

Study of the Pepsin Enzymatic Activity in *in-vitro* Dissolution Test of Bromazepam Tablets by UV/VIS Spectrophotometry

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ABSTRACT

The *in vitro* dissolution test is an essential parameter to determine the properties of biopharmaceutical formulations. Therefore, the purpose of this study was to evaluate the dissolution of solid Bromazepam formulations and different enzyme concentrations. The dissolution test for Bromazepam tablets was performed using a Vankel VK 7000, apparatus 2 (paddle), at temperature of 37.0°C. The dissolution medium simulated gastric fluid with pepsin, which was prepared with 2 g of sodium chloride, varying concentrations of purified pepsin and 7 mL of hydrochloric acid (PA) in 1000 mL of water. The pH was maintained close to 1.2. The results obtained in the dissolution test showed that the samples have been influenced by the presence of pepsin for both similar drug and reference drug. Among the various enzyme concentrations tested, the highest enzyme concentration tested showed the best results. Thus, it could be concluded that the increase in enzyme concentration increases the dissolution of both drugs, causing a decrease in the test time.

INTRODUCTION

Bromazepam is a drug that has been widely used in psychiatry disorders for four decades (Blanco, 2002; Sampaio *et al.*, 2008), with selective anxiolytic, anticonvulsant, myorelaxant and hypnotic actions (Podilskiy. *et al.* 2008; Versiani, 1997). It acts on the central neural system as an inhibitor of the neurotransmitter gamma aminobutyric acid (GABA) (Khan *et al.* 2010; Kopp *et al.*, 2004, Machado *et al.*, 2005; Puga *et al.* 2005).

It is a drug belonging to class 1,4-benzodiazepine and chemically corresponds to 7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepine-2-one, C₁₄H₁₀BrN₃O (Fig. 1) (Attas, 2009; Machado *et al.*, 2005). It is a controlled psychotropic substance-B1 class according National Agency of Sanitary Vigilance in Brazil (ANVISA), with the DCB identification numbers: 01366, DCI: 2692 and CAS: 1812-20-2.

The solid form is the widespread used and prescribed in clinical practice (Azevedo *et al.*, 2008, Rodrigues *et al.*, 2008).

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The solid form presents problems associated to the bioavailability (Rodrigues *et al.*, 2008), indeed, the absorption of oral drugs in the solid form depends on the solubility and dissolution in physiologic liquids and its permeability through the gastrointestinal tract (Ferraz *et al.* 2007; FDA, 1997; Manadas, *et al.* 2002; Pita *et al.*, 2004), factors that influence directly its bioavailability and subsequent pharmacological effects (FDA, 2000; Pezzini *et al.* 2007). The biotransformation from solid into absorbable form depends on its dissolution in organic liquids (Rodrigues *et al.*, 2008), therefore, dissolution tests became an essential parameter to determine the properties of biopharmaceutical formulations (Malesuik *et al.*, 2006; Manadas *et al.*, 2002; Oliveira *et al.* 2009; Peixoto *et al.* 2005) in order to predict their quality. The quality of pharmaceutical formulations is important in financial and ethical terms because it is directly associated with the patient's health (Nascimento *et al.*, 2010, Nascimento *et al.* 2011). Thus, there is a real need for the development of dissolution tests able to predict *in vivo* physiological conditions (Manadas *et al.*, 2002), like dissolution protocols to compare the percentage of drug dissolved versus time that represents an alternative to access solid formulations before

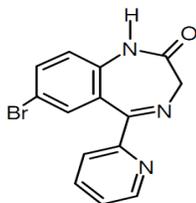


Fig. 1: Molecular structure of Bromazepam (MW=316.2).

clinical tests (Oliveira *et al.*, 2009). In this sense, dissolution tests using simulated gastric fluid (SGF) with or without pepsin or simulated intestinal fluid (SIF), with or without pancreatin, to can be used to determine the quality of different lots in the industrial production process. *The quality of a pharmaceutical product* requires full knowledge to each production stage, and dissolution is among them (Nascimento *et al.*, 2011).

