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Determination of temazepam and temazepam glucuronide by reversed-phase high-performance liquid chromatography

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ABSTRACT

A rapid and sensitive method for extracting temazepam from human serum and urine is presented. Free temazepam is extracted from plasma and urine samples using *n*-butyl chloride with nitrazepam as the internal standard. Temazepam glucuronide is analyzed as free temazepam after incubating extracts with β-glucuronidase. Separation is achieved using a C_8 reversed-phase column with a methanol–water–phosphate buffer mobile phase. An ultraviolet detector operated at 230 nm is used and a linear response is observed from 20 ng/ml to 10 μg/ml. The limit of detection is 15.5 ng/ml and the limit of quantitation is 46.5 ng/ml. Coefficients of variation are less than 10% for concentrations greater than 50 ng/ml. Application of the methodology is demonstrated in a pharmacokinetic study using eight healthy male subjects.

INTRODUCTION

Benzodiazepines are the most commonly prescribed medications for the short-term management of anxiety. Members of the 1,4-benzodiazepine class of drugs are also indicated for the relief of insomnia both secondary to and unrelated to anxiety. All the members of this class of drugs cause sedation to some degree and vary only in potency concerning this effect. Temazepam (7-chloro-2,3-dihydro-3-hydroxy-1-methyl-5-phenyl-1H-1,4-benzodiazepin-2-one) is one of the most commonly prescribed hypnotic benzodiazepines [1].

The method presented here for the quantitation of temazepam in human serum and urine is modified from methods by Gill *et al.* [2,3] and Prex [4]. A series of studies involving the effects of temazepam on human psychomotor performance are being conducted in this laboratory. As a prelude to those studies a pharmacokinetic study of this benzodiazepine hypnotic was carried out to verify the accuracy and precision of the high-performance liquid chromatographic

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(HPLC) assay and to confirm previous reports of peak plasma concentration and half-life [5–8]. Other analytical methods for the determination of temazepam and similar 1,4-benzodiazepines have previously been published. These include immunoassay [9,10], gas chromatography [11–13], gas chromatography–mass spectrometry (GC–MS) [13,14] and HPLC methods using either electrochemical detection [15,16] or ultraviolet spectrophotometric detection [4,14,17–19]. The HPLC method presented here was considered favorable because: (1) it requires only a one-step extraction; (2) sensitivity is acceptable without the necessity of pH adjustment or other steps requiring extra sample handling (*e.g.* sample derivatization); (3) the method is specific; and (4) the expense and expertise required are less than that required with a GC–MS method. This method also utilizes an internal standard, nitrazepam, that is a structurally similar benzodiazepine not available therapeutically in the USA. The limit of detection for this method is approximately 15 ng/ml and the limit of quantitation is approximately 46 ng/ml.

EXPERIMENTAL

Materials

Temazepam and nitrazepam used for the preparation of calibration standards and the internal standard were purchased from Sigma (St. Louis, MO, USA). Working solutions were prepared as 1 mg/ml solutions in methanol. Calibration standards and the internal standard solution (5 μg/ml) were prepared by dilution from the working solutions. HPLC-grade methanol, *n*-butyl chloride, dipotassium hydrogenphosphate (K_2HPO_4) and potassium dihydrogenphosphate (KH_2PO_4) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Subjects received a single 15-mg oral dose of temazepam (Restoril) purchased from Sandoz Pharmaceuticals (East Hanover, NJ, USA). Serum standards were prepared in lyophilized normal control serum (Ciba Corning Diagnostics, Irvine, CA, USA). Glusulase, a preparation of β-glucuronidase and sulfatase, was purchased from NEN Products (Du Pont, Boston, MA, USA) to liberate temazepam glucuronide for quantitative analysis.

