

# Development and validation of HPTLC method for simultaneous estimation of canagliflozin and metformin hydrochloride in bulk and tablet dosage form

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## Abstract

The discovery of new drug combinations and the ongoing update of international regulations for the safety and efficacy of pharmaceutical formulation, demand the development of new analytical methods for these combinations. Combined-dose tablet formulation containing metformin hydrochloride (MET) and canagliflozin (CANAG) has recently been introduced in the market and a literature survey revealed a limited number of high-performance liquid chromatography (HPLC) and spectrophotometric methods reported, but no high-performance thin-layer chromatographic (HPTLC) method for the simultaneous estimation of these drugs in pharmaceutical formulation. The present work describes the development and validation of an HPTLC method for the simultaneous determination of MET and CANAG in a combined dosage formulation. The chromatography was performed on pre-coated silica gel 60 F 254 plates using methanol : toluene : ethyl acetate : ammonia (2 : 4 : 4 : 0.1) as mobile phase. A thin layer chromatographic (TLC) scanner set at 254.0 nm was used for direct evaluation of the chromatograms in reflectance/absorbance mode. The drugs were satisfactorily resolved with R<sub>f</sub> 0.15 for MET and 0.50 for CANAG. The method was validated according to The International Council on Harmonisation (ICH) guidelines. The calibration plot was linear between 0.5–3.0 µg/band for MET and 50–300 ng/band for CANAG respectively. Accuracy and precision of the proposed method were evaluated by recovery studies and intra-day and inter-day precision studies respectively. In stability testing, MET and CANAG were found to be susceptible to acid hydrolysis and alkaline degradation. Because the method could effectively separate the drugs from their degradation products, it may be used as a stability-indicating method.

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## Introduction

Metformin, chemically, is *N,N*-Dimethylimidodicarbonimidicdiamide which decreases hyperglycaemia primarily by suppressing glucose production by the liver (hepatic gluconeogenesis).

Canagliflozin, chemically, is *2S,3R,4R,5S,6R*)-2-{3-[5-[4-Fluoro-phenyl]-thiophen-2-ylmethyl]-4-methyl-phenyl}-6-hydroxymethyl-tetrahydro-pyran-3,4,5-triol which is an inhibitor of subtype 2 sodium-glucose transport protein (SGLT2), which is responsible for at least 90% of the renal glucose reabsorption (SGLT1 being responsible for the remaining 10%).

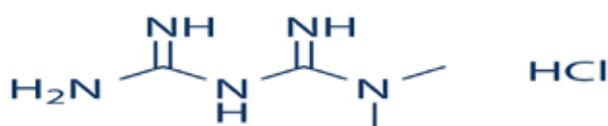


Figure 1a. Structure of metformin hydrochloride

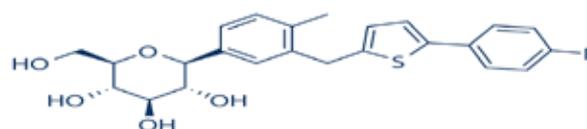


Figure 1b. Structure of canagliflozin

A literature survey reveals spectrophotometric, reverse phase high-performance liquid chromatography (RP-HPLC) and HPTLC<sup>1,2,3</sup> methods for the determination of MET either as a single or in combination with other drugs in pharmaceutical preparations. Analytical methods reported for CANAG include spectrophotometric<sup>4-8</sup> and HPLC<sup>9-13</sup> either as a single drug or in combination with other drugs. However, many resource-limited countries are deficient in laboratories equipped with HPLC. In poor countries, where such equipment is available, the high costs of HPLC-grade solvents and columns, and the lack of the possibility to analyse many samples simultaneously, significantly affects the timely release of laboratory results for action. Therefore, alternative methods are required to facilitate and reduce the time of analysis,

with relatively low cost. HPTLC represents a good alternative to HPLC as the time required for analysis and cost of analysis is exceptionally low. Additionally, HPTLC has several advantages over HPLC, such as:

1. Simultaneous processing of sample and standard can be done under similar conditions giving better analytical precision and accuracy.
2. Solvents need no prior treatment such as filtration and degassing; mobile phase consumption per sample is extremely low.
3. No possibility of interference from previous analysis as fresh stationary phase and mobile phase are used for each analysis.
4. Extreme flexibility for various steps i.e. selection of stationary phase, mobile phase, developing technique, detection with or without pre- or post-column derivatisation, corrosive and UV-absorbing mobile phase can also be used etc.

