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Application of stability-indicating HPTLC method for quantitative determination of metadoxine in pharmaceutical dosage form

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Received 27 July 2003; revised and accepted 3 January 2005

Available online 09 March 2005

Abstract

A sensitive, selective, precise and stability-indicating high-performance thin-layer chromatographic method for analysis of metadoxine both as a bulk drug and in formulations was developed and validated. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of acetone–chloroform–methanol–ammonia (7.0: 4.0: 3.0: 1.2, v/v/v/v). Densitometric analysis of metadoxine was carried out in the absorbance mode at 315 nm. This system was found to give compact spots for metadoxine (R_f value of 0.45 ± 0.02, for six replicates). Metadoxine was subjected to acid, alkali and neutral hydrolysis, oxidation, dry and wet heat treatment and photo and UV degradation. The drug undergoes degradation under all stress conditions. Also, the degraded products were well resolved from the pure drug with significantly different R_f values. The method was validated for linearity, precision, robustness, LOD, LOQ, specificity and accuracy. Linearity was found to be in the range of 100–1500 ng/spot with significantly high value of correlation coefficient $r^2 = 0.9997 \pm 1.02$. The linear regression analysis data for the calibration plots showed good linear relationship with $r^2 = 0.9999 \pm 0.58$ in the working concentration range of 200–700 ng/spot. The mean value of slope and intercept were 0.11 ± 0.04 and 18.73 ± 1.89, respectively. The limits of detection and quantitation were 50 and 100 ng/spot, respectively. Statistical analysis proves that the method is repeatable and specific for the estimation of the said drug. As the method could effectively separate the drug from its degradation products, it can be employed as a stability-indicating one. Moreover, the proposed HPTLC method was utilized to investigate the kinetics of acid and base degradation process. Arrhenius plot was constructed and activation energy was calculated respectively for acid and base degradation process.

Keywords: Metadoxine; HPTLC; Stability indicating; Method validation; Quantitative analysis; Degradation kinetics; Arrhenius plot; Activation energy

1. Introduction

Metadoxine, pyridoxine L-2-pyrrolidone-5-carboxylate (Fig. 1) is an ion pair that combines pyridoxine (vitamin B6) and pyrrolidone carboxylate (or pyroglutamate, that is intermediate in the synthesis/degradation of glutathione). Both pyridoxine and pyroglutamate are well-known agents that are safe, naturally occurring substances, present in food, and are useful in the treatment of alcoholics and alcoholic liver disease [1–4]. Metadoxine exerts several actions that are beneficial to patients with alcoholic liver disease. It increases the clearance of alcohol and acetaldehyde, reduces the damaging effect of free radicals, restores ATP and glutathione levels, reduces steatosis and prevents liver fibrosis [5–10]. After thorough survey of literature, there is not a single method reported for the analysis of metadoxine. Consequently, the implementation of an analytic methodology to determine metadoxine in pharmaceutical dosage form in presence of its degradation products is a pending challenge of the pharmaceutical analysis.

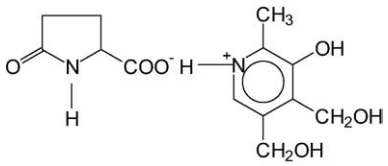

Fig. 1. Chemical structure of metadoxine.

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doi:10.1016/j.farmac.2005.01.001

To our knowledge, no article related to the stability-indicating high-performance thin-layer chromatography (HPTLC) determination of metadoxine in pharmaceutical dosage forms has ever been mentioned in literature. The international conference on harmonization (ICH) guideline entitled *Stability Testing of New Drug Substances and Products* requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance [11]. Susceptibility to oxidation is one of the required tests. Also, the hydrolytic and the photolytic stability are required. An ideal stability-indicating method is one that quantifies the standard drug alone and also resolves its degradation products. Katalin Ferenczi-Fodor et al. [12,13] explained basic acceptance criteria for evaluation of validation experiments based on practical experience for planar chromatographic procedures, which may be used at different levels either in qualitative identity testing, assays, semi quantitative limit tests or quantitative determination of impurities. The parameters for robustness testing of given procedures and quality assurance of quantitative planar chromatographic testing have been described as per ICH guidelines. According to the European pharmacopoeia [14], the profile of the impurities has been defined in relation to the sources of drug identified. The impurities detected by HPTLC are limited to 0.1%. The limits for these impurities have been fixed at the minimum level permitted by the analytical method in accordance with the requirements laid down in system conformity.

Now-a-days HPTLC is becoming a routine analytical technique due to its advantages of low operating cost, high sample throughput and need for minimum sample clean up. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis.

The aim of the present work is to develop an accurate, specific, repeatable and stability-indicating HPTLC method for the determination of metadoxine in presence of its degradation products and related impurities for assessment of purity of bulk drug and stability of its bulk dosage forms. The proposed method was validated as per ICH guidelines [15,16] and its updated international convention [17]. Acid- and base-induced degradation kinetics was investigated by quantitation of drug by validated stability-indicating HPTLC method.

2. Experimental

2.1. Materials

Pharmaceutical grade of metadoxine (batch no: S 02-614) was kindly supplied as a gift sample by Sun Pharma Ltd, Baroda, India, used without further purification and certified to contain 99.72% (w/w) on dried basis. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India.