Chromatography

A methanol–deionized water–phosphate buffer (55:25:20, v/v) mobile phase was used after filtration (0.45-μm pore filter) and degassing. Phosphate buffer was prepared by dissolving 2.78 g of K_2HPO_4 (0.008 *M*) and 25.04 g of KH_2PO_4 (0.092 *M*) in 4 l of deionized water. The mobile phase flow-rate was 2.2 ml/min using a Waters Assoc. Model 6000A solvent delivery system (Milford, MA, USA). Separation was achieved using a Supelcosil LC-8 reversed-phase column (5 μm, 15 cm × 4.6 mm I.D., Supelco, Bellefonte, PA, USA) preceded by a Supelguard LC-8 guard column (5 μm, 2 cm × 4.6 mm I.D., Supelco). A Waters Assoc. Model U6K universal liquid chromatograph injector and a Perkin-Elmer

(Norwalk, CT, USA) LC-75 spectrophotometric detector operated at 230 nm were also used. Chromatograms were generated using both a Hewlett Packard (Avondale, PA, USA) 3390A reporting integrator, for peak-height internal standard method calculations and a Hewlett Packard 3380A integrator for peak-area calculations in the internal standard method. The system was operated at ambient temperature and the operating pressure was approximately 175 bar.

Extraction

To 1 ml of serum or urine in a 100 mm × 16 mm borosilicate glass tube were added 50 μl of internal standard prepared in methanol (5 μg/ml) and 4 ml of *n*-butyl chloride. Samples were vortex-mixed for 15 s and centrifuged at 9000 *g* for 5 min. The organic layer was transferred to a clean tube and evaporated to dryness under a stream of dry air at ambient temperature. Residues were then reconstituted in 30 μl of mobile phase, and 25 μl were subsequently injected on column.

Calibration

A calibration curve was generated using serum samples spiked with temazepam in the following concentrations: 20, 50, 100, 200, 300, 400, 500, 1000, 5000 and 10 000 ng/ml. Standards were spiked using a 10 μg/ml temazepam working solution prepared from the 1 mg/ml stock solution. The concentration of that solution was verified using a UV-160 Shimadzu recording spectrophotometer (Shimadzu, Kyoto, Japan) using a Beer's–Lambert calculation (λ = 230 nm, A_{230} = 109, b = 1 cm) [3]. Curves were generated for serum samples using both peak-height and peak-area ratios between temazepam and the internal standard. Both methods of quantitation were applied in an effort to determine which gave the most reproducible and accurate results. Serum standards were analyzed on seven days for a total of nineteen samples (20–400 ng/ml) and on four days for a total of twelve samples (500–10 000 ng/ml). Within-day and between-day coefficients of variation (C.V.) were calculated as a measure of assay reproducibility. Urine standards were found to give comparable results to serum standards indicating that the matrix does not affect sample quantitation. For this reason only serum calibration curves were generated. Percentage error, [1 − (calculated concentration/actual concentration)] × 100%, was calculated for both within-day and between-day analyses as a measure of assay accuracy.

Recovery

The recovery of temazepam from serum and urine samples was assessed by comparing the peak-height ratios for spiked serum and urine samples at 200 ng/ml to a 200 ng/ml standard solution prepared in methanol. All samples and the methanolic solution were spiked with internal standard at a concentration of 250 ng/ml. The extraction procedure detailed above was performed on eight serum and urine samples. Eight 1-ml samples of the 200 ng/ml standard solution

were evaporated to dryness under a stream of air and reconstituted in 30 μl of mobile phase, and 25 μl were injected on column. Percentage recovery is defined as the mean concentration for the eight standard solutions divided by the mean of the eight analyses for either the serum standards or the urine standards multiplied by 100%. Recovery was determined only at 200 ng/ml because a standard of that concentration was used as the calibrator when quantitating samples.

