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Comparison of capillary electrophoresis and reversed-phase liquid chromatography methodologies for determination of diazepam in pharmaceutical tablets

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Abstract

Two novel analytical methodologies using capillary electrophoresis (CE) and reversed-phase high-performance liquid chromatography (RP-HPLC) for the determination of diazepam in commercial and simulated tablet formulations were developed and compared. The CE analysis was carried out in a bare fused-silica capillary with 75 μm i.d. and total length of 50 cm (28 cm to the detector) with a buffer solution containing 20 mmol L^{-1} sodium tetraborate and 20 mmol L^{-1} sodium dodecylsulfate (SDS), pH 9.23. The applied voltage was 20 kV and bromazepam was used as internal standard (IS). The RP-HPLC analysis was carried out in a LiChrospher® 100 RP-18 (5 μm) column with a mobile phase constituted of methanol, acetonitrile and water (45:25:30) with a flow rate of 0.8 mL/min, using acetaminophen as IS. In both cases, detection was carried out by ultraviolet (UV) absorption at 242 nm. Under the optimized conditions, the CE retention times for the standard diazepam and bromazepam (IS) were 4.08 and 3.43 min, respectively, and the retention times of the RP-HPLC analysis for the standard diazepam and acetaminophen (IS) were 4.86 and 1.58 min, respectively. The resolution and efficiency for CE were 7.4 and 1.18×10^5 plates/m and for RP-HPLC, 7.5 and 1.76×10^4 plates/m. Analytical curves of peak area versus concentration presented correlation coefficients of 0.9996 for CE and 0.9994 for RP-HPLC. The limits of detection (LOD) and quantitation (LOQ) were 4.24 and 12.85 μg/mL for CE and 1.44 and 4.36 μg/mL for RP-HPLC. Relative standard deviations (R.S.D.) were 1.62 and 0.98% for CE and RP-HPLC, respectively. The percentage recovery determined with CE was 100.27 ± 1.25 and with RP-HPLC was 101.12 ± 2.48. Although both methodologies were shown to be suitable for the determination of diazepam in tablets, performing in a similar manner with regards to several aspects (linearity, recovery and specificity), CE provided a faster analysis and column efficiency whereas RP-HPLC presented a superior repeatability and sensitivity.

Keywords: Capillary electrophoresis; Diazepam; Reversed-phase liquid chromatography

1. Introduction

Benzodiazepines have become the most commonly used drugs for their anti-convulsant, anaesthetic, anti-depressive, hypnotic, tranquilizer and sedative properties. They are also used both as pre-medication and for induction or general anaesthesia and are widely prescribed throughout the world [1]. However, they are frequently involved in cases of drug intoxication, and are a contributory factor in traffic accidents. Additionally, they are used by criminals to incapacitate their victims [2,3]. The determination of benzodiazepines has been extensively studied because of the need to detect and quantitate these drugs in clinical or medical-legal studies. The analysis of such compounds is thus an important operation in many pharmaceutical analytical laboratories.

Diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 1A) is the most commonly benzodiazepine drug used as hypnotic, tranquilizer, anti-convulsant and muscle relaxant [2–4]. It is also an abused

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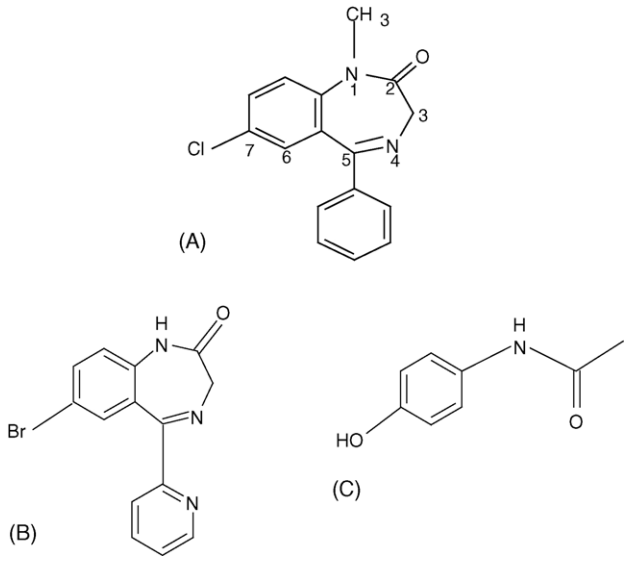

Fig. 1. Chemical structures of diazepam (A), bromazepam (B) and acetaminophen (C).

drug in which sudden withdrawal, particularly from high dosage, carries the risk of epileptic seizures.

