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Validation of a method for the determination of zolpidem in human plasma using LC with fluorescence detection

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Abstract

A sensitive and selective high-performance liquid chromatography (HPLC) method was developed for the determination of zolpidem in human plasma. Zolpidem and the internal standard (trazodone) were extracted from human plasma that had been made basic. The basic sample was loaded onto a conditioned Bond Elut C18 cartridge, rinsed with water and eluted with methanol. Forty microliters were then injected onto the LC system. Separation was achieved on a C18 column (150 × 4.6 mm, 5 μm) with a mobile phase composed of acetonitrile:50 mM potassium phosphate monobasic at pH 6.0 (4:6, v/v). Detection was by fluorescence, with excitation at 254 nm and emission at 400 nm. The retention times of zolpidem and internal standard were approximately 4.7 and 5.3 min, respectively. The LC run time was 8 min. The assay was linear in concentration range 1–400 ng/ml for zolpidem in human plasma. The analysis of quality control samples for zolpidem (3, 30, and 300 ng/ml) demonstrated excellent precision with relative standard deviations (RSD) of 3.7, 4.6, and 3.0%, respectively ($n = 18$). The method was accurate with all intraday ($n = 6$) and overall ($n = 18$) mean concentrations within 5.8% from nominal at all quality control sample concentrations. This method was also performed using a Gilson Aspec XL automated sample processor and autoinjector. The samples were manually fortified with internal standard and made basic. The aspec then performed the solid phase extraction and made injections of the samples onto the LC system. Using the automated procedure for analysis, quality control samples for zolpidem (3, 30, and 300 ng/ml) demonstrated acceptable precision with RSD values of 9.0, 4.9, and 5.1%, respectively ($n = 12$). The method was accurate with all intracurve ($n = 4$) and overall ($n = 12$) mean values being less than 10.8% from nominal at all quality control sample concentrations.

Keywords: Zolpidem; Trazodone; Human plasma; Solid phase extraction; HPLC-fluorescence detection; Automated sample processing

1. Introduction

Benzodiazepines are often prescribed for their sedative, anti-anxiety, anticonvulsant and muscle relaxant properties. It is believed that benzodiazepines modulate $GABA_A$ receptor complexes

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within the brain by attaching to at least three different $GABA_A$ receptor complexes called omega (ω) receptors. This non-specific interaction with multiple ω receptors leads to the wide range of pharmacological effects seen with the benzodiazepines.

Zolpidem (2-(4-methylphenyl)-*N*,*N*,6-trimethylimidazo[1,2-a]pyridine-3-acetamide) is used primarily as a hypnotic agent. While zolpidem is not a benzodiazepine, it does interact with the $GABA_A$ complex ω receptors. Zolpidem preferentially binds with the $GABA_A$ ω_1 receptor complex, and has very little interaction with the other ω receptor complexes. Since the $GABA_A$ ω_1 receptor complex is found primarily in the portions of the brain that affect sleep, zolpidem behaves as a sleep inducer without the muscle relaxant and anticonvulsant effects of the benzodiazepines.

Zolpidem has rapid action and a short half-life. Dosages are commonly given orally at the 5- and 10-mg levels. Doses of 5 and 10 mg, when given to healthy subjects typically yield C_{max} values of approximately 59 and 121 ng/ml, respectively, with T_{max} at 1.6 h.

Several analytical methods [1–4] have been developed for quantitation of zolpidem in human plasma using an internal standard. These methods used an analogue of zolpidem (*N*,6-dimethyl-2-(4-methylphenyl)-*N*-propylimidazo[1,2-a]pyridine-3-acetamide) as the internal standard. This compound is not commercially available, and the cost of custom synthesis is prohibitive to its use. This difficulty could be addressed by using external standard regressions. However, without an internal standard present there would be no compensation for the variability seen during extraction or injection onto the high-performance liquid chromatography (HPLC) system. Other disadvantages to the current methods include the use of liquid/liquid extraction (which wastes solvent), or on-line sample cleanup (using a precolumn and column-switching) that is complicated to set up and maintain.

A new analytical method has been developed that uses a commercially available internal standard, and solid phase extraction technology to prepare samples for analysis by HPLC using fluorescence detection [1]. The method is simple, rugged, and sensitive, with quantitation over a standard curve range 1–400 ng/ml. The sample analysis time is 8 min per sample, allowing as many as 180 injections to be made in a 24-h period. In addition, this method can be used on an automated sample preparation station that will perform the solid phase extraction on each sample and inject the eluant directly onto the HPLC system.

