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Validated HPTLC content uniformity test for the determination of Diazepam in tablet dosage form.

Miss. Vaishali P. Machale^{1*},
Mr. Avinash T. Gatade, Dr. Ramesh T. Sane.

¹Guru Nanak Institute Of Research And Development, Matunga- 400 019, Mumbai.

ABSTRACT

A simple High performance thin layer chromatography (HPTLC) Content Uniformity Test was developed and validated for the analysis of diazepam in its commercial single components tablet formulations (2mg/tablet). The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of toluene : acetone (3: 2) v/v. This system was found to give compact spots for diazepam (R_f value of 0.35 ± 0.01). Densitometric analysis of diazepam was carried out in the absorbance mode at 232 nm. The linear regression data for calibration plots showed good linear relationship with $r^2 = 0.9922$ in the concentration range of 300- 1000 mg/ml. The mean value of correlation coefficient, slope and intercept were 0.9922 ± 0.002 , 0.64 ± 0.001 and 38.09 ± 1.71 rep. the method was validated in terms of linearity (300-1000ng/spot), precision, accuracy (98% to 102%) and specificity. For Content uniformity test, Diazepam content of 10 individual tablet units of two market formulation was determined after extracting with methanol. Both the formulation complied with the USP specification of the content Uniformity test which says 10 results must lie with 85-115% and C.V must be less than 6%(R.S.D 6%). The proposed Content Uniformity Test can analyze ten tablets simultaneously on a single plate and provides a faster and cost- effective quality control tool for routine analysis of Content uniformity Test for Diazepam tablet formulations.

Correspondence to Author

Miss. Vaishali P. Machale

Guru Nanak Institute Of
Research And Development,
Matunga- 400 019, Mumbai.

Email

vaishali.khalsa@gmail.com

Key Words

Diazepam, high performance thin layer chromatography (HPTLC), Content Uniformity test, densitometric analysis.

INTRODUCTION

Most of the pharmaceutical companies today are oriented toward designing new pharmaceutical dosage forms of existing drugs rather than discovering new drug products. Utilization of the existing resource of marketed and patented drug substances with known therapeutic effects, and modification of their pharmacotherapeutic characteristics by incorporation in suitable drug delivery system, has been the target of recent pharmaceutical development [1]. Diazepam is a long-acting benzodiazepine with anticonvulsant, anxiolytic, sedative, and muscle relaxant properties [2]. It is the most widely used drug for the acute management of all types of seizures (febrile and epileptic) in both adults and children. Diazepam can be administered orally, intravenously, or rectally. Rectal administration of diazepam is the method of choice in treating and controlling acute seizures. This route of administration yields diazepam plasma levels and therapeutic efficacy comparable to that attained when the same dose is given intravenously [3, 4]. The official methods for determination of diazepam in different pharmaceutical dosage forms are prescribed in USP 26 and BP 2002. USP 26 [5] prescribes HPLC method for determination of diazepam in tablets, capsules and injections, using a reversed-phase C18 column, a mixture of methanol and water (65 : 35 v/v) as a mobile phase, and UV detection at 254 nm. In BP 2002 [6], the method for determination of diazepam in injections, tablets, oral and rectal solutions is based on spectrophotometry. A number of methods for the qualitative and quantitative analysis of diazepam in pharmaceutical dosage forms have been reported, including: UV spectrophotometry [7], derivative spectroscopy [8], comparison of TLC and HPLC [9], HPLC [10–15], and capillary electrophoresis [19]. Various HPLC methods have been developed for the determination of diazepam in different pharmaceutical dosage forms. Bakavoli et al. [10] reported an RP-HPLC method for determination of diazepam in tablets using a mixture of methanol and 0.01 M phosphate buffer, pH 7.8 (1:1 v/v) as a mobile phase, and UV detection at 254 nm.

