# Stability Indicating High Performance Liquid Chromatographic Method for The Determination of Bromazepam in the presence of its degradation products.

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**Research Article**

**Article Info:**
Received on:25/11/2015
Accepted on: 04/12/2015
Published on: 23/12/2015

QR Code for mobile

Literati Open Access

**ABSTRACT :**
A reversed-phase HPLC method was developed and validated for the determination of Bromazepam and its degradation products. The acidic hydrolysis of Bromazepam was carried out in 1N hydrochloric acid solution while the alkaline hydrolysis was carried out in 1N sodium hydroxide solution both at 95°C.Two degradation products were isolated and identified by mass spectroscopy. Spectroscopic data indicated that (2-amino-5-bromophenyl)(pyridine-2-yl) methanone was the degradation product of this acid hydrolysis, whereas 4-bromobenzene- 1,2-diol was the degradation product of the alkaline hydrolysis. A mobile phase consisting Phosphate buffer pH 3.5 Acetonitrile (82:18 v/v) was used, and separation was done on a Zorbax Column C18 dimension 250x 4.6mm, particle size 5µ. Using A flow rate of 1 ml.min-1 while detection wavelength was 240.0nm.Retention times were 3.65, 2.60 and 1.85 for intact Bromazepam, acid and alkaline induced ion products respectively. The method showed good linearity in the concentration range of 20–100 µgmL−1.
Statistical comparison between the results obtained for the analysis of Bromazepam in pure form and the reported HPLC method showed that values of the calculated t and F are less than the tabulated ones, which reveals that there is no significant difference between the compared methods with respect to accuracy and precision,
Keywords :Bromazepam; Acidic-hydrolysis; Alkaline- hydrolysis; Stability indicating method; Reversed-phase high performance liquid chromatography;

## INTRODUCTION:

Bromazepam (7-bromo-5-(2-pyridyl)-2, 3-dihydro-1H-benzo1,4-diazepin-2-one, BMZ) is a member of the 1,4-benzodiazepine series Bromazepam, a, is used for the treatment of anxiety, panic attacks and sleep disorders. This group of drugs—the benzodiazepines–constitute one of the most commonly prescribed drug types in general medical practice, as described in a survey in the State of S˜ao Paulo, Brazil, [1-3] The presence of the pyridine moiety in its molecule is responsible for its unique physicochemical properties[4] . Several methods have been reported for analytical determination of BMZ either individually or in combination with other benzodiazepines in pharmaceutical or biological fluids including spectrophotometry[5-9],LC-MS[10,11],HPLC[12-18],GC-MS[19],TLC[20]and voltammetric methods [21,22] . Also previous studies of BMZ degradation in acidic aqueous media has been reported [23-25]. The purpose of the present study was to develop a high performance liquid chromatographic method (HPLC), allowing determination of BMZ in the presence of its degradation products simultaneously and identification of degradation products.

## MATERIALS AND APPARATUS

The HPLC grade chemicals (water, acetonitrile, Phosphoric acid) used in the present study are purchased from SIGMA Aldrich, Germany, and Potassium dihydrogen phosphate from ADWIC, Egypt. Agilent 1200 HPLC system consisting of a diode array detector (DAD) (set at 240 nm) was used for quantitative estimation of Bromazepam and it is operated in isocratic mode. The stationary phase is a Zorbax C18 column (250 mm x 4.6 id, 5 µm particle size. For injecting the samples, a 20 µL Rheodyne injection port is utilized. The data is recorded using Agilent software and the obtained results are analyzed with Microsoft Excel. The degassing of the mobile phase is done by an ultrasonicator bath (Branson Model 3510), and for weighing the materials Analytical Balance Sartorius CPA225D is used. BMZ pure sample was supplied by Egydrug Company for pharmaceutical and chemical industries and Lexotanil® tablets labeled to contain 3mg of BMZ were obtained from the market.