Therefore, the aim of this study was to evaluate the dissolution of solid Bromazepam formulations in different enzyme concentrations using commercial tablets of the drug Lexotan® 3mg, a drug reference indicated by ANVISA, and a commercial similar drug with similar concentration and formulation to compare the efficiency of enzymatic Pepsin solubility against time of drug dissolution in the medium.

MATERIAL AND METHODS

The dissolution test was carried out with Powder Purified Pepsin (JTBaker) with enzymatic activity of 3200 units per milligram, determined according to methodology defined by the United States Pharmacopoeia USP 31 NF24 – 2008 [29]. To determine the potency, the following reagents were used: trichloroacetic acid (J.T.Baker), chloridric acid (Synth), bovine hemoglobin (Sigma-Aldrich).

For the dissolution medium, the following compounds were used: sodium chloride (JT Baker), hydrochloric acid (Synth) and powder purified pepsin, (JT Baker). The following equipment and glassware were used: quartz cuvettes (transparent in the range from 190 to 400 nm) with 1 cm optical path, analytical scales Sartorius model BL-210S, ultrasound Ultrasonic model: USC 2800A, dissolutor Vankel model: VK 7000 with automatic sampler collector, Varian spectrophotometer model Cary 50, and glasses with RBC calibration certificate (Brazilian Calibration Network), as required by ANVISA to Centers for Pharmaceutical

Equivalence (REBLAS). The drugs used were all within the expiration date. The dissolution test for Bromazepam tablets was performed according to the drug monograph described in the 4th edition Brazilian Pharmacopoeia, monograph no. 180.1 from ANVISA. Vankel VK 7000, apparatus 2 (paddle), temperature of 37.0 °C and stirring speed of 50 rpm were used. The dissolution medium was simulated gastric fluid with pepsin, which was prepared with 2 g of sodium chloride, varying concentrations of purified pepsin derived from pig stomach mucosa and 7 mL of hydrochloric acid (PA) in 1000 mL of water. The pH was maintained close to 1.2 and the volume used per vat was 900 mL. The sampling intervals were 5, 10, 15, 20 and 30 minutes and aliquots of 5 mL were collected and automatically returned to the vat. Six readings were determined for each sample in the UV spectrophotometer region at 239 nm using the dissolution medium to reset the equipment and concentration of 0.00033% (w/v) was prepared in the dissolution medium and filtered through Millex® membrane with 0.22 micrometers. To verify the difference and similarity between curves of reference and similar drug, f1 and f2 factors were used, respectively (FDA, 1997; Moore and Flanner, 1996).

RESULTS AND DISCUSSION

Protein activity determination

The test for determining the protein activity followed standards of the United States Pharmacopoeia (USP 31 - 2008) [29], and the manufacturer's specifications for pepsin activity between 2500 UI/mg and 4500 UI/mg. The test and control solutions were made in triplicate and analyzed three times (Table 1).

The protein activity (PA) was calculated according to method described in Pharmacopoeia (USP 31 - 2008):

$$PA = 10000(AU - AC)$$

where:

10000 = dilution factor

AU = absorbance of test solution

AC = absorbance of control solution

The average result found for the enzyme activity was 3200 UI/mg of the protein in pepsin, according to manufacturer's specification for the product as shown in Table 1.

Table. 1: Test to determine the protein activity, values obtained in the determination of test and control solutions, and the result of calculations of the pepsin enzyme activity.

