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Determination of ivermectin stability by high-performance thin-layer chromatography

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Abstract

A rapid, sensitive and stability-signifying high-performance thin-layer chromatographic (HPTLC) method was developed and validated for the quantitative estimation of ivermectin (IVM) as a bulk drug and in pharmaceutical formulations. The separation was achieved on Lichrospher TLC aluminum plates pre-coated with silica gel 60F-254 (20cm×10cm×200 µm) using *n*-hexane: acetone: ethylacetate (6.5: 3.5: 0.1 v/v/v) as mobile phase. The densitometric analysis was carried out at 247 nm wavelength. Compact spots of IVM were found at R_f = 26±0.02. For proposed procedure, linearity (r^2 = 0.9989), limit of quantification (24.9 ng spot^{-1}), limit of detection (8.22 ng spot^{-1}) recovery (98.25–100.16%), and inter as well intra-day precision (≤2.21) was found to be satisfactory. We have synthesized polymeric nanoparticles encapsulated formulation of ivermectin (IVM-NPs); utilizing micellar aggregates of cross-linked random copolymer N-isopropylacrylamide (NIPAAM) with N-vinyl-2-pyrrolidone (VP) and polyethyleneglycol monoacrylate (PEG-A) for lymphatic targeting and it was also quantified by the developed method. IVM and formulations were subjected to acid and alkali hydrolysis, oxidation and photo-degradation. The drug undergoes degradation under acidic, basic, light and oxidation conditions. This indicates that the drug is susceptible to acid- base hydrolysis, oxidation and photo-oxidation and the developed method is selective for quantifying IVM even in the presence of degradatnts. The method was applicable for routine analysis and stability testing of IVM in pharmaceutical drug delivery systems. As the method could effectively separate the said drug from its degradation products, it can be employed as a stability indicating one.

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1. Introduction

Ivermectin (IVM) is the commercially available semi synthetic macrocyclic lactone endectocide, produced by fermentation of actinomycete *Streptomyces*

avermiltilis [1-2]. IVM has broad spectrum activity against arthropod parasites located in the different layers of skin and nematode parasites located in gastrointestinal and pulmonary tracts [2-3]. It exerts its action by opening γ-aminobutyric acid (GABA) channel-I and thus widely employed in the treatment of scabies, onchocerciasis, strongyloidiasis, ascariasis, trichuriasis and enterobiasis [2-5]. Lymphatic targeting of IVM using polymeric nanoparticles holds a great potential. Stability of IVM during formulation development of polymeric nanoparticles is key to a successful formulation.

High-performance liquid chromatograpy [6-15], liquid chromatography with tandem mass spectrometry [18,19], liquid chromatography with electrospray ionization mass spectrometry [20,21], high-speed counter-current chromatography [22] and capillary electrophoresis [23] for determination of IVM as a bulk drug and in dosage forms, biological fluids as well as in human and animal body tissues has been reported in the literature. However, no published analytical technique focuses on stability testing of IVM. The present work aimed at determination of acid-base, oxidation, hydrolytic and photolytic stability of IVM by high-performance thin-layer chromatographic (HPTLC) method. The developed HPTLC method is validated for linearity, precision, accuracy, sensitivity, selectivity and robustness. The method is also applied for determination of IVM content in the prepared nano-formulation of IVM (Nano-IVM).

2. Experimental

2.1. Chemical and reagents

IVM was received as gift sample from Jubilant Organosys, NOIDA, India. All other chemicals and reagents used were of analytical grade and were purchased from Merck (Mumbai, India).

2.2. Apparatus and chromatographic conditions

Chromatography was performed as described previously [21-22]. Briefly, precoated silica gel aluminum plate Lichrospher 60F_{254} (20 cm ×10 cm, 200μm; E. Merck, Darmstad, Germany) were used as stationary phase. The samples were spotted in the form of bands of width 3.5 mm wide and 10 mm apart by with *Camag* 100 microlitre syringe using a Linomat V (*Camag*, Muttenz, Switzerland) sample applicator. The equipment parameters include constant application rate of 160 nL s^{-1}, slit dimension of 3 mm × 0.45 mm and scanning speed of 20 mm s^{-1}. Linear ascending development was carried out in a 20 cm×10 cm twin through glass chamber (*Camag*, Muttenz, Switzerland), previously saturated with mobile phase for 15 min at room temperature (25±2°C) and relative humidity of 60±5%. The development includes band space of 10 mm, chromatogram run of 8 cm, 20 ml of mobile phase and time duration of 10 minutes. The mobile phase was *n*-hexane: acetone: ethylacetate (6.5: 3.5: 0.1 v/v/v) with densitometric analysis at 247 nm in absorbance mode with *Camag* TLC scanner III, using deuterium and tungsten lamp operated by *winCATS* software (Version 1.2.0).

