Densitometric Thin-Layer Chromatographic Determination of Artemisinin and its Lipophilic Derivatives, Artemether and Arteether

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Abstract

A thin-layer chromatograpy (TLC) method is developed to analyze artemisinin (AT) and its derivatives, artemether (AM) and arteether (AE), using a silica-gel plate with a mobile phase containing pure chloroform. After development, all products are visualized after dipping in a 4-methoxybenzaldehyde dipping reagent of 1% (v/v) in an acidic solution of sulphuric acid (98%, v/v) and acetic acid (96–98%, v/v) (respectively, 2% and 10%, v/v in alcohol–water, 60:30, v/v), presenting a purple color against a slightly colored background. This TLC system is quantitatively evaluated in terms of stability of the color, precision, accuracy, and calibration. Activation is performed at 110°C. Stability of the color of both analytes is reached after 12 min. Precision, less than 5%, is obtained at two levels. Good linearity is obtained in the range of 0.5–8 µg for all analytes. Some applications show its utility in the quality control of capsules. The prederivatization technique, applying the described dipping reagent before development, reveals the presence of various reaction products, possibly isomers. These results prove that TLC can be a cheap and easy alternative for the analysis of AT and its lipophilic derivatives, AM and AE, as pure powder and in pharmaceutical-dosage forms.

Introduction

Artemesinin (AT) is a natural sesquiterpene lactone endoperoxide, isolated from the plant *Artemisia annua L.* (1). Various semisynthetic derivatives of this parent drug such as artemether (AM), arteether (AE), and others, are nowadays extensively applied for the treatment of patients with either uncomplicated or severe malaria including multidrug-resistant falciparum diseases (2). In China, the plant is listed in the Chinese pharmacopoeia as a remedy for various fevers including malaria (3,4) and contains the well-established antimalarial compound AT.

Various formulations of these compounds have been studied for oral, rectal, and parenteral administration (5). AM and AE for intramuscular injection are prepared in various oily formulations, such as fractionated coconut oil, and Cremophore solutions were experimentally applied in intravenous treatments (6–7). Many published clinical data showed the administration of tablets (5,8–9) and suppositories (5,10) prepared from these compounds. In the development of such pharmaceutical-dosage forms with the AT derivatives, easy techniques to perform identification tests, quality control, and stability of the drugs should be available.

Some analytical methods were already proposed in literature, but because of the inert nature of the molecules, rather highly specific and uncommon techniques are needed such as gas chromatography–mass spectrometry–selected ion monitoring (11), UV-derivatization (12–14), chemiluminescence (15), electrochemical detection (16–22), and light scattering (23). Many of them require intensive sample preparation or highly technical experience and are not always suitable for a great number of samples and routine analysis on pharmaceutical formulations.

Thin-layer chromatography (TLC), in general, is still of great interest to chromatographers in the clinical-toxicological field and in pharmaceutical analysis for the quality control and stability testing of pharmaceutical active compounds and pharmaceutical formulations (24). A TLC method could facilitate the investigation of drugs, especially for the analysis of AT and its derivatives, which don't present any UV chromophores and, therefore, sophisticated detection techniques are required. Moreover, another strong argument may exist for application of TLC because of its economical advantages for many pharmaceutical labs. It is rapid, relatively simple, and samples need not be very pure in comparison with high-performance liquid chromatography (HPLC) (25).

Therefore, in this paper a simple technique for the analysis of AT and its lipophilic derivatives, AM and AE, is developed, specifically a TLC method using densitometry to quantitate the drugs after derivatization.

In this study, the experimental parameters for a TLC with derivatization in pre- and postmode using dipping reagents were

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optimized for identification and quantitative analysis of AT and the two lipophylic derivatives. There is no need for highly sensitive analytical techniques in our pharmaceutical-technological studies because of the high dosage of active compound in the usual pharmaceutical preparations (tablets, injections containing 50 mg drug, and more). The TLC technique can, thus, be a good alternative.

TLC systems and detection techniques via derivatization are already mentioned in literature for the analysis of AT, but none of them managed to separate the derivatives; even more, these were only qualitatively used as an identification technique (26,27).

