ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis
18 (1998) 453–460
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

Analysis of clonazepam in a tablet dosage form using smallbore HPLC

J. Christopher Spell, James T. Stewart *

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens GA 30602-2352, USA

Received 5 January 1998; received in revised form 6 April 1998; accepted 6 April 1998

Abstract

A stability indicating, reversed phase high-performance liquid chromatographic method utilizing a smallbore HPLC column has been developed for the determination of clonazepam in a commercial tablet dosage form. The use of a small bore column results in a substantial solvent savings, as well as a greater mass sensitivity, especially in the identification of degradation peaks in a chromatogram. The method involves ultraviolet detection at 254 nm and utilized a 150 × 3.0 mm i.d. column packed with 3 μm octyldecylsilane particles with a mobile phase of water–methanol–acetonitrile (40:30:30, v/v/v) at a flow rate of 400 μl min^{-1} at ambient temperature, with and without the use of 1,2-dichlorobenzene as the internal standard. The current USP method for the analysis of clonazepam using a 300 × 3.9 mm i.d. conventional octyldecylsilane column was utilized as a comparison to the smallbore method. The retention times for clonazepam and the internal standard on the 3.0 mm i.d. column were 4.0 and 12.5 min, respectively. The intra- and interday RSDs on the 3.0 mm i.d. column were $<0.55\%$ ($n=4$) using the internal standard, and $<0.19\%$ ($n=4$) without the internal standard at the lower limit of the standard curve, 50 μg ml^{-1} and had a limit of detection of 24 ng ml^{-1}. The assay using the 3.0 mm i.d. column was shown to be suitable for measuring clonazepam in a tablet dosage form. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Clonazepam; Smallbore HPLC; Tablet dosage form; Stability indicating

1. Introduction

In Spring 1995, the United States Pharmacopeia Convention meeting in Washington, DC approved a resolution calling for efforts to reduce the effects of USP 23 compendial methods on the environment. According to the resolution, ‘The USP is encouraged to initiate a program to protect the environment by adopting standards and analytical methods for pharmaceuticals, containers, and other articles that reduce the amount of reagents and materials used in pharmacopeial tests and assays that have potential to cause harm to human health and the environment’ [1]. HPLC analysis of pharmaceutical compounds by USP 23 compendial assays have entailed the use of standard analytical columns (150–300 × 3.9–4.6 mm i.d.) along with the subsequent use of large amounts of organic solvents in mobile phases with

* Corresponding author. Tel.: +1 706 5424410; fax: +1 706 5425358; e-mail: Jstewart@rx.uga.edu

0731-7085/98/$ - see front matter © 1998 Elsevier Science B.V. All rights reserved.
PII S0731-7085(98)00058-2

flow rates typically ranging from 1 to 3 ml min^{-1}. Column technology has advanced such that shorter and smaller bore columns have equal, if not superior, performance to the standard 3.9 or 4.6 mm i.d. analytical columns. Scott and Kucera [2,3] popularized smallbore columns in their papers examining drug formulations and substances of biological origin. Fejglova et al. have reported the separation of monensin, narasin, and slinomycin on a 150 × 2.1 mm i.d. Separon SGX C18 column using a post-column colorimetric reaction with derivatives of benzaldehyde in a packed-bed reactor [4]. Holeman and Danielson have separated six tertiary amine anticholinergic pharmaceuticals (atropine, scopolamine, cyclopentolate, cyclobenzaprine, dicyclomine, procyclidine) on a 150 × 2.6 mm i.d. PRP-1 polymeric column using chemiluminescence detection [5]. There are many advantages of using smallbore and/or short length analytical columns. By reducing the diameter of a 4.6 mm i.d. column to 3.0 or 2.1 mm, a 60–80% reduction, respectively, in solvent usage can be achieved, with comparable or shorter run times than those on larger bore columns. Another facet of smallbore columns is their small peak volumes, which are related to their low volumetric flow rates. For an equal injected mass, the solute concentration at the column exit should be greater for smallbore columns due to the decreased volumetric dilution which results in an increased mass sensitivity [6]. This sensitivity increase is important in that it will allow analysts to identify degradation or metabolic products more easily in drug degradation or metabolism studies, respectively. The use of smallbore columns reduces the effect that temperature gradients have on analysis resulting in less band broadening and peak asymmetry. There is also greater compatibility with newer detection principles, such as electrospray ionization mass spectrometry. Another advantage of smallbore columns is that conventional HPLC systems require minimal modification to use the columns. Any changes required may only entail a smaller flow cell and/or smaller internal diameter tubing, as well as shorter tubing lengths, to reduce dead volumes and bandspreading due to extra-column effects. Some disadvantages are the need to dissolve analytes in diluents as close in composition to the mobile phase to avoid band broadening or peak splitting, and the higher back pressures of smaller bore columns.