Clinical pharmacokinetic study

Nine healthy volunteers received a single 15-mg dose of temazepam (Restoril). The protocol was approved by the University Institutional Review Board, and written informed consent was obtained. Blood samples (10 ml) were taken prior to dosing and again at 30, 60, 90, 120 and 150 min after dosing. Samples were obtained from the antecubital vein via an indwelling intravenous cannula (Jelco winged intravenous catheter placement unit, 20 gauge, 31.75 mm; Critikon, Tampa, FL, USA) fit with a male luer-lock stopper (Vygon, France). Samples were drawn from the stopper with a 10-cm^3 syringe fit with a 22-gauge needle (Becton Dickinson, Rutherford, NJ, USA). Normal bacteriostatic saline solution (3 cm^3, 0.9%) (LyphoMed, Rosemont, IL, USA) was used to flush the indwelling intravenous catheter between sampling. Serum was separated by centrifugation at 9000 *g* for 10 min and frozen until assayed. Urine samples were collected both before and 150 min after drug administration. Samples were subsequently analyzed for the presence of temazepam and temazepam glucuronide, the major metabolite of temazepam which is pharmacologically inactive.

Temazepam glucuronide analysis

Temazepam glucuronide was analyzed from blood and urine using the aqueous remainder following extraction of the parent compound. A deconjugation method from Greenblatt and co-workers [11,20] was used to liberate temazepam for quantitative analysis. The aqueous phase was washed by extraction with 4 ml of *n*-butyl chloride. Urine samples were buffered to pH 4.7 using 2.5 ml of 1 *M* KH_2PO_4 and serum samples were buffered to pH 4.7 using 3.5 ml of 2 *M* KH_2PO_4. Samples were incubated for 18–20 h with 30 μl of Glusulase, a preparation containing 135 000 U of β-glucuronidase and 10 000 U of sulfatase activity per ml. Following incubation, samples were buffered to pH 7.0 with 1 *M* sodium hydroxide (650 μl for urine samples and 2 ml for serum samples), extracted and quantitated as described above.

RESULTS AND DISCUSSION

Retention times for the internal standard (nitrazepam) and temazepam were 3.19 and 5.31 min, respectively. It was noted that the retention times could vary significantly (greater than ±5%) with small volumetric changes in the mobile phase composition. Recalibration was necessary on the sixth day of analyzing

serum standards because a new mobile phase was prepared and retention times shifted out of the established 5% window on both integrators. Quantitation based on peak-height ratios between temazepam and the internal standard was found to yield more consistent and reproducible results than peak-area ratio calculations, therefore only peak-height results are reported.

Potential interference was considered by analyzing solutions of commonly used benzodiazepines in the assay. Table I contains the relative retention times of a number of such drugs. Only clonazepam was found to coelute with the internal standard. The other benzodiazepines evaluated were adequately separated from both temazepam and the internal standard.

Fig. 1 shows a chromatogram of drug-free serum, drug-free lyophilized serum and a lyophilized serum sample spiked with 250 ng/ml internal standard (3.19 min) and 200 ng/ml temazepam (5.31 min). Lyophilized serum was compared to a negative serum sample (pooled patient samples assayed negative for benzodiazepines by immunoassay) and both were found to be free of interfering substances with this assay. All serum standards were subsequently prepared in lyophilized serum.

The serum calibration curve was linear over the range 20 ng/ml to 10 μg/ml (HPLC concentration = $-2.07 + 1.05 \times$ actual concentration, $r = 0.9994$). Each point on the curve was determined as the mean of nineteen samples analyzed over a seven-day period for the 50–400 ng/ml standards, sixteen samples for the 20 ng/ml standard analyzed over a seven-day period (three of the nineteen samples yielded peaks too small to integrate) and twelve samples analyzed over a four-day period for the 500–10 000 ng/ml standards. The standard error of the mean (S.E.M.) for each concentration is included in Table II. Within-day and

