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Comparative Study for Determination of Atracurium Besilate in Presence of Its Toxic Degradant (Laudanosine) by Reversed Phase HPLC and by TLC Densitometry

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Abstract: Two methods are described for the stability indicating determination of Atracurium Besylate in presence of its major degradant (Laudanosine). The first method was based on reversed liquid chromatography, C18 column (150 mm × 4.5 mm, 5 µm packing) was used for separation. The mobile phase consisted of a mixture of 0.075 M potassium dihydrogen: methanol: acetonitrile adjusted to a pH of 3.1 ± 0.2 with o-phosphoric acid (50:30:20, by volume). Flow rate of 1.0 mL/min was applied. Quantitation was achieved with UV detection at 280 nm. Linearity, accuracy and precision were found to be acceptable over the concentration range of (1-8 µg/mL) atracurium besylate (ATR). The second method was based on the thin layer chromatography (TLC) separation of the two drugs followed by densitometric measurements of their spots at 282 nm. The separation was carried out on silica gel plates using methanol: ethyl acetate (3:7, *v*/*v*) as developing system. The linear regression analysis data were used for the regression line in the range (2-18 µg/mL) of ATR. The two proposed methods were successfully applied to the determination of ATR in presence of LDS in laboratory prepared mixtures and in commercial vials. The optimized methods proved to be accurate, precise, highly selective and highly sensitive.

Key words: Reversed phase chromatography, TLC densitometry, atracurium besylate (ATR), degradation product, laudanosine (LDS).

1. Introduction

Atracurium besylate (ATR) 1-[(3,4-dimethoxyphenyl) methyl]-2-[3-({5-[(3-{1-[(3,4dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4tetrahydroisoquinolin-2-ium-2-yl} propanoyl) oxy]pentyl}oxy)-3-oxopropyl]6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolin-2-iumdi-benzenesulfonate [1] is a skeletal muscle relaxant used in general anesthesia. It undergoes chemo degradation *in vivo* by two processes: Hofmann elimination and ester hydrolysis [2]. A closer insight to the *in vivo* metabolic process of ATR, it was reported to be susceptible to degradation by Hofmann elimination as a primary route yielding its toxic metabolite Laudanosine (LDS) (Fig. 1) which has the ability to pass across the blood brain barrier increasing the risk of convulsion [3, 4].

Several analytical methods have been described for the determination of ATR in pharmaceutical and biological samples. These methods include chromatography [5-12] and electrochemistry [13]. However, no previous method has been adopted for the determination of ATR in presence of LDS with such selectivity and sensitivity, LOD (0.0539 µg/mL

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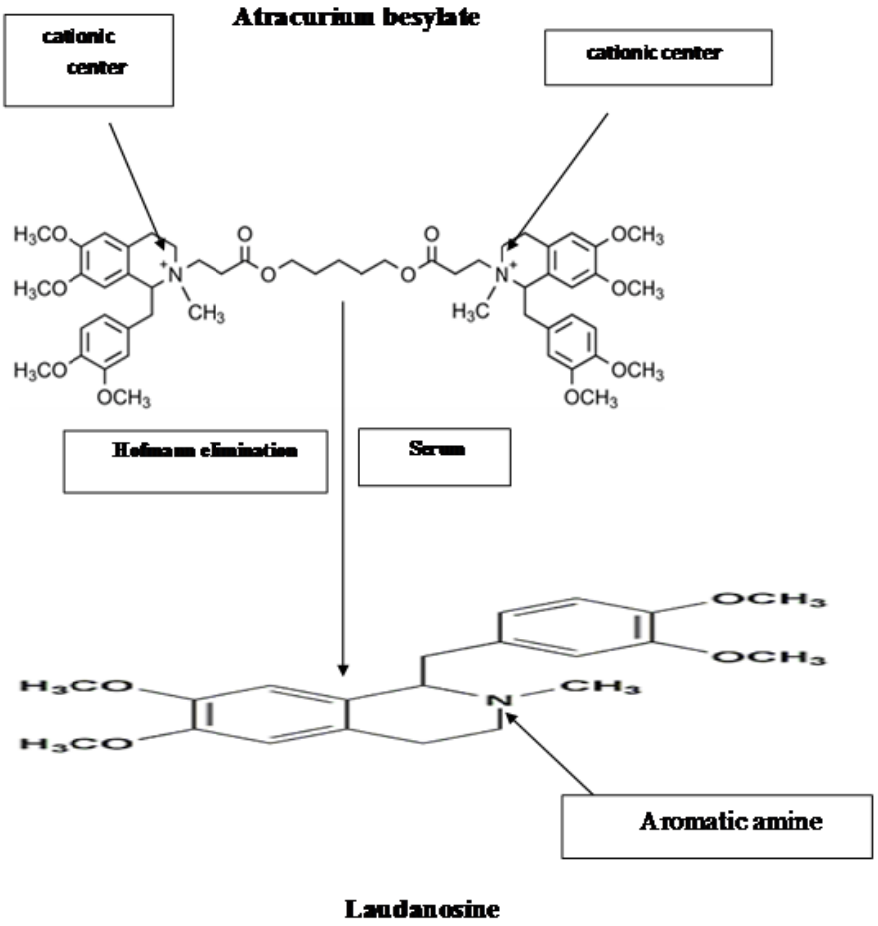