EXPERIMENTAL

MATERIALS AND METHODS

Diazepam was a gift sample from Anchrom Pvt. Ltd. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India. Two single component uncoated tablet formulations diazepam (2 mg/tablet) (formulation 1 and 2) were purchased from market silica gel aluminum plate 60 F-254, (20X10 cm with 250um thickness; E. Merck, Germany) were used as stationary phase. The mobile phase consisted of toluene: acetone (3: 2) v/v

INSTRUMENTATION

A Camag HPTLC system (Switzerland) comprising Camag Linomat 5 semiautomatic sample applicator, Hamilton syringe (100ul) Camag TLC Scanner 3, Camag win CATS software 1.4.5 covering single content uniformity option, Camag twin trough chamber (20x10 cm) and sonicator were used during the study. Densitometric scanning was performed on Camag TLC scanner3 in the absorbance mode. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. The camag winCATS software offers content uniformity test methods and specifications according to USP (XXIII). Content uniformity test was performed by single level calibration and often without replicates. It only produces correct results when true calibration function linear and runs through origin. In case of multi-level calibration win CATS offer linear and polynomial regression functions. Systematic errors can be avoided by multi-level calibration and also analytical assurance is achieved with little extra time and cost. Criteria are defined by group 1 dosage which says 10 results must lie within 85-115% and C.V. must be less than 6%. Here content uniformity test obtained with single- level and multi-level calibration using linear and polynomial regression is compared.

Preparation of standard diazepam and sample

Diazepam (10mg) was weighed accurately and transferred to 100 ml volumetric flask. It was dissolved in 50 ml of methanol and adjusted to the mark with methanol to obtain final concentration 100 ug/ ml of

diazepam. Then uncoated tablets (each containing 2 mg diazepam) were taken and each tablet was dissolved in 5ml of methanol. The solution was filtered through whatmann filter paper no 41 and the residue was washed with methanol and the volume was adjusted to 10 ml with the same solvent to have concentration of diazepam equivalent to 200 ug/ml.

A stock solution of diazepam (100ug/ul) was prepared in methanol. Different volumes of stock solutions 3,4,5,6,7,8,9 and 10 ul were spotted on the pre-washed and activated TLC plate under nitrogen stream using semiautomatic spotter to obtain concentration 300, 400, 500, 600, 700, 800, 900, 1000 ng/spot of diazepam. The TLC plate was air dried, developed and automatically analyzed as described earlier. The data of peak area vs. drug concentration was treated by linear least square regression analysis. Linearity was also determined over the range of 300-1000ng/spot.

Determination of diazepam in formulations.

Six micro liter of sample solution for formulation 1 and 2 (600 ng/ spot) were applied on TLC plates, developed and scanned as described earlier. Amount of diazepam present in the sample solution was determined by using content uniformity test obtained with single-level and multi-level calibration using linear and polynomial regression with the help of camag winCAT software.

METHOD VALIDATION

Precision-

Repeatability of sample application and measurement of peak area was carried out using six replicates of the same spot (600ng/spot of diazepam). It showed very low% RSD of peak area of diazepam.

Ruggedness

The intra-day variation was evaluated in the range of 300-1000 ng/spot three times a day. The inter-day variation was similarly evaluated over a period of 3 days.

Robustness of the Method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition like toluene : acetone (3: 2) v/v and toluene : acetone (3.2 : 1.8) v/v

were tried at three different concentration levels 400ng/spot, 800 ng/spot and 1000 ng/spot.

Limit of detection and limit of quantification

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank methanol was spotted six times. The noise level was determined. The limit of detection was calculated to be three times the noise level while ten times noise level was considered as limit of quantification.

Recovery studies.

The analyzed samples were spiked with extra 50,100 and 150 % of the standard diazepam and mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicates. This was done to check for the recovery of the drug at different levels in the formulations.

RESULTS AND DISCUSSION

Since diazepam is freely soluble in methanol, tablet powder was extracted with methanol. Sonication for 30 min helped to extract completely diazepam from tablet matrix. The mobile phase toluene : acetone (3: 2) v/v gave a sharp and symmetrical peak. Even the presaturated of TLC chamber with mobile phase for 15 min at room temperature assured better reproducibility in migration of diazepam and better resolution.

The linearity of response for diazepam was determined by analyzing corresponding standards for each concentration in the range of 300 to 1000 ng/spot in 6 replicates. It was observed that the response for various concentrations of standard diazepam were linear in the said range, with a correlation coefficient of 0.9922 ± 0.002 . The average linear regression equation was represented as $Y = 0.064(\pm 0.001) X + 38.09 (\pm 1.71)$, where X= concentration of diazepam and Y= peak area. The limit of detection and limit of quantification for diazepam were found to be 88 ng/ spot and 265 ng/ spot respectively.

Accuracy of analysis was determined by calculating recovery of diazepam by standard addition method at three levels of the calibration curve. The recovery of

99.76% (RSD= 0.45%) indicated that the method is accurate.

