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Optimized determination of lorazepam in human serum by extraction and high-performance liquid chromatographic analysis

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The present research was designated to evaluate a rapid and sensitive method for determining low concentrations of the highly active drug lorazepam in serum. Isolation of the drug from biological fluid after addition of nordazepam as the internal standard was achieved using a simple liquid-liquid extraction with dichloromethane and the extracted compounds were quantified by high-performance liquid chromatography. Chromatographic separation on a reversed phase column containing a stationary phase with low silanol activity resulted in a perfectly symmetrical peak with a tailing factor of 1.0. The limit of quantitation in serum is 2.5 ng mL^{-1} for both lorazepam and internal standard. The procedure is rapid and sensitive enough for determination of lorazepam in serum.

Keywords: lorazepam, serum, high-performance liquid chromatography, peak tailing

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Lorazepam [7-chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepine-2-one] is a short-acting benzodiazepine that produces central depression of the CNS. It is administered in the treatment of anxiety states, insomnia associated with anxiety and as an anticonvulsant. Numerous clinical studies have adequately demonstrated the importance of blood concentrations of lorazepam to its efficacy and toxicity (1–4). Consequently, knowledge of the lorazepam blood levels is helpful in therapeutic drug monitoring and in the control of overdosed patients. Unfortunately, determination of blood concentrations of lorazepam is more difficult than that of most benzodiazepines since very low levels result from low dosage (1–2.5 mg).

Several liquid chromatographic procedures have been described for the analysis of lorazepam in body fluids (5–7). The gas chromatographic method appears to be more sensitive than high-performance liquid chromatographic (HPLC) methods; however, it requires sample derivatization for drug analysis, thus increasing the complexity and time required for analysis (8). In addition, thermal instability of this compound at high

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column temperature used in gas chromatography complicates analysis. GC quantification of lorazepam without prior drivatization has been made feasible by improvements in sensitivity of the electron capture detectors, together with improved performance properties of the silicone liquid phase (9).

In this study, we report a rapid, sensitive, accurate and reproducible reversed phase HPLC method for lorazepam assay in human serum that prevent all tailing problems.

EXPERIMENTAL

Instruments and chromatographic conditions

The chromatographic system used to develop this technique is a Knauer HPLC consisting of a Wellchrom K-1001 pump and a Wellchrom K 2600 UV-Vis detector connected to Euchrom 2000 integrator (Knauer, Germany). The system is fitted with a model 7125 Rheodyne manual injector (Rheodyne, USA) and a 50 µL-sample loop. Separation was performed using an analytical Perfectsil Target ODS-3 column, 5 µm particle size, 125 × 3.5 mm (i.d.) made of stainless steel (Mainz-Analysentechnik GmbH. Germany). The wavelength was set at 254 nm. The mobile phase consisted of 0.05 mol L^{-1} ammonium dihydrogenphosphate solution (adjusted to pH 5.8 with concentrated ammonia solution) and methanol (1:1, *V/V*). The mobile phase was prepared daily and filtered through a 0.45 µm Waters (USA) membrane filter before use. It was pumped through the column at a flow rate of 1.6 mL min^{-1}.

Materials

Lorazepam was supplied by Profarmaco (B.N. 530916), Italy. The internal standard nordazepam was obtained from the European directorate for the quality of medicines, Council of Europe (Strasbourg, France). Tablets used correspond to lorazepam 2 mg (Chimidaru Pharmaceutical Company, Iran). HPLC-grade methanol and all other chemicals were obtained from Merck (Germany). Water was obtained by double distillation and purified additionally using a Milli-Q system.

Preparation of stock and standard solutions

Stock solutions of lorazepam and the internal standard nordazepam were prepared at a concentration of 10 µg mL^{-1} in methanol. The solutions were stored at 4 °C and showed no significant alterations in peak areas or heights determined daily by direct injection throughout the study. Working standards were prepared in drug-free human serum by spiking of serum with appropriate amounts of drug and internal standard stock solutions to obtain final concentrations ranging from 0–200 ng mL^{-1} of lorazepam and 30 ng mL^{-1} of internal standard.