**Forced degradation studies**:

All stress degradation experiments of BMZ were performed in accordance with the ICH guidelines in order to demonstrate the stability-indicating feature of the assay. Acid degradation was performed by using 1N HCl and refluxed for 1 hour at 90°C while alkaline degradation was performed by using 1N NaOH and refluxed for 4 hours at 90°C.

doi: 10.15272/ajbps.v5i51.763

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**Conflict of interest:** Authors reported none

**Standard stock solution and sample preparation:**

**Standard solution of Bromazepam (100µgmL$^{-1}$)**

Stock standard solution of Bromazepam having concentration of 100µgmL$^{-1}$was prepared by weighing 10mg of Bromazepam, transferring into 100-ml volumetric flask, dissolving and diluting to the volume with the mobile phase.

**Alkaline degradate stock solution (100µgmL$^{-1}$)**

An accurately weighed amount of BMZ (10mg) was transferred to 100-ml round bottom flask and 20ml 1N NaOH was added then refluxed for 4 hours at 90°C and then neutralized with 1N HCl using digital pH-meter (Jenway 3510, UK),then accurately transferred to 100-ml volumetric flask.

The volume was completed to 100ml with mobile phase to produce a concentration equivalent to 100µgmL$^{-1}$of BMZ degradation product

**Acid degradate stock solution (100µgmL$^{-1}$)**

An accurately weighed amount of standard Bromazepam (10mg) was transfered to 100-ml round bottom flask and 20ml 1N HCl were added then refluxed for 1 hour at 90°C and then neutralized with1N NaOH using digital pH-meter,then accurately transferred to 100-ml volumetric flask The volume was completed to 100ml with mobile phase to produce a concentration equivalent to 100µgmL$^{-1}$ of BMZ degradation product.

**Procedure:**

The optimal condition of the mobile phase is Phosphate buffer and acetonitrile in the ratio 82:18 v/v. This composition of the mobile phase resolved the degradates very well. The mobile phase and samples are degassed by ultra-sonication for few minutes and then filtered through 0.45 µm multipore filter paper. All the measurements are performed at constant temperature of the column. The pH value of the final mobile phase composition is 3.5. The flow rate is set to 1 mL/minute. Elutes runtime is set to 10 minutes assuring no interferences as well as influence from the excipients.

**Method validation:**

The analytical method was validated according to ICH guidelines [26]. The parameters evaluated were specificity, linearity, precision and accuracy.

**Linearity:**

Accurately measured volumes (2.00-10.00 mL) of BMZ standard solution (100µgmL$^{-1}$) were transferred in a series of 10-mL volumetric flasks, diluted to the volume with mobile phase to reach a final a concentration range of 20.00-100.00 µgmL$^{-1}$. 20uL of each solution were analyzed by HPLC under flow rate 1 mL/minute and runtime 10 minutes and constant temperature of the column. The calibration curve was obtained by plotting peak area vs. concentration and the corresponding regression equation was computed.

**Accuracy:**

The previous procedure under linearity was applied for the determination of different concentrations of Bromazepam.. The concentrations were calculated from its corresponding regression equation. The recovery percentages, the mean recovery and RSD were then calculated.

**Precision:**

To measure the degree of method repeatability and intermediate precision, samples containing (25, 45,65 µgmL$^{-1}$) of Bromazepam were injected, intradaily and on three successive days using the previously mentioned procedure under linearity , the mean recovery and the relative standard deviation were then calculated.

**Specificity**:

Specificity and selectivity, is the ability of the method to measure accurately and specifically the analyte of interest in the presence of other components such as impurities, degradation products and compounds of matrix. To examine the selectivity of the proposed method, Different laboratory prepared mixtures of intact BMZ with its alkaline degradation products or acidic degradation products were prepared and were analyzed under the same conditions mentioned under linearity. The concentration of the intact drug was calculated from its corresponding regression equation.