2. Experimental

2.1. Materials and reagents

Zolpidem was obtained from Research Biochemicals International (Natick, MA, USA) and the internal standard (ISTD), trazodone, was obtained from Sigma Chemical Company (St. Louis, MO, USA). All organic solvents used were HPLC grade and purchased from Fisher (Fair Lawn, NJ, USA) or Burdick and Jackson (Muskegon, MI, USA). Deionized water was purified by a Milli-Q system (Millipore). Anhydrous potassium phosphate monobasic was purchased from Sigma Chemical Company, potassium hydroxide pellets (analytical reagent grade) were purchased from Mallinckrodt (Paris, KY, USA), phosphoric acid (85%, HPLC grade) was purchased from Fisher, and sodium hydroxide pellets (ACS grade) were purchased from Baker (Phillipsburg, NJ, USA). Control human plasma with sodium heparin anticoagulant was obtained from Biochemed (Winchester, VA, USA).

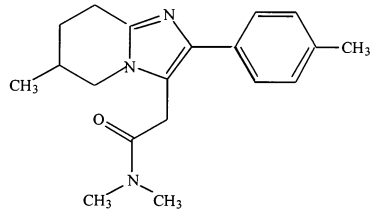
Duplicate stock solutions of zolpidem were prepared from separate weighings for standards and quality control (QC) samples. Stock solutions and subsequent dilutions were prepared in methanol and stored under refrigerated conditions. Calibration standard curves were prepared daily by adding 50 μl of 10-fold concentrated solutions into 500 μl of blank human plasma to yield standards at zolpidem plasma concentrations of 1, 2, 10, 20, 50, 100 and 400 ng/ml. Three levels of QC samples (3, 30, and 300 ng/ml) were prepared, placed into polypropylene storage vials, and stored at approximately −20°C.

2.2. Instrumentation

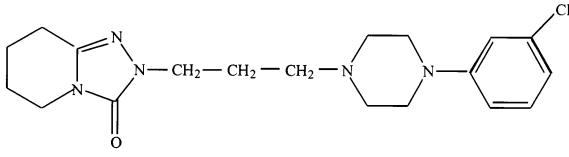
The first HPLC system consisted of a Perkin Elmer Series 200 solvent delivery system, a Rainin Dynamax Model AI-1A autoinjector and a Jasco Model FP920 fluorescence detector. The flow rate was 1.0 ml/min. Injections were filtered by a 2/0.5 μm in-line two-stage prefilter by Upchurch (Oak Harbor, WA, USA), with separation on a Shiseido CapcellPak UG C18 (150 × 4.6 mm, 5 μm) analytical column (Phenomenex, Torrance, CA, USA). The mobile phase was composed of acetonitrile:0.05 M potassium phosphate monobasic (pH 6.0) (4:6, v/v). Detection was by fluorescence, with λ_{ex} 254 nm, and λ_{em} 400 nm.

The second HPLC system (using automated sample extraction) consisted of an SSI series III Accuflow solvent delivery system, a Gilson Aspec XL sample processing station and autoinjector with a Model 402 Dilutor and a Jasco Model FP920 fluorescence detector. Analytical conditions were identical to those used on the first HPLC system.

Data collection was performed using a Waters Millennium 2020 Chromatography Manager data system (Version 2.15.3) with data storage on a DEC-Alpha 4100 server running Alpha Open VMS® and IBM compatible client workstations running Windows 95®.



Zolpidem



Trazodone

Fig. 1. Molecular structures of zolpidem and trazodone (internal standard).

2.3. Extraction procedure

Five hundred microliters of human plasma were made basic by the addition of 500 μl of 0.1 M sodium hydroxide. The sample was then loaded to a conditioned Varian Bond Elut C18 cartridge (1 cc/100 mg). After the cartridge was rinsed with water, the sample was eluted with 0.5 ml of methanol. Forty microliters were then injected onto the HPLC system.