2.3. Stock solution and QC samples

A stock solution of IVM (1000 ng μL^{-1}) was prepared in methanol. Different volumes of stock solution ranging from 0.1-5 μL^{-1} were spotted in triplicate on TLC plate to obtain final concentration range of 100-5000 ng $spot^{-1}$. The data of peak areas plotted against corresponding concentration were treated by linear-square regression. QC samples were prepared at concentration of 500 and 1000 ng $spot^{-1}$.

2.4. Method validation

2.4.1. Precision and accuracy

The intra-day precision and accuracy of the assays was evaluated by performing replicate analyses (n=6) of QC samples (500 and 1000 ng $spot^{-1}$). The inter-day precision and accuracy of the assay was determined by repeating the intra-day assay on three

different days. Precision was expressed as the percentage coefficient variation (CV, %) of measured concentrations for each calibration level whereas accuracy was expressed as percent recovery [(IVM found / IVM applied) ×100].

2.4.2. 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 of *n*-hexane: acetone: ethylacetate (6.25: 3.75: 0.1 and 6.75: 3.25: 0.1 v/v/v) was tried for two QC samples (500 and 1000 ng spot^{-1}).

2.4.3. Sensitivity and linearity

In order to estimate detection (LOD) and quantification (LOQ) limits, blank methanol was spotted six times following the same method as explained under the section 2.2 (apparatus and chromatographic conditions) and the standard deviation (σ) of the magnitude of analytical response was determined. The LOD was expressed as 3.3σ/slope of IVM calibration curve, whereas LOQ was expressed as 10σ/slope of IVM calibration curve.

2.4.4. Recovery studies

Recovery studies for the proposed method were carried out by applying the method to drug samples to which known amount of IVM corresponding to 50, 100 and 150% of the IVM label claim had been added. At each level of the amount, six determinations were performed. This was done to check for the recovery of the drug at different levels in the formulations.

2.5 Evaluation of IVM-loaded nanoparticulate formulation (nano-IVM)

2.5.1 Preparation of nanoparticles

Polymeric nanoparticles of ivermectin (IVM-NPs) were synthesized by using a cross copolymers of N-isopropylacrylamide (NIPAAM) with N-vinyl-2-pyrrolidone (VP) and polyethyleneglycol monoacrylate (PEG-A) with the aid of Spectrapore® membrane dialysis bag (12 kD cut off) yielding >85% [25]. Dyanamic light scattering studies (DLS) and high resolution transmission electron microscopy (HR-TEM) of IVM-NPs confirms mean particle size of 120 nm with low polydispersity index (*PI*<1).

2.5.2 IVM content determination

Twenty milligrams of prepared polymeric nanoparticles was added to 100 mL organic solvent. After sonication for 30 min, the resulting solution was centrifuged at 15000 rpm for 20 min and supernatant was analyzed for drug content. Five microliter (500 ng spot^{-1}) of the above solution was spotted onto the plate followed by development and scanning as described in Section 2.2. The analysis was repeated in triplicate. The drug loading capacity and encapsulation efficiency of the nanoparticles were calculated according to the following equations:

Safranal loading (%) = (Safranal encapsulated in nanoparticles / Nanoparticle weight) x 100

Safranal encapsulation (%) = (Safranal encapsulated in nanoparticles / Total safranal) x 100

The possibility of excipient interference in the analysis was also studied.

2.6. Forced degradation studies

A stock solution containing 50 mg of IVM and powdered polymeric nanoparticles (IVM-NPs) equivalent to 50 mg of IVM were separately prepared in 50 mL methanol. The stock solution (1000 µg mL^{-1}) was used for forced degradation to provide an indication of the stability indicating property and specificity of the proposed method.

