Scope of Harmonisation of Pharmacopoeial Liquid Chromatography (LC) Methods for Diazepam and Its Related Substances

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Abstract
Drug analysis is an imperative activity to check the quality of a drug compound. Pharmacopoeial monographs provide important information about the quality of a drug substance. The expected quality of a medicine during period of use is also explained in such monographs. Analytical tools such as spectroscopic and chromatographic methods have been developed for such investigations. We have analysed the purity of a well known anxiolytic drug; diazepam, by using liquid chromatographic (LC) technique. It was noticed that with Zorbax Eclipse XDB – C8 (4.6 x 150 mm, 5 μm) column and recommended mobile phase comprising acetonitrile - methanol - potassium dihydrogen phosphate (22+34+44 v/v), the desired results obtained were not according the chromatograms provided by European Pharmacopeia (EP), but by using another column (ACE – 5 – C8) (4.6 x 150 mm, 5 μm), an extra peak of diazepam degradant was obtained, which showed that by using appropriate mobile phase containing CH_3CN- CH_3OH- KH_2PO_4 (20+32+48 v/v), the better results can be achieved. The mean retention time for diazepam analysis was 2.9 minutes.

Keywords: Diazepam; British Pharmacopoeia; European Pharmacopoeia; System suitability; Liquid Chromatography.

Introduction

Analysis of pharmaceutical products is an important aspect of drug analysis for checking the purity and degradation of drug substances [1-3]. Pharmacopoeial monographs contribute to the overall quality control of pharmaceutical products by providing authoritative official reference standards for medicinal products. They also provide official statements describing the qualitys which a pharmaceutical product is expected to have at any time during its period of use [4]. Several analytical and spectroscopic methods including Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Liquid Chromatography coupled with Mass Spectrometer (LC-MS), Tandem Mass Spectrometry (MS/MS), Capillary Electrophoresis and other related techniques have been reported for their analysis [5-8]. Pharmacopoeial methods such as those found in the European Pharmacopoeia (Ph. Eur) and the British Pharmacopoeia (BP) are considered to be validated standard testing methods ensuring the highest quality of products. It is desirable to achieve as much harmonisation as possible between methods for the drug substance or active pharmaceutical ingredient (API) set out in the EP and methods for drug products in the BP, in order to bring about simplification and rationalisation of quality control methods. Wherever possible, the BP harmonises its methods and requirements for formulated preparations with

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those included for Ph. Eur monographs for the medicinal substances. Diazepam is a significant drug in the medicinal world, used in treatment of variety of diseases and disorders. It is a benzodiazepine derivative with molecular formula $C_{16}H_{13}ClN_2O$ (Fig. 1). Diazepam is a main anxiolytic drug that is used as an anticonvulsant, sedative and skeletal muscle relaxant by the patients of anxiety and depression [9-11].
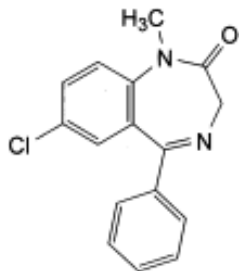

Figure 1. **Structure of diazepam**

The sedative effects of these drugs are due to their binding with the benzodiazepine site of $GABA_A$ receptors [12]. Among the sedative pharmaceuticals, Diazepam is considered as a safer drug as it does not stimulate the receptor of $GABA_A$ when endogenous GABA is not present [13]. It is also a core medicine in the World Health Organisation's (WHO) *'Essential drug list'* [14]. Considering the importance of diazepam, it was selected for the harmonisation of its pharmacopoeial methods (BP and Ph. Eur monographs). The current BP diazepam related substances (formulation) testing method is thin layer chromatography (TLC). The method involves use of TLC plates coated with silica gel GF254 and a mixture of equal volumes of ethyl acetate and hexane as the mobile phase [15]. It is necessary to replace the TLC method with an HPLC method for routine analysis because of number of disadvantages, i.e., relatively low resolving power and lack of online quantization of resolved compounds (spots).

Accordingly, in this instance, it was sought to establish whether the Ph. Eur HPLC conditions for diazepam related substances and drug substance analysis methods could also be used for the determination of related substances and API in a range of diazepam products available in the UK

market. The aim of this study was to harmonize the BP monographs of the various preparations of diazepam (tablet and injection) with that of the Ph. Eur, and to show whether or not the test method used is also suitable for assay. In this study the pure drug sample was tested, the results analysed and the actual drug products were tested under the same conditions. The results obtained were analysed and compared in the discussion below to assess the effectiveness of the harmonisation attempt.