2.2. HPTLC instrumentation

The samples were spotted in the form of bands of width 6 mm with a Camag microlitre syringe on precoated silica gel aluminium Plate 60 F-254, (20 × 10 cm with 250 μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai, India) using a Camag Linomat IV (Muttenz, Switzerland). The plates were prewashed by methanol and activated at 100 °C for 5 min prior to chromatography. A constant application rate of 0.1 μl/s was employed and space between two bands was 5 mm. The slit dimension was kept at 5 × 0.45 mm and 10 mm/s scanning speed was employed. The monochromatic bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of acetone–chloroform–methanol–ammonia (7.0: 4.0: 3.0: 1.2, v/v/v/v) and 15 ml of mobile phase were used per chromatography. Linear ascending development was carried out in 20 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) and the top of chamber was covered tightly with the lid. It was saturated (lined on the two bigger sides with saturation pads that had been soaked thoroughly with the mobile phase) and the chromatoplate development was carried out in dark. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 °C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 9 cm. The run length of 9 cm results in better apparent resolution with more convenient capability of the detecting device to perform integration of peak area. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer in wooden chamber with adequate ventilation. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust). Densitometric scanning was performed on Camag TLC scanner III in the reflectance–absorbance mode at 315 nm and operated by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was via peak areas with linear regression.

2.3. Calibration curves of metadoxine

Stock standard solution of metadoxine was prepared in methanol at 10 mg/ml. Standard solutions were prepared by dilution of the stock solution with methanol to give solutions containing metadoxine in concentration range of 100–1500 μg/ml. One microlitre from each standard solution was spotted on the TLC plate to obtain final concentration range of 100–1500 ng/spot. Each concentration was spotted six times on the TLC plate.

2.4. Method validation

2.4.1. Precision

Precision of the method was determined with the product. An amount of the product powder equivalent to 100% of the label claim of metadoxine was accurately weighed and assayed. System intra-day repeatability was determined by

six replicate applications and six times measurement of a sample solution at the analytical concentrations of 300, 500 and 700 ng/spot, respectively. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of % RSD (relative standard deviation) and SE (standard error) and found to be less than 2%. Intermediate precision was assessed by the assay of two sets of six samples on different days (inter-day precision). The intra- and inter-day variation for determination of metadoxine was carried out at three different concentration levels 300, 500 and 700 ng/spot, respectively.

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 like acetone–chloroform–methanol–ammonia (7.2: 3.8: 3.0: 1.2, v/v/v/v), (6.8: 4.2: 3.0: 1.2, v/v/v/v), (7.0: 3.8: 3.2: 1.2, v/v/v/v), (7.0: 4.2: 2.8: 1.2, v/v/v/v), (7.0: 4.0: 3.2: 1.0, v/v/v/v) and (7.0: 4.0: 2.8: 1.4, v/v/v/v) were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ± 5%. The plates were prewashed by methanol and activated at 60 °C ± 5 for 2, 5, 7 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. Robustness of the method was done at three different concentration levels 300, 500 and 700 ng/spot.

2.4.3. Limit of detection and limit of quantitation

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times following the same method as explained in Section 2.2. The signal-to-noise ratio was determined. An LOD was considered as 3:1 and LOQ as 10:1. The LOD and LOQ were experimentally verified by diluting known concentrations of metadoxine until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

2.4.4. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for metadoxine in sample was confirmed by comparing the R_f and spectra of the spot with that of standard. The peak purity of metadoxine was assessed by comparing the spectra at three different levels i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot.

2.4.5. Accuracy

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

2.5. Analysis of the marketed formulation

To determine the content of metadoxine in tablets (label claim: 500 mg/tablets), the contents of 20 tablets were weighed, their mean weight determined and finely powdered. An equivalent weight of the drug was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 30 min and diluted to 100 ml with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and supernatant was analyzed for drug content (5000 μg/ml). One microlitre of the above filtered solution was further diluted to produce 500 μg/ml solution. One microlitre of the above solution (500 ng/spot) was applied on the TLC plate followed by development and scanning as described in Section 2.2. The analysis was repeated in triplicate. The possibility of excipient interference in the analysis was studied.

2.6. Forced degradation of metadoxine

A stock solution containing 100 mg metadoxine in 100 ml methanol was prepared. This solution was used for forced degradation to provide an indication of the stability-indicating property and specificity of proposed method. In all degradation studies, the average peak area of metadoxine after application (1000 ng/spot) of seven replicates was obtained.

2.6.1. Preparation of acid- and base-induced degradation product

To 15 ml of methanolic stock solution, 15 ml each of conc. HCl and 10 N NaOH were added separately. These mixtures were refluxed for 2.0 h at 70 °C. To study the decomposition of drug in phosphate buffer pH 7.4, 15 ml of buffer solution were added to 15 ml of methanolic stock solution. It was refluxed at 70 °C for 6.0 h. The forced degradation in acidic and basic media was performed in the dark in order to exclude the possible degradative effect of light. Two microlitres of the resultant solutions (1000 ng/spot) were applied on TLC plate and the chromatograms were run as described in Section 2.2.

