Acta Poloniae Pharmaceutica – Drug Research, Vol. 65 No. 2 pp. 179–186, 2008 ISSN 0001-6837
Polish Pharmaceutical Society

A NEW UNIVERSAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF 1,4-BENZODIAZEPINES AS BULK DRUGS AND IN PHARMACEUTICAL FORMULATIONS

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Abstract: A universal high-performance liquid chromatographic method was developed for the simultaneous determination of seven frequently prescribed 1,4-benzodiazepines in bulk powder or formulated in tablets or capsules. Peak tailing commonly reported with HPLC method developed for benzodiazepines, is completely omitted with this new method. The assay procedure consisted of pulverization, extraction into methanol, filtering, diluting and injecting of aliquots of clear filtrate into a reversed phase column with a very low silanol activity. The mobile phase consisted of a mixture of methanol and 0.05 mol × L^{-1} buffer solution of ammonium dihydrogen phosphate (50:50, v/v) adjusted to pH 5.8. The effluent was monitored by UV detection at 254 nm and delivered at a flow rate of 1.6 mL × min^{-1}. The new method has been applied for quantifying of 1,4-benzodiazepines in different commercial formulations with recoveries ranging from 99.0 (±1.98) to 102.0 (±1.58)%. The excipients present in tablets and capsules did not interfere with the developed method. The applicability of the method for content uniformity and dissolution tests has been also investigated and the results were considered satisfactory. The developed method is rapid and sensitive, and is suitable for routine control of pharmaceutical dosage forms.

Keywords: 1,4-benzodiazepine, HPLC assay, pharmaceutical dosage forms, quality control, dissolution tests

The 1,4-benzodiazepines (BZD) as anxiolytic drugs are the most widely-prescribed in the world today. They are also active as hypnotic and are frequently prescribed for the treatment of epilepsy, convulsion, and related disorders (1-3).

Optimal analytical measurement of benzodiazepines has been, and is still the subject of many investigations. Several techniques have been reported for the analysis of BZD, individually in pharmaceutical dosage forms and biological fluids. Immunological assays (4), micellar electrokinetic chromatography (5), derivative spectrophotometric (6), polarographic (7), square-wave voltammetric (8), capillary electrophoretic (9), thermal desorption gas chromatographic (10) and high-performance liquid chromatographic (HPLC) procedures (6, 11-15) are examples. To the best of our knowledge, only a few published high-performance liquid chromatographic methods are applicable to the simultaneous determination of BZD. In general, the separation of benzodiazepines by HPLC is performed using reversed-phase systems composed of silica support materials, chemically bonded coating of alkyl chains (12) or on monolithic column (16) and by normal-phase chromatography with medium polarity stationary phases (13). Reviewing the literature reveals that none of these methods is universal. In addition, the reported methods could not eliminate important problems like peak tailing in HPLC chromatograms of BZD (15, 17). The significant peak tailing causes a number of problems including lower resolution, sensitivity, accuracy and precision.

The objective of this research was to develop a universal, rapid, precise, and sensitive high performance liquid chromatographic method required for quality control of seven BZD in bulk powder and in pharmaceutical dosage form. The method is adapted from our previously published method for oxazepam analysis in human blood (17).

EXPERIMENTAL

Chemicals and reagents

The BZDs investigated were: alprazolam, chlordiazepoxide, diazepam, flurazepam, lorazepam, nitrazepam, and nordiazepam as bulk powders and pharmaceutical formulations. Pharmaceutical grade chlordiazepoxide, diazepam and lorazepam reference standards were supplied by Profarmaco, Italy. Alprazolam, flurazepam and nitrazepam raw

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material were kindly donated by Jalinus Pharmaceutical Company (Iran) and were used as reference standards without further purification. Nordazepam was obtained from European directorate for the quality of medicines (Council of Europe, Strasbourg, France).

Commercial tablets and capsules of the above BZD analyzed were: chlordiazepoxide 5 mg, and diazepam 5 mg tablets provided by Sobhan Pharmaceutical Company (Iran), Apo-Flurazepam® labeled to contain 15 mg flurazepam hydrochloride per capsule from Apotex (Canada), nitrazepam B.P., 5 mg tablets from Norton (UK), Ativan® (lorazepam 1 mg tablets) from Wyeth-Ayerst (UK), Calmday® (nordiazepam 10.0 mg tablets) from Will Pharma (Belgium) and Xanax® (alprazolam 1 mg tablets) from Pharmacia (UK). HPLC-grade methanol was used and all other chemicals and reagents were of analytical grade and purchased from Merck (Germany). Water was obtained by double distillation and purified additionally with a Milli-Q purification system (Millipore, USA).