TABLE I

RELATIVE RETENTION TIMES FOR COMMON BENZODIAZEPINES

Compound	Relative retention time (min)
Nitrazepam	Internal standard
Clonazepam	Coelutes with nitrazepam
Chlordiazepoxide	−0.43
Triazolam	1.35
Alprazolam	1.47
Lorazepam	1.57
Oxazepam	1.75
Temazepam	2.12
N-Desalkylflurazepam	2.22
N-Desmethyldiazepam	4.24
Diazepam 5.52	
Midazolam	6.28
Flurazepam	12.03

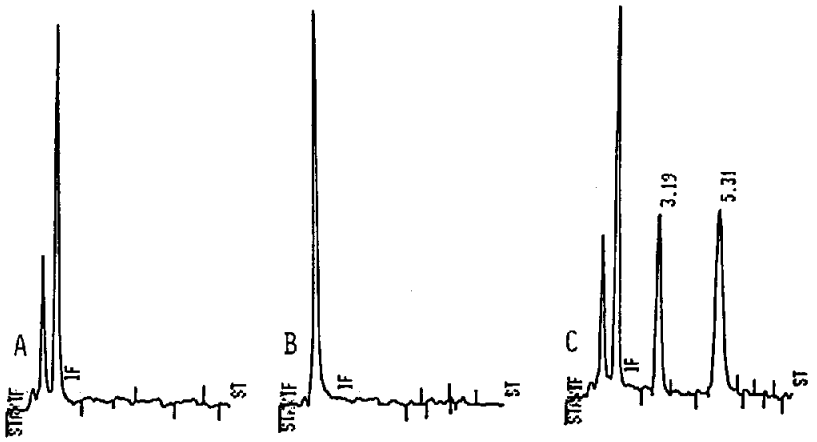

Fig. 1. (A) Chromatogram of drug-free lyophilized serum. (B) Chromatogram of drug-free serum. (C) Chromatogram of lyophilized serum spiked with 250 ng/ml internal standard (3.19 min) and 200 ng/ml temazepam (5.31 min).

between-day C.V.s were calculated and were less than 10% for both between-day and within-day calculations for standards greater than 50 ng/ml (Table II). Accuracy for the assay was good with mean values for each standard within ± 10% of the actual concentration (error for the 20 ng/ml standard was 14.95%). Urine standards were prepared in the same concentrations as the serum standards.

TABLE II

PRECISION DATA FOR TEMAZEPAM IN SERUM

Concentration added (ng/ml)	Concentration found (mean ± S.E.M.) (ng/ml)	Between-day C.V. (%)	Error (%)
20[a]	22.99 ± 2.16	37.63	14.95
50[b]	52.96 ± 2.27	18.69	5.9
100[b]	100.77 ± 2.02	8.74	0.8
200[b]	212.89 ± 4.18	8.34	6.4
300[b]	323.01 ± 6.52	8.81	7.7
400[b]	435.93 ± 7.46	7.46	8.9
500[c]	462.81 ± 3.52	2.64	7.4
1000[c]	1014.81 ± 22.25	7.59	1.4
5000[c]	4905.59 ± 53.47	3.78	1.9
10 000[c]	10 618.42 ± 77.75	2.54	6.2

[a] Mean value for seven days (n = 16). Three samples resulted in peaks indistinguishable from baseline noise and were not integrated.

[b] Mean value for seven days (n = 19).

[c] Mean value for four days (n = 12).

Results for urine standards were comparable with serum results, therefore a complete precision study was performed only with serum standards. Quantitations for serum standards using peak-area calculations resulted in larger C.V. values for the 20 and 50 ng/ml standards and a larger error for the 20 ng/ml standard than did the peak-height calculations (between-day C.V. = 57.59 and 37.63% for 20 ng/ml, 21.81 and 18.69% for 50 ng/ml and error = 38.4 and 14.95% for 20 ng/ml for peak area and peak height, respectively). C.V. values and error were similar for other concentrations, but the peak-height calculations were chosen because of better reproducibility near the limit of detection (LOD) of the assay.