A literature survey revealed no HPTLC method for the simultaneous analysis of MET and CANAG in pharmaceutical formulation. This paper describes a sensitive, precise, rapid, selective, and economical HPTLC method for the simultaneous quantification of these compounds in bulk and tablet dosage form. The proposed method was optimised and validated as per ICH guidelines.<sup>14-21</sup>

## Materials and methods

Pure drugs, metformin hydrochloride and canagliflozin were procured from IPCA laboratories, Mumbai and Manusaktteva Biopharm LLP, Ahmedabad, respectively.

## Instrumentation

Pre-coated silica gel 60F254 aluminium plates (10 x 10 cm, 250 µm thickness; Merck, Germany), automatic TLC sampler 4 (Camag, Switzerland), twin trough chamber (10 x 10 cm; Camag, Switzerland), UV chamber (Camag, Switzerland), TLC scanner 4 (Camag, Switzerland), winCATS version 1.4.6 software (Camag, Switzerland) were used in the study. Ultrasonic bath (PowerSonic405, Hwashin technology, Korea) and electronic balance Shimadzu AX200, (Shimadzu Corporation, Japan) were used in the study.

## Preparation of standard solution

Stock solutions for measurements were prepared by dissolving MET and CANAG separately in methanol to obtain a concentration of 5000 µg/ml and 500 µg/ml respectively for each compound. For calibration, by diluting the stock standard solution with methanol in 10 ml standard volumetric flasks, a series of solutions was prepared containing 0.5, 1.0, 1.2, 2.0, 2.5, 3.0 µg /band for MET and 50, 100, 150, 200, 250, 300 ng/band for CANAG.

## Preparation of sample solution

An accurately weighed quantity of 50.0 mg of MET and 5.0 mg of CANAG, respectively, was transferred to a 10.0 ml volumetric flask, 5.0 ml of methanol added and ultrasonicated for 10 minutes;

the volume was then made up to the mark with methanol (conc 5 000 µg/ml and 500 µg/ml of MET and CANAG, respectively). From this solution, 2.0 ml was diluted to 25.0 ml with methanol (conc 400 µg/ml and 40 µg/ml of MET and CANAG, respectively).

## Selection of mobile phase

A trial and error method was used to select the optimised mobile phase. The solvent system of methanol : toluene : ethyl acetate : ammonia in the ratio 2 : 4 : 4 : 0.1 (v/v/v/v) was the most appropriate mobile phase for the HPTLC analysis of MET and CANAG in methanol as solvent, as it gave good resolution of both drugs with acceptable R<sub>f</sub> values.

## Selection of wavelength

Standard stock solution C was applied on a TLC plate with the help of CAMAG LINOMAT-V automatic sample applicator; the plate was chromatographed in a twin-trough glass chamber saturated with mobile phase for 20 minutes. After chromatographic development, the plate was removed and air-dried. The separated bands on the TLC plate were scanned over the wavelength range of 200–700 nm. From the overlain spectra it was observed that both MET and CANAG exhibit significant absorbance at 254.0 nm which was selected for densitometric evaluation of separated bands.

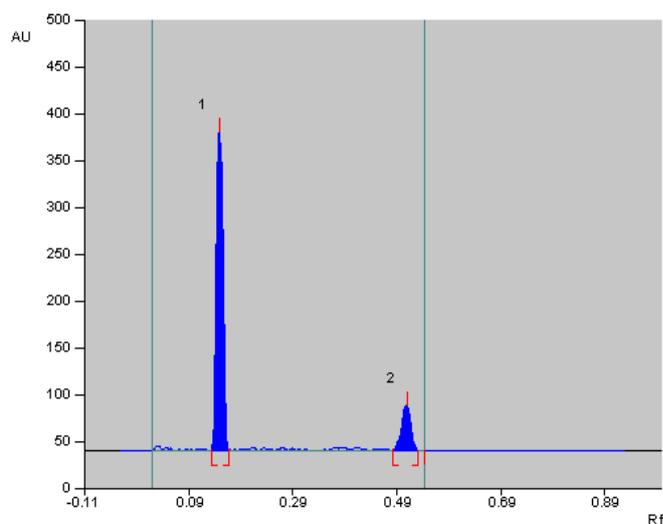


Figure 2. Typical densitogram of metformin HCl and canagliflozin.