The intra-day precision was determined by analyzing standard diazepam solutions in three concentration range of 300-1000ng/spot for the three times on the same day while inter-day precision was determined by analyzing corresponding standards daily for a period of 3 days. The intra- day and inter-day coefficient of variation were found to be 0.19 and 0.08 respectively. The smaller values intra-day and inter-day variations in the analysis indicate that the method is precise. The low values of % RSD obtained after introducing small changes in mobile phase composition indicated robustness of the method.

Repeatability of sample application was assessed by spotting 20 ul of diazepam solution on TLC plate and developing the plate. The separated spot of diazepam was scanned seven times without changing position of the plate and RS for measurement was calculated and was found to be 0.30%

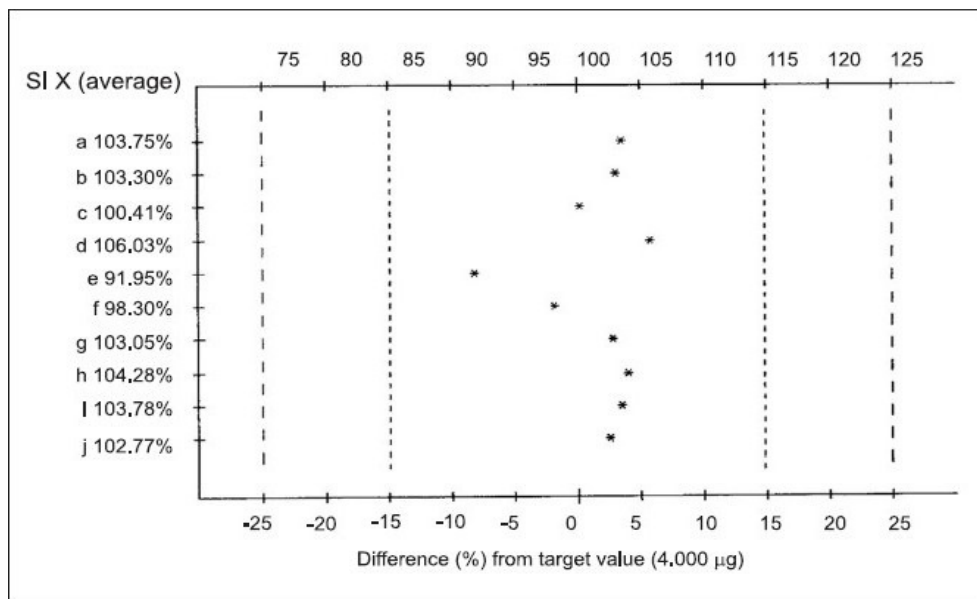
Repeatability of sample application was assessed spotting 20 ul of diazepam solution. TLC plate by semiautomatic spotter, and done by development of the plate and recording of the area for seven spots. The RSD for the peak a values was calculated and was found to be 1.80%.Both the RSD values, for measurement of the area and sample applications (1% and 3% respectively), proper functioning of HPTLC system.

Table 1: Summary of validation parameter

Parameter	Data
Linearity range	300-1000ng/spot
Correlation coefficient	0.9922± 0.002
Limit of detection	88ng/spot
Limit of quantification	265ng/spot
Accuracy (n=3)	99.76± 1.14
Precision (%RSD)	
Repeatability application (n=7)	1.80
Repeatability of measurement (n=7)	0.30
Inter-day (n= 3)	0.08
Intra-day (n=3)	0.19
Specificity	Specific

Confirm the specificity of the proposed method determined diazepam solution was spotted on the plate, developed and scanned as described earlier. It was observed that excipients present in the formulation did not interfere with peak of diazepam (Rf= 0.35±0.01). The purity of the diazepam was determined by comparing the spectrum at three different levels i.e. at peak start (S), peak apex (M) and peak end (E). Good correlation between these three spectra indicates the purity of diazepam $r(S, m) = 0.997$, $r(M, E) = 0.9992$. The spectrum of the diazepam extracted from the tablet was also compared with spectrum of standard diazepam, which showed good correlation ($r = 0.9988$). The summary of validation parameters are listed in table 1. Content uniformity test of diazepam was performed in two different market samples of single component diazepam tablets. The diazepam content of individual tablet unit (10 replicates) for both formulations was calculated by comparing the peak height and peak area of diazepam from formulation unit with that of standard diazepam. The diagrammatic representation generated using Camag CATS 4 software linear regression content uniformity option for ten units of the tablet formulations is shown in fig 1 for formulation 1 as per peak height.

