantitation of TBZ have recently been reported with regard to its use as a fungicide [1—4]. However, for two reasons, none of these methods are directly applicable to monitoring the therapeutic use of TBZ. First, they are not designed for the determination of TBZ in serum. Second and more importantly, they do not address the quantitation of the metabolites of TBZ. That monitoring of the metabolites' concentrations is important is evident from a recent study in which accumulation of the metabolites was linked with toxicity in a patient who was unable to eliminate the metabolites because of impaired renal function [5]. We report here the development of a simple and precise HPLC method for the determination of TBZ and 5-hydroxythiabendazole (5OHTBZ) in human serum, as well as the quantitation of the glucuronide and sulfate esters of 5OHTBZ following an in vitro enzymatic conversion to 5OHTBZ. We have successfully utilized the method in monitoring TBZ therapy and metabolite elimination in an anephric patient receiving hemodialysis and hemoperfusion [6].

EXPERIMENTAL

Chemicals

Purified TBZ and 5OHTBZ were kindly supplied by the Merck Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). Dibasic potassium phosphate, phosphoric acid (85%), sodium acetate and hydrochloric acid were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). β-Glucuronidase [EC 3.2.1.31] (Type B-1 from bovine liver), sulfatase [EC 3.1.6.1] (Type H-1 from Helix pomatia) and 2-methylindole were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and methanol (both glass distilled) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

HPLC mobile phase preparation

Water for the mobile phase was passed through a Milli-Q Reagent Grade Water System (Millipore, Bedford, MA, U.S.A.) until a resistance of 16—18 MΩ cm or greater was achieved. Phosphate buffer, 0.01 mol/l was prepared from K_2HPO_4 and adjusted to pH 7.00 ± 0.05 with phosphoric acid. This phosphate buffer was stored up to four weeks at 4°C. Mobile phase was prepared daily by mixing phosphate buffer with methanol. Two mixtures were employed for these studies. The first was used for TBZ and 5OHTBZ determination in normal human sera and consisted of a mixture of buffer—methanol (50:50, v/v). The second was used specifically for 5OHTBZ quantitation in serum samples from a renally compromised patient, and consisted of a mixture of buffer—methanol (55:45, v/v). After mixing, the mobile phase was filtered and degassed through a Millipore GS membrane (0.22 μm, Millipore).

HPLC apparatus

A Waters Model 6000A solvent delivery system and Model U6K injector (Waters Assoc., Milford, MA, U.S.A.) were connected to a Waters precolumn (containing 37—50-μm Bondapak C_{18}/Corasil) and a 30 cm × 4 mm μBondapak C_{18} column, particle size 10 μm (Waters Assoc.). The Model FS970 L.C. Fluorometer (Schoeffel, Westwood, NJ, U.S.A.) fitted with a GM970 excitation monochromator, a Corning 7-54 excitation prefilter and emission cutoff filters was used. Output was monitored using an Omniscribe Recorder (Houston Instruments, Austin, TX, U.S.A.). Excitation was set at 305 nm, while emission was monitored using either a 370-nm or 470-nm cutoff filter. Fluorometer settings included a time constant of 6 sec and range setting of 0.2 to 1.0 μA, depending upon the size of the eluting peaks. The flow-rate was 1.0 ml/min.

Sample preparation

The internal standard (IS), 2-methylindole [2], was dissolved in acetonitrile at concentrations of 50 μg/ml (for an emission cutoff of > 370 nm) and 2000 μg/ml (for an emission cutoff of > 470 nm). These solutions were stored at 4°C. A 50-μl aliquot of patient serum or aqueous standard was pipetted into an 0.5-ml polypropylene micro test tube and 50 μl of acetonitrile—IS were then added. The mixture was vortexed to facilitate mixing and protein precipitation. The tubes were then centrifuged for 2—4 min at 7500 *g* (Fisher Centrifuge Model 59, Pittsburgh, PA, U.S.A.). A 10—20 μl aliquot of the supernatant was injected into the chromatographic system.

Preparation of controls and standards

TBZ and 5OHTBZ were added to optically clarified [7] pooled human sera to obtain two control pools; a high and a low pool spanning the expected range of concentrations for specimens from a dialysis patient [5] (high: 6 μg TBZ per ml, 60 μg 5OHTBZ per ml; low: 1 μg TBZ per ml, 5 μg 5OHTBZ per ml). These were aliquoted (50 μl) into micro test tubes (as described above for patient sera and standards), capped and frozen at −20°C. Each day, a high and low tube was thawed, to which 50 μl of acetonitrile—IS was added.