In this study, the chromatography of clonazepam, a benzodiazepine used widely as an anticonvulsant agent in the treatment of epilepsy, was investigated on a short, nonporous silica (NPS) C18 and two smallbore columns of differing internal diameters. The USP 23 HPLC assay for the clonazepam tablet dosage form specifies a 300 × 3.9 mm i.d. C18 analytical column at a flow rate of 1 ml min^{-1} [7], which was used to perform comparison HPLC runs. The clonazepam monograph also contains TLC tests for two related compounds, 3-amino-4-(2-chlorophenyl)-6-nitrocarbostyril and 2-amino-2′-chloro-5-nitrobenzophenone, which are present in the clonazepam drug substance and dosage forms, but not quantitated. We included these two related compounds in the method development in order to show selectivity of the method.

2. Experimental

2.1. Reagents and solutions

Clonazepam, 2-amino-2-chloro-5-nitrobenzophenone and 3-amino-4-(2-chlorophenyl)-6-nitrocarbostyril standards were kindly donated by the United States Pharmacopeia (Rockville, MD). Methylparaben, ethylparaben, and 1,2-dichlorobenzene were supplied by Sigma (St. Louis, MO). Methanol and acetonitrile were supplied by J.T. Baker (Phillipsburg, NJ). Klonopin™ 1 mg tablets (Lot: 2198, Expiration: 9/1/98) were provided by a local pharmacy and were produced by Roche Pharmaceuticals.

The mobile phases used to compare the 3.0, 2.1 and 4.6 mm i.d. columns in this study were water: methanol: acetonitrile (40:30:30, v/v/v), water: methanol: acetonitrile (50:25:25, v/v/v) and water: methanol: acetonitrile (80:10:10, v/v/v), respectively. In the comparison study between the 3.9 and 3.0 mm i.d. columns, water: methanol: acetonitrile (40:30:30, v/v/v) was used as the mobile phase. The flow rates used were 1 ml min^{-1} for the 4.6 and 3.9 mm i.d. columns, 400 μl min^{-1} for

the 3.0 mm i.d. column, and 200 μl min^{-1} for the 2.1 mm i.d. column. The wavelength was set at 254 nm for the entire study.

The clonazepam stock solution was prepared at a concentration of 0.5 mg ml^{-1} in a 25 ml volumetric flask. 2-amino-2-chloro-5-nitrobenzophenone (related compound B) and 3-amino-4 - (2 - chlorophenyl) - 6 - nitrocarbostyril (related compound A) solutions were prepared as individual stock solutions at a concentration of 150 μg ml^{-1}. Methylparaben and ethylparaben standards were prepared at a concentration of 50 μg ml^{-1}. All the above solutions were diluted with water-methanol (60:40, v/v). The USP internal standard stock solution, 1,2-dichlorobenzene, was prepared by transferring 4 ml of the commercially obtained reagent into a 100 ml volumetric flask, and adding acetonitrile to volume. All clonazepam working standards (50, 100, 150 and 200 μg ml^{-1}) were prepared in 50 ml volumetric flasks by transferring 4 ml of the internal standard solution and various aliquots of the clonazepam stock solution followed by mobile phase to volume. These solutions were prepared fresh daily. In order to examine the separation of the related compounds, a separate clonzepam working standard (100 μg ml^{-1}) was prepared and both related compounds A and B were added to achieve concentrations of 50 μg ml^{-1}. All working standards were diluted with the mobile phase.