Materials and Methods
Instrumentation

Reverse Phase HPLC was carried out on an Agilent 1200 LC system with UV diode array detector (DAD) and ChemStation software. Another system comprising of pump Shimadzu LC-6A, Injector Rheodyne 7125, Shimadzu SPD-6AV UV-Vis spectrophotometric detector, integrator Shimadzu C-R5A Chromatopac was also used for data processing and execution. The following RP- HPLC conditions were used; wave length 254 nm; mobile phase flow rate 1 mL/min; sample injection volume 20 µL.

Columns and reagents

Chromatographic seperations were performed on columns; ACE-5-C8 (4.6 x 150 mm, 5 µm) Zorbax XDB- Eclipse-C8 (4.6 x 150 mm, 5 µm) and a stationary phase of octasilyl silica gel for chromatography provided by BP laboratories, Teddington, UK.

Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Sodium hydroxide was purchased from Fluka (Poole, UK) and potassium dihydrogen phosphate from Sigma-Aldrich (Poole, UK). The diazepam drug substance used was purchased from AAH pharmaceuticals (UK) and also from the Ph. Eur. Laboratory. The diazepam for system suitability was purchased from the Ph. Eur. Laboratory. The diazepam tablets ((Generics, UK) Limited, Potters Bar, England) and injection ampoules ((Generics, UK) Limited, Potters Bar, England) were supplied from AAH pharmaceuticals (UK). The related substances and pure drug substance were obtained from the Ph. Eur. Laboratory.

Preparation of mobile phase

The mobile phase was prepared according to the procedure in the Eur. Ph monograph: 22 volumes of acetonitrile 34 volumes of methanol, and 44 volumes of 0.025M solution of potassium dihydrogen phosphate, previously adjusted to pH 5 using dilute sodium hydroxide solutions were mixed. An adjusted mobile phase containing 20 volumes of acetonitrile, 32 volumes of methanol, and 48 volumes of 0.025 M solution of potassium dihydrogen phosphate, previously adjusted to pH 5 was also prepared.

Preparation of sample solutions

For system suitability: The contents of a vial of diazepam for system suitability (containing impurities A, B and E) were dissolved in 1.0 ml of the mobile phase.

From formulation (Tablet): Using pestle and mortar five diazepam tablets (each 5mg) were powdered and transferred into a 50ml volumetric flask. To the volumetric flak 0.5 ml of acetonitrile was added to dissolve diazepam and finally diluted with 50ml mobile phase.

From formulation (Injection): 5ml ampoule was mixed with 0.5ml acetonitrile and made up to 10ml with mobile phase.

Test solution for repeatability testing: The test solution for repeatability testing for the assay by LC was prepared by first agitating a 5 mg tablet in 0.5 ml acetonitrile and diluting to 10 ml with mobile phase. 2 ml of the supernatant was then made up to 10 ml with mobile phase.

Results and Discussion

In investigating the feasibility of harmonising pharmacopoeial methods, ideally full method validations for each product would be carried out but for a number of reasons (e. g. availability of products spiked with known amount of related substances) is not practical. Therefore, the approach adopted was to carry out a risk assessment involving the product and analytical method to be used, in order that effort could be focused on investigating the most likely potential problems.

The first step was to assess the critical features of the EP LC conditions hoping that it could be used in the analysis of the diazepam products available in the UK. The chromatogram provided with the EP system suitability sample (diazepam for system suitability) was obtained using a Zorbax Eclipse XDB – C8 (4.6 x 150 mm, 5 μm) column with recommended mobile phase, as mentioned above (Fig. 2a, 3a, 4a, 5a). Using these conditions, the desired resolution specified in the system suitability test was not quite good and obtained resolutions were; 2.72 between impurity A and diazepam and 2.29 between impurity A & impurity E   (Fig 2a-2b.).
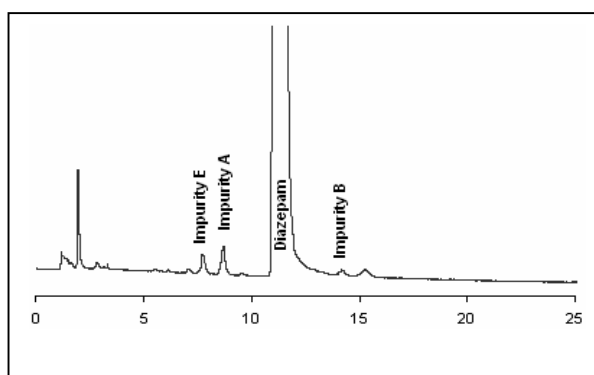