A series of aqueous standards, each containing TBZ and 5OHTBZ, were prepared from methanolic stock standards prepared gravimetrically at 100 μg TBZ per ml and 1000 μg 5OHTBZ per ml. The concentrations of the stock standards were then checked spectrophotometrically after dilution with methanol (TBZ) or 0.1 *N* hydrochloric acid (5OHTBZ) using the published extinction coefficients (TBZ: $\epsilon_{311\,nm}$ = 23,300 l/mol · cm; 5OHTBZ: $\epsilon_{318\,nm}$ = 17,794 l/mol · cm [8]) and a GCA/McPherson spectrophotometer (Acton, MA, U.S.A.). The spectrophotometrically determined concentrations were 99 μg TBZ per ml and 880 μg 5OHTBZ per ml (approximately consistent with 5OHTBZ being supplied as a monohydrate). Since recovery of both compounds from a serum matrix was virtually 100% (see Results and Discussion) the working standards were prepared in water. These aqueous standards were stored at 4°C and aliquoted fresh each day.

Quantitation of TBZ and 5OHTBZ

Only two standards were run routinely, the remaining standards being used to check the range of linearity (see Results and Discussion). Peak height was determined for TBZ and 5OHTBZ and the IS; and the ratios of peak heights, TBZ/IS and 5OHTBZ/IS, plotted vs. the spectrophotometrically determined concentrations. A linear least squares analysis was then performed using these two data points and the origin. The derived slope and intercept were used to calculate concentrations for patient or control samples.

Enzymatic degradation of the glucuronide and sulfate esters of 5OHTBZ

A modification of previously published procedures [8, 9] for the enzymatic hydrolysis of glucuronide and sulfate esters of 5OHTBZ was devised in order to minimize the dilution of sample in the reaction mixture. A mixture of β-glucuronidase and sulfatase was prepared by suspending 10 mg of β-glucuronidase (at 600,000 U/g) and 500 mg of sulfatase (21,000 U/g) in 0.1 mol/l sodium acetate, pH 5.0. A β-glucuronidase solution was also prepared by dissolving 10 mg of the enzyme in 5 ml of 0.1 mol/l sodium acetate, pH 5.0. Measurement of the 5OHTBZ peak following treatment with the combined enzyme mixture corresponded to the total 5OHTBZ metabolites (unconjugated 5OHTBZ + glucuronide ester + sulfate ester). Measurement of the 5OHTBZ peak following treatment with just β-glucuronidase yielded unconjugated 5OHTBZ + glucuronide ester. Measurement of the 5OHTBZ without any enzymatic treatment yielded the value for the unconjugated 5OHTBZ; therefore, appropriate subtraction of the various measurements gave separate values for each of the three metabolites. Sulfatase could not be used alone to measure the sulfate conjugate concentration since the Helix pomatia preparation also contains β-glucuronidase.

The conditions used for the enzymatic hydrolyses were as follows. A 50-μl aliquot of serum was pipetted into a micro test tube. To this were added 50 μl of 1.0 mol/l sodium acetate followed by 25 μl of either the combined enzyme mixture or β-glucuronidase preparations described above. The micro test tubes were capped and gently vortexed. They were then placed in a 37°C Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, IL, U.S.A.) for 18 h. Completion of the enzymatic conversion using the combined enzyme mixture was checked by noting the complete loss of conjugate peaks in a patient's serum. At the end of the incubation period the tubes were removed and stored at −20°C until analysis. At the time of analysis, the tubes were thawed, 100 μl of acetonitrile—IS were added and the mixture vortexed. These samples required centrifugation immediately prior to supernatant injection because of the ready re-suspension of fine particles. This procedure resulted in a dilution of the serum sample which was nominally 2.25 times greater than the dilution occurring with samples not taken through an enzymatic step. As a check on this dilution factor, control samples were taken through this procedure several times and found to have concentrations 1/2.32 lower than those not subjected to the procedure. Since this factor is close to that expected on the basis of the additional dilution with disodium acetate, enzyme and acetonitrile (1/2.25), we conclude that the volumes of mixing are negligible and that the concentration of unconjugated 5OHTBZ is unaffected by the treatment.

Thus, standards were not processed through this procedure. Instead, concentrations derived from the ordinary standard curve for specimens taken through an enzymatic conversion were multiplied by this dilution factor (2.32).