When the Gilson Aspec XL automated sample preparation station and autoinjector was used, the extraction procedure was performed as follows. Five hundred microliters of human plasma were made basic by the addition of 500 μl of 0.1 M sodium hydroxide. Samples were then centrifuged to eliminate particulate plasma proteins. Following centrifugation, samples, solid phase extraction cartridges, and glass collection tubes were placed on the Gilson Aspec XL system. The system was programmed to process each sample through the solid phase extraction procedure and make the injection onto the chromatograph immediately after processing.

2.4. Data regression

Chromatographic data were collected and integrated by the Millennium 2020 Chromatography Manager system. Peak height ratios of zolpidem/ISTD were calculated. The calibration curves were obtained by weighted (1/concentration) least-squares linear regression analysis. The equations of the calibration curves were then used to calculate the concentrations of zolpidem in the samples and QC samples by their peak height ratios.

3. Results and discussion

3.1. Separation

The molecular structures of zolpidem and trazodone (internal standard) are shown in Fig. 1.

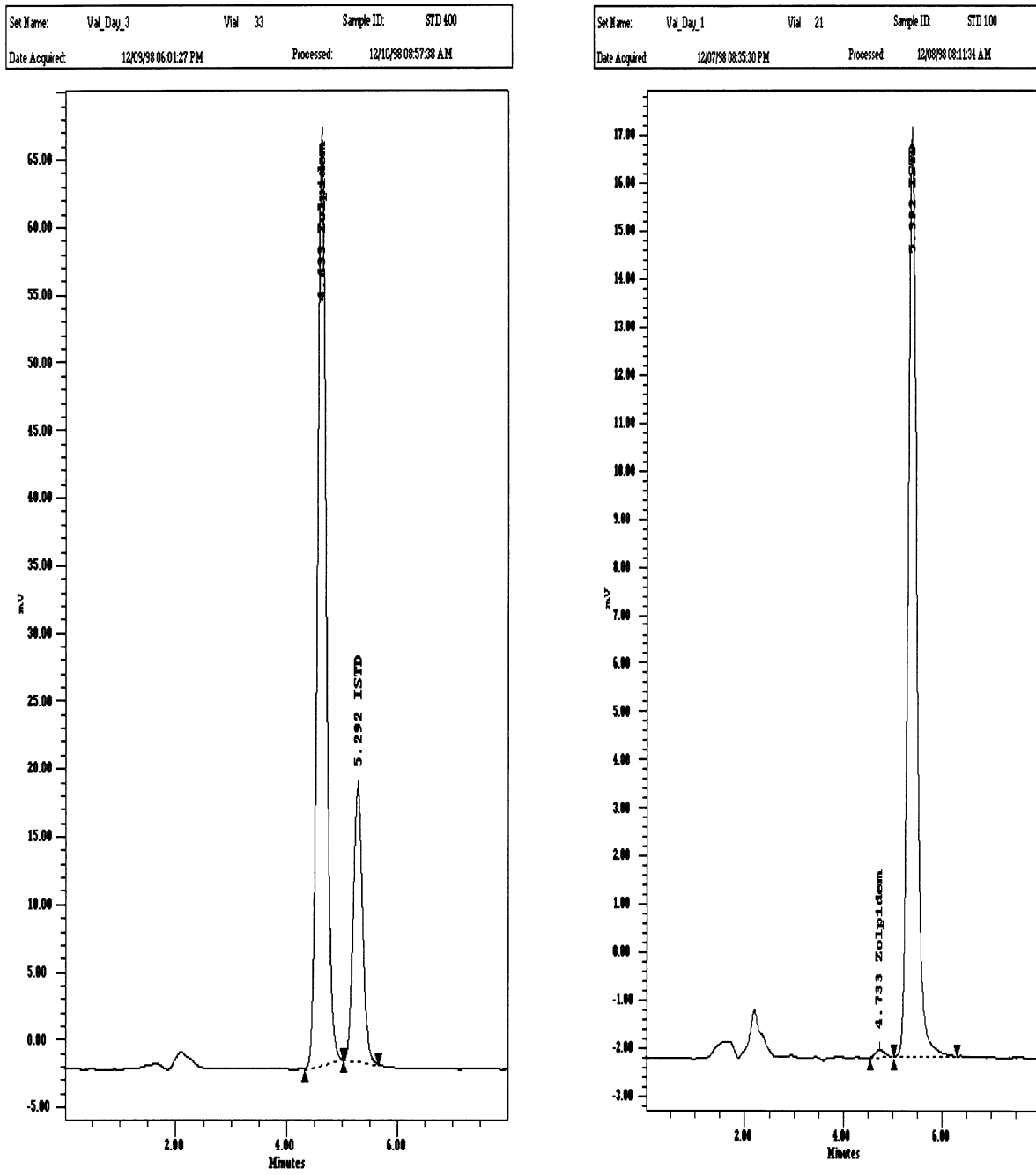


Fig. 2. Calibration standard containing 400 ng/ml (left) and 1.00 ng/ml (right) zolpidem in human plasma.