The purpose of this work is, thus, the development of a TLC method in all its aspects: selection of the most suitable stationary and mobile phase, namely a silica gel precoated plate and a mixture of organic solvents, especially methanol and chloroform. Several dipping reagents presented by Jork (28) were selected based on their ability to visualize peroxides; the most suitable one was further investigated in terms of conditions to develop a stable color after reacting with our analytes, which enables quantitative analysis. The developed technique will be investigated in depth for qualitative and quantitative analysis purposes: selectivity for the three analytes after separation and a total evaluation including precision, accuracy, detection limit, and linearization. Dosage determination of the capsules, analyzed by use of the developed TLC technique and UV-spectrophotometry with postderivatization, will be compared.

Experimental

Chemicals and reagents

AT and its derivatives were obtained from the following manufacturers: AT from ACF (Maarssen, the Netherlands), AM from Arenco N.V. (Geel, Belgium), AE from the University of Mississippi (University, MS), and artesunate (AS) and dihydroartemisinin (DHA) were respectively obtained from HelmAG (Düren, Germany) and Arenco N.V. Lactose 100 mesh from Ludeco (Brussels, Belgium) was used as filling excipient in capsules.

Chloroform (stabilized with 80 ppm 2-methyl-8-butene), methanol (gradient grade), dichloromethane, tetrachloromethane, *n*-hexane, acetic acid (96%, v/v), sulphuric acid (95–97%, v/v), ortho-phosphoric acid (85%, v/v), perchloric acid (70%, v/v) (all pro analysi), 1,1,1-trichloroethane (Selectipur), and 4-methoxybenzaldehyde were all obtained from Merck (Darmstadt, Germany). Acetonitrile was obtained from Carlo Erba Reagenti (Milan, Italy) and vanillin from Federa (Ghent, Belgium).

Chromatographic procedure

For qualitative investigations, standard solutions of each product were prepared in alcohol (94%, v/v) and always had a concentration of 0.1% (m/v). For the calibration line, series of standard solutions were prepared over a concentration range of 0.005% up to 0.1% (m/v).

A Linomat IV device from Camag (Lot, Belgium) was used to apply the test solutions on the TLC plates. Appropriate aliquots were spotted under a continuous drying nitrogen stream at 10 s/µL solution to spot in a 1-cm band at 2 cm from the bottom on the TLC plate. For quantitative analysis, 10-µL quantities of standard solutions were always applied on the plates. The development was performed in a developing chamber tank (Camag) and saturated with the appropriate eluent overnight at 25°C. The TLC layer interfaces (10 × 20 cm) applied in this investigation were RP-18 F254S and silica gel 60 F254 precoated thin layer from Merck; another set of precoated TLC plates SIL G-25HR (silica gel with gypsum) were obtained from Machery-Nagel (M&N, Düren, Germany). They all had a thin-layer thickness of 0.25 mm. The migration distance of the solvent in the TLC plate was approximately 7 cm. The following mobile phases were investigated: chloroform–methanol (varying from 25:75 to 100:0, v/v) tetrachloromethane–methanol, *n*-hexane–methanol, trichloroethane–methanol (all in a ratio of 95:5, v/v), and pure chloroform. Development of the thin-layer plate took approximately 30 min, depending on the mobile phase.

The spots were visualized by the aid of a dipping technique in dipping chambers (20 × 20 cm) (Camag). Dipping reagents containing vanillin or 4-methoxybenzaldehyde in aqueous and organic solvents were used in the derivatization test. Vanillin (1 g) was dissolved first in a mixture of water–ethanol (94%, v/v), as presented by Jork (28); later on, other mixtures were also tested, namely chloroform–ethanol (94%, v/v). All solutions were acidified with different amounts of ortho-phosphoric acid. The composition of the tested derivatization reagents is given in the "Influence of the heat on the stability of the color after derivatization" section. 4-Methoxybenzaldehyde (1%, v/v) was dissolved in a mixture of water–ethanol (94%, v/v) (ratio ranging from 40:50 to 70:20, v/v) and acidified with sulphuric acid (2%, v/v) and acetic acid (10%, v/v). The effect of the derivatization reagents was tested on different plates from different manufacturers by using the dipping technique. The entire dipping process was performed at room temperature.