RESULTS AND DISCUSSION

Fig. 1a presents a typical chromatogram of the low control made from pooled human sera. This chromatogram was run using buffer—methanol (50:50), 50 μg IS per ml acetonitrile, and the 370-nm emission cutoff filter. Retention times were 5.5 min for 5OHTBZ, 10.5 min for TBZ and 13.5 min for IS. Peak-to-trough noise level was equivalent to 0.07 μg/ml TBZ and 0.2 μg/ml 5OHTBZ per ml serum after a 1:1 dilution with acetonitrile—IS, so the detection limits could be defined as approximately twice these levels. Fig. 1b illustrates serum from a uremic patient 1 h following the patient's first dose of TBZ (25 mg/kg orally). As is clear from Fig. 1b, large amounts of fluorescent substances present in the serum of our patient made it impossible to accurately quantitate

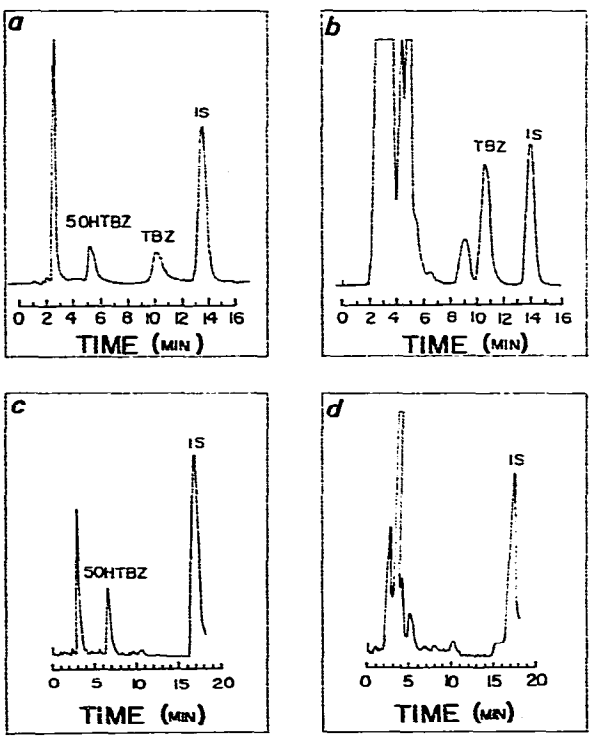

Fig. 1. Typical chromatograms of: (a) low serum control material, 1 μg TBZ per ml, 6 μg 5OHTBZ per ml, and (b) renal patient serum 1 h following an oral TBZ dose (25 mg/kg orally), TBZ concentration determined to be 5.0 μg/ml. Conditions: buffer—methanol (50:50) mobile phase, 50 μg IS per ml acetonitrile, 370-nm cutoff filter. (c) Low serum control material, 6 μg 5OHTBZ per ml, and (d) renal patient serum blank (i.e. before TBZ dosage) taken through the combined enzyme procedure described in the text. Conditions: buffer—methanol (55:45) mobile phase, 2000 μg IS per ml acetonitrile, 470-nm cutoff filter: range setting for IS is double that of the remaining chromatogram. Abbreviations: TBZ = thiabendazole; 5OHTBZ = 5-hydroxythiabendazole; IS = internal standard.

the level of 5OHTBZ. This is probably not unique to our patient: it is well known that uremic patients have significant amounts of fluorescent substances in their serum and urine [10—12]. This problem was solved by using the 470-nm cutoff filter which dramatically reduced the fluorescence from the endogenous interfering substances (reported in one study to have a fluorescence maximum emission at 430 nm following excitation at 342 nm [12]), TBZ and IS, but not that of 5OHTBZ (which has emission maxima at 425 nm and 525 nm following excitation at 325 nm [8]). In addition, a slight change in the mobile phase to a 55:45 mixture of buffer and methanol further improved the resolution of the 5OHTBZ peak in our patient serum. The IS concentration had to be increased to 2000 μg/ml for a suitable signal level using the 470-nm cutoff filter. Figs. 1c and d are chromatograms run under these modified conditions. Fig. 1c shows the low control material and Fig. 1d the same uremic patient's serum prior to any TBZ dose after having been subjected to combined enzyme treatment. It is clear that under these conditions, 5OHTBZ could be readily resolved and quantitated. Retention times under these conditions were 7.0 min for 5OHTBZ and 17 min for IS. Peak-to-trough noise level was equivalent to 1.7 μg 5OHTBZ per ml, the detection limit therefore being 3 μg 5OHTBZ per ml.

Typically then, specimens from the uremic patient were divided into eight 50-μl aliquots and frozen for later use. Two were used for duplicate TBZ quantitation under the first set of conditions described. Two each were used for 5OHTBZ quantitation under the second set of conditions, following either no enzymatic treatment, combined enzyme treatment or β-glucuronidase treatment.