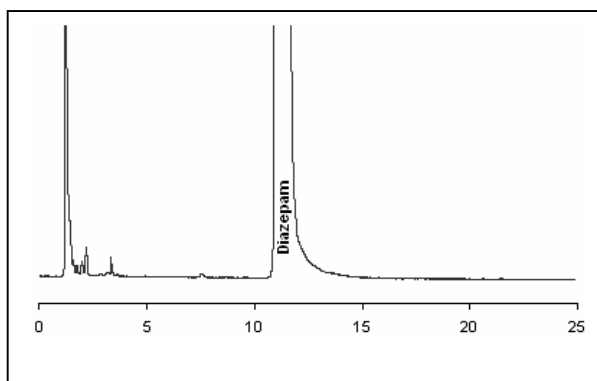*Figure 2a.* **System suitability chromatogram on Zorbax Eclipse XDB – C8 (4.6 x 150 mm, 5 μm)**

Figure 2b. **Formulation (tablet) chromatogram on Zorbax Eclipse XDB – C8 (4.6 x 150 mm, 5 μm)**

But this was put down initially to the resolution test being inappropriate (measuring resolution between peaks of very different sizes), especially since the desired resolution was also not achieved in the chromatogram provided by the EP. Therefore, by using same mobile phase on column ((ACE – 5 – C8), over an extended period, resolution obtained were; 2.29 between impurity A and diazepam and 1.71 between impurity A &

impurity E. An extra peak (subsequently shown to be a diazepam degradant) was also observed in the chromatogram just with baseline, resolved in front of the main diazepam peak (Fig 3b).
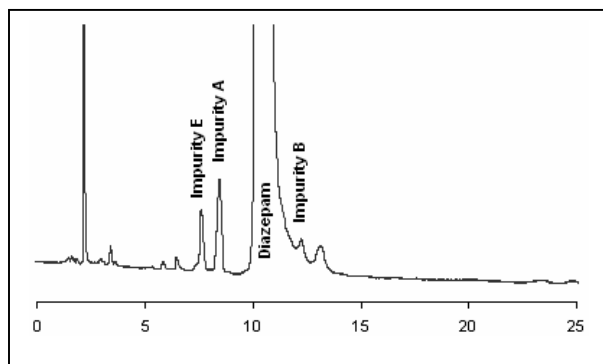

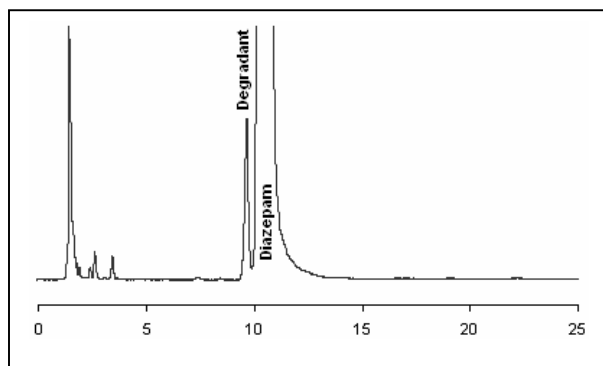*Figure 3a.* **System suitability Chromatogram on ACE 5 C8 column (4.6 x 150 mm, 5 µm)**

Figure 3b. **Formulation (tablet) chromatogram on ACE 5 C8 column (4.6 x 150 mm, 5 µm)**

No drastic change observed in the pattern of system suitability chromatogram, except better resolution between impurity A and diazepam after mobile phase adjustment. The resolution obtained were; 4.13 between impurity A and diazepam and 2.82 between impurity A & impurity E (Fig. 4a)
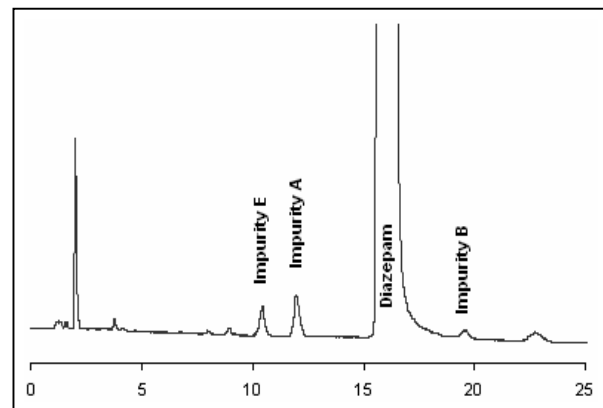

Figure 4a. **System suitability chromatogram on Zorbax Eclipse XDB – C8 (4.6 x 150 mm, 5 µm)**
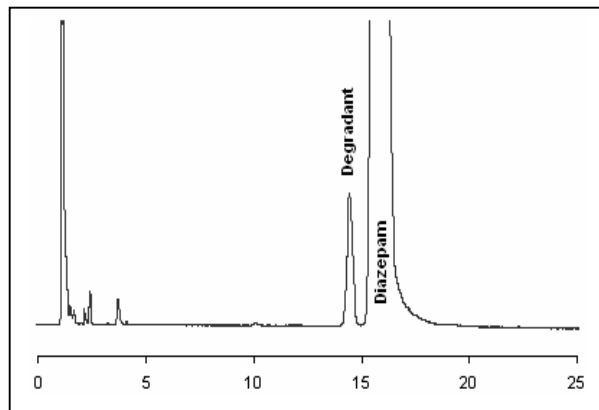

