Quantitative Thin-Layer Chromatographic Method of Analysis of Azithromycin in Pure and Capsule Forms

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Abstract

A validated stability-indicating thin-layer chromatographic (TLC) method of the analysis of azithromycin (AZT) in bulk and capsule forms is developed. Both AZT potential impurity and degradation products can be selectively and accurately estimated in both raw material and product onto one precoated silica-gel TLC plate 60F254. The development system used is *n*-hexane–ethyl acetate–diethylamine (75:25:10, v/v/v). The separated bands are detected as brown to brownish red spots after spraying with modified Dragendorff's solution. The R_f values of AZT, azaerythromycin A, and the three degradation products are 0.54, 0.35, 0.40, 0.20, and 0.12, respectively. The optical densities of the separated spots are found to be linear in proportion to the amount used. The stress testing of AZT shows that azaerythromycin A is the major impurity and degradation product, accompanied by three other unknown degradation products. The stability of AZT is studied under accelerated conditions in order to provide a rapid indication of differences that might result from a change in the manufacturing process or source of the sample. The forced degradation conditions include the effect of heat, moisture, light, acid–base hydrolysis, sonication, and oxidation. The compatibility of AZT with the excipients used is also studied in the presence and absence of moisture. The amounts of AZT and azaerythromycin A are calculated from the corresponding linear calibration curve; however, the amounts of any other generated or detected unknown impurities are calculated as if it were AZT. This method shows enough selectivity, sensitivity, accuracy, precision, linearity–range, and robustness to satisfy Federal Drug Administration/International Conference of Harmonization regulatory requirements. The method developed can also be used for the purity testing of AZT raw material and capsules, content uniformity testing, dissolution testing, and stability testing of AZT capsules. The potential impurity profiles of both active AZT material and capsule forms are found comparable. The linear range of AZT is between 5 and 30 mcg/spot with a limit of quantitation of 2 mcg/spot. The intraassay relative standard deviation percentage is not more than 0.54%, and the day-to-day variation is not more than 0.86%, calculated on the amounts of AZT RS recovered using different TLC plates.

Introduction

Azithromycin (AZT) (Figure 1) is a subclass of macrolide antibiotics. Physically, AZT as a dihydrate is a white crystalline powder. AZT in an aqueous or chloroform solution has insignificant UV absorption properties; also, it is very difficult to find a suitable active reagent to enhance its UV absorptivity with no byproducts or degradation of the intact compound. Attention should also be paid to the fact that AZT dihydrate–acidic solution is rapidly decomposed via intramolecular dehydration to form erythromycin-6,9-hemiketal and then anhydroerythromycin (1). Therefore, United States Pharmacopoeia (USP) 24 and most plasma work have used high-performance liquid chromatographic (HPLC) methods and tracing with electrochemical detectors (2,3). This official method depends primarily on the liability of certain reaction centers of AZT to oxidation without sample pretreatment. Alternatively, only one

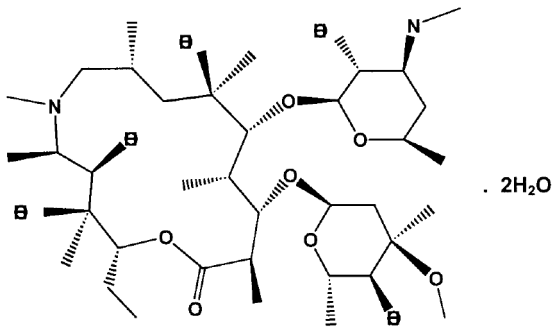

Figure 1. Azithromycin dihydrate ($C_{38}H_{72}N_2O_{12} \cdot {}^2H_2O$, fw = 785.0): (*2R,3S,4R,5R,8R,10R,11R,12S,13S,14R*)-13-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-ribo-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-α-D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one.

publication has described a precolumn derivatization procedure of AZT with 9-fluorenylmethyloxycarbonyl chloride to yield a stoichiometric amount of fluorescence derivative for separation by an HPLC method (4). This method depends on the stoichiometric reactivity of the five hydroxyl groups with the reagent.