Zolpidem and the internal standard were separated from each other, as shown in Fig. 2. The mean retention times for zolpidem and the internal standard were 4.66 and 5.32 min, respectively.

3.2. Selectivity

Blank plasma from six separate lots of human plasma was tested for endogenous interferences. Of the lots tested, all were clear of interferences in the zolpidem and internal standard regions. A representative chromatogram of the plasma blank selected for use in the validation procedure is in Fig. 3.

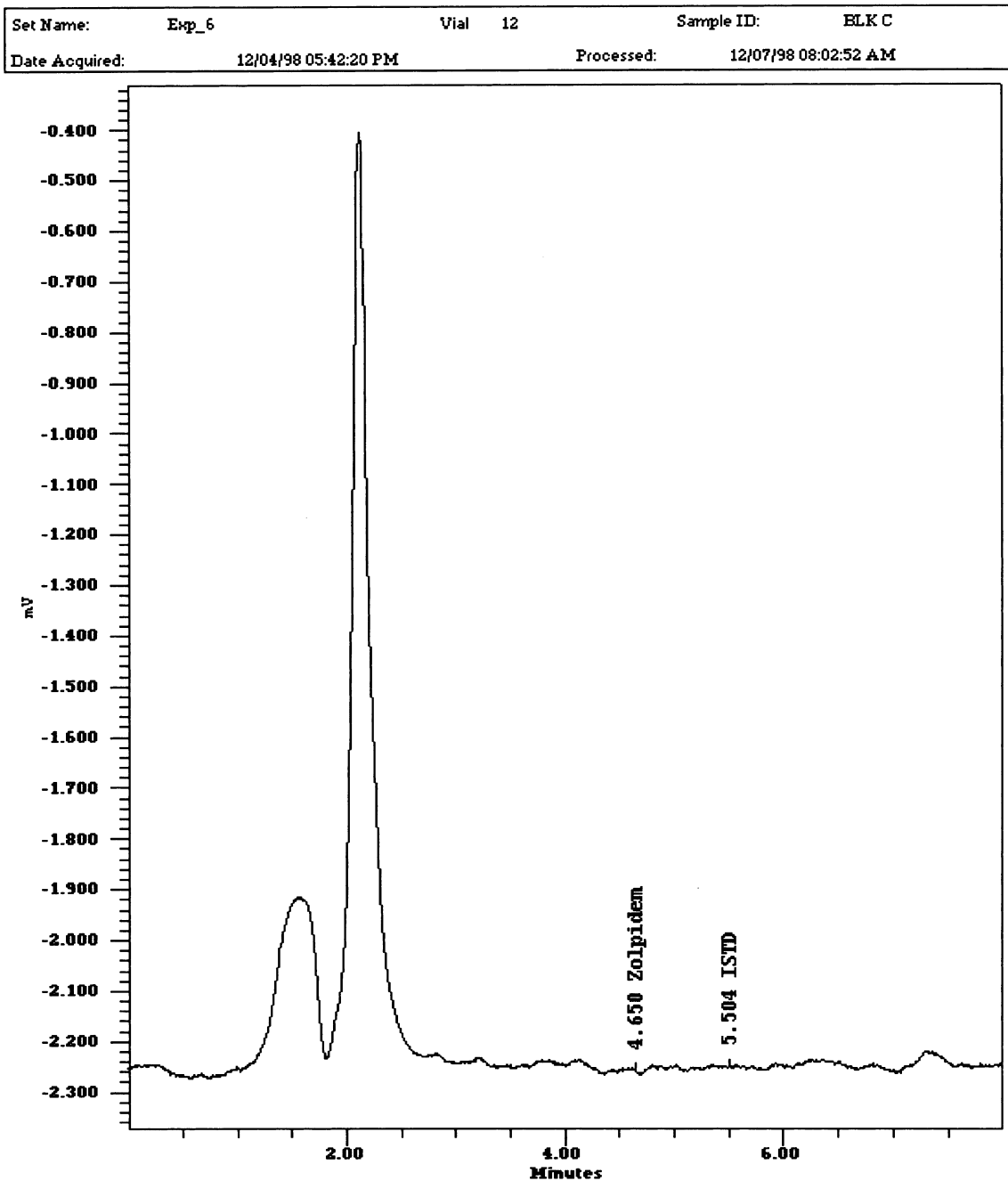


Fig. 3. Chromatogram of blank (analyte-free) human plasma.

