# Determination of Bromazepam in Pharmaceutical Formulations Using Fluorescence Enhancement by Surfactants

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## Abstract

A sequential Injection (SI) method for the determination of Bromazepam (BRZ) is described. The proposed method is based on the enhancement of BRZ fluorescence in the presence of a non-ionic surfactant Tergitol XH and lactose. The fluorescence was monitored at 423 nm with excitation at 340 nm. The optimum concentration of surfactants and lactose were determined using a univariate approach. With the optimum conditions described, linear calibration curves were obtained from 25 ppm to 200 ppm. The method was successfully applied for the determination of BRZ in pharmaceutical formulations using the standard addition technique.

**Key words**: Bromazepam, Sequential Injection, Surfactant, Fluorescence

## 1. Introduction

Bromazepam, (BRZ) [7-Bromo-1, 3-dihydro-5-(2-pyridyl)-2H-1, 4-benzadiazepin-2-one] is a member of the 1, 4-benzodiazepine family of drugs. These drugs form an important psychotherapeutic agents that act on the central nervous system. They are widely used in the treatment of psychoneuroses to reduce pathological anxiety, agitation, insomnia and epileptic convulsions due to their low toxicity and limited side effects [1]. They are often abused by the illicit drug user causing profound behavioral effects that can lead to dependence. Benzodiazipines may also cause or contribute sudden death if misused [2].

Since benzodiazepines are widely used in clinical and forensic cases their determination in biological fluids, tissues and pharmaceutical formulations is important to keep track of the metabolic effect of the drug as well as for rapid screening of illicit drug users. Many analytical methods for the analysis of these drugs have been reported [3-6]. Recently, Drummer [7] published a review on the determination of benzodiazipines in biological fluids.

Luminescence spectrometry has proven to be a sensitive and a selective technique for the determination of many compounds of pharmaceutical and biological interest. Limited work has been done on the fluorimetric determination of 1,4-benzodiazipines due to their weakly fluorescent nature. However, the emission signal can be enhanced with suitable modification of the molecule or its environment. The common method so far has been acid hydrolysis [8,1,9], photo-degradation [9], or by altering their quantum yield through incorporating into micellar media [1,10]. The low solubility of BRZ in water and the weakly fluorescence of the benzodiazipines moiety limit the application of these methods in the determination of the drugs in the biological fluids. Hence, there is a need to enhance their solubility in aqueous systems in order to facilitate their therapeutic applications. One such way is by complexation with cyclodextrin molecules or by solubilisation in micellar system[11]. On the other hand, organized media is well known for its ability to enhance the sensitivity of the spectrofluorimetric determinations, and hence surfactants have been employed in many systems to increase the molar absorbitivity or the fluorescence quantum yields [12, 13].

In this work a sequential injection (SI) spectrofluorimetric method for the determination of BRZ in pharmaceutical formulations is reported. The protocol is based on the solubilisation of the drug in various surfactants in the presence of carbohydrates whereby the natively weak fluorescence of BRZ is greatly enhanced. Non-ionic compounds such as urea, carbohydrates and lower alcohols have been known to affect the micellisation characteristics of surfactants by either increasing or decreasing the critical micelle concentration (CMC) of both ionic and non-ionic surfactants [14].

## 2. Experimental

### 2.1 Apparatus

The SI system, FIAlab3500 (FIAlab-Instruments, http://www.flowinjection.com) used in this study consisted of the following components (Figure 1): syringe pump (2.5 ml, Sunnyvale, CA, USA), 200 cm holding coil (0.73 mm ID PTFE tubing, Upchurch Scientific, Oak Harber, USA), multiposition-value (eight ports, Valco, Houston, USA), 45 cm reaction coil (0.8 mm ID PTFE tubing, Upchurch Scientific, Oak Harber, USA), and a Hellma (Type 176.753-QS, Mülheim/ Barden, Germany) cell with an internal volume of 20 μL. Aminco Bowman Series-2 Luminescence Spectrometer (SLM Instruments, NY, USA) was used for fluorescence measurements. A personal computer was used for fluid control using FIAlab for windows software (FIAlab Instruments, http://www.flowinjection.com). The SI-integrated fluidic system is connected to the computer via an RS-232C interface. The spectrofluorimeter and the data collection and evaluation were under the control of another software using OS2-operating system (SLM Instruments, NY, USA). The data was collected in the time trace mode for the SI system.

Absorption measurements were performed on Cary UV 50 spectrophotometer.