2.6.2. Preparation of hydrogen peroxide-induced degradation product

To 15 ml of methanolic stock solution, 15 ml of 50.0% w/v hydrogen peroxide were added. The solution was heated in boiling water bath for 10 min to remove completely the excess of hydrogen peroxide and then refluxed for 2.0 h at 70 °C. Two microlitres of the resultant solution (1000 ng/spot) were applied on TLC plate and the chromatograms were run as described in Section 2.2.

2.6.3. Dry heat and wet heat degradation product

The standard drug was placed in oven at 100 °C for 7 days to study dry heat degradation, and the stock solution was refluxed for 8.0 h on boiling water bath for wet heat degradation.

2.6.4. Photochemical and UV degradation product

The photochemical stability of the drug was also studied by exposing the stock solution (1 mg/ml) to direct sunlight for 3 days on a wooden plank and kept on terrace. The drug solution was also exposed to UV irradiation for 15 days in UV stability chamber. One microlitre of each solution (1000 ng/spot) was applied on TLC plate and chromatograms were run as described in Section 2.2.

2.6.5. Neutral hydrolysis

To 10 ml of methanolic stock solution, 10 ml double distilled water were added and the mixture was refluxed for 6.0 h at 70 °C to study the degradation under neutral conditions.

2.7. Detection of the related impurities

The related impurities were determined by spotting higher concentrations of the drug so as to detect and quantify them. Metadoxine (2000 mg) was dissolved in 100 ml of methanol, and this solution was termed as sample solution (20 mg/ml). One millilitre of the sample solution was diluted to 100 ml with methanol and this solution was termed as standard solution (0.2 mg/ml). One microlitre of both the standard (200 ng/spot) and the sample solution (20,000 ng/spot) was applied on TLC plate and the chromatograms were run as described in Section 2.2.

2.8. Study of acid- and base-induced degradation kinetics

Accurately weighed 100 mg of drug were dissolved in 100 ml methanol. Separate 15 ml aliquots of this standard solution were transferred into separate 100 ml of double neck round bottom flask and mixed respectively with 15.0 ml of conc. HCl and 10.0 N NaOH to get final concentration of 500 μg/ml. The flasks were refluxed at different temperatures (40, 50, 60, 70, 80 and 90 °C) for acidic and basic degradation for different time intervals. At specified time intervals, the contents of the flask (100 μl) were quantitatively transferred to 10 ml volumetric flasks with the help of microsyringe. Then 2 μl were spotted to get the final concentration of 1000 ng/spot and estimated by HPTLC method by one point standardization using external standard. The experiment was carried out in triplicate. The concentration of the remaining drug was calculated for each temperature and time interval. Data were further processed and degradation kinetics constants were calculated.

3. Results and discussion

3.1. Development of the optimum mobile phase

The TLC procedure was optimized with a view to develop a stability-indicating assay method. Both the pure drug and the degraded drug solution were spotted on the TLC plates and run in different solvent systems. Initially, acetone–chloroform–methanol in varying ratios was tried. The mobile phase acetone–chloroform–methanol (7.0: 4.0: 3.0, v/v/v) gave good resolution but R_f value for metadoxine was less. Also, the typical peak nature was missing because the spot for metadoxine was slightly diffused. Addition of 1.2 ml of conc. ammonia solution to the above mobile phase improved the spot characteristics and increased the R_f value to 0.45 ± 0.02 when densitometric scanning was performed at 305 nm. Finally, the mobile phase consisting of acetone–chloroform–methanol–ammonia (7.0: 4.0: 3.0: 1.2, v/v/v/v) gave a sharp and symmetrical peak. Resolution between spots of standard and degradates appeared better when TLC plates (pretreated with methanol and activated at 100 °C for 5 min) were saturated with conc. ammonia vapors for 30 min in TLC chamber prior to application. Well-defined spots (compact dense spots) were obtained when the chamber was saturated with the mobile phase for 30 min at room temperature (Fig. 2). It was required to eliminate the edge effect and to avoid unequal solvent evaporation losses from the developing plate that can lead to various types of random behavior usually resulting in generally lack of reproducibility in R_f values.

3.2. Validation of the method

3.2.1. Linearity

With HPTLC, the analyte interacts with the layer surface of the stationary phase where scattering and absorption tend to take place, especially with high concentrations of analyte [18]. Beer-Lambert law does not adequately describe this process, but the Kubelka Munk model [19], which relies on the idea that lights, is travelling in all directions simultaneously within the precoated TLC plate. This is approximated as a flux of light travelling upwards and a flux travelling downwards at any depth in the plate. When this flux passes through a thin layer of material, some of it passes through, some of it is scattered backwards and some of it is absorbed. Calibration graph was found to be linear that is adherence of the system to Kubelka Munk theory, which was found over the concentration range of 100–1500 ng/spot (r^2 ± SD = 0.9997 ± 1.02). Linearity was evaluated by determining six standard working solutions containing 200–700 μg/ml of metadoxine in triplicate. Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data showed a good linear relationship over the low concentration range of 200–700 ng/spot (r^2 = 0.9999 ± 0.58, slope = 0.11 ± 0.04, intercept = 18.73 ± 1.89). The linearity of calibration graphs and adherence of the system to Kubelka Munk theory were validated by high value of correlation coefficient and the SD for intercept value was less than 2. No significant difference was observed in the slopes of standard curves (ANOVA; $p < 0.05$).

