Development of a validated HPLC method for the separation and analysis of a Bromazepam, Medazepam and Midazolam mixture

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Abstract The purpose of this work was to develop a rapid, sensitive and validated HPLC method for the separation and analysis of a Bromazepam, Medazepam and Midazolam mixture. The three benzodiazepine compounds were separated on a reversed-phase C18 column at 50 °C using a mobile phase containing 25% acetonitrile, 45% methanol and 30% ammonium acetate (0.05 M). The pH was adjusted to pH = 9 by the addition of ammonia solution (35%, w/w). The samples were detected using a UV detector at 240 nm. The validation study of the method included the effect of temperature, flow rate, ratio of the components of the mobile phase and the pH of the mobile phase on the efficiency of separation. The linear range of Bromazepam and Midazolam was between 0.12 and 0.18 mg/mL, while that of Medazepam was between 0.08 and 0.12 mg/mL. The relative standard deviation for precision was less than 2%. The linearity, selectivity, accuracy and robustness of the developed method showed acceptable values. The method was applied to the analysis of the samples of raw material of the three compounds under study, and the percentage of recoveries was 99.89% ± 1.06%. It was also applied to the analysis of samples of pharmaceutical preparations of those compounds and spiked serum samples. Recoveries from serum samples ranged between 91.5% and 99.0%. The developed method is suitable for quality control of Bromazepam, Medazepam and Midazolam in their mixtures and in pharmaceutical preparations (tablets, capsules, ampoules). It can also be used to determine their concentrations in serum.

1. Introduction

Benzodiazepine compounds are used to generate a variety of pharmacological effects, including anxiolytic, sedative, tranquilizer, muscle-relaxant, anti-convulsion or hypnotic (Fig. 1). The pharmaceutical compounds belonging to this group are relatively safe when compared with barbiturates as they do not lead to coma when used in high doses [1]. The inhibitory
action of benzodiazepines on the central nervous system results from its interactions with the GABAA receptors, which are present in several brain regions [2]. Since medicinal chemist Strenbach synthesized chlordiazepoxide in 1950, a large number of benzodiazepine derivatives have been synthesized and their pharmacological effects have been investigated. At present, more than twenty of such compounds are globally marketed for clinical uses [2].

Bromazepam, Medazepam and Midazolam that were under study in this research (Fig. 2) are among the most important benzodiazepine derivatives used as anxiolytic, sedative or hypnotic drugs, let alone the wide-scale use of Midazolam as a premedicant medicine before surgery [1,3,4]. These compounds are the most commonly prescribed class of drugs in the world for the treatment of anxiety and insomnia, particularly for the elders [5]. These facts lend importance to the study of the pharmacokinetics and bioavailability of these compounds and their concentrations in serum in cases of abuse: forensic cases, drug poisoning or suicidal excessive doses [6–8].

These three components had not been included yet in the U.S. Pharmacopoeia till its 2010 edition [9]. The analytical methods described by the British, European and Japanese Pharmacopoeia in the monographs of the three studied compounds depend on the anhydrous titration by perchloric acid in the presence of acetic anhydride, and the end point is determined by a potentiometer [3,10,11].

Literature reviews have listed a number of publications on the analysis and determination of therapeutic and toxic blood concentrations [7] of Bromazepam, Medazepam and Midazolam either as raw materials or in serum [12–14]. But the analysis of the mixture, as in this research, was not found in any of these publications [15,16]. These researches have adopted several methods, including HPLC [15,17–19], LC-MS [20] and GC-MS [21], in addition to electrochemical and spectral methods [16,22,23]. The aim of this study was to develop a valid, rapid and sensitive analytical procedure using high performance liquid chromatography (HPLC) for the separation and concentration determination of a mixture of Bromazepam, Medazepam and Midazolam.