Apparatus and analytical conditions

Routine HPLC analyses were performed using a Knauer chromatograph (Knauer GmbH, Germany). The chromatographic system was composed of a K-5002 WellChrom solvent degasser, Wellchrom K-1001 pump, Rheodyne 7725i manual injector, K-2600 Wellchrom UV-VIS detector, Euchrom 2000 integrator and ChromGate™ V2.55 chromatographic data system with the Chrom Gate™ analytical software working under Windows 2000 operating system.

The separation was performed using an analytical Perfectsil Target ODS-3 column 125 × 4 mm i.d. (5 µm particle size), made of stainless steel (Mainz-Analysentechnik, Germany). The mobile phase consisted of a mixture of 0.05 mol × L^{-1} ammonium dihydrogen phosphate solution – methanol (50:50, v/v) which was adjusted to pH 5.8 with concentrated ammonia solution. The flow rate of 1.6 mL × min^{-1} and the volume injection of 50 µL were constant in all cases. The mobile phase was prepared daily and filtered through a 0.45 µm Waters (USA) membrane filter before use. The wavelength was set at 254 nm and the analyses were performed at ambient temperature (25 ± 2°C) under isocratic conditions.

The dissolution tests were performed in accordance with the USP 29, (18) in an Erweka DT800 multi-bath (n = 6) dissolution test system (Erweka, Germany).

Preparation of stock standard solutions

Stock solutions of each drug studied (20 µg × mL^{-1}) were prepared separately by transferring accurately weighed amounts of each compound into calibrated flasks and dissolving in methanol to volume. These solutions were stored at 4°C and protected from light to minimize the risk of decomposition. Standard working solutions were then prepared fresh each day by appropriate dilution in the mobile phase. The calibration standards were prepared by diluting each of the stock solutions in the mobile phase to furnish solutions with final concentrations of 0.5, 1.0, 2.0, 4.0, 8.0 and 10.0 µg × mL^{-1} for drugs studied.

Validation

Once optimal chromatographic conditions have been established, the method was validated. The validation was established with respect to linearity, intra-day and inter-day precision, accuracy, specificity and sensitivity (18, 19).

Calibration graph linearity. The calibration graphs were obtained by injecting a series of standard solutions of each BZD separately into the chromatographic system and plotting mean chromatographic peak area against the nominal concentration of each compound. Each concentration was injected in triplicate and the mean peak area value was observed over the concentration range of 0.5 – 10 µg × mL^{-1} for all investigated BZDs.

Precision. The system precision of the assays was investigated by performing five replicate analyses of added standard samples at three different concentrations (2.5, 5.0 and 10 µg × mL^{-1}) for each BZD on the same day and on three separate days and evaluated by relative standard deviations (RSD) of the peak areas of each analyte.

Accuracy (recovery method). The accuracy of HPLC method was tested by calculating the recovery of known amounts of each BZD added separately at three different concentrations (2.5, 5.0 and 10.0 µg × mL^{-1}) to samples representing the average weight of the corresponding BZD dosage forms**.** The recoveries were also confirmed by determination of these drugs in laboratory-prepared dosage formulations containing 80, 100 and 120% of active substances. At each level of the amount, six determinations were performed.

Selectivity and specificity. For the selectivity and specificity study, identification of drugs was studied, comparing the chromatogram of active drugs with chromatogram of BZD reference standards. Another study was carried out to check the absence of interference by the excipients which take

part in the pharmaceutical preparations (placebo solutions). Placebo of each tablet or capsule sample was prepared by mixing respective excipients. The following excipients were used in the preparation of placebo: starch, lactose monohydrate, carboxymethylcellulose, magnesium stearate, hydroxypropylcellulose, hydroxypropylmethylcellulose, aerosole, polyethylene glycol 400 and neutral talc. In addition, the studied 1,4-benzodiazepines were also evaluated for interference with each other. For this purpose a mixed solution containing reference standards of seven investigated BZD was determined under the proposed chromatographic conditions.