**Application to pharmaceutical formulation:**

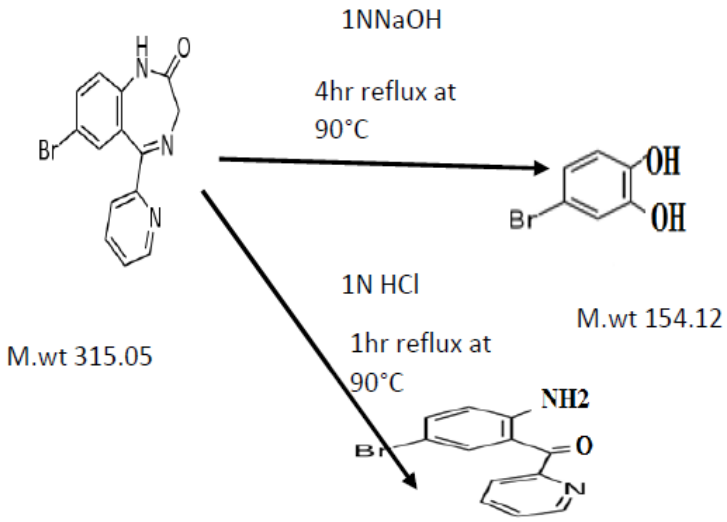
Ten tablets were accurately weighed and finally powdered. A portion of the powder equivalent to 2.5mg of BMZ was accurately weighed dissolved in 15mL of mobile phase stirred magnetically for about 30min then filtered through a filter paper into a 25-mL volumetric flask, the volume was completed after quantitative transfer with the mobile phase to have a solution of concentration of 100 µgmL$^{-1}$, 3mL of this solution were transferred into 10-mL volumetric flask and diluted to the volume by the mobile phase to give a final concentration of 30 µgmL$^{-1}$.Adopting the procedures under linearity the concentration of the pharmaceutical preparation was calculated from the regression equation.

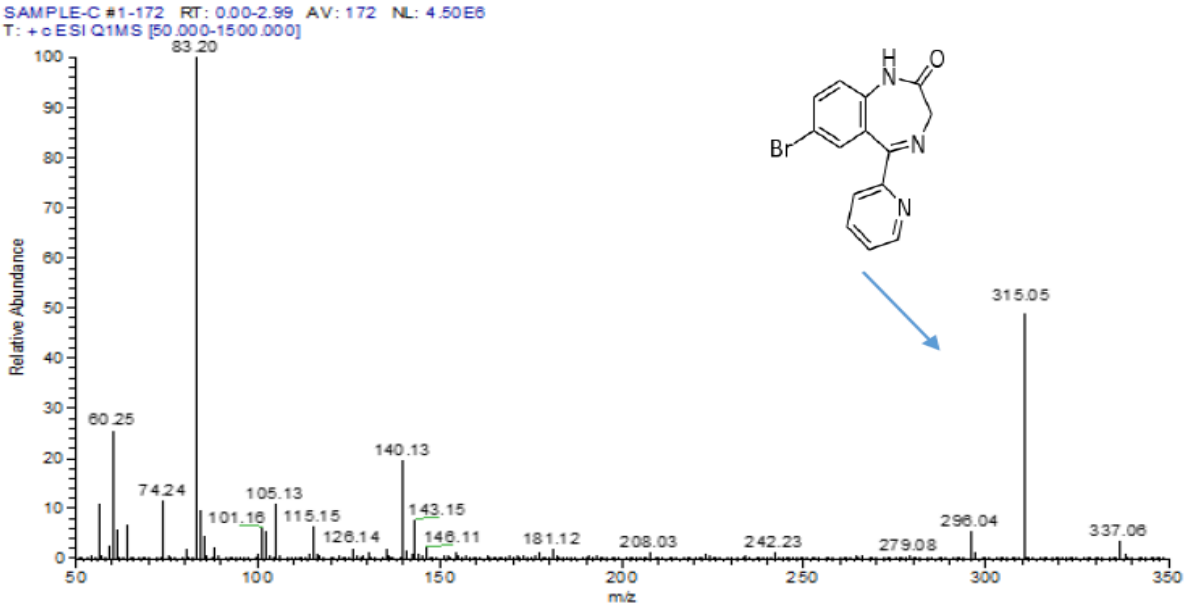
**RESULTS AND DISCUSSION**

The aim of this study is to develop a method to determine Bromazepam in the presence of its alkaline and acidic degradation products.