The LOD is the concentration that can reproducibly be differentiated from baseline noise. LOD is related to sensitivity and noise level and may be calculated as LOD = $2R_N/S$, where R_N is the peak-to-peak noise level at the retention time of the compound of interest and S is sensitivity or the slope of the calibration curve. This calculation resulted in an LOD of approximately 15.5 ng/ml. The limit of quantitation was calculated as 3 × LOD and is approximately 46.5 ng/ml [21].

Extraction efficiency was determined for both serum and urine samples spiked with 200 ng/ml temazepam and compared to a methanolic standard at the same concentration. The recovery was 102.82 ± 4.45% for the serum samples and 111.76 ± 15.11% for the urine samples.

The serum temazepam concentration profile is depicted in Fig. 2. Each point represents the mean concentration of eight patient samples ± S.E.M. at each time period after receiving a single 15-mg oral dose of temazepam. All samples were analyzed in duplicate. Data are presented for only eight subjects because it was learned that one of the subjects had recently donated plasma (one subject was dropped from the study prior to receiving the drug because of plasma donation).

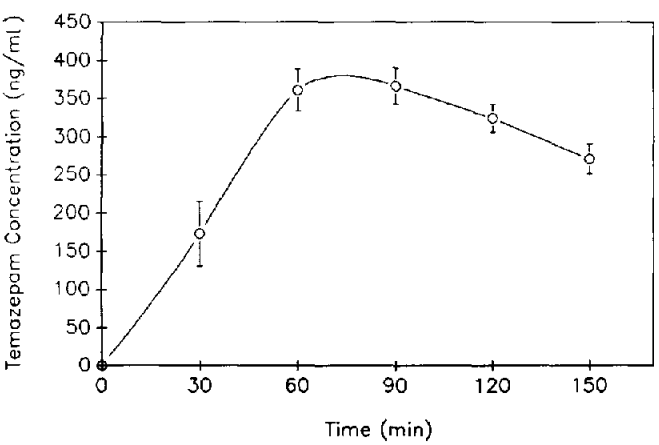

Fig. 2. Temazepam plasma concentration–time profile. Each point represents the mean ± S.E.M. for eight subjects after receiving a 15-mg oral dose of temazepam.

Investigators were not apprised of this fact until after the drug had been administered and samples analyzed. That subject's plasma values were considerably higher than anticipated (~900 ng/ml at 30 min after drug administration). Those data were not included in subsequent calculations. A loss of plasma proteins of up to 1% may be expected with plasma donation leading to increased free drug levels, especially with temazepam which is approximately 96% plasma protein-bound. Results from this study compared well with previous temazepam pharmacokinetic studies [5–8]. The peak plasma level for temazepam occurred between 60 and 90 min and was approximately 370 ng/ml. Of those samples collected during this study the sample collected at 90 min after drug administration had the highest plasma concentration (366.48 ± 23.49 ng/ml, mean ± S.E.M.). Plasma temazepam glucuronide levels also compared well with published results with approximately 45% of the drug appearing in the blood as the glucuronide metabolite at 150 min after dosing [5]. There was a large inter-subject variability in concentrations of both temazepam and temazepam glucuronide as indicated by the large standard errors in Figs. 2 and 3. Such variability between subjects has also been observed in other pharmacokinetic studies.

Temazepam glucuronide levels were determined in both urine (2.5 h after drug administration) and serum samples. Fig. 3 is the plasma time–concentration profile for the glucuronide metabolite. Each point is the mean of eight single analyses ± S.E.M. over the time course of the study. Based on kinetic data from Schwarz [5] and a treatment described in Ritschel [22] the expected amount of temazepam excreted in the urine as the glucuronide within 2.5 h was calculated to be approximately 162 μg assuming 80% of the drug is excreted in urine as the metabolite. The mean value in this study was 236.73 ± 43.94 μg. There was also a large