Limiting values. The sensitivity of the method can be determined through the limit of detection (LOD) and quantitation (LOQ). The limits of sensitivity for BZD were determined by spiking mobile phase with known amounts of each BZD separately in decreasing concentrations and then chromatographing the samples.

Analytical procedure for dosage forms

The validated method was used to assay BZD, for determination of content uniformity, dissolution rates and dissolution profiles of BZD drug formulations.

For the assay, the sample treatment was the same for all studied preparations, except the diluting steps due to different amount of each drug in formulation. Ten tablets (for flurazepam the content of ten capsules) were weighed, pulverized and thoroughly mixed. An accurately weighed amount of the dosage form powder equivalent to 1 mg of active component was transferred into a 50 mL calibrated flask, mixed with 45 mL of methanol and sonicated for 15 min. The mixture was diluted to volume with methanol and filtered using a Millipore filter. Appropriate solutions with final concentration of 5 $\mu g \times mL^{-1}$ for each BZD were prepared by taking suitable aliquots of the clear filtrates and diluting them with the mobile phase. 50 µL aliquots of solutions prepared as described under assay were injected into the chromatographic system. Similarly, the same volume of standard solutions containing 5 $\mu g \times mL^{-1}$ of corresponding BZD was then analyzed under the same conditions. The amounts of active component per tablet or per capsule were then calculated by using the following formula:

$$\frac{Ax}{As} \times 100 = \text{percent of the label claim}$$

where Ax is the sample peak area (average of three injections), and As is the average area for standard solution of identical concentration injected three times sequentially. This procedure was applied to the sample preparation of all formulations mentioned above.

Ten single whole tablets or capsules were separately subjected to the procedure for content uniformity determination according to the corresponding monographs of USP 29 (18). Individual dosage forms were weighed and each tablet (for capsules the content of each capsule) was separately dissolved in 50 mL of mobile phase using ultrasonic bath. After filtration, aliquot of each filtrate was diluted with mobile phase to obtain the concentration of 5 $\mu g \times mL^{-1}$. 50 µL aliquots of standards containing 5 $\mu g \times mL^{-1}$ of corresponding active drug were also injected.

Dissolution test has emerged in pharmaceutical field as a very important tool to characterize drug product performance. The conditions specified by the corresponding monographs of USP 29 for the determination of dissolution rate of each studied BZD formulations by the basket or paddle method were followed**.** The dissolution vessel was filled with specified volume of corresponding dissolution medium, specified in individual BZD monograph and maintained at 37 ± 0.5°C. The dissolution media for BZD are varying from degassed water to 0.1 mol $\times L^{-1}$ HCl or enzyme-free simulated gastric solution. The quantity Q, the amount of dissolved active ingredient at the specified time, required in the individual monograph and expressed as a percentage of the labeled content was determined as follows: 6 BZD tablets or capsules were individually put in the corresponding dissolution apparatus (basket or paddle) and the procedure was started. At the time specified in the individual monograph for each BZD drug formulations, 5 mL aliquots of dissolution medium were withdrawn and after filtration 1 mL samples of each filtrate were diluted up with the mobile phase to suit the calibration graph.

The dissolution profiles were acquired by withdrawing 2 mL aliquots of the dissolution media at 10.0, 20.0, 30.0, 40.0, 50.0 and 60.0 min. After filtration, appropriate solutions were prepared by taking suitable aliquots of the filtrates and diluting them with the mobile phase to suit the calibration graphs. The amount dissolved at each specified time was calculated from linear regression equations of the corresponding calibration and the percent released of each BZD was plotted against the time. Each dissolution experiment was performed in triplicate on six dosage forms.

RESULTS AND DISCUSSION

In order to evaluate an efficient and universal HPLC method, preliminary tests were performed with the objective to select adequate and optimal

conditions. Parameters, such as optimal mobile phase, optimum pH, stationary phase, detection type, detection wavelength, and flow rate were exhaustively studied. The attention was mainly focused on optimization of the mobile phase and the selection of proper column to obtain satisfactory results eliminating tailing problems. The exact composition of mobile phase that seemed to fulfill the above requirements was 0.05 mol × L^{-1} disodium hydrogen phosphate in 50% methanol adjusted with concentrated ammonia solution to a pH range of 5.6-5.8 with a flow rate of 1.6 mL × min^{-1}. Since the influence of variation of the eluent pH on the retention time of these drugs is significant, the pH of the mobile phase had to be checked before the analysis in order to ensure that it was in the range mentioned above. Lower pH values significantly decreased the retention times, while an increase in the retention times occurred at higher pH values.