Correlation was obtained between standard and sample solution of diazepam at



Diagrammatic representative (statement) of percent content in ten individual tablet units of formulation 1 (linear regression as per peak height, USP limit for drug content= 85 to 115%)

Single and multi level for content Uniformity Tests as per USP specifications for both formulations (Table 2). According to USP if the average of the limits specified in the potency definitions in the individual monographs is 100% or less unless otherwise specified in the individual monographs, the requirement for dose uniformity are met if the amount of an active ingredient in each of 10 dosage units as determined from weight

variation or content uniformity method is within the range of 85-115% of the tablet claim and RSD is less than or equal to 6%. Thus, since the content of individual tablet unit and the RSD between the content of 10 tablet units fall within the permissible limit according to USP, both formulation 1 and 2 comply with the content uniformity test of USP (Table 3)

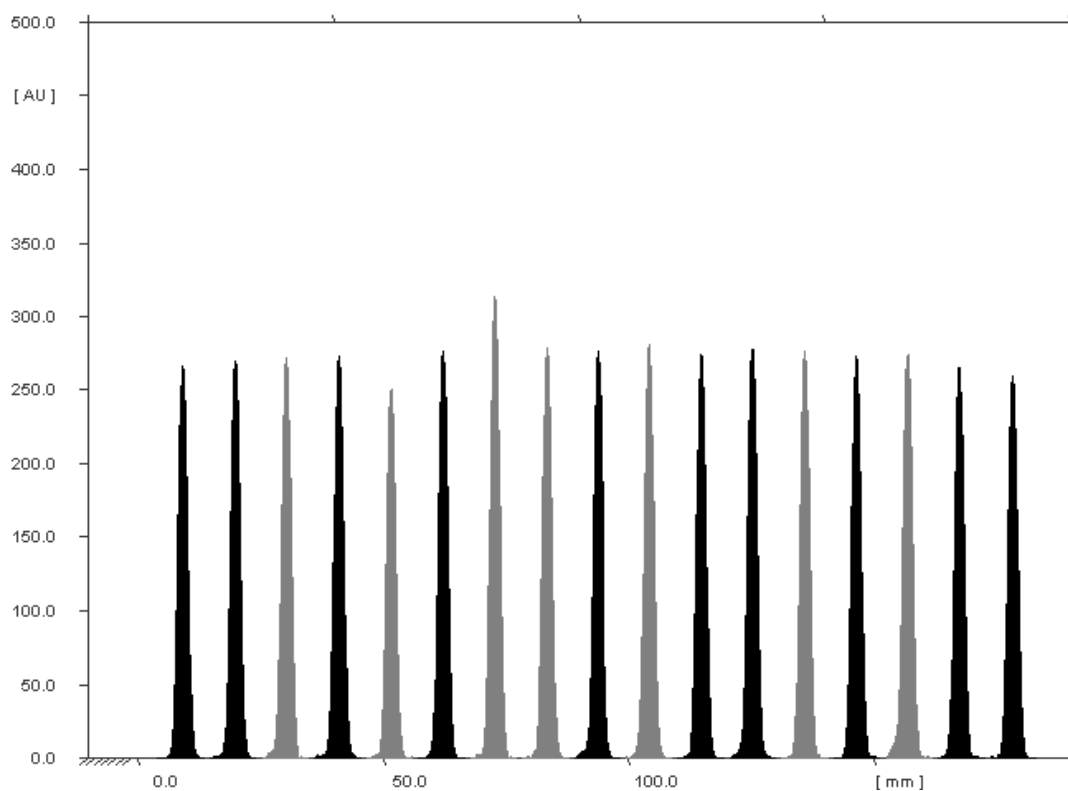
Table-2 Correlation data between standard and sample solution of folic acid for two different formulations at different levels

Level	Formulation 1				Formulation 2			
	S.D ^a		% C.V ^b		S.D		% C.V	
	Height	Area	Height	Area	Height	Area	Height	Area
Singel level	0.41	1.66	1.7	2.8	0.33	0.29	1.92	2.01
Linear regression	2.2	1.6	3.5	3.8	1.6	1.4	3.69	2.95
Polynomial regression	1	1.2	3.6	3.9	0.7	0.7	3.86	3.05