Several methods for the analysis of this drug in pharmaceuticals, body fluids and human hair have been reported, including gas chromatography [5], gas chromatography–mass spectrometry [6], ultraviolet (UV) spectrophotometry [7], liquid chromatography–mass spectrometry [8] and thin-layer chromatography [9]. High-performance liquid chromatography (HPLC) is the most used chromatographic technique for determination of benzodiazepines. Mannucci et al. [10] analyzed diazepam and related compounds in tablets with a 0.5 mmol L^{-1} sodium acetate trihydrate buffer containing 5 mmol L^{-1} 1-heptanesulfonic acid monohydrate sodium salt:methanol, 30:70 (adjusted to pH 6.0 with glacial acetic acid) as mobile phase. The method was found to be linear and reproducible. Abu-Qare and Abou-Donia [11] determined the diazepam and their metabolites in rat plasma and urine using methanol, acetonitrile and water (pH 3.2) (10:40:50) as isocratic mobile phase with an analysis time of 12 min; the average percentage extraction recovery of diazepam in spiked plasma sample was 79.1 ± 7.7. Capella-Peiró et al. [12], determined benzodiazepines in serum with micellar liquid chromatographic procedure using sodium dodecylsulfate, butanol and 10 mmol L^{-1} phosphate buffer (pH 7). The serum samples were injected directly and eluted in less than 22 min. Repeatability and intermediate precision were tested giving R.S.D. values below 10%. Azzam et al. [13] developed a HPLC method for the analysis of diazepam and its metabolites in human plasma and urine using chloroform as the extracting solvent. Methanol–acetonitrile–potassium dihydrogenphosphate buffer, 0.05 mol L^{-1} (50:10:40) (pH approximately of 3.5) was used as mobile phase. The authors obtained an average recovery of 87.7 ± 6.5% for diazepam. Bakavoli and Kaykhaii [14] determined diazepam, nitrazepam and flunitrazepam in tablets by HPLC and thin layer chromatography-densitometry (TLC) techniques. The mobile phase was a 1:1 (v/v) mixture of methanol and 0.01 M phosphate buffer (pH 7.80), the TLC plate was developed in a chromatographic chamber containing chloroform:acetate (9:1). The recoveries were of 69.0–105.1 and 72.0–108.3% for HPLC and TLC, respectively. Recently, capillary electrophoresis (CE) has emerged as a powerful new method for rapid separations of analytes. When comparing CE with high performance liquid chromatography (HPLC), it was found that in many applications, CE is superior to HPLC in efficiency, selectivity, peak symmetry and speed [15]. McClean et al. [16] identified and determined benzodiazepines by capillary electrophoresis-electrospray mass spectrometric method aiming at enhancing sensitivity and repeatability of peak area and migration time. The diazepam was identified by around 11 min. In further work, McClean et al. [17] separated and determined benzodiazepine drugs and metabolites by CE compared with high-performance liquid chromatography using ultraviolet and electrospray ionization mass spectrometry. CE was shown to be superior to liquid chromatography (LC) in terms of separation efficiency, LC–MS has proved to be the most useful tool in sensitivity terms for identification of nanogram/milligram levels of benzodiazepines in human hair. Cahours et al. [18], identified five benzodiazepines using capillary electrochromatography (CEC) method; the separation was achieved in 18 min, using Tris–HCl (pH 8)–acetonitrile mixture as mobile phase. Tomita and Okuyama [19] made a simultaneous analysis of six benzodiazepines. The best conditions were obtained using 5 mmol L^{-1} phosphate–borate buffers (pH 8.5) containing 50 mmol L^{-1} SDS and 15% methanol; diazepam was analyzed at approximately 21 min. Vanhoenacker et al. [20] analysed benzodiazepines in dynamically coated capillaries by CE–DAD, CE–MS and CE–MS^2. The system was first evaluated with a mixture of benzodiazepine standards in CE-DAD and the electrolyte composition was further optimized for CE–MS. The buffer used was CEofix, which covered a pH range from 2.5 to 9.2. The benzodiazepines were analysed at approximately 10 min. R.S.D. varied from 0.51 to 1.02% ($n = 7$) for migration times and from 4.75 to 11.80% ($n = 7$) for peak areas.