Fig. 1 The serum *in-vitro* degradation of ATR to LDS.

and 0.2946 μg/mL) for HPLC method and thin layer chromatography (TLC) densitometric method respectively and limit of quantification (LOQ) (0.1635 μg/mL and 0.8929 μg/mL) for HPLC method and TLC densitometric method respectively.

HPLC is generally considered to be the most suitable for the determination of drugs present in multi-component dosage forms, because it is the most reproducible. It is also specific, linear, precise, accurate and rapid [14]. Providing quality control procedures for determination of ATR in its pharmaceutical preparation depending on a compelling quantitative approach, alternative and competitive with HPLC, in terms of applicability, sensitivity, high throughput and reproducibility, a TLC-densitometric procedure was also adopted. The two proposed methods are simple, sensitive, reproducible and rapid. They were designed to be suitable for the quantitative determination of pure ATR, in laboratory mixture and in its pharmaceutical preparation.

2. Experimental

2.1 Reagents and Reference Samples

- Methanol, acetonitrile and ethyl acetate—HPLC grade (E. Merck, Germany).
- Bi-distilled water (Egypt Otsuka Pharmaceutical, Egypt).
- Potassium dihydrogen phosphate (Sigma-Aldrich, Labochemikalien GmbH, Germany).
- Ortho-phosphoric acid (Adwic, Cairo, Egypt).
- Sodium hydroxide, and 10 N hydrochloric acid; Adwic, Cairo, Egypt.

- Bi-distilled water (Egypt Otsuka Pharmaceutical, Egypt).

All chemicals used throughout this work were of pure analytical grade.

2.2 Instrumentation

2.2.1 For HPLC Method

A liquid chromatography system consisted of:

- An isocratic pump (Model LC H20 AT pump, Shimadzu L2026490897355 series).
- An ultra violet detector (Model SPD-M20A, Shimadzu L20154907953 AE series).
- Column C18 (Octadecyl Silane).
- Rheodyne injector (Model SH-20A) equipped with 100 μL injector.
- Spotelin-Sonorex TK sonicator (Budapest, Hungary).
- Jenway pH glass electrode (UK, No. 924005-BO3-Q11C).

2.2.2 For TLC-Densitometric Method

A thin layer chromatography system consisted of:

- CAMAG Linomat 5, autosampler (Muttens, Switzerland).
- CAMAG TLC densitometric Scanner 3S/N 130319 in the reflect anceabsorbance mode (Muttens, Switzerland).
- WINCATS software was used for densitometric evaluation (Muttens, Switzerland).
- Thin layer chromatography (HPTLC) plates precoated with silica gel 60 F254 0.25 mm thicknesses (E. Merck, Germany).