Stability of Bromazepam was studied according to ICH guidelines for stress acidic and alkaline hydrolysis using1N HCl refluxed for 1 hour and 1N NaOH refluxed for 4 hours. The resultant solution was tested for complete degradation by HPLC where a complete disappearance of Bromazepam peak was observed.

Furthermore The structure of intact and acid and alkaline induced degradation products of BMZ were elucidated by mass spectrometry where the spectral data were compared to each other showing the appearance of a peak at 315.08 m/z which is equivalent to the molecular weight of the intact While in case of alkaline degradation, a peak at 154.2 m/z which is equivalent to the molecular weight of the degradation product suggested to be 4-bromobenzene-1,2-diol and in case of acidic degradation, a peak at 264.04 m/z which is equivalent to the molecular weight of the degradation product suggested to be: (2-amino-5-bromophenyl)(pyridine-2-yl) methanone, Figures (1-3)



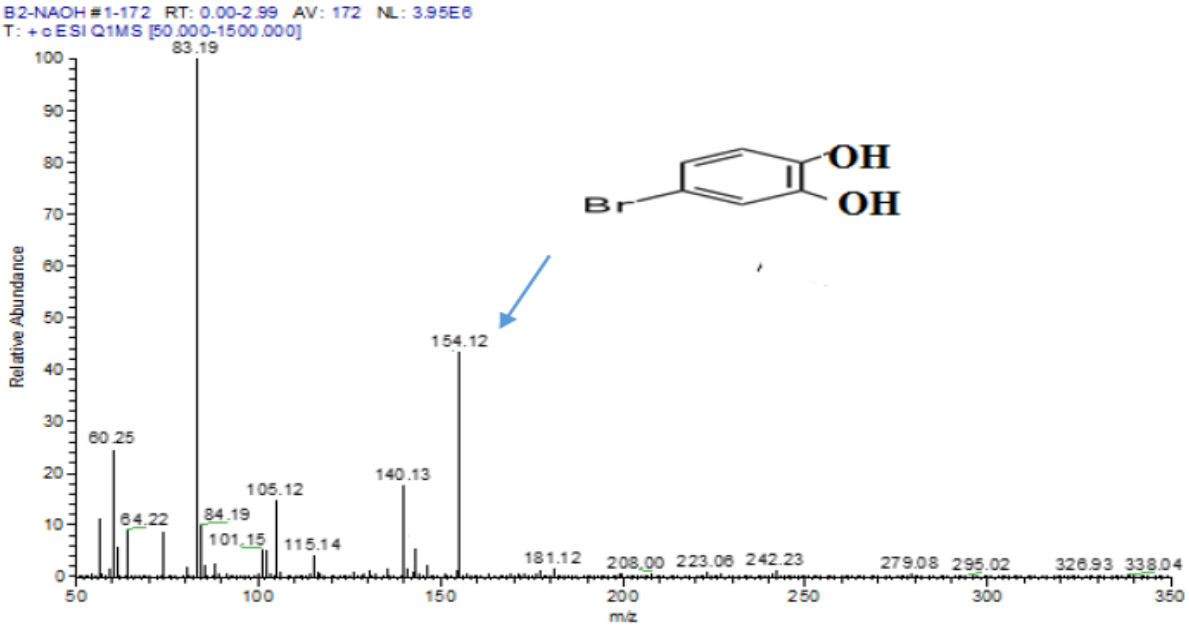




Figure (2): Mass Spectrum of Alkaline degradate of Bromazepam

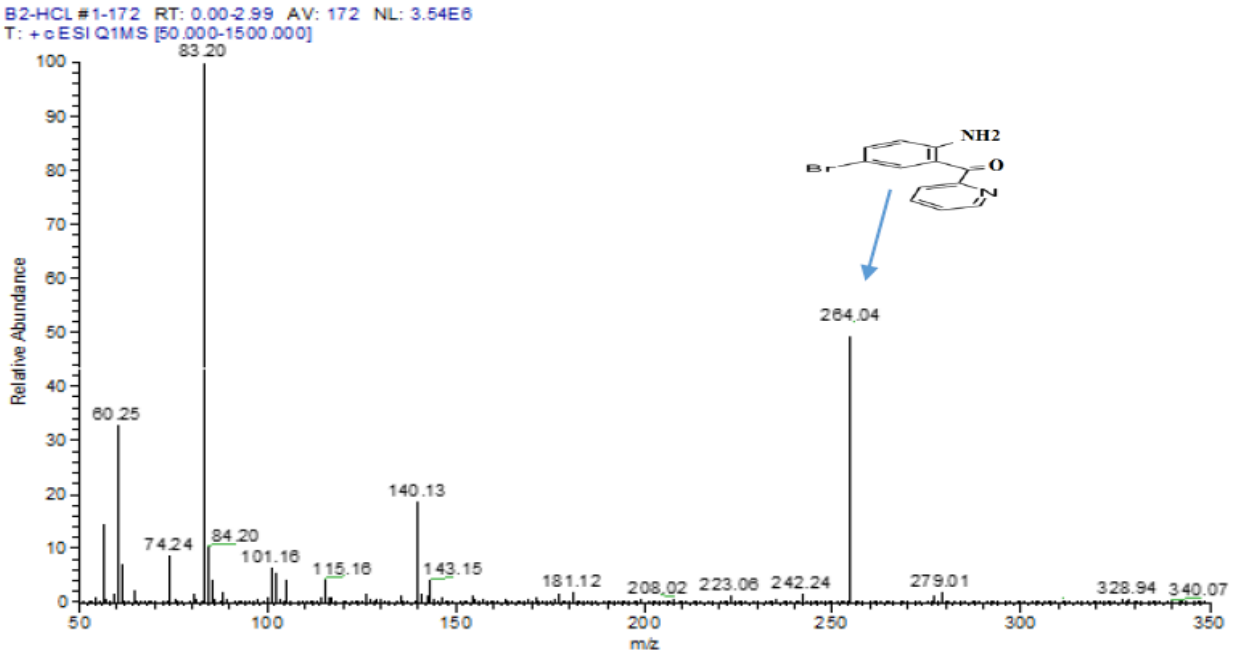



Figure (3): Mass Spectrum of Acidic degradate of Bromazepam

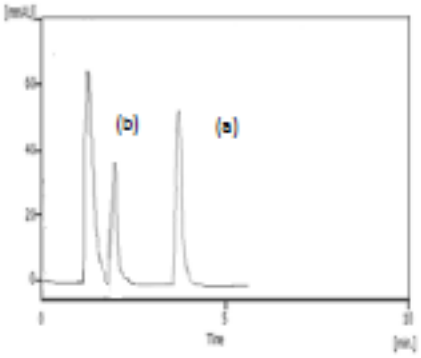



Figure (4): HPLC chromatogram of (a) intact Bromazepam 20μgmL$^{-1}$ $t_R$ = 3.95 and (b) alkaline degradate 20μgmL$^{-1}$ $t_R$ = 1.85 using the specified chromatographic conditions.

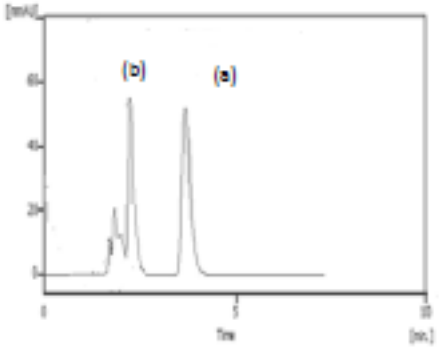



Figure (5): HPLC chromatogram of (a) intact Bromazepam 20μgmL$^{-1}$ $t_R$ = 3.65 and(b) acidic degradate 20μgmL$^{-1}$ $t_R$ = 2.60 using the specified chromatographic conditions

Table (1): Parameters of system suitability of the developed HPLC method for determination of bromazepam.