The aim of this work was to develop a rapid and simple procedure using two separation techniques, capillary electrophoresis and reversed phase-high performance liquid chromatography (RP-HPLC) for determination of diazepam in pharmaceutical formulations and to compare the performance of both techniques.

2. Experimental

2.1. Apparatus

(a) *CE system:* Capillary electrophoresis system, model 270A-HT (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA), equipped with a variable UV–vis

detector. The instrument was operated under positive polarity (injection end of the capillary).

(b) *Capillary column:* An uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 75 μm and a total length of 50 cm (28 cm to detector) was used. The capillary column when new was flushed for 30 min with filtered 1 mol L^{-1} sodium hydroxide, 15 min with deionized water and 30 min with electrolyte buffer.

(c) *CE conditions:* The solutions and the electrolyte buffer were degassed in an ultrasonic bath and filtered through 0.22 μm membrane filter (Millipore®) before use. The electrolyte buffer was prepared at the beginning of the day. The capillary was conditioned with 1 mol L^{-1} potassium hydroxide for 20 min, followed by deionized water for 10 min and then running electrolyte buffer for 30 min. Samples were introduced onto the capillary via electrokinetic injection by applying 10 kV for 2 s. A constant voltage of 20 kV was used for all experiments. Between runs, the capillary was rinsed with electrolyte solution for 3 min. At the end of the day, a final washing with 1 mol L^{-1} sodium hydroxide and water was performed. The wavelength used for recording the electropherograms was 242 nm; the capillary was thermostatted at 29.9 °C. A data acquisition and treatment software supplied by the manufacturer (Turbochrom™, PE-Nelson, Cupertino, CA, USA) was used for the peak integration and data analysis.

(d) *RP-HPLC system:* High-performance liquid chromatograph Model 480C (Instrumentos Científicos CG Ltda., Brazil), isocratic mode, equipped with a variable UV detector (model CG-435), a loop injector (20 μL) and an integrator (model CG-200).

(e) *RP-HPLC column:* An analytical column LiChrospher® 100 RP-18 (5 μm) in a LiChroCART® (4 mm × 12.5 cm) pre-column (Merck®, Darmstadt, F.R. Germany) was used.

(f) *RP-HPLC conditions:* The wavelength used for recording the chromatograms was 242 nm. All analyses were conducted under isocratic conditions and at room temperature. The mobile phase flow rate was 0.8 mL/min and the sample injection volume was 20 μL.

(g) *pH meter:* Digimed, Model TE-901 (São Paulo, Brazil).

(h) *Filtering system:* Filter Durapore (hydrophilic), Millipore catalogue number GVWP 04700, 0.22 μm (São Paulo, Brazil) for solution filtration, filter Durapore (hydrophilic), Millipore catalogue number, GVWP 01300, 0.22 μm (São Paulo, Brazil) for sample filtration.

(i) *Ultrasonic apparatus:* Thorton, Model T-14 (São Paulo, Brazil).

2.2. Reagents

(a) *Solvents:* HPLC grade methanol, (Merck, Rio de Janeiro, Brazil).

(b) *Water:* Water was purified using a Millipore O-Plus system (São Paulo, Brazil).

(c) *Acetonitrile:* Analytical grade (Merck, Rio de Janeiro, Brazil).

(d) *Sodium hydroxide pellets:* Analytical grade (Merck, Rio de Janeiro, Brazil).

(e) *Sodium tetraborate:* Analytical grade (Merck, Rio de Janeiro, Brazil).

(f) *Sodium dodecylsulfate (SDS):* Analytical grade (Eastman Organic Chemicals, Rio de Janeiro, Brazil).

(g) *CE buffer:* Tetraborate buffer solution (20 mmol L^{-1}) and SDS (20 mmol L^{-1}) (pH 9.23), prepared by dissolution of sodium tetraborate.

(h) *HPLC eluent:* Methanol:acetonitrile:water (45:25:30).

2.3. Standards

Diazepam was supplied by FURP (Fundação para o Remédio Popular, São Paulo, Brazil). Acetaminophen and bromazepam used as internal standards (IS) were obtained from Janssen-Cilag Farmacêutica Ltda. (São Paulo, Brazil) and Sintefina (São Paulo, Brazil), respectively, and they were used without further purification.