Table 1
Recovery of zolpidem and trazodone (internal standard) from human plasma

Quality control concentration (ng/ml)	Zolpidem			Trazodone (internal standard)		
	Mean extract height ($n = 6$)	Mean pure height ($n = 6$)	Mean % recovery ($n = 6$)	Mean extract height ($n = 6$)	Mean pure height ($n = 6$)	Mean % recovery ($n = 6$)
3.0	455.2	401.7	113.3	19 321.2	21 529.7	89.7
RSD (%) ($n = 6$)	5.6	5.9		2.2	4.0	
30.0	4908.8	4093.2	119.9	19 689.0	21 124.9	93.2
RSD (%) ($n = 6$)	6.9	5.0		7.3	1.0	
300	46 895.6	44 829	104.6	18 881.0	21 364.4	88.4
RSD (%) ($n = 6$)	2.6	6.8		3.7	4.6	
Overall recovery ($n = 18$)			112.6			90.4

Table 2
Calibration curve parameters and statistics for zolpidem in human plasma[a]

Curve	Slope	*y*-Intercept	Correlation coefficient
Manually processed samples			
1	8.284E-03	−7.620E-04	0.9999
2	8.815E-03	−1.875E-03	0.9998
3	8.677E-03	−1.450E-03	0.9999
4	8.821E-03	−6.200E-04	0.9999
5	8.478E-03	−6.080E-04	0.9999
6	8.628E-03	−6.610E-04	0.9998
Mean ($n=6$)	8.617E-03	−9.960E-04	0.9999
S.D.	2.0724E-04		
RSD (%)	2.4		
Samples processed by aspec XL			
1	1.288E-02	−1.613E-03	0.9993
2	1.219E-02	−2.272E-03	0.9997
3	1.192E-02	−7.400E-04	0.9999
Mean ($n=3$)	1.233E-02	−1.542E-03	0.9996
S.D.	4.9508E-04		
RSD (%)	4.0		

[a] S.D., standard deviation; RSD, relative standard deviation.

The literature methods [1–4] called for use of a zolpidem analog (*N*,6-dimethyl-2-(4-methylphenyl)-*N*-propylimidazo[1,2-a]pyridine-3-acetamide) as an internal standard. However, this compound is not commercially available, and synthesis of the compound for use as an internal standard is costly. For this reason, trazodone was selected as an alternative internal standard. Dibucaine, chloroquine, ethaverine and papaverine were also evaluated as potential internal standards, but did not have the appropriate fluorescence qualities to be useful.

Neat solutions of zolpidem and trazodone were injected onto the HPLC system. There were no extraneous peaks associated with the injection of either compound under the chromatographic conditions used.

3.3. Absolute recoveries

Absolute recoveries were determined by comparing the peak heights of extracted QC samples with the peak heights of recovery standards (unextracted equivalents of extracted QC samples). The mean recoveries for zolpidem and the internal standard were 112.62 and 90.44%, respectively (Table 1).

The recovery of zolpidem appeared to be greater than 100%. The actual elution volume of extracted samples was probably somewhat less than 0.5 ml, due to retention of a portion of the eluant by the solid phase extraction cartridge. The result was a slightly more concentrated extract and recoveries that appeared to be greater than 100%.