However, the spectrophotometric method described by Bebawy et al. (5) using 7,7',8,8'-tetracyanoquinodimethane is based on the measurement of the charge–transfer complex. All of these methods are not described as stability indicating. However, the USP 24 assay method described azaerythromycin A as the only potential impurity expected in the bulk material. In order to estimate the shelf life, the suitability of the packaging material, the compatibility with the combined excipients, or the validation of batch processing, a well-validated stability indicating assay method should be used.

In order to meet the regulatory guidance of the Federal Drug Administration/International Conference of Harmonization (ICH), the material should be forcibly degraded until 10–30% of the major compound degrades (6). The method should be able to detect and quantitatively estimate the degradation products generated. In this work, a stability-indicating assay procedure is described to estimate the potential impurity A (azaerythromycin A) and other possible degradation products. Also, the compatibility, impurities profile, and applicability are investigated. From a regulatory point of view, the method developed can also be used to fulfill the essential similarity and drug-development criteria as part of the summary of product characteristics required by notes to an applicant (7).

Experimental

Equipment

The UVP scanner and software (GelWorks 1D Advanced Version 3.01) were from Ultra Violet Products (Cambridge, U.K.). A test tube atomizer (12 mL) from Desaga GmbH (Wiesloch, Germany) was connected with the positive-pressure outlet valve of a membrane pump. A thin-layer chromatographic (TLC) spotting syringe (25 µL) was obtained from Hamilton (Reno, NV). Nylon-sample filtration discs (0.45 µm) were used. A TLC Tank (standard type) and minivials lined with a silylated tetrafluoroethylene cap (1 mL) were from Alltech (Deerfield, IL).

Materials

TLC aluminum sheets precoated with silica gel 60F254 (20 × 20 cm, 0.25-mm layer thickness) were obtained from E. Merck (Darmstadt, Germany). HPLC-grade solvents and other chemicals were of analytical grade. AZT dihydrate RS and azaerythromycin A (impurity A) RS were from USP RS (Rockville, MD). AZT dihydrate active in bulk was a free sample from Orchid Chemicals and Pharmaceutical (Chennai, India). Zithromax (250-mg capsules, batch no. 9114) (Pfizer, Cairo, Egypt) and Azalid (250-mg capsules, pilot batch) (T3A Industrial, Assiut, Egypt) were obtained directly from the manufacturer.

Chromatography

Preparation of developing system

A mixture of *n*-hexane (75 mL), ethyl acetate (25 mL), and 99% diethylamine (10 mL) was prepared and 50 mL of this mixture was poured into a TLC tank. The tank was then covered with a lined lid and presaturated with the solvent vapor system for at least 30 min at room temperature before use.

Loading

The samples were applied to the marked start edge of the TLC plate (1.5-cm height) using the specified TLC–Hamilton glass syringe (25-µL capacity). The sample volumes for the assay experiments were 10 µL, and the volumes spotted for the purity or stability testing were 20 µL. The plate was then allowed to air-dry for 10 min and then inserted into the TLC tank for development. The TLC plate was developed for no less than a 15-cm migration distance of the solvent from the start line.

Preparation of detection reagent

Forty grams of potassium iodide was dissolved in 100 mL of water (solution 1), and approximately 850 mg of bismuth subnitrate was dissolved in 50 mL glacial acetic acid (20% in water) (solution 2). Five grams of sodium nitrite was dissolved in 100 mL water (solution 3). Immediately before use, a mixture of 10 mL of solution 1, 10 mL of solution 2, and 20 mL of glacial acetic acid was prepared and diluted to 100 mL with water in a 100-mL volumetric flask (Dragendorff's reagent) (solution 4).

After drying the developed TLC plate (at room temperature for approximately 20 min), the detection reagent (solution 4) was sprayed (~ 8 mL was consumed), the plate was air-dried for 10 min, and then the plate was sprayed with solution 3 (5% sodium nitrite). The entire plate was turned a brown color.

The plate was allowed to air-dry for no less than 30 min at room temperature. The plate was then scanned and integrated to allow lanes and bands identification and quantitation. The scanning and data integration were performed by using a Gelworks 1D UVP instrument. The concentrations of the eluted spots were correlated to the light intensity (pixels).