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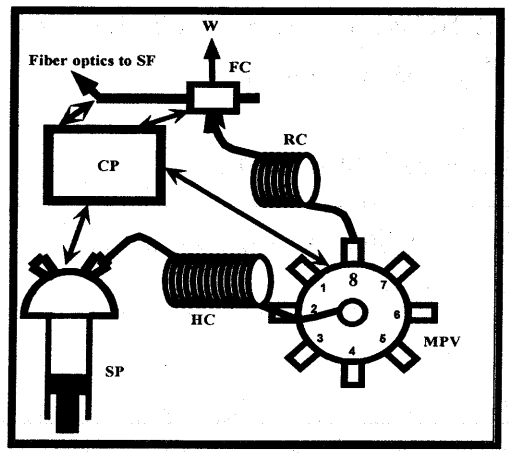


Fig. 1 Schematic diagram of the SI manifold. SP, syringe pump; CP, computer; RC, reaction coil, 45cm length and 0.8 ID; HC, 200 cm holding coil of 0.8 mm ID; MPV, eight ports selector valve; FC, flow through cell placed in fluorimeter compartment; SF, Spectrofluorometer; W, waste.

2.2 Reagents

All reagents used, were of analytical reagent grade. Ultra pure water 18.2 MΩ (Milli–Q Millipore Corporation) was used. HPLC grade methanol (Aldrich, Germany) was used in this study.

2.2.1. Preparation of standard BRZ solution.

A stock solution of BRZ (2000 ppm) was prepared by dissolving (50.00 mg) of pharmaceutical purity grade bromazepam donated by Roche (Switzerland) into enough methanol to make a final volume of 25.00 ml in a volumetric flask. Working standards were prepared by appropriate dilution with either de-ionized water or with surfactant solutions.

2.2.2. Preparation of surfactant solutions

Stock solutions of various surfactants were prepared by dissolving 1.00 g of each of the surfactant in 100.0 ml deionised water in a volumetric flask. The surfactants used in this study include Tergitol XD, Tergitol XH, Brij 58 and sodium dodecylsulphate (SDS) (All from Sigma, Germany).

2.2.3. Preparation of sugar solutions

Stock solution of various sugars were prepared by dissolving enough reagent of each of the sugars i.e lactose, glucose, sucrose, maltose in deionised water in 100.0 ml volumetric flask to make a final concentration of 0.50 M of the sugars. All Sugars used were obtained from GCC (Gaindland International Ltd., UK).

2.2.4. BRZ tablets

Three BRZ tablets (1.5 mg donated by Roche Company Switzerland) were dissolved in methanol. The tablet was then sonicated till completely disintegrated, the solution was filtered through a 0.45 μm filters into a 5.00 ml volumetric flask and completed to the mark with methanol. Each tablet was dissolved separately and analyzed in triplicate to measure its recovery.

2.3 Method

The procedure starts by nesting the BRZ solutions (position #2-7) and a mixture of XH surfactant and lactose (position #1) around the multi position valve (Fig 1). Then the syringe and the holding coils are filled by the carrier solution (deionized water). Further aspiration of appropriate volumes of the solutions by selecting one port at a time fills the tubing around the valve. The excess of the solutions introduced into the holding coil are then pumped to the waste through the detector port. FIAlab for windows software was used to program the complete procedure to run the method automatically. In this procedure 50 μl of 0.6% XH and 0.15 M lactose was aspirated via port #1 followed by aspirating BRZ solutions (75 μl) one at a time into the holding coil (HC). A flow reversal is then used to pump the composite zone through port #8 to the reaction coil (RC) and then to the detector. The fluorescence of the resultant BRZ – surfactant – lactose complex is then monitored at $\lambda_{em}$= 423 nm with the excitation at $\lambda_{ex}$=340 nm. The method requires about 75 s per sample. The peak fluorescence intensity was used as the performance criteria for the optimization study and for the quantitative analysis.

## 3. Results and Discussion

3.1. Ground State Absorption of BRZ in different surfactant solutions

The solubility of BRZ in pure water is very low, but it can be easily enhanced in the presence of surfactants. The BRZ used in this study was dissolved in methanol and was diluted to the required concentration using either deionised water or surfactant. The ground state absorption spectra revealed a well-defined band at 320 nm. On addition of surfactants, the absorbance was increased with the maximum absorbance observed in the presence of 0.4% (w/v) Tergitol XH and the minimum in the presence of SDS. No Shift in the absorption wavelength was observed in all cases (Table 1). The maximum increase in absorbance of up to 30% was observed in the case of XH as compared to that in aqueous solution.

3.2 Fluorescence of BRZ in different surfactant solutions

A study was carried out by the addition of different solutions of anionic (SDS) and non-ionic (Tergitol XD, Tergitol XH and Brij 58) surfactants to a fixed concentration of BRZ. In all cases, the maximum fluorescence intensity ($F_{max}$) of BRZ was enhanced in the presence of the surfactants. A maximum enhancement of about 70% (as compared to aqueous solution) was observed in the presence of XH, followed by XD, Brij-58 and SDS. However, the addition of all surfactants revealed an increase in the absorbance of bromazepam (Table 1), thereby causing an increase of the emitted fluorescence due to the increase of absorbed photons. Therefore, the observed maximum fluorescence intensities ($F_{max}$) were normalized to unit absorbance ($F_{max}/A$) and reported in Table 1. Clearly, maximum enhancements are obtained with Tergitol surfactants (XD and XH), whereas a moderate enhancement is observed with SDS. Practically, no noticeable enhancement is detected with Brij-58. A plausible explanation for this micellar effect on BRZ fluorescence may be due to the solubilization of BRZ in micellar microenvironment having different hydrophobicity.