## Calibration plots for metformin hydrochloride and canagliflozin

An accurately weighed quantity of 5.0 mg of CANAG and 50.0 mg of MET was transferred to a 10.0 ml volumetric flask, 5 ml of methanol added and ultrasonicated for 10 minutes. The volume was then made up to the mark with methanol (conc 500 µg/ml of CANAG and 5 000 µg/ml of MET).

The above solution containing CANAG and MET was applied on the TLC plates in the range 1–6 µl with the help of a micro syringe using LINOMAT-V automatic sample applicator. The plate was then developed and scanned under the optimised chromatographic conditions. After scanning, the peaks obtained for CANAG and

MET were integrated. The peak area was recorded for each drug concentration and the calibration curves of Concentration vs Peak area were constructed for both the drugs.

## Analysis of marketed formulation

### Preparation of standard solution

The standard stock solution was prepared as per the procedure mentioned above.

### Preparation of sample solution

Twenty tablets were weighed and crushed to obtain fine powder: the average weight of tablets was calculated. An accurately weighed quantity of tablet powder equivalent to about 50.0 mg of MET and 5.0 mg of CANAG was transferred to a 10.0 ml volumetric flask, 5.0 ml methanol was added and ultrasonicated for 20 minutes; the volume was then made up to the mark with methanol (conc 5 000 µg/ml and 500 µg/ml of MET and CANAG, respectively). From this solution, 2.0 ml was diluted to 25.0 ml with methanol (conc 400 µg/ml and 40 µg/ml of MET and CANAG, respectively).

### Method validation

The method was validated in compliance with the ICH guidelines.

The following parameters were studied:

1. Accuracy: The accuracy of a measurement is how close a result comes to the true value. Determining the accuracy of a measurement usually requires calibration of the analytical method with a known standard.
2. Precision: The term precision is used in describing the agreement of a set of results among themselves.
3. Robustness: The robustness/ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.
4. Limit of detection (LOD) and limit of quantisation (LOQ): The LOD is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the test. The LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of test.

### Accuracy

An accurately weighed quantity of pre-analysed tablet powder equivalent to about 5.0 mg of CANAG and 50.0 mg of MET was transferred individually to nine different 10.0 ml volumetric flasks. From this 2.0 ml of the above solution was added to a 25.0 ml volumetric flask. To each of the flasks the following quantities of CANAG and MET were added as 40/4 mg, 50/5 mg and 60/6 mg respectively to produce 80%, 100% and 120% levels of recovery.

All dilutions were performed with methanol. Solutions were prepared in triplicate and analysed. Accuracy was determined and expressed as percentage recovery.

### Precision

To ascertain the repeatability and reproducibility of the method, precision studies were performed. A sample solution was prepared and analysed in a similar manner to that described under analysis of the marketed formulation. Intra-day precision was determined by analysing a sample solution at three different time intervals on the same day and inter-day precision was determined by analysing a sample solution on three consecutive days. The system precision and method precision were also evaluated by applying six replicate injections of standard solution and applying six standard solutions with the same concentration on the HPTLC plate. The results of the system precision are expressed in terms of percentage relative standard deviation (% RSD) for peak area.

### Robustness

To evaluate the robustness of the proposed method, small but deliberate variations in the optimised method parameters were made. By introducing small changes in the mobile phase composition, mobile phase volume, duration of chamber saturation with mobile phase, time from spotting to development (10 min, 30 min, 45 min and 1 hr) and time from development to scanning (10 min, 25 min, 50 min and 1 hr), the effects on Rf value and peak area of drugs were examined. The composition of the mobile phase was changed slightly ( $\pm 0.1$  ml for component). TLC plates with standard and sample bands were run with mobile phases of composition, n-butanol: ethanol: ammonia (2.1:4.1:4.1:0.2 v/v/ and 1.9:3.9:3.9:0 v/v/). Mobile phase volume and duration of chamber saturation were varied at 10.0 ml  $\pm 0.1$  ml (9.1, 10 and 11.1 ml) and 20 min  $\pm 20\%$  (15, 20 and 25 min), respectively.