Procedure

Standard preparation of AZT RS solution

Approximately 52.4 mg of AZT dihydrate USP RS was accurately weighed into a 25-mL volumetric flask. It was then dissolved and diluted to the mark with dichloromethane (2 µg/µL as AZT).

Standard solution of azaerythromycin A

Approximately 20 mg of azaerythromycin A was accurately weighed into a 25-mL volumetric flask. It was then dissolved and diluted to volume with dichloromethane (0.8 µg/µL).

AZT sample solutions for assay were prepared as described under standard preparation (0.8 µg/µL).

Sample solution for purity testing

Approximately 262 mg of AZT dihydrate (equivalent to 250 mg AZT) was accurately weighed and dissolved in 5 mL

dichloromethane into a 5-mL volumetric flask (1 mg/20 µL).

Zithromax (250-mg capsule solution)

The content of one capsule was transferred into a 5-mL volumetric flask. An aliquot of approximately 4 mL dichloromethane was then added and the flask shaken for approximately 2 min. It was then diluted to the mark with the same solvent. The contents of the flask were filtered with syringe filtration disks (0.45 µm) (solution 5).

An accurate volume of 20 µL was then applied to the TLC plate for purity testing (1 mg/spot).

Two milliliters of solution 5 was then further diluted to yield a claimed concentration of 2 mg/mL of AZT. An accurate volume of 10 µL of this later solution was applied to the TLC for assay of 250-mg capsules of Zithromax.

Standard calibration of AZT solution

Five concentration levels of AZT USP RS were prepared in dichloromethane. A 10-µL volume of each level was applied to the TLC plate (5, 10, 15, 20, and 30µg/spot).

Forced degradation

Boiling

A sample solution of AZT for purity testing (1 mg/20 µL) was prepared in water, and 1 mL of this solution was heated to 100°C in capped minivials inserted in a temperature-controlled block heater for 30 min. The vials were cooled before opening, and a volume of 20 µL was applied to the TLC plate (equivalent to 1 mg AZT/spot) in order to estimate and detect the degradation products generated. Another portion of the boiled sample solution was diluted to yield a claimed concentration of 20 µg/spot of AZT. This was to estimate the remaining amounts of the intact drug.

Acid and alkali hydrolysis of AZT

A sample solution of AZT (150 mg/mL) was prepared in dichloromethane. In a minivial, an equivalent volume (0.2 mL each) of this latter solution was mixed with 0.05N hydrochloric acid. In another vial the same concentration of AZT using an equivalent volume of 1N sodium hydroxide was prepared. In both cases, the prepared solution was heated at 60°C for 30 min with intermittent shaking. The samples were cooled to room temperature, neutralized with an amount of acid or base equivalent to that previously added, and 20 µL was applied to the TLC plate (the claimed AZT concentration is 1 mg/spot). Furthermore, a portion of both solutions was diluted to get a claimed concentration of 20 µg/10 µL, and a 10-µL volume of the sample was loaded to estimate the remaining amounts of the intact drug.

Oxidation

An aliquot of 0.2 mL of an AZT sample solution (150 mg/mL prepared in CH_2Cl_2) was mixed with 0.4 mL of a hydrogen peroxide solution (10%) in a minivial capped and heated at 60°C for 30 min with intermittent shaking. A volume of 20 µL was applied to the TLC plate.

Sonication

A sample solution of AZT for purity testing (1 mg/20 µL) was prepared in water, and 5 mL of this solution was sonicated in an ultrasonic bath for 30 min (47 kHz). A volume of 20 µL was applied to the TLC plate.

Heating of 250-mg capsules of Zithromax

Two glass vials were used, each containing a content of one 250-mg capsule of Zithromax. One was mixed with 10 µL of water. Both vials were capped tightly and allowed to stand in a heating incubator at 60°C for 14 days. The vial contents were extracted by dichloromethane, filtered with syringe filtration disks, and diluted to obtain a claimed concentration of 1 mg/20 µL AZT. A volume of 20 µL was applied to the TLC plate. The same procedure was repeated but using 250 mg Azalid.