Table 1. Effect of different kinds of surfactant on Absorption and Fluorescence of BRZ

| Surfactant | $\lambda_A$ (nm) | A | $\lambda_{ex}$ (nm) | $\lambda_{em}$(nm) | $F_{max}$ | $F_{max}$ /A |
|---|---|---|---|---|---|---|
| $H_2O$ | 320 | 0.2948 | 326 | 446 | 4.00 | 13.57 |
| Bri-58 | 320 | 0.3784 | 325 | 434 | 5.15 | 13.61 |
| SDS | 320 | 0.3537 | 328 | 421 | 5.03 | 14.22 |
| Tergitol -X D | 320 | 0.3639 | 325 | 427 | 5.54 | 15.22 |
| Tergitol -X H | 320 | 0.3871 | 332 | 421 | 6.88 | 17.77 |

Accordingly, the fluorescence enhancement observed with Tergitol XH and XD micelles may reflect a more hydrophobic microenvironment of BRZ compared to that observed with SDS and Brij-58 micelles. In addition, the fluorescence enhancement may be due to a decrease in fluorescence quenching of BRZ singlet state in the presence of Tergitol XD and XH micelles. This could be due to the greater protective ability of the singlet excited state of BRZ by the block copolymers surfactant micelles (Tergitol XD and XH).

Another interesting spectral feature was observed upon incorporating BRZ in micelles, namely the hypsochromic shift of BRZ maximum fluorescence.This blue shift was observed in all cases with the maximum shift of about 25 nm in the presence of XH and SDS (Table 1).

The corresponding blue shift observed in the presence of Tergitol XD and Brig-58 were 19 nm and 12 nm, respectively. The exact location in the micelle at which BRZ solubilization occurs is dictated by the type of interactions occurring between surfactant and solubilizate (BRZ). Therefore, it seems that BRZ is experiencing a similar microenvironment in both SDS and XH micelles, owing to the similar blue shift observed in these two micellar systems (25 nm). On the other hand, the spectral blue shift of BRZ fluorescence is reflecting different solubilizate microenvironments in Tergitol XD and Brij-58 micelles.

### 3.3. Effect of Tergitol XH concentration

The optimum surfactant concentration allowing maximum sensitization was determined by adding different concentrations of the surfactant to solutions having identical concentration of BRZ and measuring the fluorescence intensity. The results are shown in Fig 2. It is clear that the fluorescence intensity increases with the surfactant concentration reaching a plateau at a concentration of 0.4% of the surfactant.

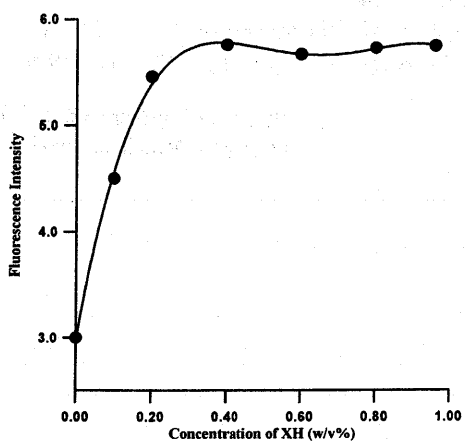


Fig. 2 The effect of XH concentration on the fluorescence intensity of BRZ solution. [BRZ] = 40 ppm.

### 3.4. Effect of Additives

The effect of excipients that are usually added to the tablet formulations such as glucose, maltose, lactose and sucrose were investigated in the presence of Tergitol XD micelles. The fluorescence intensity was enhanced in all cases, in the presence of the sugars. Lactose exhibited the maximum fluorescence enhancement followed by maltose, sucrose and glucose as shown in Fig 3. A blue shift was observed in the emission wavelength from $\lambda_{em}$ = 450 nm in water to $\lambda_{em}$ = 423 nm, while a red shift was observed in excitation wavelength from 328 nm to 340 nm in the presence of lactose. The enhancement of the fluorescence intensity of safranine-T dye in the presence of surfactants and sugars has been reported [14]. The fluorescence intensity was observed to increase with an increasing number of hydroxyl groups in the sugar. These results indicate that sugars have an effect on the micellisation characteristics of ionic and non-ionic surfactants [14]. The thermodynamic interactions for non-ionic surfactants are less complex than for ionic surfactants. The mechanisms of fluorescence enhancement are attributed to the decrease of icebergs, which are formed around the non-polar tails of the surfactants as a result of hydrophobic interaction. Therefore micelle formation is favored and the CMC is lowered. The number of OH groups in the sugar is known to usually influence the extent of micellisation. The enhancement is higher in lactose, maltose, and sucrose as these molecules have 12 O-H linkages. Similarly a less fluorescence intensity was observed in the presence of glucose as it contains only six O-H linkages.