Quality control samples (QC) were prepared in our laboratory from processed (extracted) serum at four different levels 5.0, 10.0, 50.0 and 100 ng mL^{-1} of lorazepam. Each QC sample also contained 30 ng mL^{-1} of nordazepam.

Sample preparation

The study protocol was approved by the Ethics Committee of the Tehran University of Medical Sciences and written informed consent was obtained from the volunteers. Six non-smoker male volunteers mean age 23 ± 2.4 years and mean body mass of 57 ± 4.1 kg, were included in the study. They were in good health and had not taken any medication for the last three weeks. After overnight fasting, each volunteer received a single dose of a 2 mg lorazepam tablet. Peripheral venous blood samples (5 mL) were taken 1–5 hours after drug consumption. Thirty minutes after clotting of the blood at room temperature, samples were centrifuged for 5 min at 5000 rpm. Separated serum samples were decanted in 5 mL test tubes and then frozen immediately at –20 °C until assayed (serum samples can be stored for up to 6 months at –20 °C before analysis without any change in lorazepam concentration).

Extraction procedure

Internal standard solution (300 ng mL^{-1} nordazepam, 10 μL) and 4 mL dichloromethane were added to 1 mL fresh thawed serum sample and the mixture was sonicated for 1 min and then separated by centrifugation (5 min at 5000 rpm). Approximately 4 mL of 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, vortex mixed for 30 s and 50 μL was injected onto the column.

RESULTS AND DISCUSSION

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

Peak tailing is one of serious pitfalls in benzodiazepine determinations by HPLC. It causes a number of problems, including lower resolution, sensitivity, accuracy and precision (10). These factors are due to the inability of the data system to identify exactly where a peak tailing begins and ends. Peak tailing is particularly prevalent when analyzing basic compounds such as lorazepam and, therefore, a source of persistent problem especially in low concentration samples. It 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 lorazepam in pharmaceutical and biological samples are reversed-phase columns with packing materials consisting of chemically bonded highly active silica (11–13). Use of a new polymer (14) or monolithic (15) columns has been also reported in 1,4-benzodiazepines analysis, but none of these has resulted in symmetrical peaks. Therefore, various silica stationary phases with different activity were used in this project in order to minimize or exclude the asymmetric effect. Our results indicate that there is less tailing in separating lorazepam and nordazepam

when the stationary phase exhibits lower silanol activity. It was found that the peak tailing can be completely eliminated by selecting the Perfectsil-Target ODS-3 column, which contains a stationary phase with a very low silanol activity.

We chose nordazepam as an internal standard; this compound is a diazepam metabolite (16) and has some advantages, like similar solubility, the same absorption wavelength and molar absorptivity and different retention time.

A drug-free serum sample from one of the volunteers before lorazepam administration is shown in Fig. 1a. This is followed by a serum sample containing 40 ng mL^{-1} lorazepam and 30 ng mL^{-1} internal standard (Fig. 1b). Finally, there is a chromatogram obtained from the same volunteer 5 hours after oral administration of a 2 mg lorazepam tablet, which represent a serum lorazepam concentration of 27.9 ng mL^{-1} (Fig. 1c).

As can be seen from Figs. 1b and c, under the chromatographic conditions described, lorazepam and nordazepam resulted in well resolved symmetric peaks with symmetrical factors of 1.0 and retention times of 8.0 and 13.1 minutes, respectively. The resulting symmetrical peaks and the obtained tailing factors of 1.0 are one of the novelties of our method. The tailing factor, a parameter of ICH guidelines, considered as an important factor to be controlled has a strong influence on all validation parameters. From that standspoint, most of published methods of lorazepam and other benzodiazepines analysis need revision of the validation study.