3.2.2. Precision

The repeatability of sample application and measurement of peak area were expressed in terms of % RSD and found to

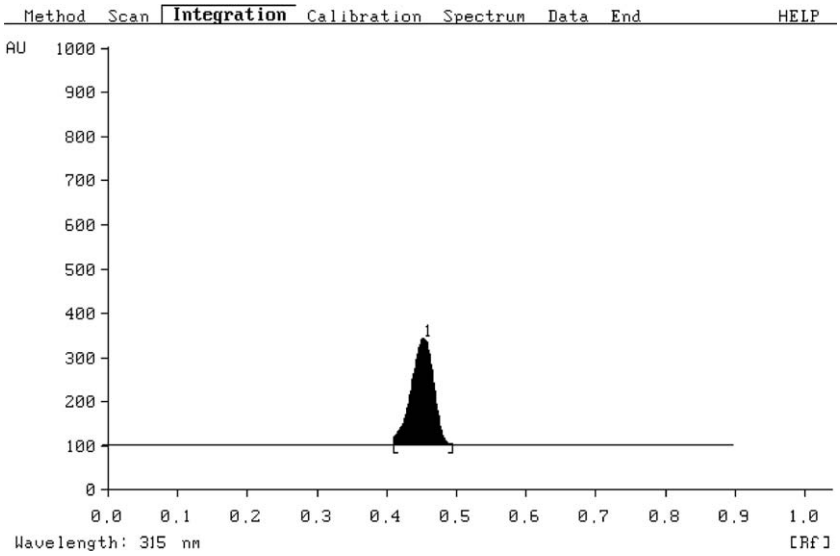

Fig. 2. Densitogram of standard metadoxine (1000 ng/spot); peak 1 (R_f: 0.45 ± 0.02), mobile phase acetone–chloroform–methanol–ammonia (7.0: 4.0: 3.0: 1.2, v/v/v/v).

be 1.54 and 1.22 respectively for six replicate determinations. The % RSD for intra- and inter-day variation of metadoxine peak area at three different concentration levels 300, 500 and 700 ng/spot is 1.21, 1.10, 1.24 and 2.41, 1.84, 1.78, respectively.

3.2.3. Robustness of the method

The standard deviation of peak areas was calculated for each parameter and RSD was found to be less than 2%. The low values of % RSD as shown in Table 1 indicated robustness of the method.

3.2.4. LOD and LOQ

The signal/noise ratios 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 50 ng/spot and 100 ng/spot, respectively.

3.2.5. Specificity

The peak purity of metadoxine was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot i.e., r^2 (S, M) = 0.9997 and r^2 (M, E) = 0.9998. Good correlation (r^2 = 0.9997) was also obtained between standard and sample spectra of metadoxine.

Table 1
Robustness testing[a]

Parameter	SD[b] of area	% RSD[c]
Mobile phase composition (± 0.1 ml)	1.58	1.34
Amount of mobile phase (± 5%)	1.47	1.20
Temperature (± 5%)	1.26	1.14
Relative humidity (± 5%)	1.54	1.29
Plate pretreatment (± 5 min)	1.05	0.98
Time from spotting to chromatography (± 20 min)	0.50	0.34
Time from chromatography to scanning (± 20 min)	1.01	0.92

[a] n = 6.
[b] = Standard deviation.
[c] = Relative standard deviation.

3.2.6. Accuracy

The proposed method when used for extraction and subsequent estimation of metadoxine from pharmaceutical dosage form after spiking with 50, 100 and 150% of additional drug afforded recovery of 99–101% as listed in Table 2.

The data of summary of validation parameters are listed in Table 3.

3.3. Stability in sample solution

Solutions of two different concentrations (500 and 700 ng/spot) were prepared from sample solution and stored at room temperature for 0.5, 1.0, 2.0, 4.0 and 24 h, respec-

Table 2
Accuracy[a]

Excess drug added to the analyte(%)	Theoretical content (ng)	Recovery (%)	RSD (%)
0	200	98.88	1.11
50	300	99.20	1.75
100	400	98.85	1.28
150	500	99.42	1.56

[a] n = 6.

Table 3
Summary of validation parameters

Parameter	Data
Linearity range	200–700 ng/spot
Correlation coefficient	0.9999 ± 0.58
Limit of detection	50 ng/spot
Limit of quantitation	100 ng/spot
Accuracy (n = 6)	99.09 ± 0.27
Precision (%RSD)	
Repeatability of application (n = 7)	1.54
Repeatability of measurement (n = 7)	1.22
Inter-day (n = 6)	2.01
Intra-day (n = 6)	1.18
Robustness	Robust
Specificity	0.9997

tively. They were then applied on the same TLC plate, after development the chromatogram was evaluated for additional spots if any. The % RSD of peak area at 500 and 700 ng/spot was found to be 1.14 and 1.54, respectively. There was no indication of compound instability in the sample solution.

3.3.1. Spot stability

The time the sample is left to stand on the solvent prior to chromatographic development can influence the stability of separated spots and is required to be investigated for validation [20]. Two-dimensional chromatography using same solvent system was used to find out any decomposition occurring during spotting and development. In case, if decomposition occurs during development, peak(s) of decomposition product(s) shall be obtained for the analyte both in the first and second direction of the run. No decomposition was observed during spotting and development.