Both post- and prederivatization techniques were applied. For both, activation was performed on a plate heater (Camag). The color development conditions were 110°C during at least 8 min, for qualitative analysis. The exact activation time needed for a stable color was investigated. After development, the plates were air dried for 5 min and sample and standard zone areas were measured by linear scanning at 565 nm with a PMQ 3 Zeiss-TLC scanner (Oberkochen, Germany). The scanning was performed vertically over the plate over a distance of 5 cm and at a rate of 5 cm/min, measuring 200 points/scanning. Densitograms were generated from transmission measurements of the samples and the blank. The respective absorbance values were calculated, and peak areas were quantitated with the aid of the Peakfit program from Jandel (Erkrath, Germany).

Applicability of the TLC method on pharmaceutical formulations and comparison with UV-spectrophotometric analysis after derivatization

To illustrate the efficacy of the developed TLC method, capsules containing AT, AE, or AM were prepared. The last ones were also analyzed by TLC and with UV after derivatization because this method was already applied in other dosage determination studies on AM in our lab. The UV method is based on a derivatization with perchloric acid, which was developed by Al-Angary (29). The capsules for this experiment were prepared in the lab as

follows. The doses prepared were selected in the range of 100 mg, which are normal doses for the presented drugs.

From each drug, 20 "Nova" no. 2 capsules (Belgica T.O.P., Turnhout, Belgium) with 130 mg AT, 150 mg AE, and 165 mg AM, respectively, were prepared in a capsule-filling apparatus (Feton International, Linkebeek, Belgium) and filled up with lactose, if needed. The average weight from each batch was defined. Six units from these batches of capsules were analyzed. The content of each capsule was dissolved separately in 100 mL ethanol (94%, v/v). Each solution was diluted three times. TLC, as presented in this study, was performed on each solution and four standards between 20 and 80 mg/100 mL.

The same solution of the AM capsules was subjected to the UV analysis after a fourfold dilution in methanol; 5 mL of these solutions were mixed with 5 mL perchloric acid reactant that contained 42 mL of perchloric acid (70%, g/g) diluted with water to a volume of 100 mL. After mixing, the solution was activated in a GFL warm water bath (Gesellschaft für Labortechnik, Burgwedel, Germany) during 15 min at 54ºC and under continuous shaking. An immediate cooling in ice avoided further degradation of the reaction products. Four standards having an end concentration that varied in the range from 0 to 5 mg/100 mL in methanol and two kinds of blanks, similarly prepared to the standards and samples, were treated under the same conditions. Measurements at 254 nm were performed with an Uvikon UV spectrophotometer (Kontron Instruments, Zürich, Switzerland). This technique was previously evaluated in our lab, showing good results for our purpose. Regression analysis revealed the following results: linearity was present in the range of 0.03 to 3 mg/mL ($Y = 13.957 \pm 0.1015X - 0.0170 \pm 0.0017$; where Y = absorbance at 254 nm and X = concentration of AM in methanol, expressed in mg/mL). Stability of the derivatized product was observed during 50 min at 25ºC and 200 min in ice.

Results and Discussion

As most of the known analytical techniques, applied on AT and its derivatives they are quite laborious and need special sample preparation; developing a reproducible and quantitative TLC method seemed to be a good alternative for the quantitative determination of these products in pharmaceutical-technological studies. To achieve this, a good TLC system (a suitable mobile and stationary phase) and a stable visualization technique were envisaged.

Qualitative results

Selection of the solvent system (mobile phase) in the TLC method

The challenge of this part of our study is focused on the separation of AT and the two lipophylic derivatives, which have a difference of just one methyl group between each other.

Very few TLC applications in the investigation of AT and its derivatives are described in the literature, being only suitable for identification or qualititative purposes. Kudakasseril et al. (27) described a method for the determination of extracted AT from plant material by use of a TLC method on silica-gel plates, and Xinyi et al. (30) performed TLC methods to detect AT in biological material. Both researchers applied normal-phase techniques in their investigation using silica-gel plates. A first set of our experiments was, thus, the selection of the best silica-gel plate that can withstand the mobile phase as well as the derivatization reactants.