With the knowledge that previously used columns (15, 20) could not eliminate peak tailing on HPLC chromatograms of benzodiazepines, we examined other stationary phases. The choice of stationary phases with low silanol activity (17) lead to symmetrical peaks for investigated BZD. The substantial innovation on developed method is the use of Perfectsil Target ODS-3 column and the choice of the mobile phase. Our results indicate that peak tailing can be completely eliminated by selecting the above mentioned column, which contains a stationary phase with a very low silanol activity. This column presents BZD chromatograms with fully symmetrical peaks and relatively short retention times, without hindering accuracy and reproducibility**.** Finally by using the Perfectsil Target ODS-3 column, peak tailing effects were totally eliminated and the best chromatograms with fully symmetrical peaks (symmetric factors close to 1), representing improvement of drug separation, detection and quantification limits were obtained.

For trace detection and determination of 1,4-benzodiazepines, HPLC separation followed by electrochemical detection (HPLC-EC), which offers enhanced selectivity and sensitivity over a UV detector by operating potential control, has been recommended (15). 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 electro oxidation of NH group on benzodiazepines. 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 (17). In this regard the only choice was the detection in UV-Vis. An UV scan of BZD solution in the used mobile phase showed that the absorption maxima in the spectra of studied BZDs are in the range of 240 to 260 nm. The UV detection at 254 nm was found to be suitable without any interference from tablets or capsules excipients and solvent. Sharper and symmetrical peaks appeared with the flow rate in the range of 1.5-1.8 mL × min^{-1}; the best flow rate was 1.6 mL × min^{-1}.

To get the regression equations, the external standard method was used. The calculated results are given in Table 1. In the regression equation $y = ax + b$, x is referred to the concentration of the standard compounds, y to the peak area, a is the intercept of the straight line with y-axis and b is the slope of the line. The r in Table 1 is referred to the correlation coefficient of the equation. All the standard compounds showed good linearity ($r > 0.995$) in a relatively wide concentration range, adequate for the requirements of analytical method.

The relative standard deviations of added standard samples were less than 2.5%, indicating acceptable precision of the method (Table 2). The precision of the developed HPLC method was also determined on the tablets and capsules samples of the same batch in five replicates. The RSD values of the assay results were less than 3.0, confirming the method precision.

The recovery results obtained in the analysis of added standard samples are shown in Table 2. The results showed the method to be adequately accurate (SE < 3.8%). Standard errors were calculated as the percentage differences of measured and added values.

As described in experimental part, the accuracy was also calculated as the percentage of the drug recovered from the formulation matrix. Mean recoveries (the mean ± SD) for BZDs from the formulations are shown in Table 3. The results indicate good accuracy of the developed method for determination of BZD in the laboratory prepared dosage formulation. It can be seen that the accuracy of HPLC method, as demonstrated by the closeness of the results of theoretical content of tablets and capsules by the low values of deviations, are acceptable for the developed method.

The determined values of detection and quantitation limits were cross-checked by actual analysis of decreasing concentrations using the proposed method. In this manner it was found that signal to noise ratio of 3 and 10 could be obtained at 0.5 (LOQ) and 2.0 ng × mL^{-1} (LOD) of the investigated drugs in the mobile phase.

Table 1. Calibration data for the standard curves of seven investigated BZDs (n = 6)[a].

Compound	Correlation coefficient (r)	Slope (mean ± SD%)	Intercept (mean ± SD%)
Alprazolam	0.995	0.004 (± 0.007)	0.031 (± 0.019)
Chlordiazepoxide	0.998	0.009 (± 0.012)	0.072 (± 0.036)
Diazepam	0.998	2.008 (± 0.096)	-0.051 (± 0.022)
Flurazepam	0.997	0.006 (± 0.017)	-0.065 (± 0.035)
Lorazepam	0.995	0.003 (± 0.018)	0.025 (± 0.019)
Nitrazepam	0.998	0.006 (± 0.009)	0.095 (± 0.059)
Nordiazepam	0.997	0.003 (± 0.010)	0.038 (± 0.021)

[a] Range: 0.5-10 μg × mL^{-1}

Table 2. Intra-day and inter-day accuracy and precision of 1,4-benzodiazepines determination.