| Parameters | Obtained values | | Reference values[97, 98] |
|---|---|---|---|
| Resolution (R ) | Acid degradate | Alkaline degradate | R> 1.5 |
| | 1.6 | 1.8 | |
| Selectivity(α) | 2.12 | 2.88 | >1 |
| Tailing factor (T) | 1.11 | 1.17 | T=1 for a typical symmetric peak |
| Capacity factor(K') | 2.55 | | 1-10 acceptabe |
| Column efficiency(N) | 3135 | | Increase with efficiency of separation |
| HETP | 0.0214 | | The smaller the value the higher the column efficiency |

Table (2):Assay parameters and method validation obtained by applying the proposed HPLC method for determination of the intact Bromazepam

| Parameters | Bromazepam | |
|---|---|---|
| Linearity(μgmL$^{-1}$) | 20.00-100.00 | |
| Accuracy (Mean±SD) | 100.11±0.251 | |
| Precision RSD%<br>Repeatability*<br>Intermediate Precision** | 0.256<br>0.756 | |
| Specificity | Alkaline degradate | Acidic degradate |
| | 100.79 ± 1.60 | 99.94± 0.864 |
| Slope | 19.043 | |
| Standard error of slope | 0.98x10$^{-3}$ | |
| Confidence limit of slope | 0.0187 ±0.0193 | |
| Intercept | 2.85 | |
| Standard error of intercept | 0.653 x10$^{-3}$ | |
| Confidence limit of intercept | -0.0226-0.02195 | |
| Correlation coefficient(r) | 0.9999 | |
| LOD | 0.876 | |
| LOQ | 2.890 | |

*The intraday (n=3), average of three concentrations (25, 45, 65 μgmL$^{-1}$) for Bromazepam repeated three times within the day

**The interday (n=3), average of three concentrations (25, 45, 65 μgmL$^{-1}$) for Bromazepam repeated three times in three successive days

*** LOD and LOQ are determined via calculations LOD = (S.D of the response/slope) x3.3 and LOQ = (S.D of the response/slope) x10

Table (3): Accuracy of the proposed HPLC method for the analysis of pure samples of Bromazepam in bulk powder

| Taken ugmL$^{-1}$ | Found* ugmL$^{-1}$ | Recovery% |
|---|---|---|
| 25.00 | 25.12 | 100.48 |
| 35.00 | 35.06 | 100.17 |
| 45.00 | 45.06 | 100.13 |
| 55.00 | 54.98 | 99.96 |
| 65.00 | 64.88 | 99.81 |
| Mean | | 100.11 |
| SD | | 0.251 |
| RSD% | | 0.251 |

Table (4): Results obtained for the analysis of laboratory prepared mixtures containing different ratios of intact bromazepam and its alkaline and acidic degradates by the proposed HPLC method.

| Intact % | Degradate % | Recovery%* | |
|---|---|---|---|
| | | Alkaline | Acidic |
| 90.00 | 10.00 | 100.50 | 100.05 |
| 80.00 | 20.00 | 101.00 | 99.87 |
| 60.00 | 40.00 | 99.60 | 101.08 |
| 40.00 | 60.00 | 101.98 | 100.05 |
| 10.00 | 90.00 | 100.89 | 98.65 |
| Mean recovery ± SD | | 100.79 ± 0.861 | 99.94± 0.864 |

Table (5): Results obtained for the pharmaceutical samples using the proposed HPLC method and application of standard addition technique

| Pharmaceutical preparation | Found* | Standard addition technique | | | |
|---|---|---|---|---|---|
| Lexotanil® tablets labelled to contain 3mg Bromazepam BN No.1812-30-2 | Recovery% | Claimed ($\mu gmL^{-1}$) | Pure added ($\mu gmL^{-1}$) | Pure found ($\mu gmL^{-}$ | Recovery%** %** |
| | 100.06±0.850 | 30.00 | 5.00 | 4.98 | 99.60 |
| | | | 30.00 | 30.09 | 100.30 |
| | | | 40.00 | 40.16 | 100.40 |
| Mean ± SD | 100.10 ±0.435 | | | | |

* The average of five separate determinations.