2.4. Samples

Sample 1: Commercially available tablet containing 10.0 mg of diazepam and excipients, sufficient quantity to one tablet.
Sample 2: Simulated sample tablet containing 10.0 mg of diazepam and excipients, sufficient quantity to 150.0 mg. The formulations were also supplied by FURP.

2.5. Solutions

(a) *Stock solutions:* Standard stock solutions of diazepam (200.0 and 300.0 μg/mL), bromazepam (500.0 μg/mL) and acetaminophen (300.0 μg/mL) were prepared in deionized water containing 10% methanol. Working standard solutions were prepared fresh daily by diluting appropriately the stock solutions with deionized water and employed as such for both CE and RP-HPLC analysis.

2.6. Analytical curves

Aliquots of 2.0, 4.0 and 5.0 mL of the standard stock solutions of diazepam (200.0 and 300.0 μg/mL) and aliquots of 1.0 mL of the standard stock solutions of bromazepam (500.0 μg/mL) (CE analysis) or 2.0 mL of acetaminophen (300.0 μg/mL) (HPLC analysis) were transferred into separate 10 mL volumetric flasks. The volumes were completed with deionized water. Concentration ranges from 40.0 to 120.0 μg/mL of diazepam, 50.0 μg/mL of bromazepam and 60.0 μg/mL of acetaminophen were obtained. The solutions were sonicated for 10 min, and filtered using a 0.22 μm

Table 1
Procedure for the recovery test (standard solution of diazepam added to commercial sample solution)

Diazepam standard solution (μg/mL)			Commercial sample solution[a] (μg/mL)		Internal standard[b] (μg/mL)	Final concentration (μg/10 mL)	
300.0	400.0	500.0	100.0	200.0	500.0	Diazepam	Internal standard
Aliquots (mL)							
1.0			3.0		1.0	600.0	500.0
	1.0			2.0	1.0	800.0	500.0
		1.0	5.0		1.0	1000.0	500.0

[a] Diazepam (tablet).
[b] Bromazepam CE method and acetaminophen for RP-HPLC method.

filter (Millipore) prior to injection. Each solution was injected in triplicate. Peak area ratios (diazepam/internal standard) were plotted versus the respective concentrations of diazepam.

2.7. Sample preparation

For the analysis of diazepam by CE, 20 tablets of each sample (samples 1 and 2) were powdered. Amounts corresponding to 20.0 mg were weighed, transferred into separate 100 mL volumetric flasks, and 10 mL of methanol was added to each flask for dissolution. The volume was completed with distilled water. The solutions were sonicated for 10 min, and filtered using a 0.22 μm filter (Millipore®), rejecting the first 10 mL filtered portion. Aliquots of 4.0 mL of these solutions and 1.0 mL of bromazepam solution (500.0 g/mL stock solution) were transferred into 10 mL volumetric flasks and volumes were completed with deionized water. The final concentrations were 80.0 and 50.0 μg/mL of diazepam and bromazepam (IS), respectively. A standard solution was prepared at the same concentration of the sample, following the procedure described above. The samples and the standard solutions were sonicated for 10 min prior to introduction onto the capillary for the CE analysis.

For the analysis of diazepam by RP-HPLC, the same procedure described above was followed except that acetaminophen (60.0 μg/mL) was used as IS. The samples and standard solutions were sonicated for 10 min prior to injection in the chromatograph loop.

2.8. Recovery test

To determinate the accuracy of the methods, recovery experiments were performed according to the Association of Official Analytical Chemists (AOAC) [21]. Diazepam standard solution was added to commercial sample solution and analyzed by the proposed methods, according to Table 1.

3. Results and discussion

Two different instrument techniques, CE and RP-HPLC, were evaluated for this work. Diazepam compound and internal standard were injected to determine elution profiles on the CE and RP-HPLC systems. An internal standard was used to minimize injection volumes fluctuations, dilution errors and errors during sample treatment. IS can substantially improve the precision of peak area determinations, especially if the injection error is the dominant source [15,22].

Under the conditions described in Section 2, the CE retention times for the standard diazepam and bromazepam (IS, Fig. 1B) were 4.08 and 3.43 min, respectively, and the RP-HPLC retention times for the standard diazepam and acetaminophen (IS, Fig. 1C) were 4.86 and 1.58 min, respectively. The proposed methodologies were simple and faster when compared to those described in the literature [10–13,16–19].