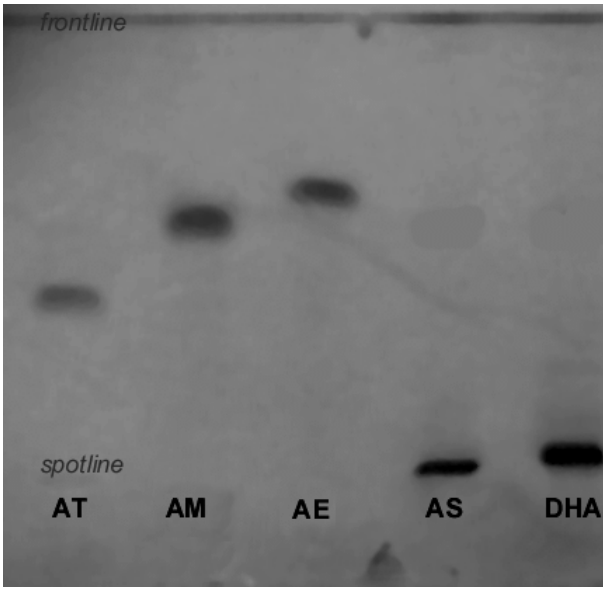

Figure 1. Separation of AT, AM, and AE from each other and AS and DHA using a silica-gel plate (Merck) and 100 mL of chloroform.

Table I. R_f Values in Different Solvent Systems*

Eluent	R_f AT	R_f AE	R_f AM	Plate
Tetrachloromethane–methanol (95:5, v/v)	0.51	0.78	0.80	TLC plates silica gel 60 254S (Merck)
n-Hexane–methanol (95:5, v/v)	0.08	0.47	0.41	TLC plates silica gel 60254S (Merck)
Trichloroethane–methanol (95:5, v/v)	0.72	0.82	0.82	TLC plates silica gel 60 254S (Merck)
Chloroform–methanol (25:75, 50:50, 75:25, 90:10; v/v)	1.00	1.00	1.00	TLC plates silica gel 60 254S (Merck)
Chloroform–methanol (95:5, v/v)	0.95	1.00	1.00	TLC plates silica gel 60 254S (Merck)
Chloroform (100 mL)	0.37	0.60	0.53	TLC plates silica gel 60 254S (Merck)
Chloroform (100 mL)	0.13	0.24	0.26	TLC plates SIL G-25HR (M&N)

* Migration distance = 6–7 cm.

Therefore, silica-gel plates of different manufacturers were tested.

Mobile phases containing methanol and other organic compounds were applied. Therefore, conditions for total saturation of the developing chamber (overnight saturation at 25°C) were consistently maintained. The TLC systems were evaluated by determining the retention factor (R_f) values of each analyte. Results are presented in Table I.

In almost all combinations of chloroform and methanol, the three analytes were migrating totally. Good separations were obtained in mobile phases containing other organic solvents than chloroform in combination with methanol and in pure chloroform. Because of its simplicity, the last one, chloroform (100%, as mobile phase), was selected for further investigation. Both silica-gel thin-layer plates from Merck and M&N showed comparable separation efficiency, despite lower R_f values with the latter.

M&N manufacturer's information mentioned the presence of an organic highly polymeric compound in combination with gypsum as binder (31), which possibly interfered in the migration of our analytes AT, AM, and AE. Results with chloroform as mobile phase are presented in Figure 1.

Figure 1 shows the separation of AT and four of its derivatives. All lipophilic analytes (AT, AE, and AM) are well separated from each other and also from the hydrophilic compounds (AS and DHA), which were almost not migrating in the presented TLC system. A suitable thin-layer method for separation of our analytes, the use of a silica-gel plate as stationary phase and chloroform (100 mL) as mobile phase, is now available to investigate all aspects for visualization and, later on, quantitation of the substances' spots.

Selection of the visualizing reagent

Because of the lack of UV chromophores in AT and its derivatives, a coloring technique to visualize the spots on the TLC is required. The use of visualizing techniques for quantitative analysis purposes necessitates a dipping method above spraying. In such a procedure, the thin-layer plate is dipped in a coloring agent, resulting in better reproducibility (32). Especially in the development of such a method, the stability of the developed color and solvent thin-layer interactions should be considered. Some reversible visualizing methods [such as iodine vapors described in literature (33)] are suitable for identification of AT and its derivatives, but will not be investigated because they are not useful for our purposes to quantitate the analytes. Reagents based on 4-methoxybenzaldehyde (27) and analogues such as *p*-dimethylbenzaldehyde (32) are described in the literature as spraying reagents; the first one was only applied in qualitative analysis of AT. Jork (28) presented this reagent, which is universal for natural products and offers the possibility for color differentiation between different analytes that results in variously colored chromatogram zones on an almost colorless background that are often fluorescent under long wavelength UV light (366 nm). The reactant has the advantage that its solvent can be modified if necessary (32), which can thus be applied in the development of a dipping solution.