2. Experimental

2.1. Materials and methods

Bromazepam, Medazepam and Midazolam as secondary standard materials were obtained from the Syrian Ministry of Health. The samples of raw materials were purchased from several local private pharmaceutical factories. The finished pharmaceutical samples (tablets, ampoules, capsules) were obtained from the community pharmacy and from several local and foreign pharmaceutical factories, including the brand company. Human serum samples were obtained from a hospital laboratory. Solvents especially for HPLC (acetonitrile, methanol, water) were purchased from the Merck, Panreac, Scharlau and Cham Lab companies. Ammonium acetate (extra pure) was purchased from Scharlau, ammonia solution (35%, w/w) was purchased from Surec. Excipient, microcrystalline cellulose (Avicel Ph 102), was purchased from Gujarat Micro Wax. Lactose crystalline, magnesium stearate and talc were purchased from Borculo.

2.2. Instruments and equipment

Cecil HPLC chromatographic system provided with Cecil adept UV/vis detector (CE4200) was used. Two pumps (CE4100), Cecil adept dual piston pump, with manual injector (20 μL loop) were used to deliver the mobile phase to the analytical column in a special oven column (CE4600); Ce11601 Column oven. Column C18 (3 μm) (250 mm × 4.6 mm) Teknokroma columns TR-013204 Ultrafast Columns Tracer Extrasil was used. An ultrasonic device, Cole-Parmer-8892, a sensitive balance, Sartorius analytic balance (CPA225D) (sensitivity of 10−5 g) and a pH-meter, Orion model 320 with glass electrode, were also used. Centrifuge, Hermle Z 230A, and Cartridge 18, RP-Adsorbex by Merck, Darmstadt. FR were employed for serum samples.

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile, methanol, and 0.05 M ammonium acetate (25:45:30, v/v/v). The pH (pH = 9) was adjusted by adding ammonia solution (35%, w/w). The
detector wavelength was 240 nm. The flow rate was maintained at 1.3 mL/min. The column temperature was set at 50 °C. The injection volume was 20 μL.

2.4. Preparation of solutions

2.4.1. Preparation of stock solutions
Stock solutions of 0.75 mg/mL Bromazepam, 0.50 mg/mL Medazepam, and 0.75 mg/mL Midazolam were prepared. 375 mg of Bromazepam was weighed and placed in a 500 mL volumetric flask. It was dissolved in an appropriate amount of the mobile phase solution, and stirred using a magnetic stirrer for a period between 15–30 min until it was completely dissolved. The 500 mL volume of the solution was filled with the mobile phase to obtain the desired concentration. Following the same procedure, 250 mg Medazepam and 375 mg Midazolam were weighed to prepare the desired stock solutions.

2.4.2. Preparation of standards
Standard solutions of 0.15 mg/mL Bromazepam, 0.10 mg/mL Medazepam, and 0.15 mg/mL Midazolam were prepared by taking 5 mL stock solution of 0.75 mg/mL Bromazepam 0.50 mg/mL Medazepam, and 0.75 mg/mL Midazolam with a calibrated pipette and placing them in a 25 mL volumetric flask. The full volume of the flask was filled with the mobile phase to get the desired concentrations.

2.4.3. Preparation of the standard mixture solution of Bromazepam, Medazepam and Midazolam
5 mL of each of the stock solutions of Bromazepam, Medazepam and Midazolam was transferred to a 25 mL volumetric flask. The full volume was filled with the mobile phase, which was stirred on an ultrasonic device for 15 min to get the mixture solution of 0.15 mg/mL Bromazepam, 0.10 mg/mL Medazepam, and 0.15 mg/mL Midazolam.

2.4.4. Preparation of solutions for validation study

2.4.4.1. Standard solutions for linearity study
Five sequential concentrations were prepared from the stock solution containing respectively 80%, 90%, 100%, 110% and 120% of the standard solution concentration. They were prepared by transferring 8, 9, 10, 11, 12 mL of the stock solution, respectively, to a 50 mL flask, filling the full volume of the flask with the mobile phase, and mixing.

2.4.4.2. Solutions for accuracy study
Tablet excipients [24] (i.e. Avesel, lactose, talc, magnesium stearate) were spiked to the standard solutions to obtain analyzed samples. Nine samples were divided into three groups containing respectively 80%, 100% and 120% of standard solution concentration.