In all of the stress cases, the same plate included the other reference spots at three levels within the calibration curve (5, 10, and 25 µg/spot) in addition to the spot of azaerythromycin A (8 µg/spot). The plate was then allowed to air-dry for 10 min before the development. After development, the plate was then allowed to dry, sprayed with both developed reagents (solution 4 and solution 3 as described previously), and the detected spots evaluated by using the UVP instrument. The gray scan and one-dimensional mode was applied. The spot lanes and bands were defined, detected, and integrated quantitatively.

Results and Discussion

AZT has insignificant UV absorbance even in high concentration. Therefore, this work was designed to separate AZT from its possible degradation products or its potential impurities. We went through three phases to achieve our goals, which were the development and optimization, validation, and applicability of the method. Several mobile systems were tried; however, a suitable separation of AZT on a TLC plate could best be achieved by using a development system as described in the "Experimental" section. In order to detect the eluted spots, freshly prepared Dragendorff's reagent was sprayed on the air-dried plate. Upon using this reagent the spots corresponded to AZT only, were colored orange, and rapidly faded to colorless bands. After spraying with Dragendorff's reagent (solution 4) and re-drying the plate, a solution of 5% sodium nitrite was sprayed. The plate then became fully brown as a result of the regeneration of iodine. After standing at room temperature for approximately 30 min, a brown band corresponding to AZT and any degradation products persisted on the plate with a light yellow background. The separated spots were almost circular with no tailing, which allowed for the accurate measuring of the R_f value. The validity of this procedure was tested for the following parameters: limit of detection (LOD), limit of quantitation (LOQ), linearity, range, precision, accuracy, selectivity, robustness, ruggedness, and sample solution stability.

Before starting the evaluation of the separated bands, the following steps have to be followed in order to get an accurate representative chromatogram using Gelworks UVP 1D software: (*a*) open the Gelworks 1D software and download the scan of TLC as a new file, (*b*) start to assign a lane for each band pathway, (*c*) start band detection, (*d*) remove the background

in order to get an accurate start and end of each band, (*e*) the obtained chromatogram can then be evaluated quantitatively, (*f*) assign standard spot band and define both the strength and measuring unit and type of quantitation (i.e., linear, nonlinear, or matching), and (*g*) use the calibration curve generated in order to calculate all other unknown or unmatched bands that were detected.

Linearity and range

The results obtained revealed a good linear calibration fit between the band volume (concentration in micrograms) and light intensity as pixels (raw volume) in the range of 5 to 30 µg/spot. The calibration curve was generated automatically once the method of calculation was defined (Figure 2). The calibration equation calculated was:

$$y = 7.79E^{-005}x + 0.05944 \quad \text{Eq. 1}$$

where y is the strength and x corresponds to the band raw volume (response) with a square regression coefficient value equal to approximately 0.9957.

The calibration curve was valid only for the calculation of an unknown AZT concentration separated on the same plate because the brown color of the bands faded proportionally and slowly.

LOD and LOQ

The LOD was estimated for nine bands that could be detected from the TLC background. The LOD found was 0.3 µg/spot.

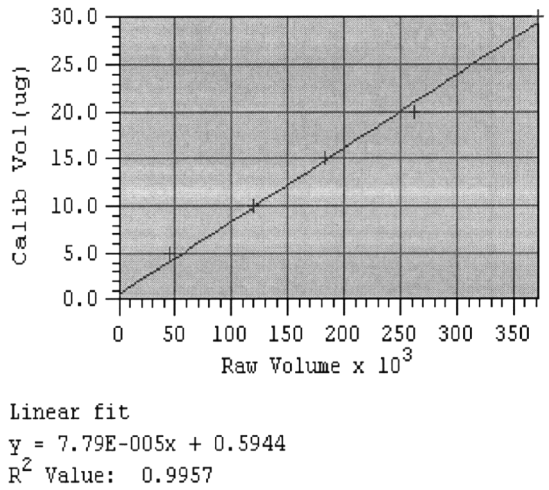

Figure 2. Generated calibration curve of onplate derivatized AZT dihydrate.

Table I. LOD and LOQ of Both AZT RS and Impurity A

Compound	LOD (µg/spot, %RSD*)	LOQ (µg/spot, %RSD*)
AZT RS	0.30, 6.2	1.00, 2.9
Impurity A	1.40, 7.3	8.00, 2.3

* %RSD of the response of nine repetitive experiments.