Figure 4b. **Formulation (tablet) chromatogram on Zorbax Eclipse XDB – C8 (4.6 x 150 mm, 5 µm)**

The resolution of this peak was only achieved on Zorbax Eclipse XDB – C8 after making an adjustment in the mobile phase (Fig 4b). The use of a similarly adjusted mobile phase with the ACE – 5 – C8 column resulted good resolution between the degradant peak and diazepam (Fig 5b).

No drastic change observed in the pattern of system suitability chromatogram, except better resolution between impurity A and diazepam after mobile phase adjustment. The resolution obtained were; 2.56 between impurity A and diazepam and 2.0 between impurity A & impurity E (Fig.5a)
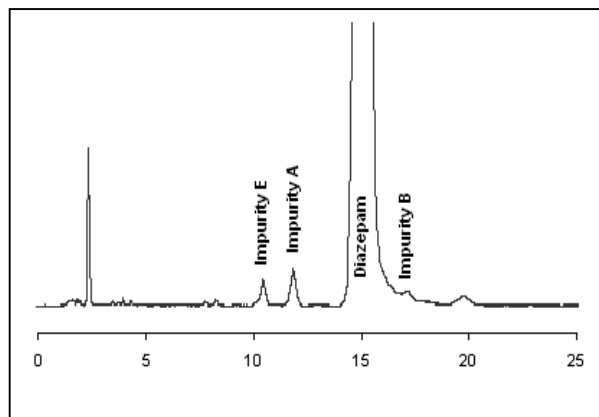

Figure 5a. **System suitability chromatogram on ACE 5 C8 column (4.6 x 150 mm, 5 µm)**

Adjustment in the mobile phase resolved the degradant peak from diazepam using modified mobile phase (Fig. 5b)
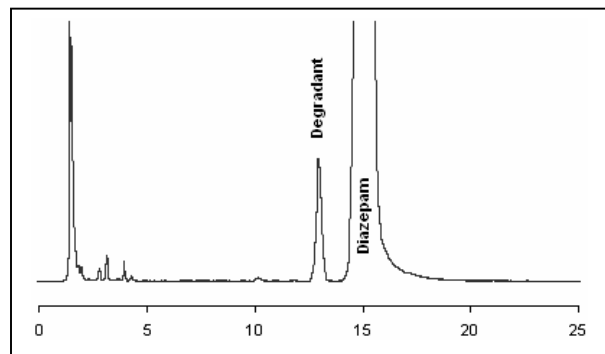
Figure 5b. **Formulation (tablet) chromatogram on ACE 5 C8 column (4.6 x 150 mm, 5 μm)**

Precision and accuracy

The precision experiments were performed to check the closeness of the results of the same samples at different intervals. It was measured in accordance with International Conference on harmonization (ICH), i.e. repeatability and intermediate precision and expressed as relative standard deviation (RSD). Three replicates of 5, 10 and 15 μg/mL were analyzed for evaluation of inter and intra-day variations. The calculated RSD's are shown below (Table 1).

Table 1. **Results of precision at different concentrations for diazepam.**

Standard solution (μg/mL) (n=3)	Recovery (%)	RSD (%)
5	99	1.77
10	97	1.23
15	98	0.92

The accuracy results for Diazepam were obtained from percent recovery and RSD of mean concentration at three different concentrations. The three standards of 5, 10 and 15 μg/mL were analyzed. The recovery and RSD results of Diazepam are summarized in (Table 2).

Table 2. **Accuracy results of diazepam concentration in the standard solutions.**

Standard solution (μg/mL)	Recovery (%)	RSD (%)
10	98	1.18
20	99	1.69
35	98	109

The results obtained (% recovery and RSD) indicate the precision of the instrument and accuracy of results.

In the context of the application of these LC conditions to the analysis of formulated products, it was demonstrated that none of the excipients in the formulated products gave rise to a peak that would interfere with diazepam or any of the related substances. In addition, there were no gross recovery problems and, critically, after extended use of the conditions for drug product analysis to check for possible build up of excipients on the column, the column performance, as evidenced by the system suitability test, did not deteriorate.

Conclusions

The aim of this study was to harmonise the BP monograph of diazepam tablet with that of the EP and was successfully achieved. The risk analysis involving drug product and EPLC method demonstrated that, given an appropriate adjustment of the mobile phase composition, diazepam EPLC conditions may be used for the analysis of diazepam like products.

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