2.3 Chromatographic Conditions

2.3.1 HPLC Method

The mobile phase consisted of a mixture of aqueous 0.075 M potassium dihydrogen phosphate adjusted to a pH of 3.1 ± 0.2 with o-phosphoric acid: methanol: acetonitrile (50:30:20, by volume). The mobile phase was filtered through a 0.45 μm Millipore filter white nylon HNWP 47 mm and degassed for 15 minutes in an ultrasonic bath prior to use. Agilent analytical column C18 (150 × 4.5 mm, 5 μm packing) was used for the separation. The flow rate of mobile phase was 1.0 mL/min and the column operated at ambient temperature (25 °C). The UV detector was set at a wavelength of 280 nm. Samples were also filtered through a 0.45 μm syringe membrane filter, and injected in volumes of 2.0 μL in triplicates. The column was equilibrated with the mobile phase until steady baseline is obtained and pressure is stabilized.

2.3.2 TLC-Densitometric Method

Different aliquots of each solution were separately applied in triplicates as spots on TLC aluminum sheets (spot width: 3 mm; spacing: 14.2 mm; 15 mm from bottom edge of the plate) using a Camag Linomat 5 applicator. Linear ascending development was performed in a chromatographic tank previously saturated with the developing system consisting of methanol: ethyl acetate (3:7, *v*/*v*) for 45 min at room temperature. The developed plates were air dried and scanned under the following conditions:

- Source of radiation: deuterium lamp;
- Scan mode: absorbance mode;
- Slit dimensions: 3 mm × 0.45 mm;
- Scanning speed: 20 mm/s;
- Output: chromatogram and integrated peak area;
- Wavelength: 282 nm.

2.4 Standard Solutions of Atracurium Besilate

An accurately weighed 100 mg of ATR was transferred into a 100-mL volumetric flask, 50 mL of methanol was added and sonicated to dissolve the drug and then the volume was completed to the mark with methanol (1.0 mg/mL-standard solution for TLC). Accurately transfer 1.0 mL into a 100-mL volumetric flask and then dilute to the mark with methanol (10 μg/mL-standard solution for HPLC).

2.5 Preparation of Alkaline Degradation Product (Laudanosine)

Twenty mL of 0.1 M NaOH solution was added to pure ATR (500 mg) in a 100-mL round-bottom flask

and left at room temperature for 15 minutes until a white precipitate is formed. To this solution, 0.1 M HCl solution was added drop wise till pH 4 and the precipitate is dissolved. Complete degradation was confirmed by TLC using ethyl acetate: methanol (70:30 *v*/*v*) as a mobile phase. Only was observed one spot different than that of ATR. The hydrolysis procedure was repeated again without the acidification step and after complete degradation; the precipitate was filtered and left to dry. Test the purity of the degradation product obtained by dissolving a small portion in methanol, applying onto TLC plates and developing using the previously mentioned solvent system. The structure of the isolated degradation product was confirmed using infrared spectroscopy and mass spectrometry.

2.6 Standard Solutions of Degradation Product (Laudanosine)

An accurately weighed degradation product (Laudanosine) prepared as mentioned in (preparation of alkaline degradation product) equivalent to 100 mg of ATR in 100 mL volumetric flask, 50 mL of methanol was added, sonicate for 5 minutes and complete to mark with methanol (1.0 mg/mL-degradation standard solution for TLC). Accurately transfer 1.0 mL to 100 mL volumetric flask, dilute to the mark with methanol (10 μg/mL-degradation standard solution for HPLC).

2.7 Sample Preparations

Ten Atrabesylate® 10 mg/mL ampoules were emptied in a beaker, and then a volume of 1.0 mL was accurately transferred into a 100 mL volumetric flask, then the volume was completed to the mark with methanol. Five milliliters were transferred accurately into a 100 mL volumetric flask and the volume was completed to mark with the mobile phase (5 μg/mL for HPLC) and twenty milliliters were transferred accurately into a 100 mL volumetric flask and the volume was completed to mark with distilled water (20 μg/mL for TLC).