2.6.1. Acid and base induced degradation

To five milliliters of methanolic stock solutions (IVM and IVM-NPs), 5 mL of 2N HCl and 2N NaOH was added and the resulting mixtures were refluxed for 3 h at 80°C in the dark in order to exclude the possible degradative effect of light. Two microliter (1000 ng spot^{-1}) of the resultant solutions were applied on the TLC plate and the chromatograms were run as described in Section 2.2. The average peak area for

IVM after application (1000 ng spot^{-1}) of six replicates was obtained.

2.6.2. Hydrogen peroxide induced degradation

Again, to 5 mL of methanolic stock solutions of (IVM and IVM-NPs) equivalent to 50 mg of IVM, 5 mL of hydrogen peroxide (H_2O_2; 30.0%, v/v) were added separately. The solutions were heated in boiling water bath for 20 min and then refluxed for 3 h at 80°C to remove excess of hydrogen peroxide. 2 µL (1000 ng spot^{-1}) of the resultant solutions were applied on the TLC plate and the chromatograms were run as described in Section 2.2. The average peak area for IVM after application (1000 ng spot^{-1}) of six replicates was obtained.

2.6.3. Photochemical induced degradation

To 5 mL of methanolic stock solutions of (IVM and IVM-NPs) equivalent to 50 mg of IVM, 5 mL of methanol was added and solution was exposed to direct sunlight for 3 days (from 09:00 to 17:00 h at 30°C, total 24 h) and UV irradiation at 254 nm for 8 h in a UV chamber. 2 µL (1000 ng spot^{-1}) of the resultant solutions were applied on the TLC plate and the chromatograms were run as described in Section 2.2. The average peak area for IVM after application (1000 ng spot^{-1}) of six replicates was obtained same as previous degradation studies.

2.7. Detection of related impurities

The related unknown impurities were precisely determined by spotting some higher concentration of drug. IVM solution was prepared at concentration of 1000 µg mL^{-1} in methanol; and this solution was specified as "sample solution". One milliliter of the sample solution was diluted to 5 mL of methanol termed as "standard solution" (200 µg mL^{-1}). Two microlitres of the sample solution (2000 ng spot^{-1}) and 2 µL of the standard solution (400 ng spot^{-1}) were gently spotted on TLC plate in triplicates (n=3) and chromatography was performed as previously discussed under section 2.2 ("Apparatus and chromatographic conditions").

3. Results and discussion

3.1. Mobile phase optimization

TLC procedure was optimized with a view to develop a stability-indicating assay method. Initially, *n*-hexane and acetone in varying ratios was tried and later on ethylacetate was added for compactness of spots. The mobile phase *n*-hexane: acetone: ethylacetate (6.75: 3.25: 0.1 v/v/v) gave good resolution with R_f value of 0.26 for IVM but typical peak nature was missing. Finally, the mobile phase consisting of *n*-hexane: acetone: ethylacetate (6.5: 3.5: 0.1 v/v/v) gave a sharp and well-defined peak at R_f value of 0.26 (Fig. 2).

3.3. Method validation

3.3.1. Precision and accuracy

Table 1 summarizes the intra- and inter-day precision, as coefficient of variation (CV, %) and accuracy of the assay determined at IVM concentration of 500 and 1000 ng spot^{-1}. The intra-day precision (n = 6) was ≤ 1.78%. The inter-day precision over three different days was ≤ 2.21%. The intra-day and inter-day accuracy were in the range of 99.26–101.2 and 98.72–99.25% respectively. The repeatability of the method was studied by assaying six samples of prepared polymeric nanoparticles at same concentration under the same experimental conditions and the coefficient of variation was found to be 0.98. The values were within the acceptable range and the method was accurate, reliable and reproducible.

3.3.2. Robustness of the method

Table 2 describes the robustness of the proposed method. The % RSD of the peak areas was calculated for the change in mobile phase composition at concentration level of 500 and 1000 ng spot^{-1} in triplicate. The low values of % RSD (<2.0) obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method. There was no significant variation in the slope values (ANOVA; P>0.05).

3.3.3. Linearity and sensitivity

Under the described chromatographic conditions, IVM was well resolved at R_f 0.26±0.02. The linear regression data for the calibration curves of IVM (n = 6) showed a good linear relationship (correlation coefficient, r^2 = 0.9989±0.0012) over the concentration range 100–5000 ng spot^{-1} with respect to the peak area. The coefficient of variations of slope for IVM was found lower than 5%, which indicates a high precision of the assay. LOD and LOQ determined by the standard deviation method were found to be 8.22 and 24.9 ng spot^{-1} respectively, indicating adequate assay sensitivity of the proposed method.