Samples	Analysis	Absorbance of Control Solution	Mean absorbance	Absorbance of Test Solution	Mean absorbance	Amount of protein in pepsin (UI/mg)
1	1	1.1001	1.1002	1.4202	1.4205	3203
	2	1.1014		1.4208		
	3	1.0991		1.4205		
2	1	1.1025	1.1021	1.4204	1.4205	3184
	2	1.1018		1.4206		
	3	1.102		1.4204		
3	1	1.0989	1.0993	1.4205	1.4207	3214
	2	1.0997		1.4208		
	3	1.0992		1.4207		
Mean			1.1005		1.4206	3200
Standard deviation			0.0014		0.0002	15.18
Variation coefficients (%)			0.13		0.01	0.47

In vitro dissolution test

For the *in vitro* dissolution test, the dissolution medium described in the Brazilian Pharmacopoeia monograph 180.1 [30], was used i.e., 3.2 g of purified pepsin with activity of 1650 IU/mg added of other reagents and completing the volume to 1,000 mL. Different enzyme activity concentrations were also tested for evaluating the solubility of the drug in the dissolution medium, being one concentration lower than 800 IU / mg, and two above 2500 and 3300 IU / mg. For this, different amounts of pepsin were weighed to obtain the expected concentrations, taking into consideration the power previously determined and weighting amounts predetermined as calculated below and shown in Table 2.

Table. 2: Pepsin equivalent, calculated mass and pepsin concentration in the medium.

Equivalent amount (UI/mg)	Mass (g)	Pepsin in 1 L of dissolution medium
800	0.8	2.560.000
1650	1.65	5.280.000
2500	2.5	8.000.000
3300	3.3	10.560.000

$$\text{Mass (g) for each liter of dissolution medium} = \left(\frac{3,2 \times Q}{Pot} \right)$$

where:

3.2 = amount (g) of Pepsin

Q = amount (IU/mg) to be used

Pot = power determined on the protein activity assay

The results obtained in the dissolution test (Table 3 and Table 4) showed that the samples have been influenced by the presence of pepsin in the highest concentrations (Figs. 2 and 3) for both similar and reference drugs. The absorption of a drug in the gastrointestinal tract depends on its solubility and dissolution in physiological conditions (Ferraz *et al.*, 2007; Manadas *et al.*, 2002; Pita *et al.*, 2004); the tests with pepsin better mimicked the gastric

juice *in vitro* tests. According Brazilian Pharmacopoeia, 4th Edition (AVISA, 2005), the tolerance is not less than 80% of Bromazepam dissolved in the dissolution medium after 20 minutes. The results with pepsin show that even with the lowest enzyme concentrations recommended, 800 IU/mg in half the time (10min.), dosages greater than 80% of Bromazepam dissolved in the dissolution medium were obtained, therefore, a better dissolution profile was obtained with the addition of pepsin in this test.

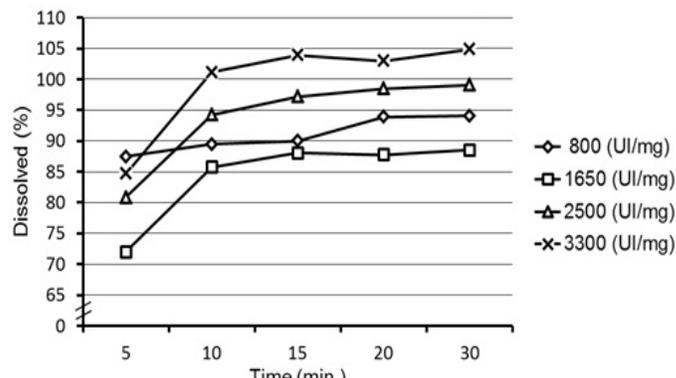


Fig. 2: Percentage of reference drug dissolved by dissolution time with different enzyme concentrations.

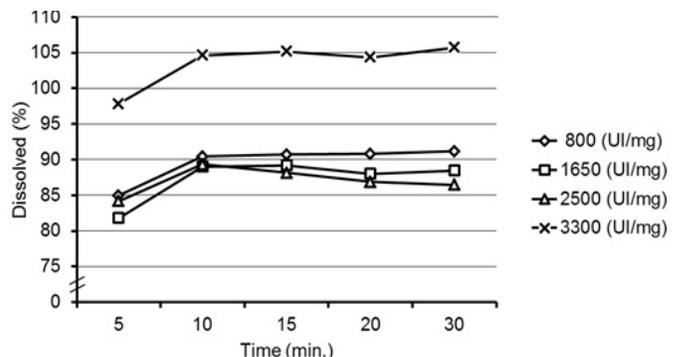


Fig. 3: Percentage of similar drug dissolved by dissolution time with different enzyme concentrations.