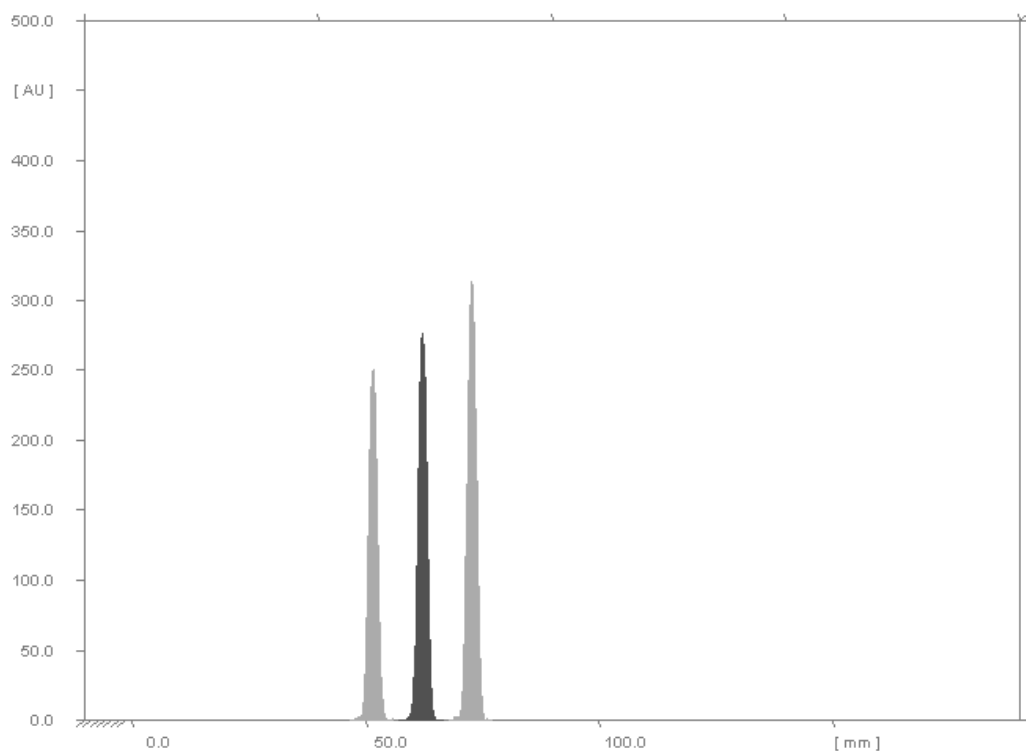
a= Standard deviation
b= Coefficient variance

Table-3 Statistical representation of the data obtained in Tablet dosage form for single level, Multi level linear and polynomial regression content uniformity for Formulation 1 and 2 (Label claim 2mg/tablet).

Parameter	Single level				Linear regression				Polynomial regression			
	Height		Area		Height		Area		Height		Area	
	F1 ^a	F2 ^b	F1	F2	F1	F2	F1	F2	F1	F2	F1	F2
Amount found ⁿ (mg)	1.97	2	1.99	2	2.06	2.074	2.03	2.05	1.95	2	1.99	2
S.D	0.44	0.04	0.07	0.04	0.08	0.07	0.1	0.06	0.08	0.07	0.1	0.06
%C.V	2.1	1.92	3.81	2.01	4.33	3.69	5.17	2.95	4.34	3.86	5.22	3.05
Confidence interval ^c	1.70- 2.24	1.98- 2.02	1.95- 2.04	1.98- 2.03	2.00- 2.11	2.02- 2.12	1.97- 2.09	2.02- 2.09	1.91- 2.00	1.96- 2.05	1.93- 2.06	1.97- 2.05

Fig. 2 Percentage content in ten individual tablets of marketed formulation Diagrammatic representation of percentage content in ten individual tablet of units of marketed formulation (linear regression as per peak area diazepam , USP limits for drug content is 85 -115%)

Densitogram of diazepam standards (light) and the samples (dark) Tracks identification: Standard (100%) :3,8,10,13,15, Standard (85%): 5 and Standard (115%): 7, samples: 1,2,4,6,9,11,12,14,16,17



Densitogram of diazepam standard & sample, Standard (85%): track 5, Sample: track 6. Standard (115%): track

Since to perform content uniformity test employing other methods, like HPLC or UV/VIS spectrophotometry, one has to analyze the specified number of dosage form units serially, this proves to be time consuming. On the other hand, using proposed HPTLC Content uniformity Test on can analyze 10 tablets on a single plate simultaneously. Thus the proposed test proves to be very fast and cost- effective and can be employed in pharmaceutical industry for determination of content uniformity of single component diazepam tablet dosage forma on routine basis.

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