3. Procedure

3.1 Linearity and Repeatability

3.1.1 HPLC Method

Aliquots of ATR (1.0-8.0) equivalent to 10.0-80.0 μg were transferred separately from its standard solution into a set of 10-mL volumetric flasks and the volume was then completed to the mark with the mobile phase to have concentrations 1.0-8.0 μg/mL. A volume of 2.0 μL of each solution was injected in triplicates and chromatographed under conditions including the mobile phase at a flow rate 1.0 mL/min, detection at 280 nm and run time program for 20 min. A calibration curve was constructed by plotting the peak area ratios of ATR to that of concentration 5 μg/mL as a function of concentrations. The repeatability of the method was assessed by analyzing a laboratory prepared mixture containing 7.0-1.0 μg/mL of ATR and 1.0-7.0 μg/mL of degradation product (n = 7). The precision (RSD) for ATR was calculated.

3.1.2 For TLC-Densitometric Method

Aliquots (2-18 μL) of ATR standard solution equivalent to (2-18 μg/mL) were spotted in triplicates on TLC plate, using Camag Linomat auto sampler with micro syringe (100 μL), and the previously mentioned chromatographic conditions were applied. The scanning profile for ATR was obtained, and the calibration curve relating the optical density of each spot to the corresponding concentrations of ATR was constructed. The regression equation was then computed. The repeatability of the method was assessed by analyzing a laboratory prepared mixture containing 2.0-12.0 μg/spot of ATR and 12.0-2.0 μg/spot of degradation product (n = 6). The precision (RSD) for ATR was calculated.

3.2 Assay of Atrabesylate®10 mg/mL amp

3.2.1 HPLC Method

A volume of 2.0 μL of sample preparation (5 μg/mL) was injected in triplicates and chromatographed under

conditions including the mobile phase at a flow rate 1.0 mL/min, detection at 280 nm and run time program for 20 min. The procedure under linearity was applied. The concentrations of ATR were calculated in reference to the standard solution under the previously mentioned HPLC chromatographic conditions.

3.2.2 TLC-Densitometric Method

Transfer aliquots (1, 2, 3, 4, 5, 6, 7, 8, 9 mL) of ATR sample preparation (20 µg/mL for TLC) into a series of 10-mL volumetric flasks, and complete to mark with distilled water (2-18 µg/mL), 10 µL were spotted in triplicates and the plate was developed under the previously described chromatographic conditions. The procedure under linearity was applied. The optical density of each spot was measured at 282 nm for ATR and its concentrations in the samples were determined in reference to the standard solutions developed on the same plate under the same TLC chromatographic conditions.

4. Results and Discussion

A high-performance liquid chromatographic method was described for the determination of ATR in presence of its degradation product without prior separation [15]. Several experimental conditions were examined with the aim of optimization of the proposed HPLC method. The pH of the aqueous portion of mobile phase has a significant role in attaining an optimal separation between ATR and its degradation product and improving selectivity, peak shape, and retention time [16]. The peak shape improved dramatically with the increase in the percentage of aqueous solution 0.075 M of potassium dihydrogen phosphate previously adjusted to pH 3.1 with o-phosphoric acid [17]. At higher organic solvents concentration, separation occurred but with excessive tailing and increased retention time of ATR. The method has been optimized on using a mobile phase of 0.075 M Potassium dihydrogen phosphate (adjusted to pH 3.1): methanol: acetonitrile (50:30:20, by volume) with 1 mL/min flow rate and UV detection at 280.0 nm. HPLC chromatogram of ATR and its major degradation product (LDS) reveals a retention time of 2.34 ± 0.02 min for LDS and 19.33 ± 0.02 min for ATR.

Modern instrumental planar chromatography, with precise and accurate sample application and computer-controlled quantification of the developed chromatograms, was considered to be an effective and reproducible tool for purity control and quantitative determination [18]. Simple, selective, sensitive and time saving stability indicating TLC densitometric method was developed and validated for determination of ATR in bulk powder, in presence of its degradation products and in its pharmaceutical dosage form.