3.1. Method validation

Before a method is routinely used, it must be validated. Validation is the process of proving that the method is acceptable for this intended purpose. This is decided by using a number of performance characteristics, such as accuracy, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, range and robustness [22–25]. In the present work, CE and RP-HPLC methods were validated with respect to linearity, limit of detection, limit of quantitation, specificity, precision and accuracy.

3.2. Linearity, limit of detection and limit of quantitation

The calibration curves for diazepam were linear for CE and RP-HPLC methods over the concentrations range of 40.0–120.0 μg/mL. The results showed an excellent linearity ($r^2 > 0.999$) between peak area ratios (diazepam/internal standard) and concentration. The coefficients of correlation and the regression equations were calculated using linear least-squares regression analysis [26]. Acceptable coefficients of correlation (≥ 0.99) and an intercept close to the origin should be achieved [24]. The limits of detection for CE and RP-HPLC analysis for diazepam were 4.24 and 1.44 μg/mL, respectively, and the limits of quantification were 12.85 and 4.36 μg/mL for CE and RP-HPLC, respectively. RP-HPLC proved to be more sensitive. The criterion used to determine the LOD and LOQ was based on standard deviation (S.D.) of response and the calibration curve slope (S) in accordance with the formulas LOD = 3.3 (S.D./S) and

Table 2
Analytical curve for the capillary electrophoresis (CE) and reverse-phase high-performance liquid chromatography (RP-HPLC) methods in the analysis of diazepam (standard solutions)

Statistical Data	CE	RP-HPLC
Concentration range (μg/mL)	40.0–120.0	40.0–120.0
Intercept	0.01318	−0.02221
Slope	0.02351	0.0196
Correlation coefficient, r^2	0.9996	0.9994
Standard error estimate (S_e) ($n = 5$)	0.0241	0.01583
Limit of detection (μg/mL)	4.24	1.44
Limit of quantitation (μg/mL)	12.85	4.36

Method validation regarding linearity, limit of detection and limit of quantitation.

LOQ = 10 (S.D./*S*) for LOD and LOQ, respectively [25]. The standard deviation of response was determined from the *y* intercept standard deviation of the regression line. The calibration curves consisted of five points and three replicate injections of standards at each concentration level were performed. Statistical data are showed in Table 2.

3.3. Specificity

The specificity of an analytical method is its ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix [24]. The specificity of the methods, CE (Fig. 2) and RP-HPLC (Fig. 3), was demonstrated by the non-interference between diazepam and excipients from the samples, criterion defined in the *USP* 26 for assays [24]. To identify the interference by these excipients, a mixture of the inactive ingredients (placebo), before (Fig. 2A and Fig. 3A) and after being spiked with standards (Fig. 2D and Fig. 3D), and the commercial samples of diazepam (Fig. 2C and Fig. 3C) were analyzed by the proposed methodology. As it can be observed, the tablet excipients interfere in the analysis of diazepam, establishing therefore the method specificity.

3.4. Precision

Precision can be defined as the degree of agreement among individual test results, when the procedure is applied repeatedly to multiple sampling of a homogeneous sample [24]. Within-day variability was deter-