### Limit of detection (LOD) and limit of quantisation (LOQ)

LOD and LOQ were separately determined based on the standard deviation of response of the calibration curve. The standard deviation of y-intercept and slope of the calibration curves were used to calculate the LOD and LOQ, using the formula  $LOD = 3.3 \sigma/S$  and  $LOQ = 10 \sigma/S$ .

### Forced degradation studies

In forced degradation studies, intentional degradation was tried by exposing a sample to following stress conditions: acidic (0.1 M HCl), alkaline (0.1 M NaOH), and oxidation (3% H<sub>2</sub>O<sub>2</sub>). For intentional degradation, contents of the flasks were refluxed in a water bath at 80 °C for 3 hrs. For heat and photo degradation, a sample was kept at 60 °C and in UV light (254 nm) for 24 hrs, respectively. After the respective time intervals all the flasks were removed and allowed to cool. The samples were then analysed in similar manner as described under analysis of MET and CANAG in formulation.

## Result and discussion

### Optimisation of procedures

Different proportions of toluene, methanol, benzene, ethyl acetate, ammonia were tried for the selection of the mobile phase. Among the different mobile phase combinations, methanol : toluene : ethyl acetate : ammonia (2 : 4 : 4 : 4 : 0.1 v/v/v/v) was finalised as mobile phase. The bands developed were dense and compact with acceptable R<sub>f</sub> values for MET (R<sub>f</sub> = 0.15) and CANAG (R<sub>f</sub> = 0.50). Peaks were symmetrical in nature and no tailing was observed when plates were scanned at 288 nm (Figure 2).

### Linearity

Peak areas were found to have good linear relationship with the concentration. MET and CANAG were found to give linear detector response in the concentration range of 0.5–3.0 µg/band (Figure 3) and 50–300 ng/band (Figure 4), respectively. The coefficient of correlation for MET and CANAG calibration curves was found to be 0.9961 and 0.9989 respectively which was comparable to the reported HPLC method (MET: 0.99 and CANAG: 0.99).

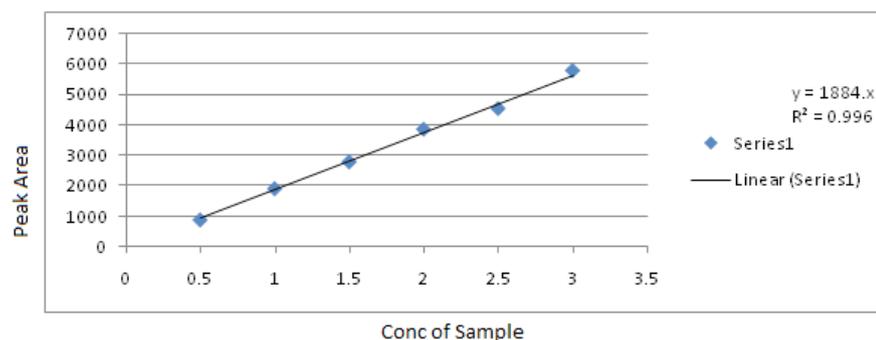


Figure 3. Calibration curve of metformin hydrochloride at 254.0 nm

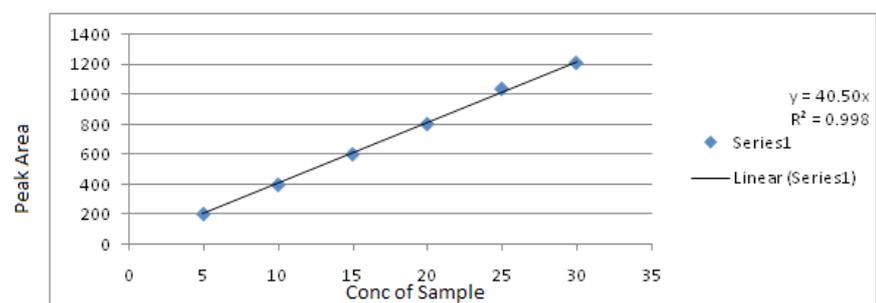


Figure 4. Calibration curve of canagliflozin at 254.0 nm

### Analysis of marketed formulation

An analysis of the marketed formulation containing MET equivalent to 50.0 mg and CANAG equivalent to 5.0 mg was carried out and the results are expressed as a percentage amount of the label claim. There was no interference from the excipients. The MET and CANAG content was found to be close to 100% and the results are summarised in Table I. The low RSD value indicated the suitability of this method for routine analysis.