	Intra-day			Inter-day		
Added concentration (μg×mL^{-1})	Measured concentration (μg×mL^{-1})[a]	Accuracy (SE %)[b]	Precision (RSD %)	Measured concentration (μg×mL^{-1})[a]	Accuracy (SE %)[b]	Precision (RSD %)
Alprazolam						
2.50	2.48 ± 0.04	0.8	1.5	2.46 ± 0.06	– 1.6	2.4
5.00	4.83 ± 0.06	3.4	1.3	4.82 ± 0.11	-3.6	2.3
10.00	10.21 ± 0.06	2.1	0.6	9.65 ± 0.10	-3.5	1.1
Chlordiazepoxide						
2.50	2.56 ± 0.04	2.4	1.6	2.58 ± 0.05	3.2	2.0
5.00	5.12 ± 0.69	2.4	1.3	5.17 ± 0.08	3.4	1.6
10.00	10.21 ± 0.08	2.1	0.7	10.35 ± 0.12	3.5	1.2
Diazepam						
2.50	2.51 ± 0.05	0.3	2.1	2.57 ± 0.04	2.8	1.6
5.00	5.04 ± 0.05	0.8	1.0	5.14 ± 0.07	2.8	1.4
10.00	10.38 ± 0.05	3.8	0.4	10.31 ± 0.12	3.1	1.1
Flurazepam						
2.50	2.53 ± 0.03	1.2	1.3	2.56 ± 0.05	2.4	2.0
5.00	5.12 ± 0.04	2.4	0.8	5.17 ± 0.01	3.4	1.9
10.00	9.71 ± 0.05	-2.9	0.6	10.35 ± 0.18	3.5	1.7
Lorazepam						
2.50	2.46 ± 0.05	-1.6	1.9	2.45 ± 0.05	-2.0	2.0
5.00	5.13 ± 0.07	2.6	1.4	5.13 ± 0.08	2.7	1.6
10.00	10.26 ± 0.13	2.6	1.2	10.3 ± 0.14	3.0	1.4
Nitrazepam						
2.50	2.57 ± 0.06	2.8	2.4	2.58 ± 0.06	3.2	2.4
5.00	5.05 ± 0.08	1.0	1.7	5.09 ± 0.11	1.8	2.2
10.00	10.29 ± 0.18	2.9	1.7	10.35 ± 0.21	3.5	2.0
Nordiazepam						
2.50	2.53 ± 0.05	1.2	1.8	2.54 ± 0.03	1.6	1.2
5.00	5.14 ± 0.08	2.8	1.6	5.10 ± 0.05	2.0	1.0
10.00	10.30 ± 0.15	3.0	1.4	10.32 ± 0.09	3.2	0.8

[a] Mean ± SD (n = 5). [b] SE = 100 × (measured conc. – added conc.) / added conc.