2.2. Equipment

A Micromeritics model 760 HPLC pump, an Alcott model 708 autosampler equipped with a 10 μl loop, a Varian model 2550 variable wavelength UV/Vis detector with an 8 μl flow cell and a Hewlett-Packard model 3392A Integrator constituted the HPLC system used in this study. A Waters 996 photodiode array detector was also used in this study to confirm purity of the clonazepam peaks in the stability study. The HPLC system utilized 0.007" tubing throughout.

Four columns were studied: (1) a Phenomenex Bondex 10 C18 column (300 × 3.9 mm i.d.) with 10 μm porous particles (Rancho Palos Verdes, CA), (2) a Micra Scientific NPS ODS-I column with nonporous 1.5 μm particles (33 × 4.6 mm i.d.) (Northbrook, IL), (3) a YMC Slimbore C18 column with 3.0 μm porous particles (150 × 3.0 mm i.d.) (Wilmington, NC), and (4) an Alltech Solvent Miser C18 column with 5.0 μm porous particles (150 × 2.1 mm) (Deerfield, IL).

3. Results and discussion

3.1. Preliminary experiments with the three smallbore/short columns

In the initial phase of the study, a separation of clonazepam and related compounds A and B was developed on a 4.6 mm i.d. ODS nonporous silica, and 3.0 and 2.1 mm i.d. ODS columns to show selectivity of the method and to identify the column with the best efficiency and most acceptable chromatographic factors such as retention factor, retention time, plate numbers, resolution, and separation factor. The HPLC system was easily modified to use 0.007" instead of 0.010" tubing to reduce dead volumes and decrease extra-column effects within the system. The tubing length was also kept to a minimum to ensure a decrease in band broadening. The analytical figures of merit and typical chromatograms for the drug and related compounds on each column are shown in Table 1 and Fig. 1a–c. Upon examination of the data, the 3.0 and 2.1 mm i.d. columns had similar chromatographic characteristics, except that the tailing factors on the 3.0 mm i.d. column were slightly lower. The 4.6 mm nonporous silica ODS column gave very short retention times (≤ 3 min), which lead to less solvent usage. Of the three columns examined, the 3.0 mm i.d. column was chosen for further study of clonazepam. The reasons for its selection were (1) the 3.0 mm i.d. smallbore column gave better peak shapes, lower tailing factors, and shorter retention times than the 2.1 mm i.d. column and (2) there was the effect of solvent mismatch using the 4.6 mm i.d. ODS nonporous column on the peak shape of clonazepam due to a density separation of the internal standard solution when diluted with the mobile phase. 1,2-Dichlorobenzene was utilized as the internal standard, since it was specified in the USP 23 HPLC assay for

Table 1
Chromatographic data of the separation of clonazepam and its related compounds on the 3.0, 2.1 and 4.6 mm internal diameter ODS columns

Column type	Analyte	T_f^a	k	t_r (min)	N^b	R_s	α
3.0 mm i.d. ODS	Related compound A	1.1	0.83	2.48	828	7.37	2.41
	Clonazepam	1.1	2.00	4.30	1795	16.32	2.29
	Related compound B	1.2	4.58	8.29	7676		
2.1 mm i.d. ODS	Related compound A	1.3	1.45	3.28	1010	8.50	2.06
	Clonazepam	1.2	3.00	5.43	2681	28.80	3.18
	Related compound B	1.0	9.55	14.55	8283		
4.6 mm i.d. ODS	Related compound A	1.2	0.50	0.35	25	6.88	12.00
	Clonazepam	1.0	6.00	1.72	335	16.36	4.00
	Related compound B	1.2	24.00	6.23	2049		