2.4.4.3. Solutions for precision study
Tablet samples were analyzed. Nine samples were prepared and divided into three groups containing respectively 80%, 100% and 120% of standard solution concentration.

2.4.4.4. Solutions for selectivity study
A drug-free sample was prepared from the excipients (Avesel, lactose, talc, magnesium stearate). Three tablet samples containing 100% of standard solution concentration were also analyzed.

2.4.4.5. Solutions for robustness study
Three tablet samples containing 100% of standard solution concentration were analyzed. The first sample was injected after adjusting the column oven temperature to 48 °C, second to 50 °C and the third to 52 °C.

2.4.5. Preparation of samples

2.4.5.1. Raw materials
Samples were prepared in the same way as for the preparation of standard solutions of Bromazepam, Medazepam and Midazolam.

2.4.5.2. Tablets
Bromazepam tablet samples were prepared by weighing 20 tablets individually and the average weight per tablet was calculated. The tablets were ground to get a fine powder. The powder equivalent to 30 mg of Bromazepam was weighed and placed in a 100 mL volumetric flask. The powder was dissolved with the mobile phase, and mixed on a magnetic stirrer for half an hour. The full volume of the flask was filled with the mobile phase and mixed. The solution was filtered through Buchner’s funnel with 0.45 μm filters. 50 mL of the filtrate was placed into a 100 mL volumetric flask and the full volume was filled with the mobile phase to obtain 0.15 mg/mL solution of Bromazepam. Midazolam sample solutions were prepared in the same way to obtain a concentration of 0.15 mg/mL. For Medazepam, the powder equivalent to 25 mg of Medazepam was weighed and treated the same way. 50 mL of the filtrate was placed into a 100 mL volumetric flask and the full volume was filled with the mobile phase to eventually obtain 0.10 mg/mL solution of Medazepam.

2.4.5.3. Capsules
Bromazepam capsule samples were prepared by weighing 20 capsules individually and then calculating the average weight per capsule. The equivalent of 25 mg of Bromazepam was weighed from the powder content of the capsules, which was placed in a 100 mL volumetric flask. The powder was dissolved with the mobile phase and mixed on a magnetic stirrer for half an hour. The full volume of the flask was filled with the mobile phase and mixed. The solution was filtered through Buchner’s funnel with 0.45 μm filters. 60 mL of the filtrate was placed into a 100 mL volumetric flask and the full volume was filled with the mobile phase to obtain 0.15 mg/mL solution of Bromazepam.

2.4.5.4. Ampoules
Midazolam ampoule samples were prepared from (50 mg/10 mL) ampoule solution. The ampoule solution was mixed for 5 min and 3 mL of the solution was placed into a 100 mL volumetric flask. The full volume was filled with the mobile phase and mixed well to obtain 0.15 mg/mL solution of Midazolam.

2.4.6. Preparation of a series of standard solutions to determine the recovery from the serum
A series of standard solutions were prepared in the following concentrations (12–15–20–25–30 μg/mL) of Bromazepam, Medazepam and Midazolam by diluting their standard solutions (0.15 mg/mL for both Bromazepam and Midazolam and 0.10 mg/mL for Medazepam).

2.4.7. Preparation of a series of serum standard solutions
The above-mentioned series of standard solutions were prepared with doubled concentrations. Each 1 mL of the solutions was added to 1 mL drug-free serum to obtain a new
series of serum standard solutions with the same concentrations of the said series. The serum standard solutions were centrifuged for half an hour.

2.4.8. Extraction
The serum solution was extracted by the liquid/solid extraction method using C18 cartridge in the following way:
- Precondition cartridge with 3 mL of methanol, withdraw.
  Then with 3 mL of water, withdraw.
- Apply the serum solution.
- Benzodiazepines were eluted with 10 mL of a mixture of methanol and acetonitrile (1:1). The eluates were left to dry for 15 min. Dry residues were reconstituted with mobile phase. The solutions were filtered through 0.45 μm HPLC filters before injected directly to HPLC.