Keeping in mind the green analytical chemistry (GAC), several experiments have been tried to reach the optimum chromatographic operational conditions for ATR separation in the presence of its alkaline degradation product. Many developing systems were tried but most of them failed to attempt proper separation. Finally, convenient and symmetric peaks were obtained using methanol: ethyl acetate (3:7, *v*/*v*) as a developing system. Good separation between ATR and its major degradation product (LDS) with sufficient difference in their *Rf* values without tailing was obtained. Defined spots were obtained when the chamber was pre-saturated with the mobile phase for 45 min at room temperature. The *Rf* values were 0.50 ± 0.02 and 0.06 ± 0.002 for ATR and LDS, respectively. Detection at 282 nm was suitable for providing good sensitivity for ATR with minimum noise.

For the above considerations, the aim of this work was to develop, simple, selective, accurate, reproducible and very sensitive reversed-phase HPLC and TLC-densitometric methods for the stability indicating determination of ATR in presence of its major degradation product (LDS) in laboratory prepared mixture.

5. Method Development

5.1 HPLC Method

For the separation of ATR and LDS, various reversed phase columns, isocratic mobile phase systems were attempted. Different ratios of the aqueous and organic phases were tried. All trials revealed poor peak shape with obvious tailing, as an alternative to reduce tailing and improve resolution; several experimental conditions were examined with the aim of optimization of the proposed HPLC method. Importantly, pH of the aqueous portion of mobile phase has a significant role in attaining an optimal separation between ATR and LDS, and improving selectivity and peak shapes.

At higher organic solvents concentrations, separation occurred with decreasing retention times but with excessive tailing. The peak shapes improved dramatically with increasing the percentage of aqueous solution of 0.075 M potassium dihydrogen phosphate previously adjusted to pH 3.1 ± 0.2 with o-phosphoric acid [18]. The final mobile phase consisted of a mixture of aqueous 0.075 M potassium dihydrogen phosphate adjusted to a pH of 3.1 ± 0.2 with o-phosphoric acid: methanol: acetonitrile (50:30:20, by volume). The mobile phase was filtered and degassed for 15 minutes in an ultrasonic bath prior to use. Samples were also filtered through a 0.45 μm syringe membrane filter, and injected in volumes of 2.0 μL in triplicates. The column (C_{18}) was equilibrated at ambient temperature (25 °C) with the mobile phase until steady baseline is obtained and pressure is stabilized. Detection was carried out at 280 nm, because high detector sensitivity was achieved at this wavelength. HPLC chromatogram of ATR and its degradation product (LDS) reveals a good separation of ATR and LDS with good peak shapes with retention time of 2.34 ± 0.02 min for LDS and 19.33 ± 0.02 min for ATR (Fig. 2).

In the course of optimizing the conditions of the proposed method, different parameters affecting the chromatographic separation were studied. The system suitability tests were used to verify the conditions of the chromatographic system for resolution and hence for analysis [19]. System suitability was checked by calculating the capacity factor (*K*), tailing factor (*T*),

