Oxazepam, (Seresta®, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepine-2-one) is a short-acting benzodiazepine. It is the active metabolite of a wide range of 1,4-benzodiazepines. Oxazepam is useful in the short term management of anxiety, agitation, insomnia, tension and related symptoms commonly seen in patients with psychoneurotic or psycho physiological disorders (1).

In the previous studies on healthy volunteers, due to low oxazepam serum concentration achieved after oral intake, double or triple doses (30-45 mg) were administered. The reported pharmacokinetic parameters varied considerably (2-5). This is possibly due to high assay sensitivity required for detection of low serum oxazepam concentration by high-performance liquid chromatography. The sensitivity is mostly affected by complex extraction procedure, low blood concentration, relatively high limit of detection and especially peak asymmetry. Peak asymmetry continues to be a common complaint in the reversed phase HPLC analysis of oxazepam and most of others benzodiazepines (5). The considerable and significant peak tailing causes a number of problems including lower resolution, sensitivity, accuracy and precision. These factors are affected due to inability of data systems to identify exactly where a peak tailing begins and ends.

The present article describes a sensitive, accurate and reproducible reversed phase HPLC assay method with chromatograms having fully symmetrical peaks. This method is capable of measuring low concentrations of oxazepam in serum and can be used in bioequivalence and pharmacokinetic studies. Although the previously published gas and liquid chromatographic methods with mass spectrometric detection (6-9) are sensitive and specific methods with sub nanogram limit of quantitation (0.25 ng × mL⁻¹), but these equipments need highly trained personnel and may not be available in all pharmaceutical laboratories and research centers. In addition, in published methods, extraction of oxazepam involved many steps and time consuming procedures, while for large scale single dose pharmacokinetic and bioequivalence studies a simple and convenient procedure is required.

**EXPERIMENTAL**

**Chemicals and reagents**

Oxazepam powder was supplied by Profarmaco Italy, B.N. 530916. The internal standard nordazepam was obtained from European directorate for the quality of medicines, Council of Europe, Strasbourg, France. 10 mg oxazepam tablets used as...
test in this project were obtained from Jalinus pharmaceutical company (Tehran, Iran) and Seresta® tablets as reference product from Wyeth-Lederle (Paris, France). HPLC grade methanol and all other chemicals were obtained from Merck (Darmstadt, Germany). Water was obtained by double distillation and purified additionally with a Milli-Q purification system (Millipore, Bedford, MA, USA).

Apparatus and chromatographic conditions

Liquid chromatography was performed with a Knauer HPLC system (Berlin, Germany) consisting of Wellchrom K-1001 pump, Rheodyne 7125 injector and K 2600 UV detector connected to Euchrom 2000 integrator. The separation was performed using an analytical Perfectsil Target ODS-3 column, 5 µm particle size, 125 × 3.5 mm (inner diameter), made of stainless steel (Mainz-Analysetechnik GmbH, Germany). The wavelength was set at 254 nm. The mobile phase consisted of 0.05 M ammonium dihydrogen phosphate solution and methanol (50:50 v/v), adjusted to pH 5.8 with concentrated ammonia. It was pumped through the column at a flow rate of 1.5 mL×min⁻¹. A Millipore filtration system (Millipore, Bedford, MA, USA) with type HV Millipore filters (0.45 µm) was used for degassing the mobile phase under vacuum. The mobile phase was prepared daily and filtered through a 0.45 Waters membrane filters before use.

Calibration standards

Stock standard solutions of oxazepam and internal standard were prepared by dissolution of each compound in methanol to obtain a concentration of 10 µg×mL⁻¹ oxazepam and 500 ng×mL⁻¹ of internal standard, respectively. The solutions were stored at 4°C and showed no significant alterations in peak areas or heights determined daily by direct injection throughout the course of the study. Biological calibration standards were prepared at concentrations of 5, 20, 80, 160 and 320 ng×mL⁻¹ of oxazepam and at final concentration of 50 ng×mL⁻¹ for internal standard, by diluting the appropriate aliquots of the stock solutions with drug-free serum. Then the samples were prepared for analysis as described in the sample preparation procedure. Calibration data were acquired by plotting the ratio of oxazepam peak area to internal standard peak area against the concentration of the calibration standards, followed by a linear regression analysis.