[a] Calculated at 5% peak height.
[b] Calculated as 5.54 $(t_r/t_w)^2$.

clonazepam tablets to compare the 3.0 mm i.d. ODS column to a conventional 3.9 mm i.d. ODS column. As shown in Table 2, the analytical figures of merit were similar except for the number of theoretical plates for the internal standard and the tailing factors on the 3.0 mm i.d. column, which showed an improvement over the 3.9 mm i.d. column. The separations of clonazepam and internal standard on each column are shown in Fig. 2a–b. Both columns met the minimum USP resolution requirement of 10 as stated in the clonazepam monograph. However, the most significant differences that existed between these two columns were the flow rate and total amount of mobile phase needed to achieve separation. The 3.0 mm i.d. smallbore column was operated at 400 µl min^{-1} compared to 1.0 ml min^{-1} for the 3.9 mm i.d. column. For a 17 min run time, this translated into 17 ml of mobile phase consumed per run for the 3.9 mm i.d. column versus only 6.8 ml for the 3.0 mm i.d. column, a solvent usage reduction of 60%. The run time of the 3.0 mm i.d. column was also shorter at 13.5 min compared to 16.0 min for the 3.9 mm i.d. column. The limit of detection for clonazepam ($S/N = 3$) was 24 ng ml^{-1} on the 3.0 mm i.d. column. It was also noted that the 3.0 mm i.d. column operated at a higher back pressure related to its smaller bore and particle size. This is similar to an observation made by Fiedler and Plaga in their study of amino-acids and antibiotics using smallbore columns [8].

3.2. Method development using the 3.0 mm I.D. smallbore column

3.2.1. Linearity and precision

To obtain inter- and intraday linearity and precision data, three standard curves with concentrations of 50, 100, 150 and 200 µg ml^{-1} were prepared on 2 different days. Least squares regression was used for evaluation of the linearity and precision. Regression data was calculated with and without quantitation of the internal standard to determine the effect of internal standard on the assay. The data is shown in Table 3. The intraday RSDs using the internal standard ranged from 0.20 to 0.80%, with correlation coefficients of 0.9998 and 0.9990 ($n = 16$ for each curve). The intraday RSDs of clonazepam without the use of the internal standard ranged from 0.12 to 0.34%, with correlation coefficients of 0.9990 and 0.9998 ($n = 16$ for each curve). On day 2, the RSDs using the internal standard ranged from 0.29 to 0.60%, with a correlation coefficient of 0.9990 ($n = 16$). The RSDs of the clonazepam without internal standard ranged from 0.11 to 0.28%, with a correlation coefficient of 0.9990 ($n = 16$). The continued use of