Another expedient reagent known for reacting with diverse groups of compounds (32,34–38) and also described for identification studies of AT (39) is the vanillin dipping solution. Presented as dipping solution in the work of Jork (28), it contains 1 g reactant in a solution of 35 mL phosphoric acid and 50 mL water–ethanol (94%; 1:1, v/v) (Solution I). We suggest that the oxidative characteristics of our analytes, being sesquiterpenes with a peroxide bridge in their heptane ring, can play a role in the development of colors. For the dipping reagents containing 4-methoxybenzaldehyde or vanillin, it is well known that they are irreversibly changing the chemical structure of the substances analyzed and are, therefore, useful for quantitative analysis. Both products can be employed on silica gel as well as RP plates (28).

Both presented dipping reagents suitable for color development on peroxides in TLC, as stated by Jork (28), will be further investigated on our analytes AT, AM, and AE.

In a first step, the best solvent for both reactants in the dipping reagent containing methanol–water or chloroform was investigated. Therefore, the effects on the spots (strong and equal coloration of the spot, which is required for the quantitative determination), as well as the influence on the binder's components in the thin-layer plate were studied.

Concerning the 4-methoxybenzaldehyde reagent, different ratios of water and ethanol (94%, v/v; 40:50; 30:60, and 70:20) were used. The best results were obtained for the first, which lead to well-formed and fine spots. More aqueous solvents were not suitable because calcium salts added as binder in the thin-layer plate are soluble in water; they can cause the removal of the silica-gel layer from the plate (32).

As described in Table II, different colors for the natural product AT and its two semisynthetic derivatives (AM and AE) were noticed.

Some preliminary tests with the vanillin reactant of Jork (28) containing a high content of water and ortho-phosphoric acid (40:35, v/v) resulted in bad-colored and too-big spots, being too diffuse for quantitative analysis. The plate dried very slowly and the thin layer was destroyed.

In comparison with 4-methoxybenzaldehyde, no improvements to visualize the spots were noticed with the vanillin reactant. Changes in the solvent composition of the vanillin reagent such as lowering the acid content in the dipping reagents, for example, in solution II (water 25 mL; ethanol, 94%, v/v, 25 mL; and ortho-phosphoric acid, 7 mL) and solution III (chloroform, 25 mL; ethanol, 94%, v/v, 35 mL; and 7 mL of the acid) were investigated. Solution II did not improve the noticed difficulties.

Table II. Color Development of AT, AE, and AM Colored with the 4-Methoxybenzaldehyde and Vanillin Reagent

Analyzed product	Colors of spots in daylight	Color of spots at 366 nm
4-Methoxybenzaldehyde reagent		
AT	Purple-red	Fluorescent orange
AE and AM	Dark gray	Fluorescent orange
Vanillin reagent		
AT	Blue-green	No colors
AE and AM	Pink	No colors

It still destroyed the thin layer, despite the lower acid content in comparison with the original reagent. Although with solution II, well-formed and fine spots having a pink to blue-green color (Table II) were obtained, but instabilities in the reactant were observed (changed into yellow after 1 day). This phenomenon was already observed in earlier studies in our lab (40). Moreover, it does not have fluorescent characteristics. So, for practical reasons the vanillin reagent was excluded from further investigation. The 4-methoxybenzaldehyde reagent seemed to be the best choice for quantitative purposes.

Prederivatization method on TLC plates RP-18 F254S

Because none of our analytes are UV detectable, a lucid identification based on one technique of such compounds is a great challenge with simple techniques. Identification of these compounds is, therefore, mostly executed with specialized techniques such as IR and mass spectrometry. Therefore, in this part of the study we tried to develop a TLC technique especially for qualitative purposes from which more information could be obtained than from the classic TLC methods, presenting a product as one spot. As it was suggested in literature that AT and its derivatives were composed of several isomers, we would like to find a method to separate them. From earlier results, we did not observe any of them with the presented normal thin-layer techniques. Hence, we tried a prechromatographic derivatization, in which the chemical reaction, prior to the development, is performed directly on the plate (starting zone).