3.4. Analysis of the marketed formulation

A single spot at R_f 0.45 was observed in the chromatogram of the drug samples extracted from tablets. There was no interference from the excipients commonly present in the tablet. The drug content was found to be 99.01% ± 1.56 with a % RSD of 0.94 for six replicate determinations. It may therefore be inferred that degradation of metadoxine had not occurred in the marketed formulations that were analyzed by this method. The good performance of the method indicated the suitability of this method for routine analysis of metadoxine in pharmaceutical dosage form.

3.5. Stability-indicating property

3.5.1. Acid- and base-induced degradation product

The chromatograms of the acid- and base-degraded samples for metadoxine showed additional peaks at R_f value of 0.10, 0.60, 0.75 and 0.28, respectively (Fig. 3). Degradation studies performed using phosphate buffer (pH 7.4) showed additional peak at 0.28. The concentration of the drug was found to be changing from the initial concentration indicating that metadoxine undergoes degradation under acidic and basic conditions.

3.5.2. Hydrogen peroxide-induced degradation product

The sample degraded with 50.0% w/v hydrogen peroxide showed additional peaks at R_f value of 0.24 and 0.56. The spots of degraded products were well resolved from the drug spot.

3.5.3. Dry heat and wet heat degradation product

The samples degraded under dry heat and wet heat conditions (Fig. 4) showed additional peaks at R_f values of 0.05, 0.14, 0.32, 0.60 and 0.68, respectively. The spots of degraded products were well resolved from the drug spot. Under wet heat conditions, the peak area of the parent drug was considerably decreased thus indicating the conversion of standard metadoxine to non-chromophoric compound.

3.5.4. Photochemical and UV degradation product

The photo degraded sample showed one additional peak at R_f 0.13 when drug solution was left in daylight for 3 days. The drug was degraded when exposed to UV irradiation for 15 days and showed additional peaks at R_f value of 0.10 and 0.52. The spot of UV degraded product was well resolved from the standard.

3.5.5. Neutral degradation

The HPTLC densitogram for neutral degradation showed decrease in peak area of standard without corresponding rise in new peak indicating the conversion of standard metadoxine to non-chromophoric compound.

This indicates that the drug is susceptible to acid–base hydrolysis, oxidation dry and wet heat degradation, photo and

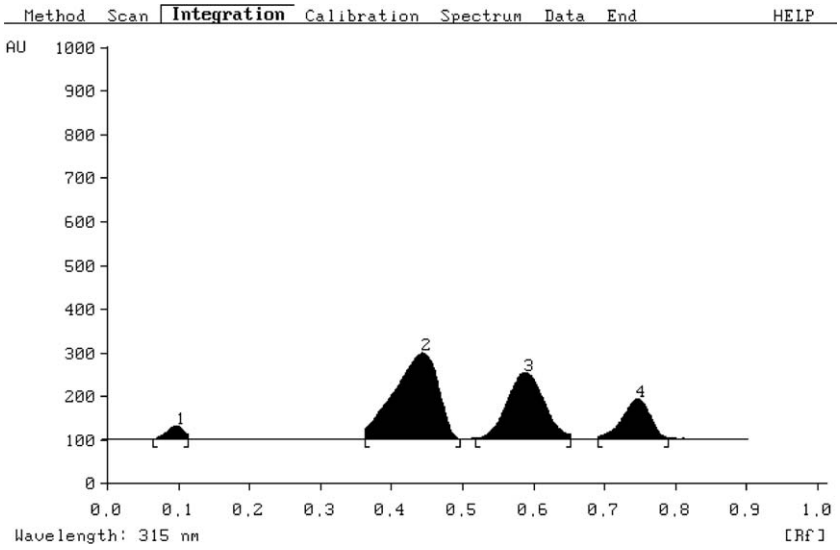

Fig. 3. Densitogram of acid (conc. HCl, reflux for 2.0 h, temp 70 °C) treated metadoxine; peak 1 (degraded) (R_f: 0.10), peak 2 (metadoxine) (R_f: 0.45), peak 3 (degraded) (R_f: 0.60), peak 4 (degraded) (R_f: 0.75).