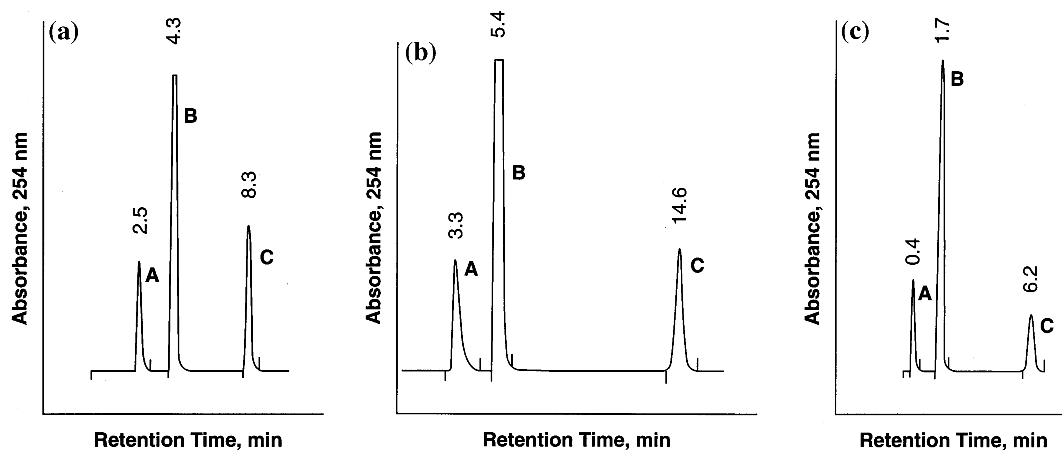

Fig. 1. (a) Separation of A) related compound A, B) clonazepam and C) related compound B on the YMC Slimbore ODS column (3.0 mm i.d.). See Section 2 for details. (b) Separation of A) related compound A, B) clonazepam and C) related compound B on the Alltech Solvent Miser column (2.1 mm i.d.). See Section 2 for details. (c) Separation of A) related compound A, B) clonazepam and C) related compound B on the Micra NPS ODS–I column (4.6 mm i.d.). See Section 2 for details.

the internal standard in the current USP 23 assay does not seem justified, since it added more variablility to the analysis and is structurally unrelated to clonazepam.

3.2.2. Selectivity

In order to assure the selectivity of the method on the 3.0 mm i.d. smallbore column, four-100 μg ml^{-1} clonazepam standards were stressed with 0.1 N HCL (ambient temperature and 80°C), 0.1 N NaOH (ambient temperature and 80°C), 15%(v/v) H_2O_2 (ambient temperature and 80°C) and heating in water at 80°C for 15, 30, 45, 60, 90, and 120 min. At both ambient and elevated temperatures, clonazepam did not undergo degradation in peroxide solution or heating at 80°C over 120 min. For the acid and base stressed samples, degradation proceeded very slowly at ambient temperature. However the acid stressed sample at 80°C showed approximately 40% degradation at 120 min, with degradation peaks appearing at 2.03 and 7.12 min, while the base stressed sample showed about 30% degradation at 30 min with one degradant peak appearing at 2.23

Table 2
Chromatographic data of the separation of clonazepam and the USP internal standard (1,2-dichlorobenzene) on the 3.0 mm and 3.9 mm i.d. columns[a]

Column type	Analyte	T_f^b	k	t_r (min)	N^c	R_s	α
3.0 mm i.d. ODS	Clonazepam	1.1	1.00	4.15	1518	28.0	5.38
	IS	1.1	5.38	12.50	6404		
3.9 mm i.d. ODS	Clonazepam	2.0	2.20	4.90	2000	22.3	3.95
	IS	1.2	8.70	14.18	2982		

[a] See Section 2 for details.
[b] Calculated at 5% peak height.
[c] Calculated as 5.54 $(t_r/t_w)^2$.

min, followed by complete degradation at 60 min. None of the degradant peaks seen in the acid or base stressed samples showed any interference with the clonazepam or internal standard peaks as confirmed by second derivative studies with a photodiode array detector.

Testing was also performed with the 3.0 mm i.d. column using methylparaben and ethylparaben to assure that these common tablet dosage form preservatives did not interfere with the peaks of interest. The methylparaben peak eluted at 2.90 min, whereas the ethylparaben peak eluted at 3.56 min. This data indicated that these preservatives did not interfere with the clonazepam or the internal standard peaks, so the selectivity of this method was considered good.