However, the lower LOQ was estimated after the spotting of AZT RS (1.0 µg/spot) nine times and calculation of the relative standard deviation (RSD) value of the response (raw volume). The RSD value of the band raw volume was no more than 3%. The LOD value of azaerythromycin A was 1.4 µg/spot, and the LOQ was estimated on the basis of the expected maximum limit (which was 0.8%) to correspond with 8 µg/spot (Table I).

Precision

System suitability testing was performed on TLC plates using a standard solution of AZT prepared in dichloromethane (20 µg/spot). The RSD of the raw volumes (pixel intensity) of the bands separated was no more than 1.22% (Figure 3). Also, the %RSD of the calculated R_f value was close to zero.

Selectivity

The proposed method was able to discriminate completely between the major compound and its potential impurity A and other possible degradation products. The following lanes were developed and matched together: sample AZT RS, premelted AZT sample solution, spiked AZT with impurity A, and impu-

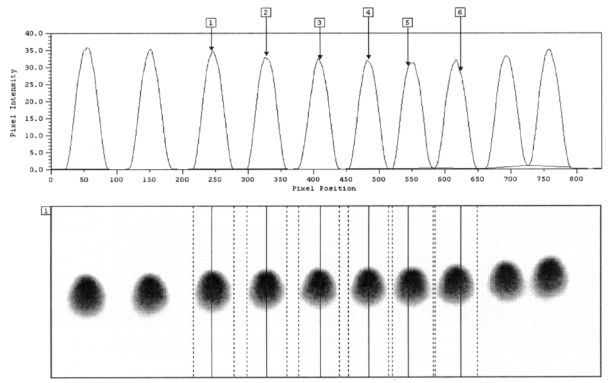

Figure 3. The repeatability of peak pixel intensity related to the corresponding spot.

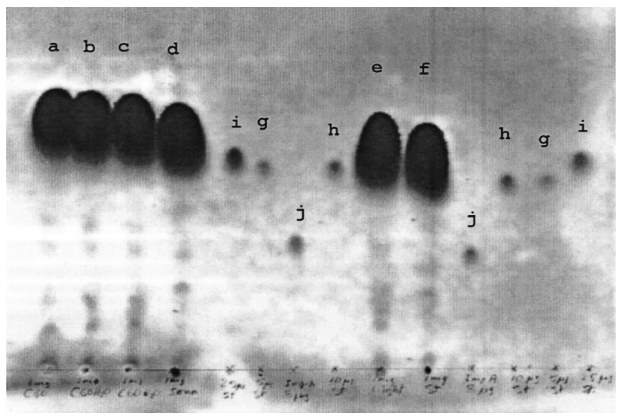

Figure 4. Thin-layer chromatogram of pure and degraded AZT: (A) effect of heat on Zithromax capsule, (B) effect of heat on Zithromax capsule, (C) effect of moisture on Zithromax capsule, (D) effect of sonication, (E) effect of light, (F) AZT raw material (all stressed and unstressed AZT concentrations used were 1 mg/spot), (G) 5 µg/spot AZT RS, (H) 10 µg/spot AZT RS, (I) 25 µg/spot AZT RS, and (J) 8 µg/spot impurity A RS.

rity A RS. The strength of all samples was 1 mg/spot, and impurity A RS loading was 8 µg/spot. The same chromatographic profile of the degradation products of the stressed AZT was obtained in order to confirm method selectivity.

Accuracy

Five concentration levels of AZT RS (including LOQ) were prepared and analyzed by the proposed method and calculated from the calibration curve derived on the same TLC plate. Because the recovered amounts of AZT RS were within the acceptable range of 100% ± 5% of the claimed amount, the method was deemed to be accurate (Table II).

Robustness

In order to measure the extent of the method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged, and in parallel the chromatographic profile was observed and recorded. The chromatographic parameters (including spot area, raw volume, migration distance, and resolution of the intact drug from its degradation products) were observed. The parameters interchanged (in the range of 10%) were included as well as the composition of the mobile system alternatively, spray amounts of Dragendorff's reagent, the amount and strength of sodium nitrite solution, drying time, and drying temperature. The resolution and detection of the analyzed spot material was relatively acceptable in all conditions, except that the drying time should be adequate before development, derivatization, and scanning. Also, the plate should be left to dry in the open air at room temperature without any heating. If the plate were to be heated in an oven at approximately 40°C, the spot color and background will fade, but nonproportionally.