Table. 3: Percentage of drug dissolved (%) by dissolution time with different enzyme concentrations for reference drug.

Enzymatic Activity (UI/mg)	Reference Drug									
	Collection time									
	5		10		15		20		30	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
800	87.45	4.45	89.45	3.95	89.99	1.58	93.88	1.70	94.07	2.22
1650	72.02	6.98	85.74	5.76	88.05	5.06	87.76	5.24	88.54	5.95
2500	80.82	5.27	94.25	6.28	97.18	3.82	98.48	3.25	99.06	2.09
3300	84.67	2.65	101.17	5.23	103.89	3.23	102.97	1.31	104.87	1.43

Table. 4: Percentage of drug dissolved (%) by dissolution time with different enzyme concentrations for similar drug.

Enzymatic Activity (UI/mg)	Similar Drug									
	Collection time									
	5		10		15		20		30	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
800	84.95	4.71	90.45	3.92	90.70	1.48	90.81	2.05	91.16	2.99
1650	81.81	3.42	89.00	1.81	89.19	2.20	87.99	2.71	88.49	1.88
2500	84.17	3.86	89.35	2.43	88.16	1.70	86.85	2.11	86.48	2.44
3300	97.84	4.06	104.60	0.86	105.19	0.95	104.35	1.90	105.72	2.00

A fact observed in relation to dissolution with higher enzyme concentration was that, with increasing amounts of enzyme, there was an improvement in the drug solubility, yielding satisfactory results with the drug stability test, since, over time, the drug undergoes adsorption with excipients from its formulation, thus preventing its solubilization in the dissolution medium.

Difference (f1) and similarity (f2) factors

The analysis of the dissolution profiles of reference and similar drugs were made through f1 and f2 factors (FDA, 1997; Moore; Flanner, 1996;), calculated using the following equations:

$$f1 = \{[\sum_{t=1}^n |Rt - Tt|] / [\sum_{t=1}^n Rt]\} * 100$$

$$f2 = 50 * \log \{[(1+1/n) \sum_{t=1}^n (Rt - Tt)^2]^{0.5} * 100\}$$

Where **n** is the number of collections, **Rt** is the percentage of the reference product dissolved at time **t** and **Tt** is the percentage of test product dissolved at time **t**.

Table 5: Results of tests of f1 and f2 factors for curves with enzymatic activities of 800, 1650, 2500 and 3300 IU/ mg .

Enzymatic Activity (UI/mg)	Test	
	f1	f2
800	2,24	80,32
1650	3,43	66,17
2500	8,83	52,02
3300	4,05	60,24

The values found showed similarity between the curves of both drugs at each enzyme activity concentration as shown in (Table 5). The values cited in literature indicate that there is similarity between curves if the f1 values were from 0 to 15 and f2 values from 50 to 100 [28]. Therefore, the presence of pepsin affects both reference and similar drugs. The results of Bromazepam quantification obtained by the pharmacopeia method described in this work were satisfactory, but the amount of pepsin contained in the dissolution medium influenced the drug release. Indeed, the best results were obtained with the highest enzyme concentrations. At the highest concentration, the amount of drug was forced to solubilize, generating results that could be different, in *in vivo* and *in vitro* analyses. Therefore, the use of an intermediate concentration is recommended and further studies on Bromazepam correlations need to be carried out.

In general, this kind of test could demonstrate more acuity in the verification of parameters between *in vitro* and *in vivo* analyses, therefore, more reliability in the understanding of the dissolution process and bioavailability of drugs.

CONCLUSION

In conclusion, the concentration of enzymes in the dissolution medium increases the dissolution of reference and similar drugs, thus generating a decrease in the drug dissolution time, which makes it advantageous to use this enzyme for

dissolution tests, performed in laboratories and pharaceutical industries.

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