The extraction recoveries of lorazepam and that of the internal standard from sera were separately quantitated using the drug standards (5, 10, 25, 50, and 100 ng mL^{-1}) and 30 ng mL^{-1} of nordazepam. The analytical recovery was measured by adding the

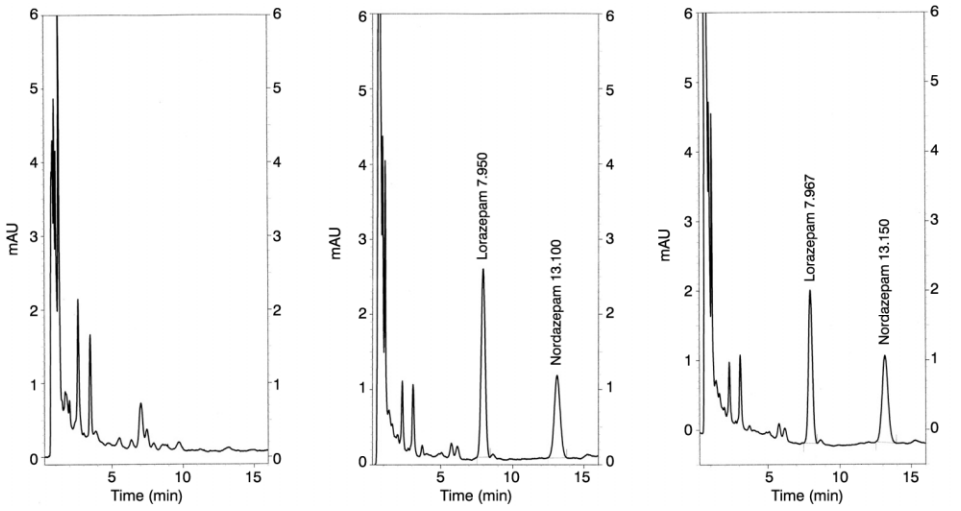

Fig. 1. Representative LC-UV chromatogram of: a) extracted drug-free serum, b) extracted serum spiked with 40 ng mL^{-1} lorazepam and 30 ng mL^{-1} nordazepam, c) extracted volunteer serum obtained 5 h after oral administration of a 2 mg lorazepam tablet and spiked with 30 ng mL^{-1} nordazepam.

Table I. Analytical recovery obtained with spiked serum samples

Added concentration (ng mL^{-1})	Measured concentration (ng mL^{-1})[a]	Extraction recovery (%)	RSD (%)
Lorazepam			
5	4.6 ± 3.8	92.9 ± 5.1	5.4
10	9.8 ± 1.2	98.0 ± 3.5	3.6
25	24.6 ± 3.5	98.8 ± 4.1	4.1
50	48.9 ± 3.9	98.4 ± 3.9	4.0
100	97.5 ± 5.1	97.9 ± 3.6	3.7
Nordazepam (I.S.)			
30	28.7 ± 5.0	95.7 ± 3.8	3.4

[a] Mean value ± SD (n = 6).

drug or internal standard to drug-free serum (six replicates for each standard) to achieve the concentration shown in Table 1. The recoveries were calculated by comparing the observed concentrations obtained from the processed standard samples to direct injections of standard solutions. Recovery of lorazepam ranged from 93.9 ± 5.1 to 98.8 ± 4.1% (n = 6). The internal standard recovery was found to be consistent from all the six pools tested; averaging 98.3 ± 3.8%. As mentioned above, the recoveries of lorazepam and nordazepam after extraction with dichloromethane were less than 100%, but high enough to determine these substances at the nanogram levels in serum. The choice of dichloromethane as extracting solvent provided adequate purity to serum samples over several other used organic solvents, such as chloroform (12), butyl chloride (15), diethyl ether (13) and solvent mixtures (11, 16). It did not demonstrate any interference with other 1,4-benzodiazepines, minimized the endogenous interfering peaks and noisy baseline and led to minimal handling time for evaporation. However, extraction of lorazepam from serum using solvents such as ether or chloroform leads to background interferences and a noisy baseline.

The method's linearity was studied by analyzing, in triplicate, the calibration curve (0 to 200 ng mL^{-1}). Calibration data were acquired by plotting the ratio of the lorazepam peak area to the internal standard peak area against the concentration of the calibration standards, followed by a linear-regression analysis. The constructed graph was linear and the average corresponding regression equation was $y = 0.00406\gamma - 0.025$ with a correlation coefficient of 0.995, where y is the peak area ratio of lorazepam to nordazepam and γ is the lorazepam concentration (ng mL^{-1}).