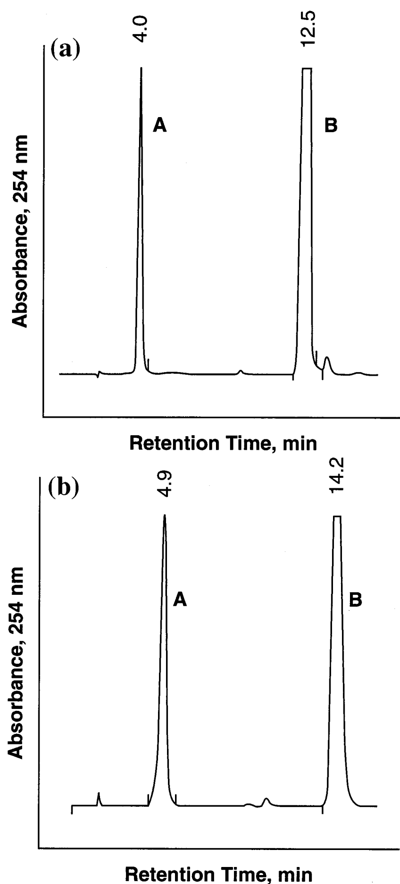

Fig. 2. (a) Separation of A) clonazepam and B) 1,2-dichlorobenzene internal standard on the YMC Slimbore ODS column (3.0 mm i.d.). See Section 2 for details. (b) Separation of A) clonazepam and B) 1,2-dichlorobenzene internal standard on the Phenomenex ODS column (3.9 mm i.d.). See Section 2 for details.

3.2.3. Accuracy

Accuracy data was obtained diluting the clonazepam stock standard and by constructing a standard curve at 50, 100, 150, and 200 μg ml^{-1} of clonazepam. Further dilutions of the stock standard were made at 75, 110, and 175 μg ml^{-1} and these were used as spiked solutions. All solutions were diluted with mobile phase and a comparison was made with and without the use of the internal standard. Recoveries of clonazepam from spiked solutions were determined by least squares analysis of the equation derived from the standard curve data. The correlation coefficients using internal standard versus no internal standard were identical, 0.9998 ($n = 16$). Spiked % recoveries using the internal standard were $101.02 \pm 0.61\%$ ($n = 3$, RSD = 0.60%) at 75 μg ml^{-1}, $102.10 \pm 0.42\%$ ($n = 3$, RSD = 0.41%) at 110 μg ml^{-1}, and $104.21 \pm 0.13\%$ ($n = 3$, RSD = 0.12%) at 175 μg ml^{-1}. Recoveries not utilizing the internal standard were $99.78 \pm 0.04\%$ ($n = 3$, RSD = 0.04%) at 75 μg ml^{-1}, $100.69 \pm 0.17\%$ ($n = 3$, RSD = 0.17%) at 110 μg ml^{-1}, and $100.12 \pm 0.20\%$ ($n = 3$, RSD = 0.20%) at 175 μg ml^{-1}. As the data shows, the recoveries and RSDs from quantitation with the internal standard were slightly higher compared to external standard methodology.

3.2.4. Label claim recoveries from 1 mg clonazepam tablets

3.2.4.1. Dosage form recovery on 3.0 mm i.d. smallbore column. Six 1 mg clonazepam tablets were individually weighed and the average mass of one tablet was found to be 170.92 mg. All six tablets were then crushed to obtain a homogenous grind and the equivalent of 1 mg of clonazepam was weighed into a 10 ml volumetric flask. Two millilitres of the internal standard solution was added along with mobile phase to volume. The solution was mixed and sonicated for 10 min. After sonication, an aliquot was removed from the flask, filtered, and placed into an autosampler

Table 3
Interday and intraday linearity and precision data for clonazepam on the 3.0 mm i.d. smallbore column with and without the internal standard

Day	$r^{2\,b}$	% RSD (50 μg ml^{-1})	% RSD (100 μg ml^{-1})	% RSD (150 μg ml^{-1})	% RSD (200 μg ml^{-1})
1-IS[a]	0.9990	0.41	0.80	0.63	0.30
	0.9998	0.55	0.45	0.20	0.32
1	0.9990	0.14	0.26	0.34	0.12
	0.9998	0.14	0.16	0.12	0.16
2-IS[a]	0.9990	0.29	0.41	0.35	0.59
2	0.9990	0.19	0.10	0.16	0.28

