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**SIMPLE LIQUID CHROMATOGRAPHY METHOD WITH UV DETECTION FOR DETERMINATION OF BROMAZEPAM IN SOLID PHARMACEUTICAL DOSAGE FORMS**

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**Abstract:** Bromazepam is a psychoactive drug belonging to class of benzodiazepines with well-known hypnotic and sedative effects. It acts on the central neural system as an inhibitor of the neurotransmitter gamma aminobutyric acid. It is frequently prescribed for treatment of severe anxiety, to reduce tension, agitation and depression. Dissolution testing (the process by which a solid solute enters in to a solution) is a requirement for all solid oral dosage forms and is used in all phases of research and development for product release and stability testing. Tablet dissolution test is a standardized method for measuring the rate of drug release from a dosage form and it simulates the percentage of active substance that can be absorbed into the blood circulation. The direct determination of Bromazepam in pharmaceutical dosage forms using HPLC with UV detector to carry out dissolution test, have not yet been described. Development of HPLC method with UV detection for direct determination of in-vitro dissolution test of Bromazepam tablets, which can be used in the same time as method for determination of assay of Bromazepam in Bromazepam tablets, can make analytical procedure easier and quicker. A simple, selective, linear, precise and accurate RP-HPLC method has been developed and validated for assay and in-vitro dissolution test of Bromazepam tablets. The method was validated according to the guidelines set by the International Conference of Harmonization for validation of analytical procedures. The chromatographic separation was carried out using reversed phase HPLC LiChrospher RP Select B column (125 x 4.0 mm i.d.; 5 $\mu$ m) at temperature of 50°C. Mobile phase was consisting of the mixture of methanol, acetonitrile and potassium dihydrogen phosphate buffer (pH 7.0, adjusted with 0.5M Potassium hydroxide), with the ratio of 45:5:50 (v/v/v) and flow rate of 1.0 ml/min. The detection was carried out at 239 nm. System suitability tests were performed through evaluation of different parameters (retention time, tailing factor, retention factor and selectivity) on freshly prepared standard solution of bromazepam. The retention time of bromazepam in 0,1M HCl was 3.5 min. High percentage of recovery shows that the method is free from the interferences from excipients in test samples. Linearity of response was calculated as a ratio of peak areas of bromazepam vs. concentration in 0,1M HCl and spiked tablets in the concentration range of 0.0018 – 0.016 mgmL<sup>-1</sup>. The response was linear over the concentration range of 0.0018 – 0.016 mgmL<sup>-1</sup> and coefficient of correlation was greater than 0.99. Good linearity shows that the proposed method may be useful for quickly and routinely determination of the percentage of dissolved bromazepam from bromazepam tablets and it can be a method of choice for assay determination in the same time.

**Keywords:** Bromazepam; Dissolution; HPLC Determination.

**INTRODUCTION**

Bromazepam, chemically described as 7-Bromo-5-(2- pyridyl)-3H-1, 4-benzodiazepin-2(1H)-one (Figure 1.) is white or yellowish crystalline powder, practically insoluble in water and slightly or sparingly soluble in organic solutions (ethanol and methylene chloride)<sup>[1]</sup>.

It is a psychoactive drug belonging to class of benzodiazepines, with well-known hypnotic and sedative effects. It acts on the central neural system as an inhibitor of the neurotransmitter gamma aminobutyric acid (GABA)<sup>[2]</sup>. The biotransformation is carried out in the liver to 3-hydroxybromazepam through oxidative biotransformation<sup>[3]</sup>. It is frequently prescribed for treatment of severe anxiety, to reduce tension, agitation and depression<sup>[4-5]</sup>.

According to the literature data, several methods are available for the determination of Bromazepam in pharmaceutical dosage forms and biological fluids such as spectrophotometry<sup>[6-8]</sup>, flow injection analysis (FIA)

methods<sup>[9]</sup>, differential pulse voltammetry<sup>[10]</sup> and combination of HPLC-MS<sup>[11]</sup>. Spectroscopy method based on second derivative absorption method for the determination of bromazepam in pharmaceutical formulations<sup>[12]</sup> and various HPLC methods were also reported for determination of bromazepam<sup>[13-18]</sup>.

The direct determination of Bromazepam in pharmaceutical dosage forms using HPLC with UV detector to carry out dissolution test, have not yet been described.