Pharmacokinetic and bioequivalence study

The developed method was applied to evaluate the pharmacokinetic parameters and the bioequivalence of two different tablet formulations of oxazepam in healthy volunteers. The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences and written informed consent was obtained from each volunteer. Twelve adult non smoker male volunteers with a mean age of 25 ± 3.6 and mean weight of 60.9 ± 8.3 kg were selected for the study after assessment of their health status by clinical evaluation and laboratory tests. The volunteers had not taken any medication for the last two weeks. After overnight fasting, they received either 10 mg oxazepam tablets as test formulation or 10 mg Seresta® tablets as reference product. Two hundred milliliters of water were given immediately after drug administration and the volunteers then fasted for 4 h, after which period a standard lunch was served.

Blood samples and sample preparation

To prepare spiked samples, peripheral venous blood from volunteers was taken before drug administration. Samples (5 mL) from the same volunteers using oxazepam tablets were taken 1-40 h after drug administration at suitable intervals. Sixty minutes after clotting of the blood at room temperature, samples were centrifuged for 5 min at 5000 g. The separated serum samples were decanted in 5 mL test tubes and then frozen immediately at -20°C until assayed. The stability of oxazepam in serum stored at -20°C was assessed during all the storage steps of the analytical methods. No change in the stability over the period of at least two months and following three freeze (-20 ± 5°C)-thaw (room temperature) cycle was observed.

To 400 µL of fresh thawed serum sample, 10 µL of internal standard solution and 5 mL of dichloromethane were added and the mixture was sonicated for 2 min and then separated by centrifugation (5 min at 5000 g). The organic layer was transferred to a second tube and evaporated at 45°C under a gentle nitrogen stream. The dry residue was reconstituted in 100 µL of mobile phase, vortexed for 30 s and 50 µL was injected onto the column.

RESULTS

Once optimal chromatographic conditions have been established, the validated method was carried out for quantitation of oxazepam in human sera. Accuracy, intra- and inter-day precision of the proposed method was assessed with spiked serum during seven consecutive days. This was done using six samples at five different concentrations 5, 20, 80, 160 and 320 ng×mL⁻¹. The samples were pretreat-
ed according to the sample preparation procedure. Selection of concentrations for analysis was made to provide for definition of precision at low, medium, and high concentrations of the linear range. Accuracy was assessed by actual concentration obtained divided by target concentration, then multiplied by 100. Precision is expressed as the percent coefficient of variation (CV %) for the drug. The intra-day precision ranged from 1.4 to 6.3%, and the accuracy ranged from 96.0 to 100.9% (Table 1). The inter-day precision in serum samples was similarly evaluated over a period of seven consecutive days. Precision ranged from 0.6 to 6.2% and accuracy from 96.1 to 101.2%.

The average recovery of oxazepam from serum was estimated at concentration ranges of 5, 20, 80, 160 and 320 ng × mL⁻¹ by comparing the analyte peak areas obtained from spiked serum samples with those from the same concentrations of unextracted standard solutions in mobile phase. The recovery of internal standard from serum was determined at a concentration of 50 ng × mL⁻¹ by the same method. Recovery data, as well as reproducibility of recovery (CV %) from serum samples supplemented with oxazepam and internal standard are referred in Table 2. The relative recovery of oxazepam ranged from 86.0 to 97.8%. The internal standard recovery was found to be consistent from all six pools tested, averaging 98.0 ± 3.6%.

The method’s linearity was studied by analyzing in triplicate the calibration curve and determining the peak area ratios by dividing the average value of oxazepam peak area by that of internal standard. The average regression equation was: 

\[
y = 0.00219 (± 0.0037) x + 0.0398 (± 0.023),
\]

where \( y \) = peak area ratio of oxazepam/internal standard and \( x \) = serum concentration of oxazepam (ng × mL⁻¹). The slopes of three calibration curves had a CV of 5.3% and intercepts were close to zero and not statistically different. The constructed graph showed excellent linearity over the concentration range studied with correlation coefficient of 0.996.