2.4.9. Preparation of mobile phase
2.4.9.1. Preparation of 0.05 M ammonium acetate solution. 3.854 g of anhydrous ammonium acetate (molecular weight = 77.08 g) was weighed and placed into a 1 L volumetric flask. Distilled water was added and the mixture was stirred until complete dissolution. The full volume of the flask was filled with distilled water and the resulting solution was filtered through 0.45 μm filters.
2.4.9.2. Preparation of one liter of mobile phase. In 1 L volumetric flask was added 250 mL acetonitrile, 400 mL methanol and 300 mL of a fresh solution of 0.05 M ammonium acetate. The flask was filled with methanol to 1 L and mixed well. Several drops of ammonia solution (35%, w/w) was added to adjust pH to 9 under stirring. The resulting solution was filtered through 0.45 μm filters.

3. Results
3.1. Method development
3.1.1. Wavelength selection
The ultraviolet spectra [25] of Bromazepam, Medazepam and Midazolam showed the maximum absorption wavelength at 239 nm for Bromazepam, 253 nm for Medazepam and 220 nm for Midazolam. Therefore, 240 nm wavelength was selected after comparing the spectra to achieve the highest sensitivity for the studied compounds [15].
3.1.2. Selection of mobile phase and experimental conditions
Many different combinations of the mobile phase were tested until the suitable phase, namely, acetonitrile, methanol, 0.05 M ammonium acetate (10:57:33, v/v/v/v), was reached. The obtained chromatogram showed a separation process and the appearance of three separated peaks representing Bromazepam, Midazolam and Medazepam (Fig. 3) with retention times at 5.12, 13.61 and 26.65 min, respectively. For confirmation, a solution of each compound was injected separately.
3.1.3. Selection of flow rate and column temperature
Increasing the column temperature from 25 °C to 50 °C led to a decrease in the total time required for the separation process. Also, increasing the flow rate from 1 mL/min to 1.5 mL/min showed a similar decrease in the retention time. Sufficient flow rate of 1.3 mL/min was chosen to avoid overlap between peaks and the loss of its acceptable resolution values (Fig. 4).
3.1.4. Ratios of the mobile phase components
We examined the effect of a gradual increase in acetonitrile from 10% to 25%, which led to a decrease in the total time required for the separation to 9 min (Fig. 5).
In order to improve the shapes of the peaks and to improve the separation between Bromazepam and Midazolam, we improved the previous experimental conditions and started to change the mobile phase pH using an ammonia solution (35%, w/w).
3.1.5. **Effect of mobile phase pH**

We studied the effect of varying the pH between 7.57 and 9, using ammonia solution (35%, w/w). We observed that the best separation results were achieved at pH = 9 (Fig. 6).

3.2. **Validation of the method**

Identity of each peak was confirmed by the retention time. Method compatibility with the requirements of system suitability according to the standards of U.S. Pharmacopeia [9] was performed. Linearity for Bromazepam and Midazolam covering the range between 0.12 to 0.18 mg/mL and 0.08 to 0.12 mg/mL for Medazepam was verified. The calibration curve linearity was examined by studying the correlation coefficient between the concentrations and the response area of each concentration.

Accuracy was assessed by the recovery percentage. Relative standard deviations for selectivity, repeatability, intermediate precision and robustness were less than 2%. Table 1 features the most important constitutional standards in the chromatogram of a mixture of Bromazepam, Midazolam and Medazepam. Table 2 summarizes the results of the method validation tests of the three compounds.

3.3. **Sample test results**

3.3.1. **Raw materials**

The percentage of active substance in each sample was calculated from the ratio of the peak areas of sample and standard solution. The results are shown in Table 3.

3.3.2. **Pharmaceutical preparations**

Tablet and capsule samples of Bromazepam were analyzed. The percentage of active substance in each sample was calculated from the peak areas of samples and standard solutions. Results of Bromazepam samples are shown in Table 4.