Practically, the analytes were first spotted on the thin-layer plate and then dipped in the 4-methoxybenzaldehyde reactant and activated under the same conditions as for the postderivatization treatment; all these steps were, thus, performed before development in the mobile phase. One important advantage of such "prechromatographic derivatization" technique, which is already extensively used in the separation of amino acids as dansyl derivatives (41), is that selectivity, as well as detection sensitivity can be enhanced (42).

In the first set of experiments on normal-phase plates, any separation of our analytes into different spots was not achieved. Therefore, in a second set-up, the same method was performed on reversed-phase plates. To select a suitable mobile phase, we based this on HPLC methods described in literature. In most of them, methanol–water and acetonitrile–water mixtures were applied on C_{18} columns (43,44). These mobile phases were investigated on RP-C_{18} thin-layer plates under the same conditions as for the normal-phase tests, achieved as described in the chromatographic procedure.

Two eluents, methanol–water (50:50, v/v) and acetonitrile–water (66:33, v/v) lead to the separation of our analytes into several bands with different colors on a migration distance of 6.5 cm. Table III and Figure 2 represent, respectively, the results under UV light at 366 nm and visible light. Under UV, several bright fluorescent colors were observed, whereas in daylight the spots were only visualized as a blue color.

Four spots having an R_f value between 0.8 and 1.0 were observed in daylight for the methanol eluent. Even more bands were observed with the mobile phase containing acetonitrile (Figure 2), and a higher number could be detected at 366 nm (Table III) because of a higher sensitivity. Six bands were observed for AT and 7 for AM and AE, all visible as fluorescent colored spots. Great differences in R_f values were visually noticed for AT compared with AE and AM (Figure 2), but not between the last ones because they have a similar structure formula, only differing in one methyl group.

Possibly, some of these isomers were observed, or the reaction with 4-methoxybenzaldehyde caused different reaction products. Therefore, the spots should be subjected to other analysis techniques such as NMR, IR, etc. after scraping off the thin layer. Nevertheless, the prederivatization technique can be used additionally to the main TLC method with postderivatization for the identification of our analytes.

Quantitative results

Influence of the heat on the stability of the color after derivatization

Quantitative analysis on TLC after derivatization necessitates a stable color to analyze our spots. As described in literature (40), the derivatization process with 4-methoxybenzaldehyde derivatization reagent needs to be activated under the following conditions: 110°C as activation temperature and 10-min activation

Table III. R_f Values and Color of Spots after Prederivitization on AT, AE, and AM in UV Light*

AT		AE and AM	
R_f	Color	R_f	Color
0.61	Bright orange	0.76	Yellow
0.58	Blue	0.61	Green
0.49	Orange	0.55	Yellow-green
0.38	Yellow	0.49	Pink
0.33	Green	0.38	Purple
0.00	Brown orange	0.30	Blue

* The mobile phase is acetonitrile–water (66:33, v/v).

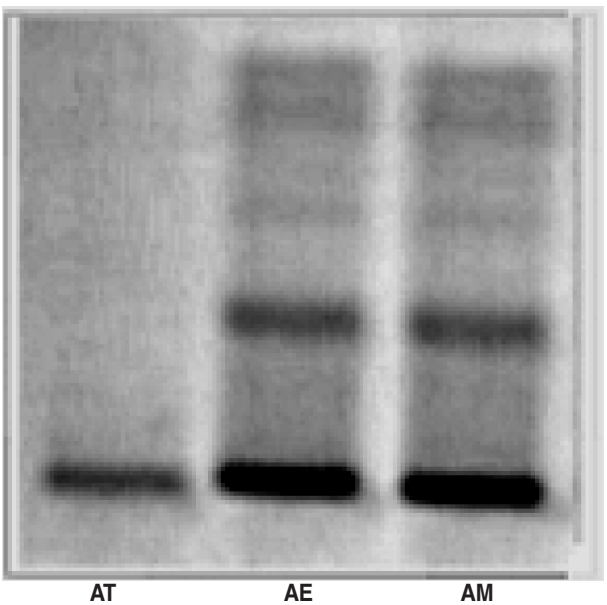

Figure 2. Spots in daylight after prederivatization using an RP-C_{18} plate and acetonitrile–water (66:33, v/v).

time. This activation period is sufficient for qualitative detection, but is not necessarily satisfactory for quantitative purposes because of the possible instability of the color on the spots.