As previously mentioned, lactose exhibited the maximum fluorescence enhancement in Tergitol XD micelles, concomitant with blue shift of the maximum fluorescence wavelength and a red shift of the excitation wavelength. To confirm that lactose will have similar effect in the presence of Tergitol XH micelles, the fluorescence intensity was measured in the presence of 0.6% XH while the concentration of lactose was varied from 0.02 to 0.2 M. Figure 4 clearly shows that the fluorescence intensity increases with an increase in the concentration of lactose reaching a maximum at a concentration of 0.15 M lactose. Hence analytical application of the method was carried out in the presence of Tergitol XH (0.6%) and lactose solution (0.15 M).

### 3.5. Analytical features

The analytical performance of the proposed system was studied using the optimum conditions discussed above. A series of BRZ standards were aspirated in duplicate into the sequential injection system. The fluorescence intensity, *I*, versus BRZ concentration *C* was found to be linear over the range between 25 ppm and 200 ppm. The calibration equation was: I = 0.01041 C + 0.365 with a correlation coefficient ($R^2$) of 0.998. The detection limit of signal to noise ratio of 3 was 12 ppm. The

reproducibility of the method was 0.84% ($n$ =7) showing excellent precision. The maximum percentage of methanol was maintained at 15% in all cases in order to avoid breaking of the micelles, which may occur due to a high percentage of organic solvents in the system.

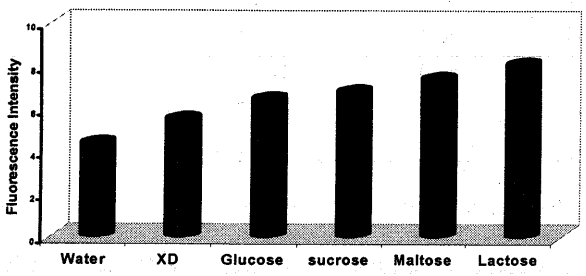


Fig. 3 The effect of various sugars on the fluorescence intensity of BRZ solution. [BRZ] = 40 ppm; [XD] = 0.6%; [sugars] = 0.10 M.

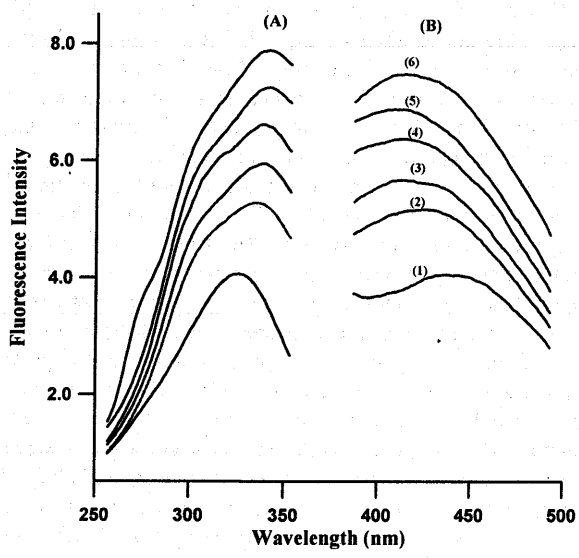


Fig. 4 The influence of different concentrations of lactose on the fluorescence intensity of BRZ solution. [BRZ] = 40 ppm; [XH] = 0.6%; (1), 0 M Lactose; (2), 0.02 M Lactose; (3), 0.04 M Lactose; (4), 0.06 M Lactose; (5), 0.10 M Lactose; (6), 0.15 M Lactose. A, excitation; B, emission.

3.6. Determination of BRZ in pharmaceutical preparation

The proposed SI method was applied for the determination of BRZ in commercially available pharmaceutical formulation. Since the typical excipients found in the formulations gave a positive interference, direct calibration curve could not be used for this determination. Hence standard addition method was employed for the determination of BRZ in the tablet form. The results obtained in 3 separate individual samples prepared in different days gave recovery values of 104.0±0.8%, 108±1.0% and 100±0.9%.

## 4. Conclusion

The proposed method can be used for the determination of BRZ in pharmaceutical formulation. The advantage of this method is that it is simple and does not involve ring opening or photodegradation of the molecule. The detection limit although high is adequate for drug analysis. The method can be extended further to indicate trace analysis of BRZ in biological fluids when combined with a solid phase extraction step.

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