According to Biopharmaceutics Classification System (BCS) of active substances, demonstration of in vivo Bioavailability (BA) or Bioequivalence Studies (BE) may not be necessary for drug products containing class 1 and class 3 drug substances, as long as the inactive ingredients used in the dosage form do not significantly affect absorption of the active ingredients<sup>[19-20]</sup>.

A systematic study of the development of a BCS-based provisional classification, based on the revised aqueous solubility and the apparent permeability across Caco-2 cell monolayers, which displays a high correlation (overall 76%) with the provisional BCS classification published by World Health Organization (WHO), classifies bromazepam in class I<sup>[21]</sup>.

Therefore, our objective was to develop a sensitive, reproducible, rapid, and cost-effective RP-HPLC method with UV detection for in-vitro dissolution test of Bromazepam tablets, which can be used in the same time as method for determination of assay, also.



**Figure 1.** Chemical structure of Bromazepam.

## EXPERIMENTAL PART

### *Chemicals and Reagents*

Bromazepam as a Reference Standard was supplied from Merck (B4144 Sigma-Aldrich). Methanol and acetonitrile were HPLC grade provided from Sigma Aldrich. All other chemicals were analytical reagent grade. Redistilled water was used to prepare solutions for mobile phase.

### *Apparatus*

Tablet dissolution test was performed on ERWEKA DT 700, apparatus 2 (paddle).

Chromatography was performed on Shimadzu Nexera HPLC system equipped with: binary pump, micro vacuum degasser, standard autosampler, column compartment and diode array detector (DAD).

### *Chromatographic Conditions*

The separation was performed at the temperature of 50°C, using an isocratic method and reversed-phase HPLC LiChrospher RP Select B column (125 x 4.0 mm i. d.; particle size 5 µm) from Merck KGaA (Darmstadt, Germany). The choice of solvent for the preparation of standard and test solutions was made considering the solubility of bromazepam.

The mobile phase containing a mixture of methanol, acetonitrile and potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) buffer (pH 7.0, adjusted with 0.5M Potassium hydroxide), in the ratio 45:5:50 % v/v/v and flow rate of 1.0 ml min<sup>-1</sup> gave the best separation of the peak of bromazepam without interference with other components from the pharmaceutical formulation.

The detection was carried out at 239 nm.

The injection volume of samples was 20 µl.

Chromatographic data were analyzed using Shimadzu software following the requirements for chromatographic analysis. Regression calculations were done with Microsoft Excel.

### *Preparation of KH<sub>2</sub>PO<sub>4</sub> buffer*

11,33 g of KH<sub>2</sub>PO<sub>4</sub> were weighted and transferred into 1000- ml flask, and then water was added to the mark. pH was adjusted to 7.0 with 0.5M Potassium hydroxide.

### *Standard Solutions and Calibration Curves*

The standard stock solution of bromazepam was prepared by dissolving of Bromazepam in methanol (conc.0,5mg/ml) and diluting with 0,1M hydrochloric acid (HCl) to achieve the concentration of bromazepam in the range of 0.0018–0.016 mg mL<sup>-1</sup>. The calibration curve was constructed by plotting the ratio of the peak area of the drug against the drug concentration. All solvents and solutions for HPLC analysis were filtered using a membrane filter (0.45 µm pore size) and were vacuum degassed before use.

**Validation of the Method**

The proposed method was validated according to the guidelines set by the International Conference of Harmonization for validation of analytical procedures [22,23]. The precision and reproducibility of the proposed method were evaluated by performing replicate analysis of the standard solution to determine intra-day and inter-day variability (within day (*n* = 5) and between days (*n* = 5) for three different concentrations. Relative standard deviations were calculated to obtain the precision of the method. The stock solution of Bromazepam was prepared by dissolving 25mg of Bromazepam reference standard in methanol and diluted with methanol up to 25 ml. Working standard solutions were prepared by appropriate dilution with 0,1M HCl to achieve final concentrations of bromazepam (0.0018 mgmL<sup>-1</sup>, 0.0084 mgmL<sup>-1</sup> and 0.016 mgmL<sup>-1</sup>).