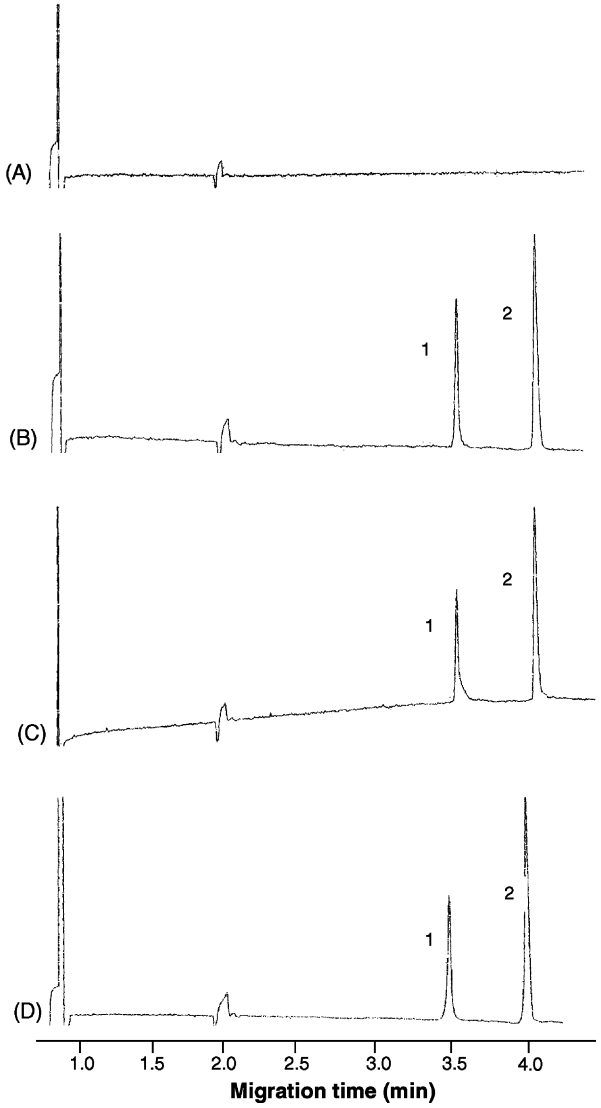

Fig. 2. CE analysis of diazepam in pharmaceutical tablets. (A) Placebo of simulated sample; (B) standards: (1) bromazepam (50.0 mg/mL), IS, (2) diazepam (80.0 mg/mL); (C) commercial sample tablet; (D) simulated sample tablet. Conditions: capillary column with 75 μm i.d. and total length of 50 cm (28 cm to the detector); buffer solution: 20 mmol L^{-1} SDS, 20 mmol L^{-1} sodium tetraborate, pH 9.23; detection at 242 nm.

Table 3
Determination of diazepam in commercial sample (1) and simulated sample (2), using capillary electrophoresis (CE) and reversed-phase high-performance liquid chromatography (RP-HPLC)

Parameters	CE		HPLC	
	Sample 1	Sample 2	Sample 1	Sample 2
Amount declared (mg/tablet)	10.00	10.00	10.00	10.00
Amount found (mg/tablet)	10.02	10.35	10.11	9.67
Relative standard deviation (R.S.D.)[a] (%)	1.62	0.90	0.98	0.36
Confidence limit (purity) ($P = 95\%$)	100.00 ± 1.16	103.49 ± 0.94	101.09 ± 0.71	99.68 ± 0.26

Method validation regarding precision.
[a] Average of 10 determinations.

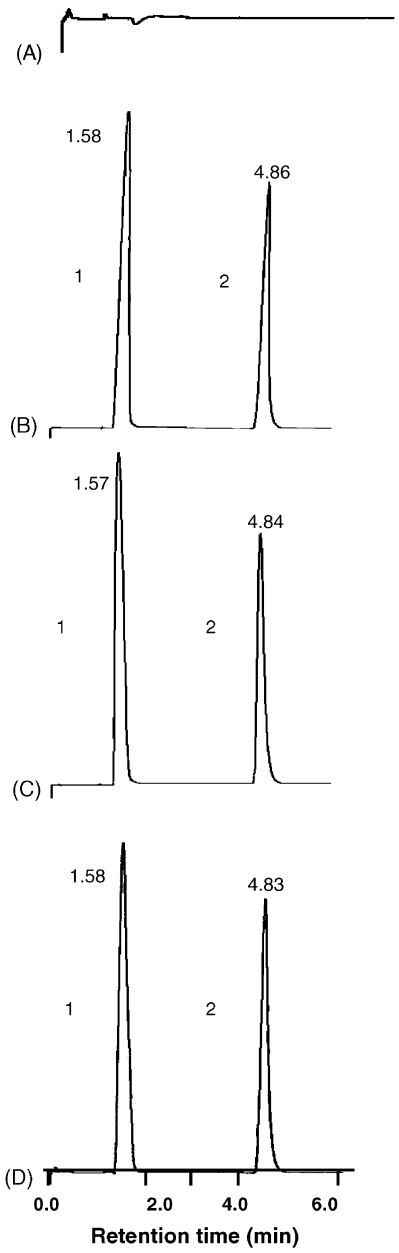

Fig. 3. HPLC analysis of diazepam in pharmaceutical tablets. (A) Placebo of simulated sample; (B) standards: (1) acetaminophen (60.0 mg/mL), IS, (2) diazepam (80.0 mg/mL); (C) commercial sample tablet; (D) simulated sample tablet. Conditions: Lichrospher® 100 RP-18 column; flow rate: 0.8 mL/min; mobile phase: methanol:acetonitrile:water (45:25:30); detection at 242 nm.