3.3.4. Recovery studies

The proposed method when used for estimation of IVM after spiking with 50, 100 and 150% of additional drug afforded recovery ranging from 98.25–100.16% for polymeric nanoparticles (IVM-NPs) as listed in Table 3. The R.S.D. of recovery IVM-NPs was in the range 1.45–1.94. The absence of interference peaks of degradation products, impurities and excipients indicate specificity of the method.

3.3.5. Analysis of the prepared polymeric nanoparticles

A single spot at R_f 0.26 was observed in the chromatogram of the IVM-NPs extracted samples. There was no interference from the polymers present in formulation. The IVM content was found to be 98.95% with a %RSD of 0.93. It may therefore be inferred that degradation of IVM had not occurred in the formulations that were analyzed by this method. The low %RSD value indicated the suitability of this method for routine analysis of IVM in pharmaceutical dosage forms.

3.5. Forced degradation studies

The chromatograms of samples degraded with stress conditions of acid, base. The peak heights showed well-separated spots of the pure drugs as well as some additional peaks at different R_f values. The spots of the degraded products were well resolved from the drug spots as shown in Fig. 3-12. The peak of IVM was not significantly shifted in the presence of degradation peaks, affording stability indication property of the proposed method. The number of the degradation products with their corresponding R_f values are listed in Table 4.

3.6. Stability indicating property

The chromatograms of the samples treated with acid, base, hydrogen peroxide, photochemical and UV_{254} light showed well separated spots of pure ivermectin as well as some additional peaks at different R_f values. The degradants identification was based on the comparison of UV spectra of stressed samples with that of the standard solution. The number of degradation products with their corresponding R_f values, contents of IVM remained and percentage recovery were calculated and listed in Table 4.

3.6.1. Acid and Base induced degradation products

Drug recovery at the level of 83.53% and 77.70% from the acid stressed samples respectively for IVM and IVM-NPs (Fig. 3 and 4), suggests significant (P<0.05) degradation of IVM in acidic conditions (Table 4). However drug recovery from base stressed sample for both IVM and IVM-NPs (Fig. 5 and 6) afforded zero signifying complete degradation of IVM in alkaline media. The chromatogram of acid degraded samples of IVM and IVM-NPs showed four additional peaks at R_f values of 0.16, 0.20, 0.32, 0.36 (Fig. 3) and 0.16, 0.20, 0.36, 0.47 (Fig. 4) respectively. Conversely, no chromatograms of base degraded samples appeared when subjected to alkaline degradation of IVM as well as IVM-NPs (Fig. 5 and 6). Therefore it may conclude that the drug can little afford acidic exposure but cannot afford alkaline conditions.

3.6.2. Hydrogen peroxide induced degradation products

The chromatogram of the sample of IVM and IVM-NPs (Fig. 7 and 8) treated with 30% (v/v) H_2O_2 showed two additional peaks other than ivermectin

peak at Rf values of 0.08, 0.13 for IVM and three additional peaks for IVM-NPs at R_f 0.08, 0.17, 0.31 (Table 4) conferring ivermectin is susceptible towards the oxidation induced degradation. The drug recovery was afforded in range of 55.02% and 48.22% respectively for IVM and IVM-NPs, suggested it's significant (P<0.05) oxidation in hydrogen peroxide.

3.6.2. Photochemical degradation products

Drug recovery at the level of 48.22% and 39.15% (Fig. 9 and 10) for the UV_{254} nm exposed samples respectively for IVM and IVM-NPs suggests significant (P<0.05) degradation of IVM in these conditions. However when exposed to sunlight drug recovery levels 64.65% and 61.63% (Fig. 11 and 12) respectively for IVM as well as IVM-NPs, reveals its susceptibility towards UV irradiation than sunlight degradation. The UV light treated IVM samples provided seven additional peaks at R_f values of 0.09, 0.11, 0.16, 0.33, 0.38, 0.70, 0.72 (Fig. 9) and eight additional peaks for IVM-NPs at R_f 0.07, 0.11, 0.17, 0.33, 0.37, 0.40, 0.47, 0.52 (Fig. 10, Table 4). Whereas the sunlight degraded samples showed three additional peaks at R_f values of 0.08, 0.52, 0.53 for IVM (Fig. 11)and R_f 0.18, 0.22, 0.51 for IVM-NPs (Fig. 12, Table 4).