Replicate samples (n = 6) spiked at four control concentrations 5, 10, 50 and 100 ng mL^{-1}, were used to assess intra-day (within-a-day) precision as well as accuracy of lorazepam assay in serum. These parameters were evaluated by processing a set of quality control samples (actual values). Selection of concentrations for analyses was made to allow defining the precision at low, medium and high concentrations of the linear range. Precision is expressed as RSD of repeated analyses. Accuracy is expressed as the percentage difference of measured and actual values. The intra-day precision ranged from 3.8 to 8.9%, and the accuracy ranged from –4.0 to 1.4% (Table II). The inter-day precision

Table II. Intra-day and inter-day accuracy and precision of lorazepam determination in serum[a]

Concentration (ng mL^{-1})	Intra-day [b]			Inter-day [b]		
	Concentration found (ng mL^{-1})	Accuracy (%)	Precision (%)	Concentration found (ng mL^{-1})	Accuracy (%)	Precision (%)
5	4.8 ± 0.4	–4.0	8.9	4.6 ± 0.4	–8.0	9.1
10	9.8 ± 0.8	–2.0	8.5	9.9 ± 0.9	–1.0	9.0
50	51.6 ± 2.9	3.2	5.6	50.7 ± 1.6	1.4	3.2
100	101.4 ± 3.8	1.4	3.8	97.3 ± 3.2	–2.7	3.3

[a] All spiked serum standards contained nordazepam (internal standard) 30 ng mL^{-1}.
[b] Mean value ± SD (n = 6).

in serum samples was similarly evaluated over a period of 4 weeks; it ranged from 3.2 to 9.1% and accuracy ranged from –8.0 to 1.4%.

Various important 1,4-benzodiazepines commonly used, like alprazolam, chlordiazepoxid, diazepam, flurazepam, oxazepam and nitrazepam, were evaluated for interference with the present lorazepam assay. Human serum was spiked with therapeutic concentrations of these drugs, followed by extraction and analysis as described above. There was no interference, except for the closeness of retention time of oxazepam (t_R = 7.5 min).

The limit of detection (*LOD*), defined as the lowest concentration of analyte that can be clearly detected above the baseline signal, was estimated as three times the signal to noise ratio. *LOD* was determined after extraction of spiked serum, by injection, with the drug and internal standard in decreasing concentrations. The limit of quantitation (*LOQ*) was obtained by the same procedure used for *LOD*, but estimated as ten times the signal to noise ratio. Each experiment was repeated 6 times. The lower limit of detection was 1 ng mL^{-1}, while the lower limit of quantification was 2.5 ng mL^{-1} for both lorazepam and nordazepam.

Although many different HPLC assays for lorazepam can be found in the literature, thus far no method resulted in a symmetric peak, which would enable its precise use in drug monitoring, forensic toxicology and clinical pharmacokinetic studies. The assay described in this paper is an optimization of the existing methods (5–7) for quantification of lorazepam in human sera. Our study has conformed that, in contrast to most of the published high-performance liquid chromatographic methods, good purification and separation conditions, especially using a stationary phase with low silanol activity, allow an accurate determination of low lorazepam concentrations without tailing problems.

CONCLUSIONS

In conclusion, the analytical methodology developed in this report is simple, rapid, sensitive and specific. It can be used for monitoring serum lorazepam and nordazepam concentrations in clinical, forensic and pharmacokinetic studies. Potential applications

of this method in the qualitative and quantitative analysis of other 1,4-benzodiazepines, especially in the field of clinical emergency, forensic toxicology and pharmacokinetics, are currently under investigation in our department.