### Recovery studies

To ascertain the accuracy of the proposed method, recovery studies were carried out using the standard addition method and the results are expressed as percentage recovery. The mean percentage recovery for each compound was calculated at each concentration level and reported with its standard deviation. The percentage recovery at three levels (80%, 100% and 120%) for both the drugs was found to be satisfactory (Table II) indicating the accuracy of the developed method.

### Precision

Precision was evaluated by carrying out six independent sample preparations of a single lot of formulation. Standard deviation (SD) and % RSD were found to be less than 2% for intra-day and inter-day precision (Table III) indicating the repeatability and reproducibility of the developed method. The % RSD for mean peak area of six replicate injections of standard solution and six samples of standard solution applied on an HPTLC plate was found to be less than two, indicating the system precision and method precision, respectively which was compared with the reported HPLC method (RSD: 0.38 to 0.6).

### Robustness

The effect of change in mobile phase composition ( $\pm 0.1$  ml), chamber saturation period ( $\pm 20$  %), time from application to development (10, 30, 45, 1 hr), time from development to scanning (10, 25, 50, 1 hr) on the R<sub>f</sub> value of drugs was studied. The method was found to be unaffected by small changes in method parameters with % RSD for R<sub>f</sub> values under varied method parameters less than 2%. The developed method is considered to be robust.

Table I. Results of analysis of marketed formulation

Serial no	Drug	Weight of tablet powder taken (mg)	Label claim (mg)	Amount of drug estimated (mg/tablet)*	% Label claim*	SD ( $\pm$ )	RSD
1.	MET	67.00	500.00	489.53	97.90	1.386	1.411
2.	CANAG	67.00	50.00	49.01	98.83	1.535	1.550

\*mean of six determinations

**Table II.** Results of recovery studies

Level of recovery	% Recovery*		SD ( $\pm$ )		RSD	
	MET	CANAG	MET	CANAG	MET	CANAG
80%	100.51	97.49	1.607	0.793	1.598	0.813
100%	99.57	100.36	1.459	1.869	1.465	1.459
120%	99.95	99.83	0.835	1.592	0.835	1.594

\*mean of six determinations

**Table III.** Results of precision studies

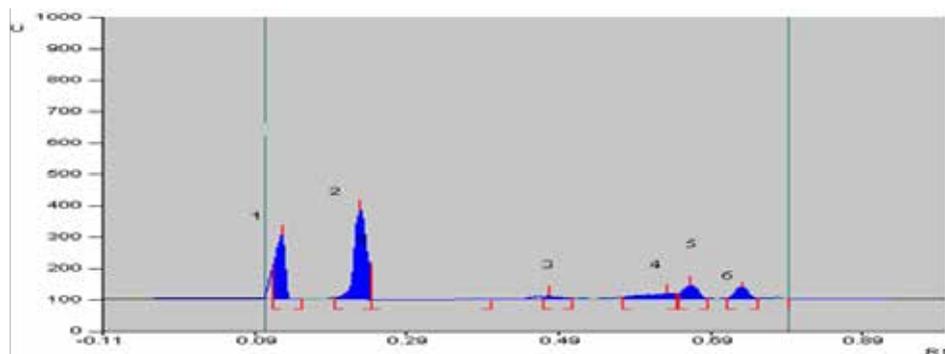
Serial no	Intra-day precision			Inter-day precision		
	% Label claim*	SD ( $\pm$ )	RSD	% Label claim*	SD ( $\pm$ )	RSD
MET	98.55	1.335	1.354	99.50	0.719	0.722
CANAG	98.68	1.451	1.470	99.73	0.802	0.804

**Table IV.** Results of robustness studies

Serial no	Factor	RSD for peak area	
		MET	CANAG
1.	Mobile phase composition ( $\pm$ 0.1 ml)	1.235	1.342
2.	Duration for chamber saturation ( $\pm$ 2 min)	1.347	1.412
3.	Spotting to development	1.201	1.369
4.	Development to scanning	1.234	1.205
5.	Volume of mobile phase ( $\pm$ 1 ml)	0.581	0.875
6.	Stability of solution	1.135	1.769