3.7. Detection of related impurities

The spots other than the principal spot for IVM from the sample solution were not more intense than the principal spot from the standard solution (Table 5). The sample solution showed three additional spots at R_f values of 0.11, 0.16 and 0.39 (Fig. 13) for ivermectin. However the area of the additional spots was found significantly smaller than the spot area of IVM from the standard solution. The impurities were well resolved from the drug and the degradants and therefore, they will not interfere with the analysis of IVM by developed HPTLC method of stability indicating standard.

4. Conclusion

The developed HPTLC technique is precise, specific, accurate and stability indicating. Statistical analysis proves that the method is repeatable and selective for the analysis of ivermectin as bulk drug and in pharmaceutical formulations, including nanoparticles. The method can be used to determine the purity of the drug available from various sources by detecting the related impurities. It may be extended to study the degradation kinetics of ivermectin and for its estimation in plasma and other biological fluids. As the method separates the drug from its degradation products, it can be employed as a stability indicating one.

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Table 1: Precision and accuracy of HPTLC method for IVM

Nominal concentration (ng spot^{-1})	Concentration found[a] (ng spot^{-1})	Precision[b] (CV, %)	Accuracy[c] (%)
	Intra-day		
500.0	496.3	1.78	99.26
1000.0	1012.0	1.41	101.2
	Inter-day		
500.0	493.6	2.21	98.72
1000.0	992.5	1.73	99.25

[a] Mean of six determinations (n = 6)
[b] Precision as coefficient of variation (CV, %) = standard deviation divided by concentration found × 100
[c] Accuracy = concentration found / nominal concentration × 100

Table 2: Method robustness with varying mobile phase composition[a]

S. No	Amount (ng spot^{-1})	Mobile phase composition	
		n-Hexane: acetone: ethylacetate (6.25: 3.75: 0.1 v/v/v) (%RSD)	*n*-Hexane: acetone: ethylacetate 6.75: 3.25: 0.1 v/v/v) (%RSD)
1	500	1.81	0.93
2	1000	0.68	1.15

[a] Mean of three determinations (n = 3)

Table 3: Recovery studies of IVM from samples with known concentration

Formulation	Theoretical (mg[a])	Added (mg)	Detected[b] (mg)	Recovery[b] (%)	R.S.D. (%)
Ivermectin nanoparticles (IVM-NPs)					
50 %	10	5.0	14.76	98.41	1.45
100 %		10	19.65	98.25	1.94
150 %		15	25.04	100.16	1.60

[a] Units: mg in nanoparticles
[b] Mean of six determinations (n = 6)

Table 4: Forced degradation of ivermectin and ivermectin polymeric nanoparticles

S. No.	Exposure conditions	No. of degradation products (R_f value)	Ivermectin remained (ng/1000ng) (±S.D., n=6)	Recovery (%)	Fig. No.
1	IVM- Acid, 2N HCl	4 (0.16, 0.20, 0.32, 0.36)	835.32 (± 1.65)	83.53	3
2	IVM-NPs- Acid, 2N HCL	4 (0.16, 0.20, 0.36, 0.47)	777.08 (± 2.23)	77.70	4
3	IVM - Base, 2N NaOH	0	Not detectable	0	5
4	IVM-NPs- Base, 2N NaOH	0	Not detectable	0	6
5	IVM - H_2O_2, 30% v/v	2 (0.08, 0.13)	638.34	63.34	7
6	IVM-NPs - H_2O_2, 30% v/v	3 (0.08, 0.17, 0.31)	550.23	55.02	8
7	IVM - UV_{254} nm	7 (0.09, 0.11, 0.16, 0.33, 0.38, 0.70, 0.72)	482.24	48.22	9
8	IVM-NPs - UV_{254} nm	8 (0.07, 0.11, 0.17, 0.33, 0.37, 0.40, 0.47, 0.52)	391.59	39.15	10
9	IVM -day light	3 (0.08, 0.52, 0.53)	646.59	64.65	11
10	IVM-NPs - day light	3 (0.18, 0.22, 0.51)	612.34	61.23	12

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