** The average recovery of three separate determinations

Table (6): Statistical comparison of the results obtained by applying the proposed HPLC method and the reported method [27] for the determination of bromazepam in pure form.

| Items | Proposed method | Reported method* |
|---|---|---|
| Mean | 100.11 | 99.74 |
| SD | 0.251 | 0.590 |
| RSD% | 0.251 | 0.600 |
| n | 5 | 5 |
| Variance | 0.063 | 0.361 |
| Student's t-test (2.30) ** | 1.271 | |
| F-test (6.39)** | 5.70 | |

*HPLC method was performed on a reversed phase ODS C-8 column (250x4.6 mm,5µm) the mobile phase, methanol: acetonitrile: mixture of potassium dihydrogen phosphate, $5x10^{-3}$ M, and ammonium acetate 0.1 M adjusted to pH 6.2 with glacial acetic acid (26.5:21.5:52, v: v: v), A flow rate of 0.8 $ml.min^{-1}$[59]

** The values in the parenthesis are the corresponding theoretical values of t and F at (p=0.05).

The RP-HPLC method development started with preparation of suitable mobile phase and its composition, pH values, flow rate and detection wavelength. The pure drug Bromazepam is injected into the HPLC system and run in different solvent systems. Different mobile phase solvents that are widely used like methanol, water and acetonitrile and their combinations were tried in order to find the best conditions for Bromazepam and its degradates separation. It is observed that Phosphate buffer and acetonitrile gave satisfactory results compared to other combinations. The Phosphate buffer and acetonitrile mobile phase system were tried with different proportions and with different flow rates to improve the peak shape, signal to noise ratio and to minimize the shift of the baseline. Finally, the optimal condition of the mobile phase was chosen as Phosphate buffer and acetonitrile in the ratio 82:18 v/v. This composition of the mobile phase resolved the degradates very well. All the measurements are performed at constant temperature of the column. The pH value of the final mobile phase composition is 3.5. The flow rate is set to 1 mL/minute after trying different flow rates. After several trials, elutes runtime is set to 10 minutes assuring no interferences as well as influence from the excipients.

The system used in this procedure gave an excellent resolution and sensitivity for Bromazepam with its alkaline and acid degradates, where its alkaline degradate peak appeared at $t_R$ = 1.85 as shown in figure (4) while acid degradate peak appeared at $t_R$ = 2.60 as shown in figure (5).

System suitability parameters for HPLC method were tested by calculating capacity factor, tailing factor, sensitivity factor and resolution. Good results were obtained as in table (1).

The results of assay validation obtained by applying the proposed chromatographic method for the determination of Bromazepam are listed in table (2).

The accuracy of the proposed method for the assay of the raw material of Bromazepam is represented in table (3).

The proposed method was tested for selectivity by analyzing different samples of intact BMZ in the presence of varying amounts of its alkaline and acid induced ion products, the results are demonstrated in table (4) and reveals that BMZ could be determined by the suggested method without any interference from its degradate.

Lexotanil® tablets were analyzed by the proposed method and the validity of the method is assessed by applying standard addition technique as shown in table (5) which showed that the method was applicable for analysis of the tablet without interference of any additives or excipients.

Statistical comparison between the results obtained for the analysis of Bromazepam in pure form and the reported HPLC method [27] was shown in table (6). The values of the calculated t and F are less than the tabulated ones, which reveals that there is no significant difference between the compared methods with respect to accuracy and precision, respectively.

**CONCLUSION**

The results of assay validation of the HPLC method showed that this method is accurate, precise and specific over the specified range; therefore it can be applied in quality control laboratories for routine analysis.

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**Cite this article as:**
Maha A. Hegazy, Nagiba Yahia, Yosra Z. Sewilam, Soheir A. Weshahy. Stability Indicating High Performance Liquid Chromatographic Method for The Determination of Bromazepam in the presence of its degradation products. Asian Journal of Biomedical and Pharmaceutical Sciences, 5(51), 2015,13-20.