In order to achieve stable spots, measurements on the same spot were repeated at different times after different activation times (8, 10, and 12 min). The following procedure was employed: after dipping the plate in the dipping solution, the residues of the solution at the backside of the plate were wiped off and dried at ambient temperature. The plate was then put on the plate heater at 70°C (a higher temperature can break the glass plate), while it was increasing to 110ºC. The activation time started when this temperature was reached. For each compound, at least a 12-min activation was required to terminate the reaction. The color then remained stable for more than 140 min. As can be noticed from Figure 3, the first points measured after activation were higher than after 30 min. The same phenomenon was even observed in other investigations in our lab (40). It can be explained as follows: the thin layer is very hot and dry after heating to 110°C. While cooling down, the background color was changing visually, possibly because of the hydration by the air. It seems necessary to wait at least 30 min after activation before measuring the first spot. The analysis needs to be finished within 2.5 h to ensure stability of the colored spots.

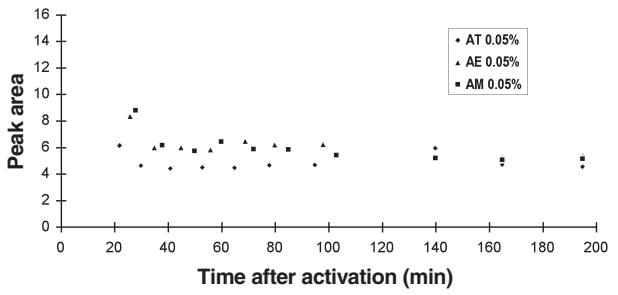

Figure 3. Stability of the color of 5 µg of AT, AE, and AM (0.05%, g/v, 10 µL spotter) after 12 min of activation at 110°C.

Precision

Ten-microliter aliquots of a sample containing 0.05% (g/v) active compound were analyzed according to the proposed procedure. Precision was investigated at several levels. In order to control the measurements with the Zeiss densitometer, one spot was analyzed several times. By spotting and analyzing the same amount several times, the precision of the automatical spotting device, as well as the uniformity of the derivatization technique, was evaluated. As presented in Table IV, the relative standard deviation (RSD) for the analysis of ten replicates indicated good precision for the proposed TLC method (RSD was consistently less than 5%).

Linearization

Calibration curves were constructed by plotting the peak areas obtained from the analysis of the drug versus the corresponding concentrations in ethanolic solution, ranging from 0.002% to 0.1% (g/v). The spotting volume was 10 µL. The results are presented in Table IV.

Regression analysis of the TLC plots revealed good correlation (at least 0.99). Graphs of the peak areas versus the amount of active compound on the plate showed intercept values tending to zero and are described by the regression equation:

$$Y = aX + b \qquad \text{Eq. 1}$$

where Y is the peak area, a is the slope, b is the intercept, and X is the spotted amount of analyte on the thin-layer plate expressed in micrograms.

Analogous calibration ranges were obtained, which could be expected because the AT derivatives react similarly with 4-methoxybenzaldehyde.

Table IV. Precision and Regression Data of the TLC Method Performed on AT, AE, and AM

Parameter	AT	AE	AM
Precision data			
Average ± SD*	7.7431 ± 0.139	10.0245 ± 0.078	11.7706 ± 0.199
Average ± SD†	8.1749 ± 0.303	9.4713 ± 0.263	11.2498 ± 0.531
RSD*	1.81	0.78	1.69
RSD†	3.71	4.32	4.72
Regression equation‡			
Slope (*b*) ‡	1.404	1.640	2.108
Intercept (*a*) ‡	1.356	0.783	0.856
Concentration range	0.5–8 µg	0.5–8 µg	0.25–8 µg
Correlation coefficient (*r*)	0.998	0.993	0.995

* S = 1, *n* = 10; one spot is measured ten times.
† S = 10, *n* = 1; ten spots are measured once.
‡ *Y* = peak area, *b* = slope, *a* = incercept, and *X* = the spotted amount of analyte on the thin-layer plate. Expressed in micrograms (µg).