**Table V.** Results of forced degradation study

Serial no	Stress condition	Temperature and time	Percentage assay of active substance		Rf values of degraded peaks	
			MET	CANAG	MET	CANAG
1.	Acid (0.1 M HCl)	80 $^{\circ}$ C for 3 hrs	70.53	82.75	0.13	0.48, 0.63, 0.73
2.	Alkali (0.1 M NaOH)	80 $^{\circ}$ C for 3 hrs	84.91	87.19	0.12	0.57, 0.71
3.	Oxide (3% H <sub>2</sub> O <sub>2</sub> )	80 $^{\circ}$ C for 3 hrs	91.04	91.16	0.01	0.63
4.	Hydro (H <sub>2</sub> O)	80 $^{\circ}$ C for 3 hrs	88.09	88.93	0.03	0.74
5.	Photo degradation	24 hrs	91.80	92.75	0.31	0.63
6.	Thermal	24 hrs	88.95	86.15	0.11	


**Figure 5.** Densitogram of acid (0.1 M HCl) treated sample

### Stability-indicating property

Intentional degradation of MET and CANAG was tried under different stress conditions such as acid hydrolysis, alkaline hydrolysis, oxidation, heat and exposure to UV radiations. Both MET and CANAG were found to degrade in acid, alkaline, oxidation, heat and UV radiation stress conditions. The percentage assay of active substance and the Rf values of degradation products are given in Table IV. The densitograms of treated samples are shown in Figures 5 to 10 respectively.

### Conclusion

Introducing HPTLC in pharmaceutical analysis represents a major step in terms of quality assurance. Since HPTLC has several advantages over HPLC, the HPTLC method represents a good alternative to the reported HPLC methods for simultaneous estimation of MET and CANAG in pharmaceutical formulation. The proposed HPTLC method gives well-resolved peaks for MET and CANAG. As the sample preparation is simple, there is no need of solvent pre-treatment such as filtration and degassing, considering chamber saturation time (20 minutes) analysis can be finished within 30 minutes and hence the method is rapid. As the amount of mobile phase required for analysis per sample is only 10 ml, it can be concluded that the method is economical. Based on the results obtained, it is concluded that the method is sensitive, accurate, precise and reproducible, where MET and CANAG can be determined in bulk and in pharmaceutical formulation without interference from each other and with their excipients. The proposed HPTLC method was also able to selectively quantitate MET and CANAG in the presence of the degradation products obtained in forced degradation study. Hence, the method can be employed as a stability-indicating one. ICH guidelines were followed throughout the method validation and the suggested method can be applied for routine quality control analysis of pharmaceutical formulation containing these drugs.