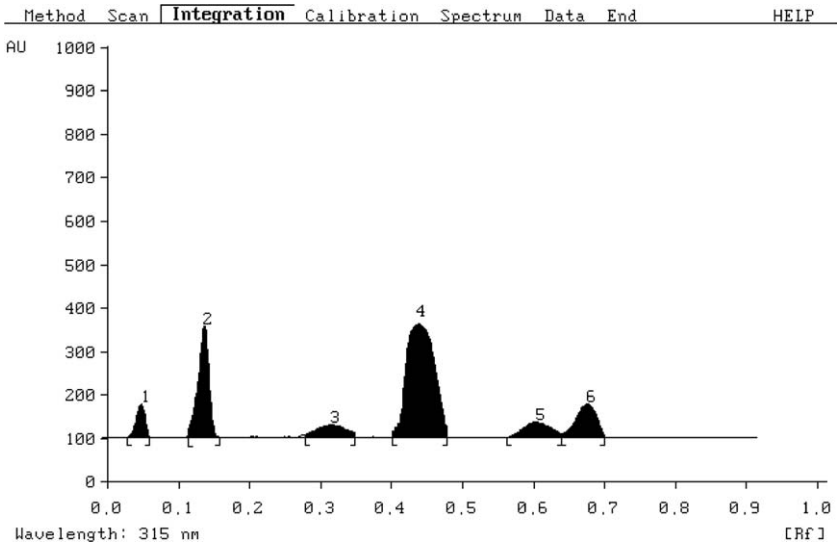

Fig. 4. Densitogram of dry heat degraded metadoxine (100 °C for 7 days); peak 1 (degraded) (R_f: 0.05), peak 2 (degraded) (R_f: 0.14), peak 3 (degraded) (R_f: 0.32), peak 4 (metadoxine) (R_f: 0.45), peak 5 (degraded) (R_f: 0.60), peak 6 (degraded) (R_f: 0.68).

Table 4
Degradation of metadoxine

Condition	Time (h)	% Recovery	R_f value of degradation products
Conc. HCl, ref (70 °C)[a]	2.0	47.31	0.10, 0.60, 0.75
10.0 N NaOH, ref	2.0	53.61	0.28
Phosphate buffer (pH 7.4)	6.0	22.81	0.28
H_2O_2 50% w/v, ref	2.0	48.05	0.24, 0.56
Dry Heat (100 °C)	168.0	41.74	0.05, 0.14, 0.32, 0.60, 0.68
Wet heat (100 °C)	8.0	25.91	—
Neutral	6.0	36.27	—
Daylight	72.0	2.07	0.13
UV	360.0	69.04	0.10, 0.52

[a] Refluxed.

UV degradation. The lower R_f values of base, buffer degraded, photo degraded, first component of acid, peroxide, UV degraded product, and first three components of dry heat degraded product indicated that they were more polar than the analyte itself. The R_f values of acid degraded products, dry heat degraded, second component of peroxide and UV degradation product that are greater than 0.45 indicated that they were less polar than the analyte itself. The results are listed in Table 4.

3.6. Detection of the related impurities

The spots other than the principal spot (metadoxine) from the sample solution (peak area = 56610.94) were not intense than the principal spot from the standard solution. The sample solution showed one additional spot at R_f 0.13 (Fig. 5). However, the peak area of the additional spot (445.88) was found

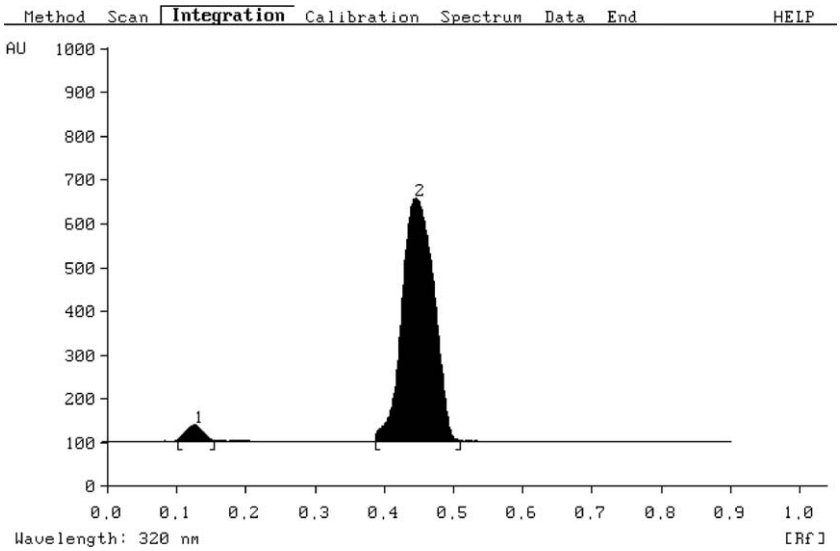

Fig. 5. Densitogram of metadoxine and its impurity; peak 1 (impurity) (R_f: 0.13), peak 2 (metadoxine) (R_f: 0.45).

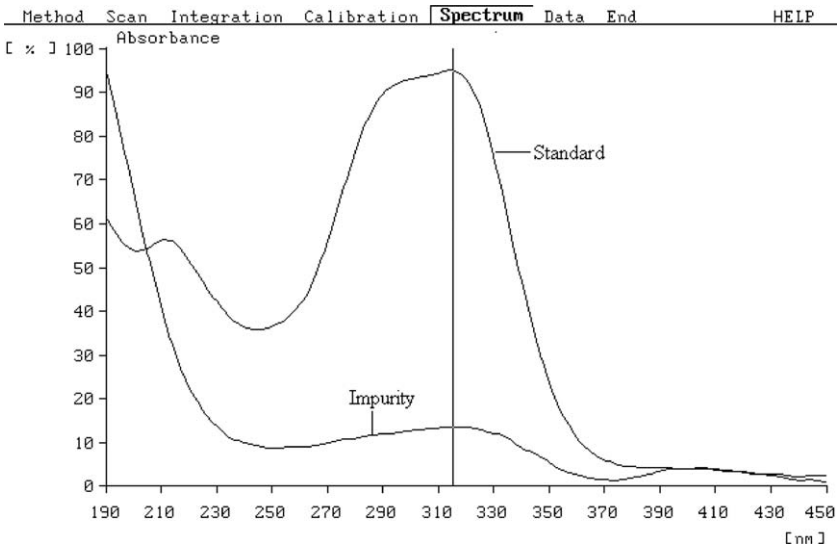

Fig. 6. In situ overlain spectrum of standard (λ_{max} = 315 nm) and its unknown impurity (λ_{max} = 320 nm) measured from 190 to 450 nm.

to be much less as compared to the peak area of principal spot (1467.54) from the standard solution. The λ_{max} of impurity was found to be 320 nm (Fig. 6).