Ruggedness

The ruggedness of the method was evaluated by applying the analysis of Zithromax capsule solutions using two different TLC plates from two different manufacturers [Merck and Fluka

Table II. Accuracy of the Developed Method Using AZT RS

Spiked concentration (µg/spot)	Measured concentration* (µg/spot, mean ± SD)	%RSD	%Deviation†
1.0	0.95 ± 0.05	5.26	5.00
5.0	4.88 ± 0.21	4.30	2.40
10.0	9.85 ± 0.30	3.04	1.50
20.0	19.80 ± 0.27	1.36	1.00
30.0	30.31 ± 0.27	0.89	1.03

* Mean of 3 experiments.
† %Deviation = [(spiked concentration – mean measured concentration) × 100] / spiked concentration.

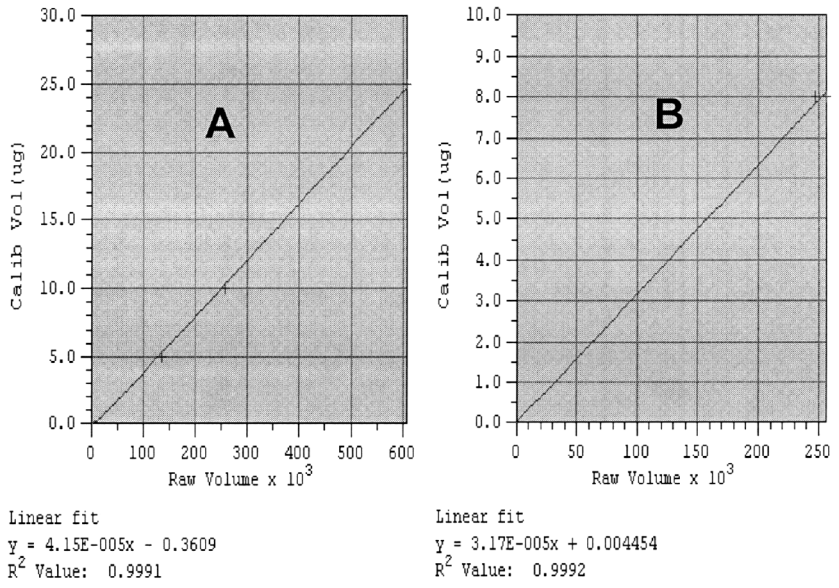

Figure 6. Linear calibration curve of AZT (A), and one-level calibration fit of standard impurity A (B). The data of both curves were retrieved from the same TLC plate.

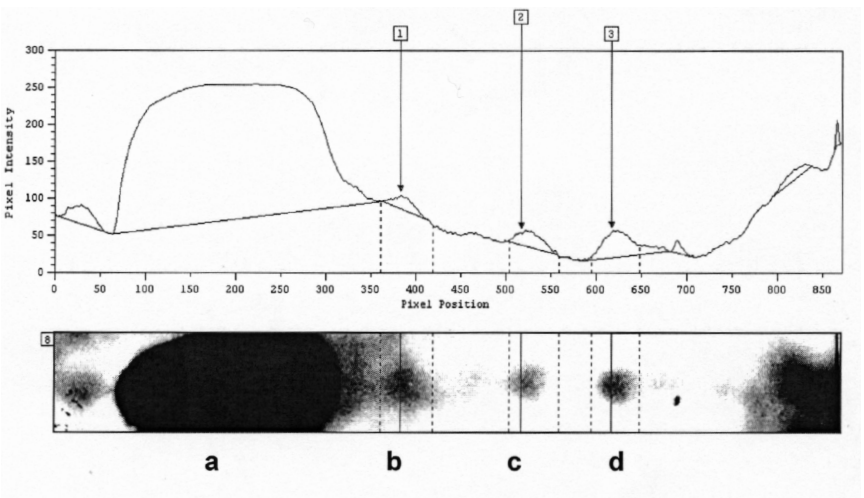

Figure 5. Representative one-lane chromatogram of forced degraded AZT (effect of sonication): (A) AZT, (B) unknown impurity, (C) degradation product matched with impurity A, and (D) unknown impurity.