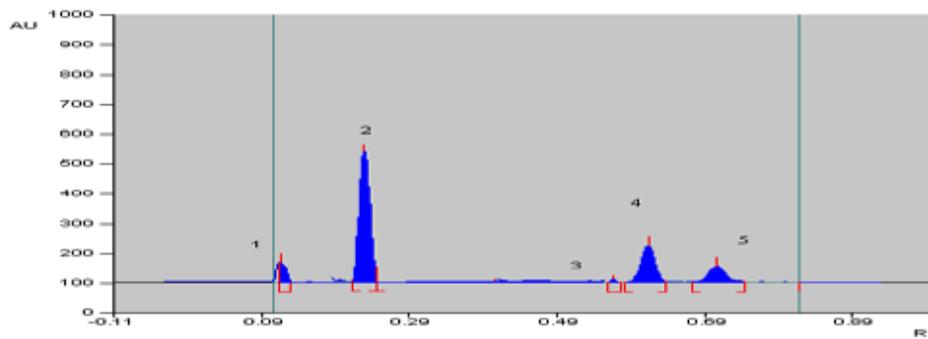


Figure 6. Densitogram of alkali (0.1 M NaOH) treated sample

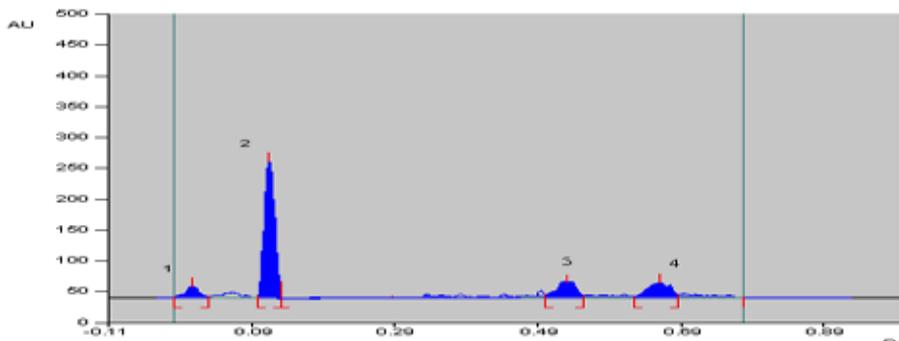


Figure 7. Densitogram of oxide (3% H<sub>2</sub>O<sub>2</sub>) treated sample

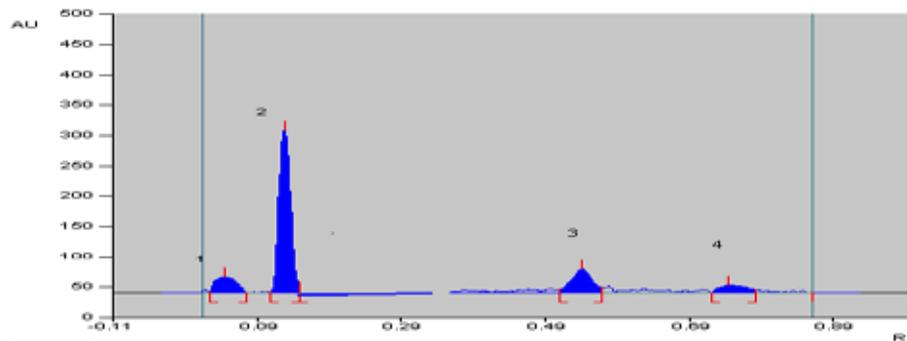


Figure 8. Densitogram of H<sub>2</sub>O (water)-treated sample

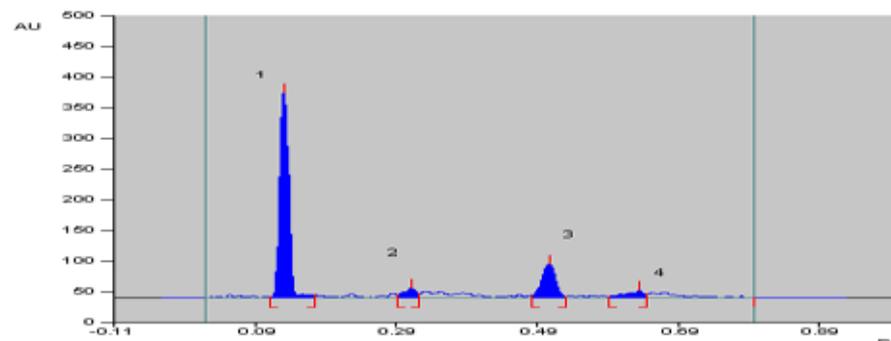


Figure 9. Densitogram of sample exposed to UV radiations

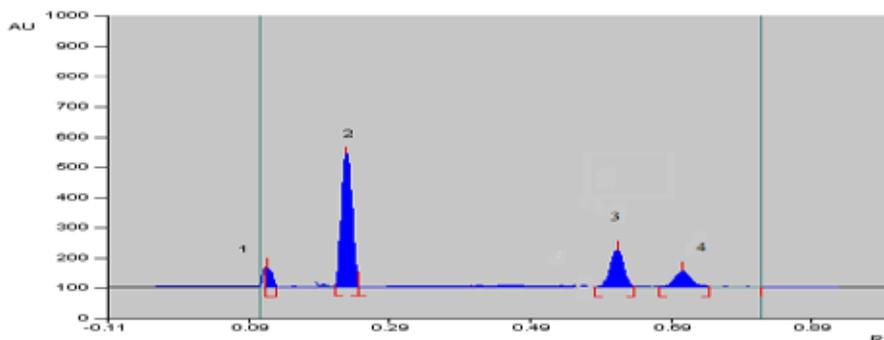


Figure 10. Densitogram of sample exposed to thermal

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## References

1. Thomas AB, Patil SD, Nanda RK, Kothapalli LP. Stability-indicating HPTLC method for simultaneous determination of nateglinide and metformin hydrochloride in pharmaceutical dosage form. *Saudi Pharmaceutical Journal* (2011)19, 221-231.
2. Rajasekaran A, Kavitha R, Arivukkarasu R. Development and validation of HPTLC method for simultaneous estimation and stability indicating study of metformin HCl and linagliptin in pharmaceutical formulation. *World Journal of Pharmaceutical Sciences* ISSN (Print): 2321-3310; ISSN (Online): 2321-3086.
3. Malgundkar SS, Mulla S. Validated HPTLC method for simultaneous determination of metformin hydrochloride and glibenclamide in combined dosage form. *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)* e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 9, Issue 2 Ver. V (Mar-Apr. 2014), PP 54-59.
4. Panigrahy UP, Reddy SK. A novel validated RP-HPLC-DAD method for the simultaneous estimation of metformin hydrochloride and canagliflozin in bulk and pharmaceutical tablet dosage form with forced degradation studies. *Oriental Journal of Chemistry* ISSN: 0970-020 X CODEN: OJCHEG 2015, Vol. 31, No. (3): Pg. 1489-1507.
5. Jani BR, Shah KV, Kapupara PP1 Development and validation of uv spectroscopic method for simultaneous estimation of dapagliflozin and metformin hydrochloride in synthetic mixture. *International Journal of Research and Development in Pharmacy and Life Sciences*, April-May, 2015, Vol 4, No 3, pp 1569-1576.
6. Beckett AH, Stenlake JB. *Practical Pharmaceutical Chemistry. Part-2.* 4th ed. New Delhi: CBS Publishers and Distributors; 2002. p. 275-325.