Tablet and ampoule samples of Midazolam were analyzed. The percentage of active substance in each sample was also calculated. Results are shown in Table 5.

Tablet samples of Medazepam were analyzed. The percentage of active substance in each sample was also calculated. Results are shown in Table 6.

3.3.3. **Serum samples**

The method was applied to analyze serum samples. Standard serological solutions were injected using the chromatographic conditions of our separation method. Linearity was acceptable in the range of 12–30 μg/mL. Table 7 shows the recovery results of the serum samples for each concentration after comparing areas of the standard serological series peaks with areas of standard solution series peaks. Fig. 7 shows the chromatogram of a serum sample containing a mixture of 0.025 mg/mL of Bromazepam, Midazolam and Medazepam.

4. **Discussion**

A sensitive, accurate and rapid analytical method has been developed in this study. It can be used for the analysis and separation of a mixture of three benzodiazepine compounds; Bromazepam, Medazepam and Midazolam. The chromatographic conditions were: reversed phase C18 column was used.

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**Table 1** The most important constitutional standards in the chromatogram of the mixture.

| Compound | Area  | Theoretical plates | Tailing factor | Resolution | Retention time |
|----------|-------|--------------------|---------------|------------|---------------|
| Bromazepam | 19974 | 3348               | 2.530         | –          | 2.95          |
| Midazolam  | 14163 | 5766               | 1.250         | 8.83       | 5.52          |
| Medazepam  | 12246 | 5139               | 0.911         | 9.31       | 8.65          |
and the column temperature was set at 50°C; the mobile phase consisted of 25% acetonitrile, 45% methanol and 30% 0.05 M ammonium acetate; the pH \( \equiv 9 \) was adjusted by adding an ammonia solution (35%, w/w); the flow rate was 1.3 mL/min and UV detector wavelength was set at 240 nm.

Up to their latest editions, pharmacopoeias (USP 2010 [9], BP 2009 [3], E.P 2007 [10], JP 2006 [11]) had not yet described any HPLC method to analyze any of our studied compounds. International references have adopted many HPLC methods for the separation and the analysis of several benzodiazepine mixtures [15,17–19] but our mixture was not found in any of them. Mobile phases containing one solvent or two did not achieve the separation of the three compounds. Separation succeeded when a mixture of acetonitrile, methanol and 0.05 M ammonium acetate (25%:45%:30%) was used [15].

Raising column temperature up to 50°C led to a decrease in the retention times of the studied compounds. The total analysis time was reduced from 30 to 16 min and the peak shapes were also improved. Increasing the acetonitrile ratio from 10% to 25% reduced the retention times of the three compounds to a total of 9 min. It also improved the symmetry of Medazepam and Midazolam peaks.

Raising the mobile phase pH to 9 improved particularly the symmetry of the three peaks. The tailing factors ranged between 0.9 and 2.5. Statistically, the symmetry of Midazolam and Medazepam peaks became acceptable and their tailing factors were 1.25 and 0.91, respectively. Bromazepam peak symmetry did not improve much and some tailing remained. The tailing factor value was 2.53 (Fig. 6, Table 1). These changes, after raising the mobile phase pH to 9, could be explained by the transformation of the majority of

### Table 2 Method validation results.

| Compound   | Linearity Equation | Recovery (%) | Detection limit (µg/mL) | Quantification limit (µg/mL) |
|------------|--------------------|--------------|-------------------------|-------------------------------|
| Bromazepam | \( Y = 128.7X + 120.4 \) | 100.11       | 1.20                    | 4.20                          |
| Midazolam  | \( Y = 84.45X + 1655 \) | 101.53       |                         |                               |
| Medazepam  | \( Y = 115.1X + 1055 \) | 101.11       |                         |                               |

| Compound   | Equation | Correlation coefficient | Accuracy | Selectivity | Precision | Robustness |
|------------|----------|-------------------------|----------|-------------|-----------|------------|
| Bromazepam | \( Y = 128.7X + 120.4 \) | 0.985       | 99.65    | 98.91       | 99.76     | 100.11     |
| Midazolam  | \( Y = 84.45X + 1655 \) | 0.994       | 99.50    | 99.81       | 101.09    | 100.93     |
| Medazepam  | \( Y = 115.1X + 1055 \) | 0.994       | 100.37   | 99.62       | 100.64    | 100.21     |