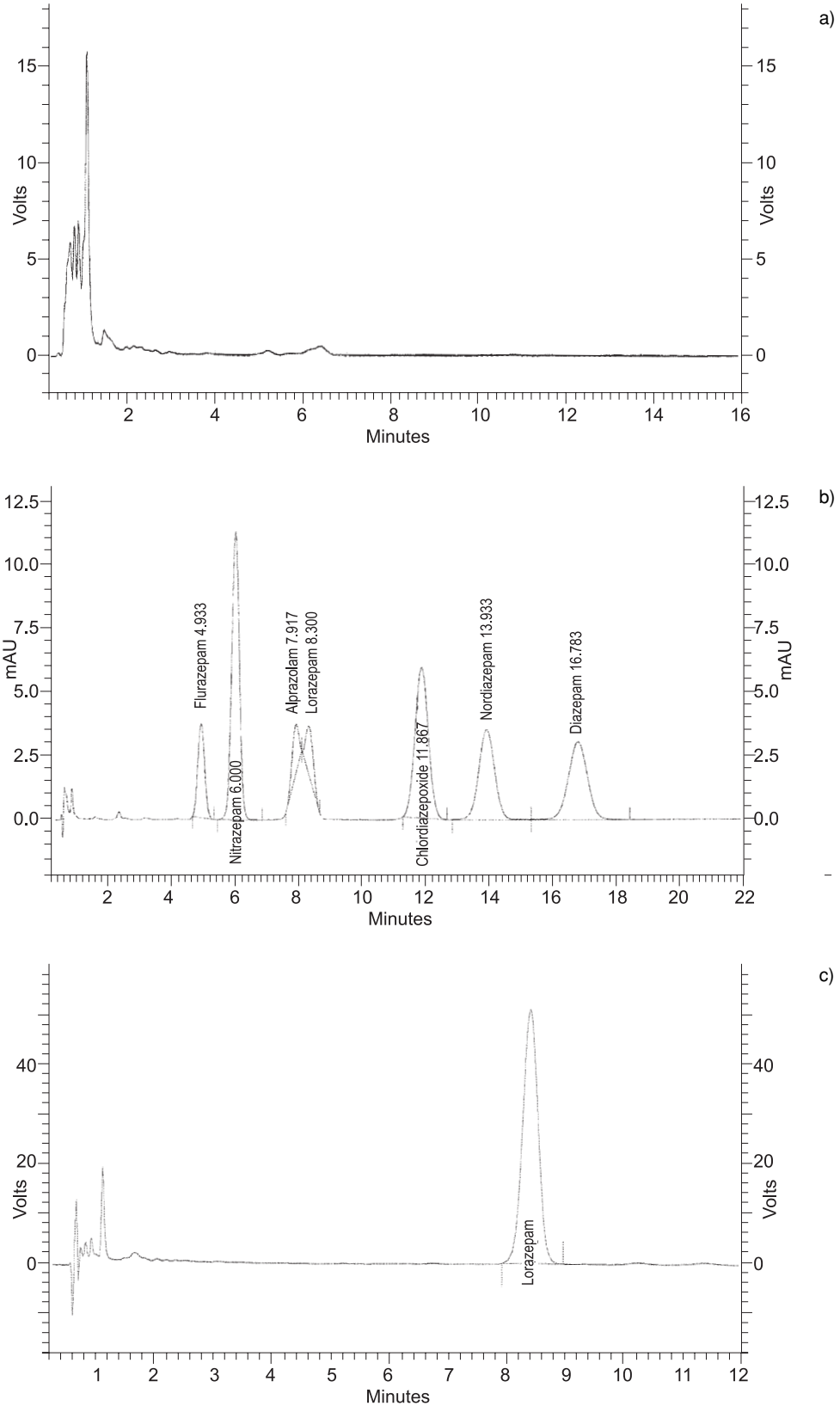

Figure 1. Representative HPLC-UV chromatogram obtained for a mixture of: a) excipients used in BZD formulation, b) studied BZD standards, c) lorazepam tablets. Chromatographic conditions are as described in the text.

Table 3. Recovery obtained with laboratory prepared dosage formulations (n = 6).

Label claim %	Mean recovery ± SD (%)						
	Alprazolam	Chlordiazepoxide	Diazepam	Flurazepam	Lorazepam	Nitrazepam	Nordiazepam
80	79.6 (± 2.08)	80.1 (±1.23)	79.8 (±1.21)	81.3 (±2.11)	79.2 (±1.20)	79.7 (±1.29)	79.8 (±1.32)
100	99.8 (± 2.03)	101.3 (±1.19)	100.0 (±1.13)	102.5 (±1.56)	99.1 (±1.14)	99.8 (±1.14)	99.8 (±1.24)
120	118.9 (± 1.12)	119.9 (±1.28)	120.2 (±1.19)	121.0 (±1.01)	118.9 (±1.63)	119.6 (±2.10)	119.4 (±1.16)

Table 4. Content uniformity of BZDs in dosage forms (x = 10).

	Alprazolam (mg)	Chlordiazepoxide (mg)	Diazepam (mg)	Flurazepam (mg)	Lorazepam (mg)	Nitrazepam (mg)	Nordiazepam (mg)
Mean	0.50	5.10	5.05	15.23	0.99	4.92	9.97
SD	0.02	0.19	0.14	0.12	0.03	0.12	0.2

n = 3

The chromatogram of excipients used in BZD dosage forms (tablets and capsules) shows the absence of interferences for pharmaceutical preparation (Figure 1a). It was concluded that the developed method is selective in relation to the excipients of the final preparations.