7. Chatwal GR, Sharma AK. *Instrumental methods of chemical analysis.* 5<sup>th</sup> ed. Mumbai: Himalaya Publishing House; 2002. p. 2.149-2.184.
8. Skoog DA, Holler FJ, Nieman TA. *Principles of instrumental analysis.* 6th ed. Thomson Brooks/Cole; 2007. p. 762-63,816-827.
9. Sethi PD. *High performance liquid chromatography, quantitative analysis of pharmaceutical formulations.* 1st ed. New Delhi: CBS Publishers and Distributors; 2001.p. 3-11, 116-120.
10. The International Council on Harmonisation (ICH), (Q2R1), Harmonized tripartite guideline, validation of analytical procedures: Text and Methodology, ICPMA. Geneva; 2005. p. -13.
11. ICH, (Q2A), Harmonized Tripartite Guideline, Text on Validation of Analytical Procedures. ICPMA. Geneva; 1994. p. 1-5.
12. ICH, (Q2B), Harmonized tripartite guideline, validation of analytical procedures: Methodology, Text and Methodology, ICPMA. Geneva; 1996. p. 1-8.
13. ICH, Guidance on analytical method validation, In: *Proceeding of the International Convention on Quality for the Pharmaceutical Industry.* Toronto, Canada; 2002.
14. Nash RA, Wachter AH. *Pharmaceutical process validation.* 3rd ed. New York: Marcel Dekker Inc; 2003. p. 1-10.
15. *Indian pharmacopoeia*, Govt of India Ministry of Health and Family Welfare. Vol.-II. Gaziabad: published by the controller of publication; 2007. p. 714-716.
16. Willard HH, Meritt LL, Dean JA, Settle F. *Instrumental method of analysis*, 7th ed. Delhi: CBS Publishers and Distributors; 2001.p.14.
17. *Instruction Manual Pharmaspec, UV 1601 Series, Operation Guide, Shimadzu Spectrophotometer.* Japan: Shimadzu Corporation Koyoto; 2001. p. 5.16, 8.2.
18. Weston A, Brown PR. *High performance liquid chromatography, and CE – Principles and practice.* 1st ed. California: Academic Press; 1997. p. 1-4, 8-11, 24-26, 29-32, 71.
19. Khopkar SM. *Basic concept of analytical chemistry.* 2nd ed. New Delhi: New Age International Ltd. Publishers; 1998. p. 1, 178-179.
20. *Instruction Manual Pharmaspec, UV 1601 Series, Operation Guide, Shimadzu Spectrophotometer.* Japan: Shimadzu Corporation Koyoto; 2001. p. 5.16, 8.2.
21. Swarbrick J, Boylan JC. *Encyclopedia of pharmaceutical technology.* Vol-1. 3rd ed. New York: Marcel Dekker Inc; 1997. p. 538-540.