Table V. Analysis of Pharmaceutical-Dosage Forms of AT, AE, and AM with TLC and UV-Spectrophotometry after Derivatization

Preparation	AT	AE	AM
Label claim (mg/cap)	130 mg	150 mg	165 mg
Average weight of the units	261 mg (*n* = 20, RSD = 4.58)	315 mg (*n* = 10, RDS = 5.33)	323 mg (*n* = 20, RDS = 5.50)
TLC method	99.4% (RSD = 4.58, 95.0%–104.7%)	101.9% (RSD = 2.18, 98.0%–104.2%)	100.7% (RSD = 2.58, 96.6%–103.8%)
UV method			97.7% (RSD = 4.75, 93.4%–105.0%)

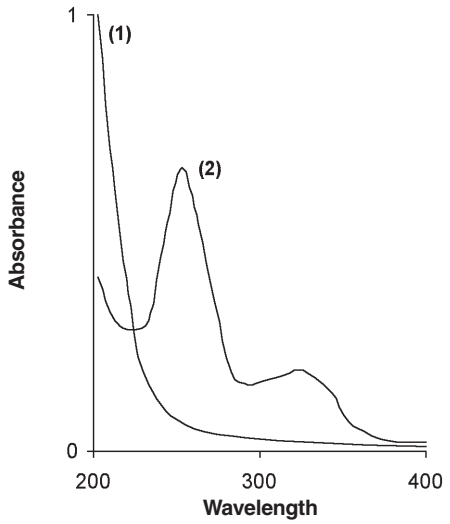

Figure 4. UV spectrum of AM without (1) and after (2) derivatization with perchloric acid. (1) Concentration = 100 mg/mL. (2) Starting concentration = 5 mg/100 mL.

Applicability of the TLC method in the analysis of dosage forms containing AT, AM, and AE

Six units of the capsule batches containing 130 mg AT, 150 mg AE, and 165 mg AM, respectively, were subjected to the quantitative analysis with TLC. All preparations containing AM were also analyzed with UV-spectrophotometry after a perchloridic acidic derivatization based on the method of Al-Angary (29). Figure 4 shows the UV spectrum of AM without and after derivatization.

The results of the experimentally measured dosage (%) and the range wherein they vary are given in Table V.

Comparing the results for both analytical techniques applied in our experiments, the TLC method showed good results, reaching a mean dosage of 100%, which suggests a normal spreading of the dosage measurements. The results of the analysis of the AM capsules with the TLC and UV derivatization method were compared statistically by the Student *t*-test and the variance ratio F-test. The Student *t*-values at 95% confidence level did not exceed the theoretical values, indicating that there was no significant difference between the precisions of the evaluated methods. It was also noticed that the variance ratio F-values calculated for $p = 0.05$ did not exceed the theoretical values, indicating there was no significant difference between the precision of both methods. The results are shown in Table V. Although, less variation was noticed for the AM capsules when performing the TLC technique in comparison with the UV method. Notice that no special sample treatment was necessary for the analysis with TLC. So, this experiment showed the usefulness of a quite simple TLC method in the quantitative analysis of dosage forms of AT and its derivatives.

Conclusion

The proposed normal-phase method using a silica-gel thin-layer plate, pure chloroform as mobile phase, and a 4-methoxybenzaldehyde reactant under specified conditions provides a first approach for qualitative and quantitative analysis of AT and its derivatives in pharmaceutical dosage forms. The prederivatization technique, in which the derivatization with the same reactant was performed before development on reversed-phase thin-layer plates with acetonitrile–water as mobile phase, can be used additionally for the identification and qualitative control of AT and AM in syntheses. If solutions with lower concentrations need to be analyzed, fluorometric densitometry can be developed, based on our observations in UV light.

Acknowledgments

We are grateful to Dr. Ir.C.B. Lught from ACF-holding B.V., N-Maarssen, and to Dr. Mc Chesney from the University of Mississippi for supplying samples of AT and derivatives.

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Manuscript accepted June 3, 2003.