mined by analyzing 10 replicate samples containing diazepam (80.0 μg/mL) and bromazepam (50.0 μg/mL, IS) for the CE method and 10 replicate samples containing diazepam (80.0 μg/mL) and acetaminophen (60.0 μg/mL, IS) for the RP-HPLC method. The precision was given in terms of relative standard deviation (%). Data in Table 3 indicate a good agreement among the individual test results obtained.

Table 4
Recovery of a standard diazepam solution added to commercial sample using capillary electrophoresis (CE) and reverse-phase high-performance liquid chromatography (RP-HPLC)

Methods	Standard amount added (μg/mL)	Standard amount found (μg/mL)	Recovery[a] (%)
CE	30.00	30.10	100.32
	40.00	40.60	101.50
	50.00	49.00	97.06
HPLC	30.00	29.49	98.26
	40.00	41.04	102.60
	50.00	51.26	102.52

Method validation regarding accuracy.
[a] Average of three determinations.

3.5. Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. Typically, accuracy is represented and determined by recovery studies [24]. Table 4 shows the accuracy of the CE and RP-HPLC methods expressed as percentage. The recoveries for diazepam ranged from 99.00 to 101.50% and from 98.26 to 102.60% for CE and RP-HPLC, respectively, thus indicating that the methods provide sufficient accuracy.

3.6. Comparison of precision and accuracy of the CE and RP-HPLC methods

Significance tests were carried out to evaluate the obtained experimental results from CE and RP-HPLC methods. These tests are used to verify whether there is a statistically significant difference between the results obtained from distinct methods [26,27]. To compare the accuracy and precision of the two proposed methods, the *t*-test and the *F*-test were used, respectively. The results obtained by comparison of accuracy and precision can be observed in Tables 5 and 6. Means and variances were found to be statistically different, at a confidence level of 95%.

Table 5
Statistical parameters used in the comparison of accuracy and precision of capillary electrophoresis (CE) and reversed-phase high-performance liquid chromatography (RP-HPLC) methodologies in the analysis of diazepam tablets

Samples	Statistical parameters	CE	RP-HPLC
1	$\bar{X}$	100.15	101.09
	S	1.62	0.99
	n	10	10
2	$\bar{X}$	99.77	99.68
	S	0.31	0.36
	n	10	10

Sample 1: commercial sample of diazepam tablet; sample 2: simulated sample of diazepam tablet; $\bar{X}$ = mean value (diazepam content in the tablet, %); S = standard deviation and n = number of determinations.

Table 6
Comparison of accuracy and precision between the capillary electrophoresis (CE) and reversed-phase high-performance liquid chromatography (RP-HPLC) methods in the analysis of diazepam tablets

Samples	Accuracy calculated *t*-value	Precision calculated *F*-value
1	1.56	2.68
2	0.59	1.35

Sample 1: commercial sample of diazepam tablet; sample 2: simulated sample of diazepam tablet; tabulated Student *t*-value with $P = 95\%$ and 18 degrees of freedom, $t = 2.101$ (ref. [17]); Tabulated Snedecor *F*-value with $P = 95\%$, $F_{9/9} = 4.026$ (ref. [17]).

4. Conclusions

Diazepam was determined with high efficiency by both CE and RP-HPLC proposed methodologies. The sample excipients (matrix peaks) did not interfere with the analyte.

The two methods (CE and RP-HPLC) have been successfully validated and may be considered for routine analysis of diazepam in any industrial laboratory. Comparison data for CE and RP-HPLC methods revealed similar results in terms of accuracy and precision although the RP-HPLC method was more sensitive.

It was demonstrated that capillary electrophoresis appears to be an adequate choice for drug quality control. CE determination of diazepam excels RP-HPLC in that it is faster and provides results with substantial advantages, not only in expeditiousness but also in ease of operation. Furthermore, relatively low volumes of electrolyte solution are required for the electrophoretic run. Therefore, as it is already well accepted, CE is capable of representing a viable alternative to RP-HPLC for many pharmaceutical assays.

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