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REFERENCES

1. C. Tsui-Ling, C. Kuan-Wen, L. Yih-Dar and F. Kang, Determination of benzodiazepines in clinical samples; Comparative evaluation of REMEDI system, aca analyzer and conventional high-performance liquid chromatography, *J. Clin. Lab. Anal.* **13** (1999) 106–111.
2. S. K. Gupta, E. H. Ellinwood, A. M. Nikaido and D. G. Heatherly, Simultaneous modeling of the pharmacokinetic and pharmacodynamic properties of benzodiazepines I: Lorazepam, *J. Pharmacokin. Pharmacodyn.* **18** (1990) 89–102.
3. H. Zhu and J. Luo, A fast and sensitive liquid chromatographic-tandem mass spectrometric method for assay of lorazepam and application to pharmacokinetic analysis, *J. Pharm. Biomed. Anal.* **39** (2005) 268–274.
4. D. J. Greenblatt, Clinical pharmacokinetics of oxazepam and lorazepam, *Clin. Pharmacokin.* **6** (1981) 89–105.
5. T. Kondo, D. C. Buss and P. A. Routledge, A rapid determination of lorazepam by high-performance liquid chromatography, *Ther. Drug Monit.* **15** (1993) 35–38.
6. S. Gunawan, N. Y. Walton and D. M. Treiman, Analysis of lorazepam in rat brain using liquid-liquid and solid-phase extraction in combination with high performance liquid chromatography, *Biomed. Chromatogr.* **4** (1990) 168–170.
7. C. Pistos and J. T. Stewart, Direct injection HPLC method for the determination of selected benzodiazepines in plasma using a Hisep column, *J. Pharm. Biomed. Anal.* **33** (2003) 1135–1142.
8. S. Higuchi, H. Urabe and Y. Shiobara, Simplified determination of lorazepam and oxazepam in biological fluids by gas chromatography-mass spectrometry, *J. Chromatogr.* **164** (1979) 55–61.
9. D. J. Greenblatt, K. Franke and R. I. Shader, Analysis of lorazepam and its glucuronide metabolite by electron-capture gas-liquid chromatography. Use in pharmacokinetic studies of lorazepam, *J. Chromatogr.* **146** (1978) 311–320.
10. L. A. Berrueta, B. Gallo and F. Vicente, Biopharmaceutical data and high-performance liquid chromatographic data of 1,4-benzodiazepines in biological fluids, *J. Pharm. Biomed. Anal.* **10** (1992) 109–136.
11. J. M. Egan and D. R. Abernethy, Lorazepam analysis using liquid chromatography. Improved sensitivity for single-dose pharmacokinetic studies, *J. Chromatogr.* **380** (1986) 196–201.
12. E. Tanaka, M. Terada, S. Misawa and C. Wakasugi, Simultaneous determination of twelve benzodiazepines in human serum using a new reversed phase chromatographic column on a 2-μm porous micro spherical silica gel, *J. Chromatogr. B. 682* (1996) 173–178.
13. U. R. Tjaden, M. T. H. A. Meeles, C. P. Thys and M. Van Der Kaay, Determination of some benzodiazepines and metabolites in serum, urine and saliva by high-performance liquid chromatography, *J. Chromatogr.* **181** (1980) 227–241.
14. X. P. Lee, T. Kumazawa, J. Sato, Y. Shoji, C. Hasegawa, C. Karibe, T. Arinobu, H. Seno and K. Sato, Simple method for the determination of benzodiazepines in human body fluids by high-performance liquid chromatography-mass spectrometry, *Anal. Chim. Acta* **492** (2003) 223–231.

15. A. Bugey and C. Staub, Rapid analysis of benzodiazepines in whole blood by high-performance liquid chromatography: Use of monolithic column, *J. Pharm. Biomed. Anal.* **35** (2004) 555–562.
16. J. Atta-Politou, M. Parissi-Poulou and D. A. Koutselinis, A modified simple and rapid reversed phase liquid chromatographic method for quantitation of diazepam and nordiazepam in plasma, *J. Pharm. Biomed. Anal.* **20** (1999) 389–396.

S A Ž E T A K

Optimizirano određivanje lorazepama u humanom serumu visokotlačnom tekućinskom kromatografijom

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U radu se ocjenjuje brza i osjetljiva metoda za određivanje niskih koncentracija lorazepama u serumu. Lorazepam je izoliran iz seruma ekstrakcijom diklormetanom, zajedno s nordazepamom, koji je upotrebljen kao interni standard i zatim određivan visokotlačnom tekućinskom kromatografijom. Kromatografskim razdjeljivanjem na reverzno-faznoj koloni sa stacionarnom fazom s niskom aktivnošću silanola dobiveni su potpuno simetrični signali s faktorom završnog povlačenja 1,0. Granica određivanja u serumu je 2,5 ng mL^{-1} za lorazepam i interni standard. Metoda je brza i dovoljno osjetljiva za određivanje lorazepama u serumu.

Ključne riječi: lorazepam, serum, visokotlačna tekućinska kromatografija, završno povlačenje

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