### Table 3 Raw materials sample results.

| Compound   | Factory | Sample peak area | Average of standard peak area | Percentage of the active substance (%) |
|------------|---------|------------------|-------------------------------|----------------------------------------|
| Bromazepam | A       | 19920            | 19984                         | 99.68                                  |
| Bromazepam | B       | 19846            | 19984                         | 99.31                                  |
| Medazepam  | A       | 12195            | 12266                         | 99.42                                  |
| Medazepam  | B       | 12090            | 12266                         | 98.57                                  |
| Midazolam  | A       | 14377            | 14193                         | 101.30                                 |
| Midazolam  | B       | 14339            | 14193                         | 101.03                                 |

### Table 4 Results of pharmaceutical preparation samples of Bromazepam.

| No. | Factory | Sample preparation (mg) | Sample peak area | Average of standard peak area | Percentage of the active substance (%) |
|-----|---------|-------------------------|------------------|-------------------------------|----------------------------------------|
| 1   | A       | tab/6                   | 20391            | 19958                         | 102.17                                 |
| 2   | B       | tab/6                   | 19734            | 19958                         | 98.88                                  |
| 3   | C       | cap/1.5                 | 20698            | 19958                         | 103.71                                 |
| 4   | D       | tab/1.5                 | 21007            | 19958                         | 105.76                                 |
| 5   | E       | tab/3                   | 20257            | 19958                         | 101.50                                 |

### Table 5 Results of pharmaceutical preparation samples of Midazolam.

| No. | Factory | Sample preparation (mg) | Sample peak area | Average of standard peak area | Percentage of the active substance (%) |
|-----|---------|-------------------------|------------------|-------------------------------|----------------------------------------|
| 1   | A       | amp/5                   | 14512            | 14172                         | 102.40                                 |
| 2   | B       | tab/7.5                 | 14852            | 14172                         | 104.80                                 |
| 3   | C       | tab/7.5                 | 13789            | 14172                         | 97.30                                  |
| 4   | D       | tab/7.5                 | 16397            | 14172                         | 115.63                                 |

### Table 6 Results of pharmaceutical preparation samples of Medazepam.

| No. | Factory | Sample preparation (mg) | Sample peak area | Average of standard peak area | Percentage of the active substance (%) |
|-----|---------|-------------------------|------------------|-------------------------------|----------------------------------------|
| 1   | A       | tab/10                  | 12187            | 12285                         | 99.20                                  |
| 2   | B       | tab/5                   | 12584            | 12285                         | 102.43                                 |
| 3   | B       | tab/10                  | 12480            | 12285                         | 101.59                                 |
Medazepam (pKa = 0.025 mg/mL of Bromazepam, Midazolam and Medazepam.

Figure 7 Chromatogram of a serum sample containing a mixture of 0.025 mg/mL of Bromazepam, Midazolam and Medazepam.

Medazepam (pKa = 6.2) and Midazolam (pKa = 6.2) molecules to non-ionized form, which increased its affinity to the stationary phase and thus improved peak separation and symmetry [26]. While for Bromazepam (pKa = 2.9, 11), pH = 9 transformed the majority of its molecules to ionized form. This in turn may explain the tailing observed in the Bromazepam peak.

Nevertheless, values of detection and quantification limits for the three compounds indicate a good sensitivity of the method. Examined raw material samples were acceptable according to the pharmacopeia [3, 11] for the three studied compounds; the active substance percentage values ranged within the pharmacopeial accepted range (85%–115%) [3, 11]. Examined pharmaceutical preparation samples of Bromazepam and Midazolam and accurate for the quantitative determination of three benzodiazepines for older adults: a qualitative study, J. Gen. Intern. Med. 22 (3) (2007) 303–307.

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