Once optimal chromatographic conditions have been established, the method was carried out for the simultaneous detection and quantitation of these compounds. The representative chromatogram of a BZD mixture obtained under the optimal conditions chosen is shown in Figure 1b. This chromatogram indicates the appropriate resolution between the compounds investigated. No interfering of peaks was observed in the samples studied, except the close retention time of alprazolam and lorazepam. This can influence the selectivity of simultaneous determination of these two drugs.

If we compare the chromatograms obtained in this work with those presented in earlier HPLC work on BZD (11-16), it can be concluded that the use of Perfectsil-ODS3 column leads to maximal optimization of previously used stationary phases.

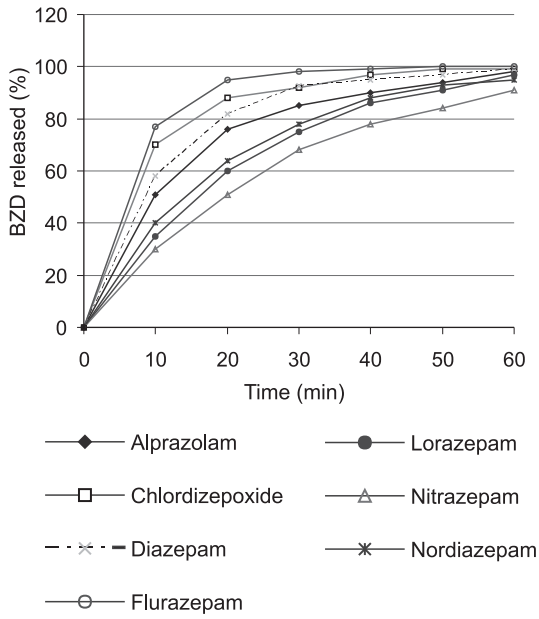

Figure 2. Dissolution profiles of investigated BZD dosage forms (n = 6).

Determination of BZD in tablets and capsule

The method developed was applied to determine these compounds in pharmaceutical formulations. The amount of BZD in each dosage form was quantitated by comparing the mean peak area obtained for the sample chromatograms and the standard solutions having the same known concentrations of individual BZD. The results indicate that the variation between the amounts detected in this study and the amounts claimed by the manufacturers are in the range of USP 29 standard (18). The assay was applied successfully to different commercial brands and proved to be free of interference from excipients normally used in these formulations (Figure 1c).

The method was also utilized for a comparative study of content uniformity and dissolution rate of the tablets and capsules studied. The test for uniformity of content of single-dose preparation like BZD tablets or capsules in official pharmacopoeia is based on the assay of the individual content of active ingredient of a number of single-dose units to determine whether the individual contents are within limits set with reference to the average content of the sample (18). The test for uniformity of content of ten individual single-dose preparations was carried out separately by triplicate injections of solution, prepared as described in the experimental section. The content uniformity of studied BZD dosage forms was between 98.4 ± 0.12 and 102.0 ±0.19 % (mean ± SD) of the claimed manufacturer's value (Table 4).

Dissolution test provides measurements of the bioavailability of a drug as well as demonstrates bioequivalence from batch to batch. Besides, dissolution is a requirement for regulatory approval for product marketing and is a vital part of overall quality control program. The developed method was also used for BZD quantitation in dissolution testing. The amount dissolved at each specified time was calculated from linear regression equations of the corresponding calibration and the percent released of each BZD was plotted against the time. Figure 2 shows the mean release profiles for studied BZD formulations in the dissolution media proposed by USP 29 (18).

The dissolution profiles are considered satisfactory. Therefore the dissolution test developed is adequate for its purpose.

The present study confirms that good extraction and separation conditions, especially using a stationary phase with low silanol activity, allow for an accurate determination of BZD without tailing problems and without any interference from excipients and other studied 1,4-benzodiazepines.

CONCLUSIONS

Based on the studied parameters, the developed method is applicable for simultaneous detection and determination of most 1.4-benzodiazepines. In conclusion, the analytical methodology developed in this research is simple, rapid, accurate, sensitive and specific and can be used in routine analysis in quality control laboratories. Therefore it can be applied to assay, for content uniformity and dissolution testing of most used BZD formulations. At present, further pharmacokinetic studies are being carried out on healthy volunteers at our institute and will be the subject of a future publication.

Acknowledgments

The financial support of Deanship and Research Council of Tehran University of Medical Sciences is acknowledged. The technical assistance of Miss Maryam Fattahi is greatly appreciated.

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Received: 14. 09. 2007