Chemie GmbH (Reisenhofen, Germany)] of different layer thicknesses (0.25 and 0.20 mm). Both plates yielded the same resolution efficiency and R_f values; however, the thinner-layer plate required a longer time for the solvent to reach the marked front. A compact band zone without tailing was observed in both cases.

The percentage recoveries of the capsule content using two different TLC plates were found comparable (~ 100% ± 1.5%). Awareness should be paid to the amount of samples delivered from the spotting syringe because this step expresses the material concentration. A calibrated autospotting device could be useful to avoid sample volume variation. However, the loaded volume area variation did not make any difference in the results.

Sample solution stability

As per the experimental procedure described, the development of approximately 20 samples on one plate will consume approximately 30 min, which is not enough time to allow for much material degradation. The proposed procedure did not show any degradation products resulting from experimental error or run time. However, freshly prepared samples were used within 2 h, and the sample solutions should not be allowed to stand until analysis.

Forced degradation of AZT

A working standard of AZT was used for this study. This was to confirm that the proposed method was able to detect and analyze the major compound in the presence of any possible degradation product or in-process impurities resulting from the manufacturing procedure. Both raw material and capsule form were included in this study. A relatively high concentration of AZT was prepared in dichloromethane or water, following the stress conditions applied. As described in the "Experimental" section, different stress cases were applied in order to include the effect of boiling, heat, acid, base hydrolysis, sonication, oxidation, and excipient compatibility with the drug. A chromatogram of stressed samples illustrated in Figure 4 shows that the degradation products and impurity profiles in all cases were similar with the complete separation of degradation products from the major drug spot. In order to estimate the unknown degradation products, a three-point calibration curve was used on the same plate. Also, in order to estimate the known impurity (azaerythromycin A), one level concentration was used to estimate the matched detected impurity A released from the raw material or product. Figure 5 is an example of how to detect and evaluate the one-lane degradation profile. Also, as in Figure 6, the calibration curves were calculated on the same plate. From this investigation, it was clear that AZT was very sensitive toward acid, and if the hydrolysis time or acid strength increased, the entire compound would be completely degraded with no spot corresponding to AZT. In contrast, the compound was relatively stable in an alkali medium. Table III shows the recovered amounts of impurity A and total impurities in all stress conditions. As per ICH guidelines and drug dose, the limit of impurity A should not be more than 1% (0.8% was used as an acceptable limit), unknown impurities no more than 0.5%, and total impurities no more than 2.0%.

Two strengths of AZT were prepared from the same Zithromax and Azalid capsules or raw material stock solutions. The higher strength (1 mg/spot) was spotted to evaluate the purity level of the sample. However, the diluted solution (20 µg/spot) was spotted to assay the content of material that remained.

The stress testing results revealed that AZT was compatible with the combined excipients and degraded to a limit of no more than 3.55% for 250-mg capsules of both Zithromax and Azalid.

Table III. Calculated Amount Percentages of Impurity A and Total Impurities*

Stress condition	Calculated amounts percentage	
	Impurity A	Total impurities
Boiling	2.32	14.28
Acid hydrolysis	26.66	38.36
Alkali hydrolysis	1.47	11.84
Oxidation	2.64	14.86
Sonication	0.26	0.81
Light	0.53	0.72
Heat, dry capsule content	1.22	2.11
Heat, moisture, capsule content	2.30	3.55
Unstressed sample (1 mg/spot)	0.14	0.26

* The amount of total impurities was calculated from the calibration curve of AZT RS, and the amount of impurity A was calculated from the calibration fit of impurity A RS.

Conclusion

The method developed was valid for purity testing, a stability-indicating assay, and content-uniformity testing. Also, this method could be used for the quantitation of pure material and capsule form. The developed method was able to estimate the dissolution profile of Zithromax capsules applying the USP method, but using 500 mL dissolution media instead of 900 mL. This method could be useful for the study of pure material and product shelf life. All proposed analytical procedures should be completed at room temperature. This is to avoid inconsistency of the developed brown color.

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