The assays for both compounds were found to be linear over the ranges encompassed by the aqueous standards (0—7.4 μg TBZ per ml; 0—132 μg 5OHTBZ per ml; 0—88 μg 5OHTBZ per ml using the 470-nm cutoff filter). In order to present this wide range of peak heights, the range setting on the fluorometer was often varied within a single chromatogram. Peak heights were then multiplied by the range setting to normalize all heights to the same range setting. Table I presents the slope and intercept data obtained using all the standards. 2-Methylindole solutions were slightly unstable so that over a period of weeks, the IS peak would diminish, causing an elevated slope in the standard curve. However, this slow degradation of IS never presented a problem during a single day's run.

TABLE I

LINEARITY DATA FOR AQUEOUS STANDARDS

Analyte	Cutoff filter (nm)	Intercept (a)	Slope (b) (ml/μg)	r	n	IS—acetonitrile concn. (μg/ml)
TBZ	370	0.0199	0.197	0.991	8	50
5OHTBZ	370	0.0879	0.0584	0.998	8	50
5OHTBZ	470	−0.0288	0.194	0.997	7	2000

Recovery from a serum base after protein precipitation was determined by spiking pooled human sera volumetrically to concentrations equal to those of the aqueous standards using the stock TBZ and 5OHTBZ solutions. Absolute recovery was determined by comparing peak heights for identical injection volumes of aqueous standards and serum specimens having the same concentration. These recoveries (%R = 100 × serum peak height/aqueous standard peak height, 7 concentrations) were found to average 103 ± 13% (S.D.) for 5OHTBZ and 108 ± 5% for TBZ. Relative recoveries were determined by measuring concentrations from the standard curve. These recoveries (%R = 100 × measured serum concentration/volumetrically spiked concentration, 7 concentrations) were found to average 91 ± 12% (S.D.) and 104 ± 18% for TBZ and 5OHTBZ, respectively. Thus, if the serum base materials were used as standards, similar slope and intercept parameters would be obtained (TBZ: $Y = 0.026 + 0.20X$, $r = 0.986$; 5OHTBZ: $Y = -0.014 + 0.056X$, $r = 0.996$: 370 nm cutoff filter; compare with parameters in Table I). Therefore, aqueous standards could be used throughout the study.

Table II presents precision data obtained for within-run and between-run studies. All the precision data were judged to be acceptable for clinical utility. The two instances of high coefficients of variation (43% and 19% for 5OHTBZ at 6 μg/ml) are of no great concern clinically because of the low 5OHTBZ concentration: precision is significantly better at the more important higher levels where toxicity may result. The low and high controls were also taken through the enzymatic conversion steps, but precision for 5OHTBZ was not significantly affected by these steps. We obtained coefficients of variation of 11.7% and 4.6% ($n = 5$) for the low and high 5OHTBZ controls, respectively.

Table III presents those drugs which were tested and found not to interfere with these assays. All were checked at concentrations greater than expected to

TABLE II

ANALYTICAL PRECISION PARAMETERS FOR SERUM CONTROL MATERIALS

Control	Analyte	$\overline{X}$ (μg/ml)	S.D. (μg/ml)	C.V. (%)	n	Cutoff filter (nm)
Within-run precision						
Low	TBZ	1.0	0.027	2.7	20	370
	5OHTBZ	4.5	0.14	3.1	20	370
High	TBZ	4.1	0.077	1.9	21	370
	5OHTBZ	53	1.2	2.2	21	370
Between-run precision						
Low	TBZ	1.1	0.083	7.3	14	370
	5OHTBZ	5.9	2.6	43	14	370
	5OHTBZ*	5.6	1.1	19	13	470
High	TBZ	4.9	0.55	11	16	370
	5OHTBZ	57	4.5	7.9	14	370
	5OHTBZ*	57	2.7	4.7	13	470

*These samples were not taken through the enzymatic conversion steps.

TABLE III

SUBSTANCES CHECKED FOR INTERFERENCES

Propranolol	Lidocaine
Quinidine	Guanethidine
Procainamide	Disopyramide
N-Acetylprocainamide	Diazepam
Prazosin	Benztropine
Minoxidil	Caffeine
Phenytoin	Chlorothiazide
Phenobarbital	Acetaminophen
Carbamazepine	Theophylline
α-Methyldopa	Nordiazepam
Furosemide	Propoxyphene
Hydralazine	Salicylic acid

be observed in clinical specimens. This does not rule out the possibility that metabolites of these drugs may interfere with these assays.

In summary, we have devised a method for the simultaneous quantitation of TBZ and 5OHTBZ which is simple and precise enough for clinical monitoring of TBZ therapy. A micro method for the enzymatic conversion of the glucuronide and sulfate esters of 5OHTBZ was developed to obtain estimates of the various metabolite fractions. Since monitoring would be most appropriate for patients unable to eliminate the potentially toxic metabolites, we also have devised a modified method which should eliminate interferences from endogenous fluorescent substances which can be expected to be present in the sera of uremic patients.

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