[a] Denotes internal standard used in linear regression calculations.
[b] Based on $n = 16$ for each curve constructed.

vial. The four clonazepam standards (50, 100, 150, and 200 μg ml^{-1}) were injected four times each with and without internal standards to obtain a standard curve. The correlation coefficients for the internal and external standard curves were identical, 0.9998 ($n = 16$). The tablet solution was injected four times and the data subjected to linear regression analysis. In the quantitation with internal standard, the percent label claim found was 98.36 ± 0.65% ($n = 4$, RSD = 0.66%) or 0.9836 mg/tablet. In quantitating without internal standard, the percent label claim was found to be 98.48 ± 0.09% ($n = 4$, RSD = 0.10%) or 0.9848 mg/tablet.

3.2.4.2. Comparison quantitation using the USP 23 method. As a comparison, the USP 23 HPLC method in the clonazepam monograph was used to quantitate clonazepam in the tablet dosage form using the conventional 3.9 mm i.d. C18 column. Samples of clonazepam in tablet dosage form were prepared for assay according to the USP monograph for clonazepam tablets. A clonazepam working standard was also prepared and injected according to the USP 23 monograph. The tablet solution was injected four times and the percent label claim was found to be 98.97 ± 0.07% ($n = 4$, RSD = 0.07%), identical to that determined above with the 3.0 mm i.d. smallbore column.

4. Conclusions

In conclusion, the advantages of using a 3.0 mm i.d. column in stability indicating methods were noted from the assay development. First, with the flow rates utilized, the subsequent waste solvent produced was reduced by 60%. Second, the peak shapes of clonazepam and internal standard were better on the 3.0 mm i.d. smallbore column compared to the 3.9 mm i.d. conventional column and degradant peaks were easy to identify. Third, all columns are commercially available and can be utilized with easy modification of an existing HPLC system. Fourth, the frequency and cost of disposal of HPLC waste solvent was decreased considerably. Last, the reproducibility of assay results on the smallbore column was excellent. The results obtained in this study demonstrated that the internal standard described in the USP 23 assay did not enhance or improve the analysis of clonazepam. The use of an internal standard introduced slightly more variablility in the accuracy data with the 3.0 mm i.d. column.

There were some disadvantages with the use of the 3.0 mm i.d. column. Solvent mismatch, which leads to poor peak shapes should be avoided by dissolving the analytes in mobile phase prior to injection whenever possible. Another aspect is that, during our studies, higher operating back pressures due to the smaller diameter and particle size of the column were always present. However, no detrimental effects on column life were noted over a 3–4 month period.

It appears that smallbore HPLC columns have the potential to replace larger, conventional 3.9 and 4.6 mm i.d. analytical columns currently used in industry and in USP 23 monograph assays.

Acknowledgements

The author wishes to thank the United States Pharmacopeia for providing drug substances and related compounds and for a USP fellowship which funded this study.

References

[1] United States Pharmacopeial Convention, Washington, DC, Spring 1995.

[2] R. Scott, P. Kucera, J. Chromatogr. 186 (1979) 475–487.

[3] R. Scott, P. Kucera, J. Chromatogr. 185 (1979) 27–41.

[4] Z. Fejglova, J. Dolezal, A. Hrdlicka, K. Frgalova, J. Liq. Chromatogr. 17 (1994) 359–372.

[5] J. Holeman, N. Danielson, J. Chromatogr. Sci. 33 (1995) 297–302.

[6] C. Poole, S. Poole, Chromatography Today, Elsevier, Amsterdam, 1993, pp. 71–76.

[7] United States Pharmacopeia 23, Clonazepam Tablets Monograph, Rand McNally, Taunton, MA, 1995, pp. 402–403

[8] H. Fiedler, A. Plaga, J. Chromatogr. 386 (1987) 229–241.