**Recovery Studies**

To establish the accuracy and reliability of the proposed method, recovery experiments were carried out by adding the known quantities of bromazepam in placebo. Tablets were previously dissolved in methanol and then diluted with 0.1M HCl. Calculations were made after five repeated injections. The amount of bromazepam in spiked placebo samples was calculated from the linear regression equation.

**Limit of Detection and Limit of Quantification**

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using following formula: LOD = 3.3 SD/S and LOQ = 10 SD/S, where SD is the standard deviation of the response (peak area) and S is the slope of the calibration curve obtained.

**Experimental in-vitro dissolution test**

In-vitro dissolution test was performed on 6 tablets on ERWEKA DT 700 apparatus. As dissolution medium 500ml of 0,1M HCl was used. After the equilibration to 37°C ± 0.5°C, each tablet was place in the dissolution medium and apparatus was immediately switched on 75 rpm for 45 minutes. Samples before injection into HPLC column were filtered through a 0.45 µm filter.

**RESULTS AND DISCUSSION**

Tablet dissolution test is a standardized method for measuring the rate of drug release from a dosage form<sup>[24]</sup>.

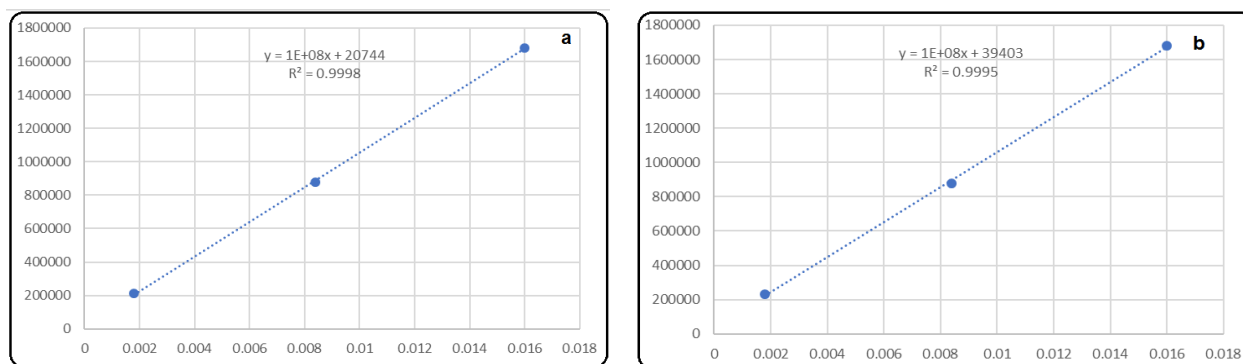
Initial experiments were carried out using the mobile phase consisting of KH<sub>2</sub>PO<sub>4</sub> buffer, methanol and acetonitrile in different proportions and at different pH values. Mobile phase composition of mixture of methanol, acetonitrile and potassium KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.0, adjusted with 0.5M Potassium hydroxide) was found to be optimal for good peak resolution for bromazepam. The optimum wavelength for detection was 239 nm.

System suitability tests were performed through evaluation of different parameters (retention factor, tailing factor, and selectivity) on freshly prepared standard solution of bromazepam (conc. 0.0084 mgmL<sup>-1</sup>). The retention time of bromazepam in 0,1M HCl was 3.5 min. Tailing and retention factors for bromazepam were obtained as 1.08 and 1.13. The variation in retention time of bromazepam among five replicate injections of standard solution in 0,1M HCl was very slight, giving the relative standard deviations (RSD %) of 0.25% calculated on peak area and 0.33% calculated on retention time.

The calibration characteristics and validation parameters of the proposed method are shown In Table 1. Linearity of response was calculated as a ratio of peak areas of bromazepam vs. concentration in 0,1M HCl and spiked tablets in the concentration range of 0.0018 – 0.016 mgmL<sup>-1</sup>. Coefficient of correlation was greater than 0.99 for both. The Figure 2 shows a calibration curves from bromazepam standard solution (a) and bromazepam in spiked placebo tablets (b).

**Table 1.** Characteristics of the linear regression analysis

	0,1M HCl	Spiked tablets
Linearity range (mgmL <sup>-1</sup> )	0.0018–0.016	0.0018 -0.016
Slope	1E + 08x	1E + 08
Intercept	20744	39403
Determination coefficient (r <sup>2</sup> )	0.9998	0.9995
Detection limit (mgmL <sup>-1</sup> )	0.0075	0.0088
Quantification limit (mgmL <sup>-1</sup> )	0.0172	0.0232



**Figure 2.** Calibration curves from bromazepam standard solution (a) and bromazepam in spiked placebo tablets (b). The results of precision, accuracy (recovery), and reproducibility (assay) of the method are shown in Table 2. They demonstrate a good precision (determined as the RSD %), accuracy, and reproducibility.