From Table 4, it can be observed that the R_f values of photo degraded, first component of acid and UV degraded, and second component of dry heat degraded product closely matches with the R_f value of impurity present in the drug. Therefore, it might be possible that during processing or storage the drug may have undergone hydrolysis or oxidation to a little extend.

3.7. Degradation kinetics

The kinetic of degradation of metadoxine was investigated in conc. HCl and 10.0 N NaOH, since the decomposition rate of metadoxine at lower strength of HCl and NaOH was too slow to obtain reliable kinetic data. Each experiment was repeated three times at each temperature and time interval. The mean concentration of metadoxine was calculated for each experiment. A regular decrease in the concentration of metadoxine with increasing time intervals was observed. At the selected temperatures (40, 50, 60, 70, 80 and 90 °C for acidic and alkaline degradation), the degradation process followed pseudo first-order kinetic (Figs. 7 and 8). From the slopes of the straight lines, it was possible to calculate apparent first degradation rate constant, half life ($t_{1/2}$) and t_{90} (i.e., time where 90% of original concentration of the drug is left) at each temperature for acidic and alkaline degradation processes determined by HPTLC method (Table 5). Data obtained from first-order kinetics treatment were further subjected to fitting in Arrhenius equation:

$$\text{Log } K = \text{Log } A - \frac{Ea}{2.303\,RT} \quad (1)$$

Where K is rate constant, A is frequency factor, E_a is energy of activation (Cal mol^{-1}), R is gas constant (1.987 cal/deg mol) and T is absolute temperature (^oK). A plot of (2 + log K_{obs}) values versus ($1/T \times 10^3$) the Arrhenius plot was obtained (Fig. 9), which was found to be linear in the temp. range of 40–90 °C. The activation energy the Arrhenius frequency factor was calculated respectively for acidic and alkaline degradation processes determined by HPTLC method. The method of accelerating testing of pharmaceutical products based on principles of chemical kinetics was used to obtain a measure of the stability of the drug under said conditions [21,22]. The degradation rate constant at room temperature (K_{25}°) is obtained by extrapolating to 25 °C (where $1000/T = 3.356$) by inserting this into Eq. 1 and $t_{1/2}$ and t_{90} are calculated respectively for different stress conditions (Table 6).

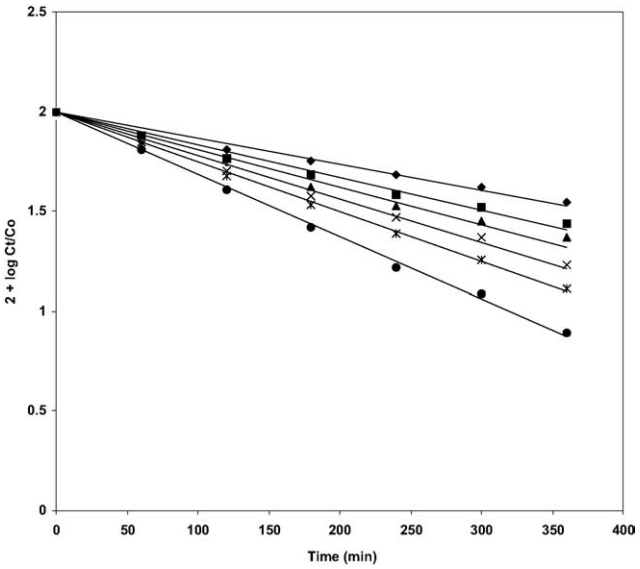

Fig. 7. Pseudo first-order plots for the degradation of metadoxine with conc. HCl at various temperatures using HPTLC method. Key: 90 °C (♦), 80 °C (■), 70 °C (▲), 60 °C (×), 50 °C (*), 40 °C (●), C_t, concentration at time t; C_0, concentration at time zero.

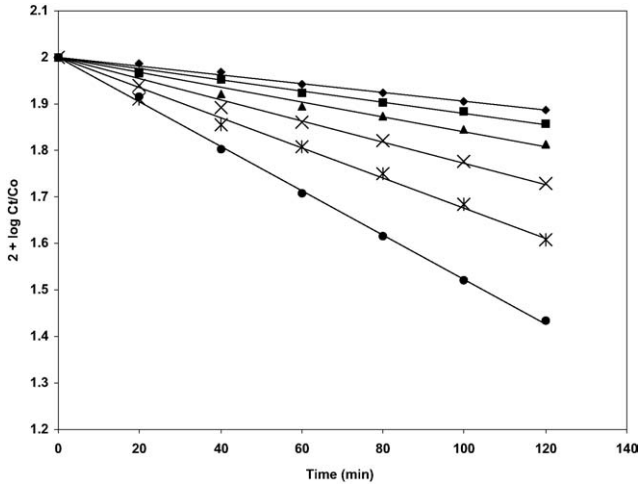

Fig. 8. Pseudo first-order plots for the degradation of metadoxine with 10.0 N NaOH at various temperatures using HPTLC method. Key: 90 °C (♦), 80 °C (■), 70 °C (▲), 60 °C (×), 50 °C (*), 40 °C (●), C_t, concentration at time t; C_0, concentration at time zero.