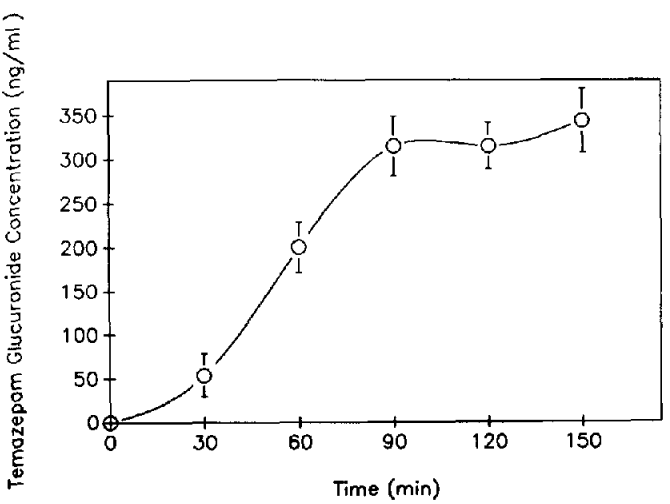

Fig. 3. Temazepam glucuronide plasma concentration–time profile. Each point represents the mean ± S.E.M. for eight subjects after receiving a 15-mg oral dose of temazepam.

between-subject variability in these values as occurred in all of the measurements in this clinical trial.

In analyzing urine and plasma samples from a subject not included in the results of the pharmacokinetic analysis, it was discovered that ibuprofen (retention time 4.37 min) is an assay interferent. The subject had a blood temazepam level of approximately 770 ng/ml 30 min after receiving the 15-mg dose. Plasma values declined over time from that value. The subject reported taking 400 mg ibuprofen approximately 16 h before receiving temazepam. The unexpectedly high plasma temazepam levels may be related to the use of ibuprofen. Plasma and urine were collected again from that subject after one week of taking 400 mg ibuprofen every 4 h and no other medications. Ibuprofen was detected in a urine specimen obtained approximately 1 h after a 400-mg dose, but was not detected in a serum sample drawn at the same time. The serum and urine ibuprofen peaks were not quantitated but the retention time matched the retention time of an ibuprofen extract. The extract did not coelute with either the internal standard or temazepam, but was very close to the triazolam peak. Results here are sufficient to presume that the peak in both the urine and serum samples is ibuprofen based on the subject's medication history and the presence of an ibuprofen extract peak near the suspected ibuprofen peak. No further qualitative or quantitative work was performed on ibuprofen, but further work is necessary to confirm the serum ibuprofen peak. Controlled studies are also necessary to evaluate the potential interaction between ibuprofen and temazepam because both are commonly used medications, especially among the elderly.

CONCLUSION

The method presented is a simple and rapid technique that allows for the quantitation of temazepam and temazepam glucuronide in serum and urine. The one-step extraction procedure requires only 1 ml of serum or urine but provides only one possible analysis from that sample ("one-shot assay"). Serum and urine levels of both temazepam and the glucuronide metabolite showed a large variability between subjects, but means correlated well with expected results. Due to the short time course of sample collection accurate measurements of half-life and volume of distribution were not obtainable. The limit of detection of the assay was determined to be 15.5 ng/ml with a limit of quantitation of 46.5 ng/ml. Since plasma temazepam levels fall below 100 ng/ml within 10–12 h after a single therapeutic dose [7,8,12,23–25] this assay is limited to analysis of plasma samples during the redistribution phase of the drugs kinetic profile. Overdose levels of temazepam and urine analysis of temazepam may be adequately handled by this method beyond that time period. Increased sensitivity of this method would probably require samples sizes greater than 1 ml. The assay was found to yield reproducible and accurate results for concentrations within the expected therapeutic range of temazepam: 356 ± 52 ng/ml at 90 min after a 15-mg dose, 382 ± 192 ng/ml at 2.5 h and 26 ng/ml at 24 h after a 30-mg dose [6,19].

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