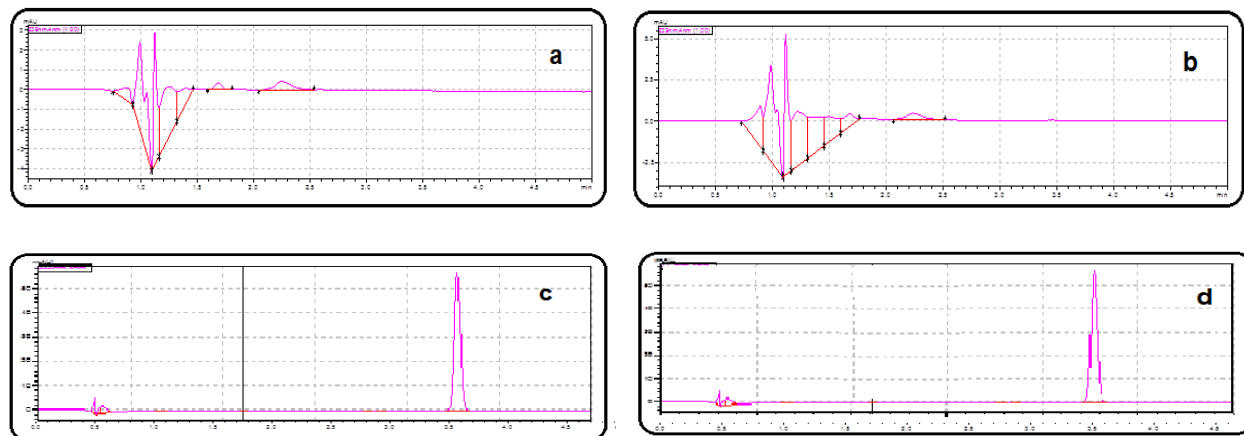
**Table 2.** Precision and accuracy of the method

Concentration added (mg mL <sup>-1</sup> )	Measured concentration (mg mL <sup>-1</sup> ) <sup>a</sup>	
	In 0,1M HCl	
	Intra-day	Inter-day
	Mean (mg mL <sup>-1</sup> ) ± RSD (%)	Mean (mg mL <sup>-1</sup> ) ± RSD (%)
0.0018	0.0525 ± 0.75	0.0584 ± 1.13
0.0084	0.2722 ± 0.88	0.2713 ± 0.72
0.016	0.3431 ± 1.13	0.3447 ± 1.45

<sup>a</sup> Mean value of five determinations

The Figure 3-a shows a typical chromatogram of the placebo. The Figure 3-b shows chromatogram of 0,1M HCl used as solvent, the Figure 3-c shows chromatogram of bromazepam standard solution in 0,1M HCl, while the Figure 3-d shows chromatogram of test solution. There are no any extraneous peaks from excipients in chromatograms obtained in test samples.

All obtained results can confirm that the proposed method can be applied for in-vitro dissolution testing of bromazepam tablets. In the Table 2 we present the results obtained by HPLC analysis of the spiked samples with bromazepam.



**Figure 3.** Chromatograms of placebo (a), 0,1M HCl used as solvent (b), bromazepam in 0,1M HCl (c) and test solution (d)

## CONCLUSION

The aim of this paper was to develop HPLC method with UV detection which can be used in the same time for determination of assay and in-vitro dissolution of bromazepam tablets, bearing in mind that direct determination of Bromazepam in pharmaceutical dosage forms using HPLC with UV detector to carry out dissolution test, have not yet been described.

The proposed RP-HPLC method is a simple, accurate, precise and rapid for determination of assay and in-vitro Dissolution test of Bromazepam tablets. The developed HPLC method was fully validated by evaluation of the validation parameters. High percentage of recovery shows that the method is free from the interferences from excipients in test samples. Good linearity shows that the proposed method may be useful to determine the percentage of dissolved bromazepam from bromazepam tablets and it can be a method of choice for assay determination in the same time.

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