The specificity of the method was evaluated by analysis of twelve blank serum samples. In addition, various commonly used 1,4-benzodiazepines, like alprazolam, chlordiazepoxid, clonazepam, diazepam, flurazepam, lorazepam and nitrazepam were evaluated for interference with the present assay of oxazepam. Human serum was spiked with therapeutic concentrations of the above mentioned drugs followed by extraction and analysis as described. No endogenous serum components were observed at the

| Table 1. Intra-day and inter-day accuracy and precision of oxazepam in serum*.
<table>
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<tr>
<td></td>
<td>Precision %</td>
<td>Accuracy %</td>
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<td>Accuracy %</td>
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<tr>
<td>Inter-day</td>
<td></td>
<td></td>
<td></td>
<td>Intra-day</td>
<td></td>
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<tr>
<td>6.1</td>
<td>98.0</td>
<td>4.9 ± 0.3</td>
<td>6.3</td>
<td>96.0</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>6.2</td>
<td>97.0</td>
<td>19.4 ± 1.2</td>
<td>4.7</td>
<td>96.0</td>
<td>19.2 ± 0.9</td>
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<tr>
<td>5.3</td>
<td>96.1</td>
<td>76.9 ± 4.1</td>
<td>3.9</td>
<td>97.9</td>
<td>78.3 ± 3.1</td>
</tr>
<tr>
<td>2.8</td>
<td>99.6</td>
<td>159.3 ± 4.5</td>
<td>3.2</td>
<td>100.9</td>
<td>161.5 ± 5.2</td>
</tr>
<tr>
<td>0.6</td>
<td>101.2</td>
<td>323.8 ± 2.1</td>
<td>1.4</td>
<td>100.4</td>
<td>321.2 ± 4.4</td>
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</table>

* All spiked serum standards contained nordazepam (internal standard) to 50 ng x mL⁻¹.

Mean values for six different serum samples for each concentration ± standard deviations.

| Table 2. Analytical recoveries of oxazepam and nordazepam obtained with spiked serum samples*.
<table>
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<tr>
<td>Added concentration (ng x mL⁻¹)</td>
<td>Measured concentration (ng x mL⁻¹)</td>
<td>Analytical recovery (%)</td>
<td>CV (%)</td>
<td></td>
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<tr>
<td>Oxazepam 5</td>
<td>4.3 ± 3.8</td>
<td>86.0 ± 5.1</td>
<td>5.9</td>
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<tr>
<td>20</td>
<td>18.9 ± 2.9</td>
<td>94.3 ± 3.5</td>
<td>3.7</td>
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<tr>
<td>80</td>
<td>76.1 ± 3.5</td>
<td>95.1 ± 4.1</td>
<td>4.3</td>
<td></td>
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<tr>
<td>160</td>
<td>157.6 ± 1.7</td>
<td>97.8 ± 3.9</td>
<td>4.0</td>
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<tr>
<td>320</td>
<td>312.3 ±1.2</td>
<td>97.5 ± 2.9</td>
<td>3.0</td>
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<tr>
<td>Nordazepam 50</td>
<td>49.0 ± 4.0</td>
<td>98.0 ± 3.6</td>
<td>3.7</td>
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</table>

* Mean value of six assays ± standard deviation
retention times corresponding to the analyte and internal standard and there was no interference with other studied 1,4-benzodiazepines, except the close retention time of lorazepam. The retention times of the analyzed drugs studied for possible interferences are: flurazepam 5.5, nitrazepam 6.8, clonazepam 7.0, alprazolam 8.6, lorazepam 9.1, chlordiazepoxide 12.9, nordazepam 15.1 and diazepam 17.8 min.

The limit of quantitation (LOQ) was determined by repeated injections (n = 6) of spiked sera with oxazepam in decreasing concentration after pretreatment. Then, the LOQ was evaluated as the lowest concentration that could be measured with acceptable accuracy (R.S.D. ≤ 20%). The determined lower limit of quantitation for oxazepam was 2.0 ng x mL⁻¹, allowing the measurements for pharmacokinetic and bioequivalence studies.

A drug-free serum sample from a volunteer before oxazepam administration is shown in Figure 1A. This is followed by a zero serum sample, representing a standard containing 70 ng x mL⁻¹ oxazepam and 50 ng x mL⁻¹ internal standard. (Figure 1B). Finally, there is a chromatogram obtained from the same volunteer 15 h after oral administration of a 10 mg oxazepam tablet (Figure 1C).