3.4. Method performance

Calibration curve parameters for zolpidem are in Table 2. Results were calculated using peak height ratios. Calibration curves for zolpidem in human plasma were linear using linear regression weighted $1/c$ in the concentration range from 1 to 400 ng/ml, with correlation coefficients greater than or equal to 0.9998 for all curves when samples were manually processed, and greater than or equal to 0.9993 when the Gilson Aspec XL was used to process and inject samples. Precision for zolpidem in QC samples over the course of the validation using manual extraction ($n=18$) was indicated by an RSD of $\leq 4.6\%$, with deviations of mean values from nominal ($n=18$) of $\leq 3.9\%$ (Table 4). In calibration standards, RSD ($n=6$) was $\leq 4.8\%$, with deviations of mean values from nominal ($n=6$) of $\leq 4.5\%$ (Table 3). Precision for zolpidem in QC samples over the course of the validation with automated extraction done on the Gilson Aspec XL ($n=12$) was indicated by an RSD of $\leq 9.0\%$, with deviations of mean values from nominal values ($n=12$) of $\leq 5.1\%$ (Table 4). In calibration standards, RSD ($n=3$) was $\leq 9.3\%$, with deviations of mean values from nominal values ($n=3$) of $\leq 5.2\%$ (Table 3). The lower limit of quantitation (LLOQ) for zolpidem in human plasma was set at 1.00 ng/ml. At the LLOQ, the RSD ($n=6$) of the measured concentration was 6.0%, and the deviation of the mean of the measured concentrations from the nominal value was 4.5% (Table 5).

Table 3
Calibration curve data and statistics for zolpidem in human plasma[a]

	Theoretical concentration (ng/ml)						
	1.0	2.0	10.0	20.0	50.0	100	400
Manually processed samples							
Mean ($n=6$)	1.05	2.05	9.67	19.6	49.4	99.9	402
S.D.	0.050	0.082	0.110	0.40	1.08	2.75	3.3
RSD (%)	4.8	4	1.1	2.0	2.2	2.8	0.8
DMT (%)	4.5	2.3	−3.4	−2.1	−1.1	−0.1	0.4
Samples processed by aspec XL							
Mean ($n=3$)	1.02	2.10	9.99	19.6	48.0	98.0	404
S.D.	0.095	0.067	0.28	1.40	1.60	3.56	4.0
RSD (%)	9.3	3.2	2.80	7.1	3.3	3.6	1.0
DMT (%)	2.0	5.2	−0.1	−2.0	−4.1	−2.0	1.1

[a] S.D., standard deviation; RSD, relative standard deviation; DMT, deviation of mean value from nominal.

3.5. Stability

QC samples containing 3, 30, and 300 ng/ml zolpidem in plasma were subjected to three freeze/thaw cycles and to storage for 24 h at ambient temperature prior to extraction. Another set of aliquots of each QC sample were taken, fortified with internal standard and 0.1 M sodium hydroxide, and allowed to stand for approximately 24 h before being extracted.

Freezing and thawing of the QC samples up to three times, as well as storing QC samples at ambient temperature for up to 24 h prior to analysis, with or without addition of sodium hydroxide and internal standard, appeared to have little effect on the quantitation of zolpidem in plasma (Table 6).

QC samples stored in a freezer set to maintain −10 to −30°C remained stable through the course of the validation (7 days).

Calibration standards and duplicate QC samples were injected onto the HPLC after the extracts were allowed to stand at ambient temperature for at least 24 h prior to injection. Storing the extracted samples under these conditions prior to injection appeared to have no effect on quantitation of the standard curve or QC samples (Table 7).

Stock standard solutions remained stable for at least 25 days when stored in a refrigerator set to maintain 2–8°C. This conclusion is based on comparison of peak heights of a stock solution that had been stored for 25 days to those of a freshly prepared stock solution.

3.6. Using the aspec automated sample processing unit

The Gilson Aspec XL sample processing unit can be used as described in the sample preparation procedure. It is important to centrifuge the sample before placing it on the aspec. This removes particulate protein material from the sam-

Table 4
Quality control sample data and statistics for zolpidem in human plasma[a]

	Theoretical concentration (ng/ml)		
	3.00	30.0	300
Manually processed samples			
Overall mean ($n=18$)	2.88	29.0	294
S.D.	0.108	1.33	9.0
RSD (%)	3.7	4.6	3.0
DMT (%)	−3.9	−3.3	−2.0
Samples processed by aspec XL			
Overall mean ($n=12$)	2.85	28.5	287
S.D.	0.255	1.38	14.7
RSD (%)	9.0	4.9	5.1
DMT (%)	−5.1	−5.0	−4.4

[a] S.D., standard deviation; RSD, relative standard deviation; DMT, deviation of mean value from nominal.