Table 5
Degradation rate constant (K_{obs}), half-life ($t_{1/2}$) and t_{90} for metadoxine in presence of conc. HCl, 10.0 N NaOH and 50% w/v H_2O_2 determined by HPLC method

Temperature (^{0}C)	K_{obs} (h^{-1})	$t_{1/2}$ (h)	t_{90} (h)
In conc. Hydrochloric acid			
40	0.0029	3.98	0.60
50	0.0037	3.12	0.47
60	0.0044	2.63	0.39
70	0.0050	2.31	0.35
80	0.0058	1.99	0.30
90	0.0071	1.63	0.25
In 10.0 N Sodium hydroxide			
40	0.0027	4.28	0.65
50	0.0038	3.04	0.46
60	0.0048	2.41	0.36
70	0.0059	1.96	0.29
80	0.0076	1.52	0.23
90	0.0097	1.19	0.18

4. Conclusion

Introducing HPTLC in pharmaceutical analysis represents a major step in terms of quality assurance. The developed HPTLC technique is precise, specific, accurate and stability indicating. Statistical analysis proves that the method is suitable for the analysis of metadoxine as bulk drug and in pharmaceutical formulations without any interference from the excipients. This study is a typical example of development of a stability-indicating assay, established following the recommendations of ICH guidelines. It is one of the rare studies where forced decomposition was done under all different suggested conditions and the degradation products were resolved. The method can be used to determine the purity of the drug available from various sources by detecting the related impurities. It is proposed for the analysis of the drug and degradation products in stability samples in industry. Further, it can be concluded that the impurity present in the drug

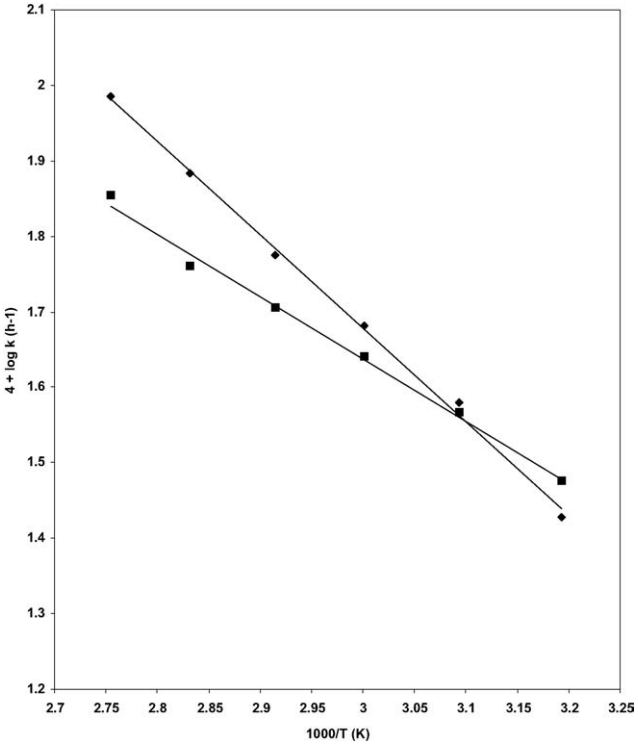

Fig. 9. Arrhenius plot for the degradation of metadoxine in presence of conc. HCl (■)and 10.0 N NaOH (♦).

Table 6
Summary of degradation kinetic data at 25 °C using HPLC method

Parameters	In conc. HCl	In 10.0 N NaOH
E_a(Kcal/deg mol)[a]	3.78×10^{-3}	5.64×10^{-3}
K_{25} (h^{-1})[b]	2.20×10^{-2}	1.74×10^{-2}
$t_{1/2}$ (h)[c]	31.50	39.00
t_{90} (h)[d]	4.77	6.03
A[e]	216	66.63

[a] = Activation energy.
[b] = Degradation rate constant.
[c] = Half-life.
[d] = Time left for 90% potency.
[e] = Arrhenius frequency factor.

could be due to hydrolysis or oxidation during processing and storage of the said drug. The above results showed the suitability of proposed method for acid- and base-induced degradation kinetic study of metadoxine. The degradation rate constant, half-life and t_{90} of metadoxine can be predicted for acid and base degradation process. It may be extended for quantitative estimation of said drug in plasma and other biological fluids. The method, however, is not suggested to establish material balance between the extent of drug decomposed and formation of degradation products. As the method separates the drug from its degradation products, it can be employed as a stability-indicating one.

Acknowledgements

The authors thank Dr. S. Bhowmick, General Manager, Sun Pharma Advanced Research Centre, Baroda, India for

providing gift sample of standard metadoxine. Authors are also thankful to Mr. Dilip Charegaonkar (Managing Director, Anchrom HPTLC specialists, Mumbai, India) for providing facilities during research work.

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