As can be seen from Figures 1B and 1C, under the chromatographic conditions used, oxazepam and internal standard resulted in well resolved symmetric peaks with symmetrical factors of 1.0. The resolution between the two peaks was 1.71. The observed retention times of oxazepam and internal standard were 8.8 (± 0.5 %) and 15.0 (± 0.7 %) min, respectively.

The developed HPLC method was applied to evaluate the pharmacokinetic parameters of oxazepam. These included the maximum serum concentration (Cmax), the time to reach this concentration (tmax), the area under the concentration time curve during the sampling period (AUC0-40), the area under the concentration time curve from zero to infinity (AUC0-∞), and the elimination half life (t1/2). These parameters were calculated using Mirfazaelian pharmacokinetic software (Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran).

Typical serum concentration-time profiles for two preparations are presented in Figure 2 and calculated pharmacokinetic parameters are shown in Table 3. The pharmacokinetic data are in accordance with the parameters reported by Greenblatt et al. (2, 3).

Figure 2 and Table 3 indicate that the proposed method can be successfully applied to pharmacokinetic and bioequivalence studies of oxazepam in human after oral administration. The bioavailability of oxazepam is determined using the AUC0-40 h value from test (oxazepam) versus that from reference (Seresta®) to the last collected data point (40 h) and found to be 90.2% (n = 12). Statistical test to assess bioequivalence of oxazepam formulations was
DISCUSSION AND CONCLUSION

The aim of this investigation was to develop a sensitive, accurate and precise HPLC method for oxazepam analysis in biological samples. There are three important requirements for the HPLC assay of oxazepam in human blood. Firstly, the method should be sensitive enough for very low serum concentration of oxazepam, which rarely exceeds $100 \text{ ng} \times \text{mL}^{-1}$. Secondly, the method should be accurate and rapid enough for the actual needs. Thirdly, the symmetrical chromatographic peaks should be achieved.

Peak tailing is one of the serious pitfalls in benzodiazepine determinations by HPLC. It is particularly prevalent when analyzing basic compounds such as oxazepam and therefore, is a source of persistent problem especially in low concentration samples. This is observed most often when using HPLC columns with stationary phases that have significant silanol activity. The HPLC columns normally used for the analysis of oxazepam in pharmaceutical and biological samples are reversed-phase columns with packing materials consisted of chemically bonded highly active silica (10-12). Using a new polymer (13) or monolithic (14) columns has been also reported in 1, 4-benzodiazepines analysis, but none of these have been able to correct unsymmetrical configuration of peaks. In this project, various silica stationary phases with different activities were used in order to minimize or eliminate the asymmetric presentation of these peaks. The results indicate that there is less tailing in separating oxazepam and nordazepam when the stationary phase exhibits less silanol activity. It was found that the peak tailings can be completely prevented by selecting Perfectsil-Target ODS-3 column, which contains a stationary phase with very low silanol activity.

Different mobile phase composition was used to curtail the tailing of the peaks. It was apparent that the ionic strength of the buffer solution played a major role in the resolution of the chromatograms. In fact, increasing the molarity of the used ammonium dihydrogen phosphate buffer from 0.01 to 0.05 M improved the resolution of oxazepam and internal standard from endogenous peak in serum. The effect of mobile phase pH to curtail the tailing was also investigated. Varying this parameter did significantly alter the retention times as well as the peak area. The best reproducible results were achieved by the pH range of 5.5-5.9. Increasing this pH range does significantly increase the retention times as well as the peak area, while decreasing pH toward strong acidic medium leads to hydrolysis of booth benzodiazepines.