Table 5
Evaluation of lower limit of quantitation (1 ng/ml)for zolpidem in human plasma[a]

	Replicate	Calculated concentration (ng/ml)	DEV (%)
	1	1.10	10.0
	2	1.07	7.0
	3	1.11	11.0
	4	0.953	−4.7
	5	1.05	5.0
	6	0.986	−1.4
With-in day mean ($n=6$)		1.04	
S.D.		0.063	
RSD (%)		6.0	
DMT (%)		4.5	

[a] S.D., standard deviation; RSD, relative standard deviation; DMT, deviation of mean value from nominal; DEV, deviation of single value from nominal.

ple and prevents clogging of the needle. This is also the reason that internal standard and 0.1 M sodium hydroxide are added before the sample is loaded to the aspec. Addition of these reagents by the aspec would necessitate mixing, and it is likely that the plasma proteins would then be drawn into the needle, clogging it.

Results generated using the aspec were acceptable, but not as reproducible as those generated using the manual extraction. This is most likely due to two reasons. The aspec injector variability is greater than that of a standard autoinjector due to the large bore needle used to make the injections. Also, allowing the samples to stand in 0.1 M sodium hydroxide appears to introduce some minor variability to the results, especially at lower concentrations of zolpidem in plasma.

3.7. Use of the upchurch 2/0.5 μm inline prefilter

Fine particulate matter from the Bond Elut cartridges appeared to be present in the final extract. An Upchurch 2/0.5 μm in-line prefilter was used (as opposed to a standard 0.5 μm in-line prefilter) to remove particulates before they reached the analytical column. This prefilter offers the advantage of a 2-μm prefilter immediately before the 0.5-μm prefilter, to remove larger particulates before they reach the 0.5-μm filter. This set-up gave excellent results, with little or no backpressure increase over the course of an analytical run.

Because these samples are known to contain particulate matter, prefilters should be changed prior to each analytical run. Buildup of particulate matter on prefilters, even if not accompanied by an increase in system backpressure, could compromise peak shape and overall results.

Table 6
Evaluation of the stability of zolpidem in human plasma quality control samples after three freeze/thaw cycles, after storage at ambient temperature for 24 h, and after storage at ambient temperature for 24 h following the addition of 0.1 m NaOH and internal standard[a]

	Theoretical concentration (ng/ml)		
	3.00	30.0	300
Three freeze/thaw cycles			
Mean ($n=3$)	2.93	29.7	299
S.D.	0.04	0.96	2.5
RSD (%)	1.4	3.2	0.8
DMT (%)	−2.2	−1.1	−0.2
Thawed for 24 h at ambient temperature			
Mean ($n=3$)	2.87	29.5	302
S.D.	0.032	0.55	6.5
RSD (%)	1.1	1.9	2.2
DMT (%)	−4.2	−1.8	0.6
Thawed for 24 h at ambient temperature following addition of 0.1 M NaOH and internal standard			
Mean ($n=3$)	3.25	29.2	278
S.D.	0.361	0.42	8.5
RSD (%)	11.1	1.4	3.1
DMT (%)	8.4	−2.8	−7.2

[a] S.D., standard deviation; RSD, relative standard deviation; DMT, deviation of mean value from nominal.

Table 7
Stability of zolpidem extracted from human plasma and stored at ambient temperature for at least 24 h[a]

Theoretical concentration (ng/ml)	Calculated concentration (ng/ml)	DEV (%)
Calibration standards		
1	1.08	8
2	1.93	−3.5
10	9.64	−3.6
20	19.7	−1.5
50	52.3	4.6
100	94.5	−5.5
400	404	1.0
Quality control samples		
3.00	2.88	−4.0
3.00	2.92	−2.7
30.0	29.3	−2.3
30.0	29.1	−3.0
300	301	0.3
300	294	−2.0
Correlation	0.9996	
y-Intercept	−3.890E-04	
Slope	8.861E-03	

[a] DEV, deviation of single value from nominal.

4. Conclusions

The objective of this study was to validate a simple, rugged method for the determination of zolpidem in human plasma using a commercially available internal standard. Use of a solid phase extraction procedure allowed much of the process to be easily automated, as was demonstrated in the comparisons of manual and automated extraction results. This method will allow as many as 180 injections to be made in a 24-h period, permitting rapid sample analysis in support of drug interaction studies. The extraction procedure and the chromatographic set-up is simple, reliable and consistent.

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