Table 3. Pharmacokinetic parameters (mean ± SD), confidence limits and bioequivalence (F) of oxazepam formulations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Oxazepam (test)</th>
<th>Seresta® (reference)</th>
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<tbody>
<tr>
<td>$C_{\text{max}}$ (C.I*) (ng × mL$^{-1}$)</td>
<td>260.5 ± 2.1 (259.4-261.6)</td>
<td>255.1 ± 5.1 (252.5-257.7)</td>
</tr>
<tr>
<td>$\text{AUC}_{0-40}$ (C.I*) (ng × h × mL$^{-1}$)</td>
<td>4375 ± 10.9 (4368-4382)</td>
<td>4761 ± 8.1 (4755-4765)</td>
</tr>
<tr>
<td>$\text{AUC}_{0-\infty}$ (ng × h × mL$^{-1}$)</td>
<td>4529 ± 11.8</td>
<td>5006 ± 6.2</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>3.0 ± 3.8</td>
<td>3.2 ± 3.5</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>10.5 ± 2.0</td>
<td>$F = 90.2%$</td>
</tr>
<tr>
<td>F = 90.2%</td>
<td>11.3 ± 2.9</td>
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</table>

* 90% confidence interval

Figure 2. Mean serum concentration-time profiles of the drug for two oxazepam preparations in 12 volunteers after administration of a single 10 mg oral dose of: test (oxazepam tablet) and reference (Seresta® tablet).
Most internal standards for benzodiazepines are compounds of the same family. Thus, diazepam has been used as internal standard for oxazepam (15, 16). However, in pharmacokinetic and bioequivalence studies, a more suitable internal standard would be any 1,4-benzodiazepine that is not frequently used in clinical settings. To select an ideal internal standard a number of similar compounds considering their UV spectrum, retention time, solubility and resolution from oxazepam and endogenous peaks were tested. Based on the collected data, nordazepam was selected.

The previous reported recoveries of oxazepam ranged between 80-99% (17) and that from nordazepam 71-93% (18), respectively. Hence, the recoveries obtained with our method were comparable with available methods. As observed, the mean extraction recovery of oxazepam using dichloromethane was less than 100% (86.0-97.8%). In spite of this fact, the choice of dichloromethane as extracting solvent provided adequate purity to serum samples over several other used organic solvents such as chloroform (11), diethyl ether (12, 19), n-butyl chloride (14) and mixtures of hexane-isooamyl alcohol (10), or dichloromethane-n-pentane (20). The extraction with dichloromethane did not demonstrate any interference with other medically used 1,4-benzodiazepines. It minimized the endogenous interfering peaks and irregular baseline.

For trace detection and determination of these compounds, HPLC separation followed by electrochemical detection (HPLC-EC) has been recommended (5). It has been suggested by other investigators to optimize the method with an electrochemical detector, which offers enhanced selectivity and sensitivity over a UV detector by operating potential control (21). Based on these suggestions we attempted to increase the sensitivity of the assay using oxidative electrochemical mode. However, our attempt has failed due to high potential required for electrooxidation of oxazepam NH-group. We found that for an accurate and sensitive HPLC-EC assay the required oxidative potential is higher than 1.4 V, however, the available electrodes are not able to provide the required positive potential without influencing the accuracy.

An UV scan of the drug showed maximal absorptivity at three wavelengths of 215, 230 and 254 nm. The wavelength of 254 nm was selected, because more interfering peak emerged from endogenous compound in serum at lower wavelength, although the molar absorptivities for both compounds are higher at 230 nm.

The assay described in this paper is an optimization of existing methods for quantification of oxazepam in human sera. Our study have confirmed that in contrast to the most published high-performance liquid chromatographic methods, using a stationary phase with low silanol activity allow for an accurate determination of low oxazepam concentration without tailing problems. This method results in fully symmetrical peaks, which enable us to use it in clinical pharmacokinetic studies and drug monitoring of oxazepam.

In conclusion, the analytical methodology developed in this report is simple, rapid, sensitive and specific. It can be used for monitoring serum oxazepam and nordazepam concentrations in clinical, forensic and pharmacokinetic studies. This new method has been demonstrated to be suitable for use in pharmacokinetic studies with suitable LOQ. We are aware of the fact that our developed assay for oxazepam is less sensitive than that using GC- or LC-MS. However, GC- and LC-MS for quantification are far more expensive and time consuming than the HPLC-UV method. The potential application of this technique to establish the qualitative and quantitative analysis of other 1,4-benzodiazepines is currently under investigation in our department. In our next projects, we are especially interested to apply this developed method in the field of clinical emergency and forensic toxicology.

Acknowledgments

This work has been financially supported by the Research Council of Tehran University of Medical Sciences. The authors wish to thank Mrs. M. Fattahi for technical assistance.

REFERENCES


Received: 1.12.2006