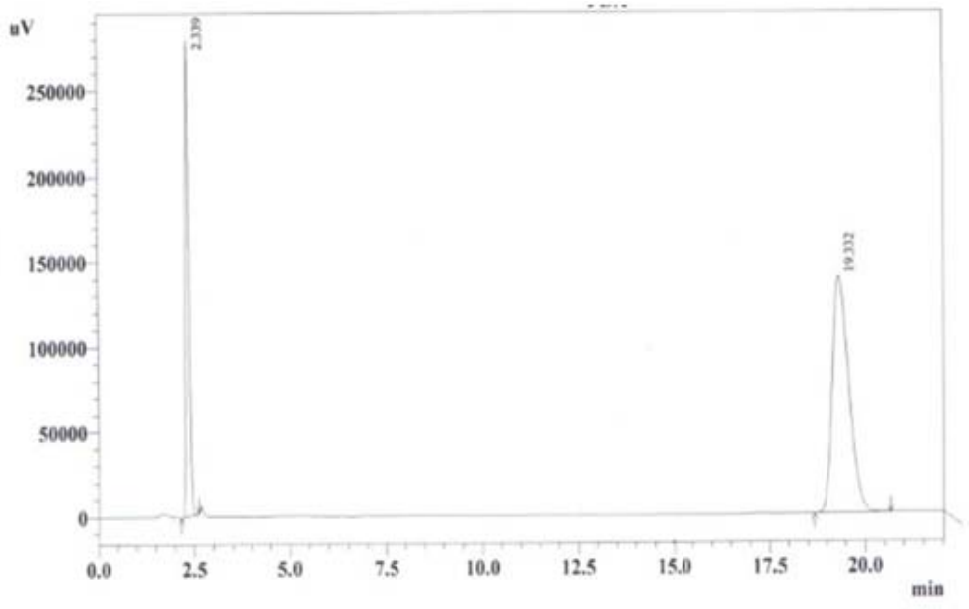

Fig. 2 The x-coordinate represents the retention time (min) and the y-coordinate represents detector response. HPLC chromatogram of a resolved mixture of ATR (4 μg/mL) and its degradation product (LDS) (4 μg/mL), column C_{18} (150 × 4.5 mm, 5 μm packing). The flow rate was 1.0 mL/min and the column operated at ambient temperature (25 °C). The UV detector was set at a wavelength of 280 nm.

Table 1 Parameters required for system suitability test of HPLC method.

Parameter	Obtained value		Reference value
	LDS	ATR	
R (resolution)		35.29	$R > 0.8$
T (tailing factor)	1.34	1.35	$T \leq 2$ $T = 1$ for a typical symmetric peak
α (selectivity factor)		2.91	> 1
K (column capacity)	1.11	3.23	1-10 acceptable
N (column efficiency)	3,252	9,362	Increases with increasing the efficiency of separation
HETP	0.104	0.034	The smaller the value, the higher the column efficiency

column efficiency (N) and the selectivity factor-resolution (α), where the system was found to be suitable (Table 1).

5.2 TLC-Densitometric Method

Several developing systems and chromatographic conditions, comprising those reported in the literature [20] were tried to separate ATR and LDS. Initially, a mixture of chloroform: methanol in the ratio of 7.0:3.0, *v*/*v* was tried for separation of ATR and its degradation product. A main problem was encountered; tailing of LDS. By replacing chloroform by ethyl acetate and the use of a mixture of ethyl acetate: methanol in the ratio of 7.0:3.0, *v*/*v* resulted in *Rf* values of 0.06 and 0.50 for LDS and ATR, respectively. The spots developed were dense, compact at *Rf* of 0.06 ± 0.002 and 0.50 ± 0.02 for LDS and ATR, respectively. Typical peak nature for both drugs with no tailing was observed when plates were scanned at 282 nm (Fig. 3). To improve the spot shapes, particularly when drug of interest migrates near the solvent front, a prewashing step was introduced [21]. The plate was developed with the mobile phase before sample application, and then, activated at 100 °C for 30 min prior to spotting. The saturation of the chamber atmosphere with developing solvent vapor is recommended to obtain reproducible migration distances. To establish the optimum conditions for the analysis of LDS and ATR, the saturation time was studied, and an influence of this parameter on the spot shapes was observed. An increase in the saturation time produced an improvement in the spot shapes due to the decreased layer activity. However, no further improvements were observed when using saturation times longer than 30 min. To maintain a constant saturation degree in the chamber, the developing system was replaced after developing one plate. It should be noted that an important advantage of using an automated sample applicator is that variable volumes of the sample can be accurately applied; obtaining equalized initial zones that lead to an accurate and precise densitometric determination [22].

5.3 Method Validation

Both methods were validated in compliance with the International Conference on Harmonization (ICH) Guidelines [23].

5.4 Linearity

Linearity was studied and a linear relationship between the ATR concentrations and the corresponding relative peak area was obtained.

The regression equation was computed and found to be (HPLC):

$$A = 0.197\ C + 0.005,\ r = 0.9995$$

where A is the relative peak area (to that of a concentration of 5 μg/mL of ATR), C is the concentration in μg/mL for ATR and r is the correlation coefficient.

And the regression equation was computed and found to be (TLC):

$$A = 0.098\ C + 0.218,\ r = 0.9990$$

where A is the relative peak area (to that of a concentration of 10 μg/spot of ATR), C is the concentration in μg/spot for ATR and r is the correlation coefficient. In total 8 and 9 concentrations

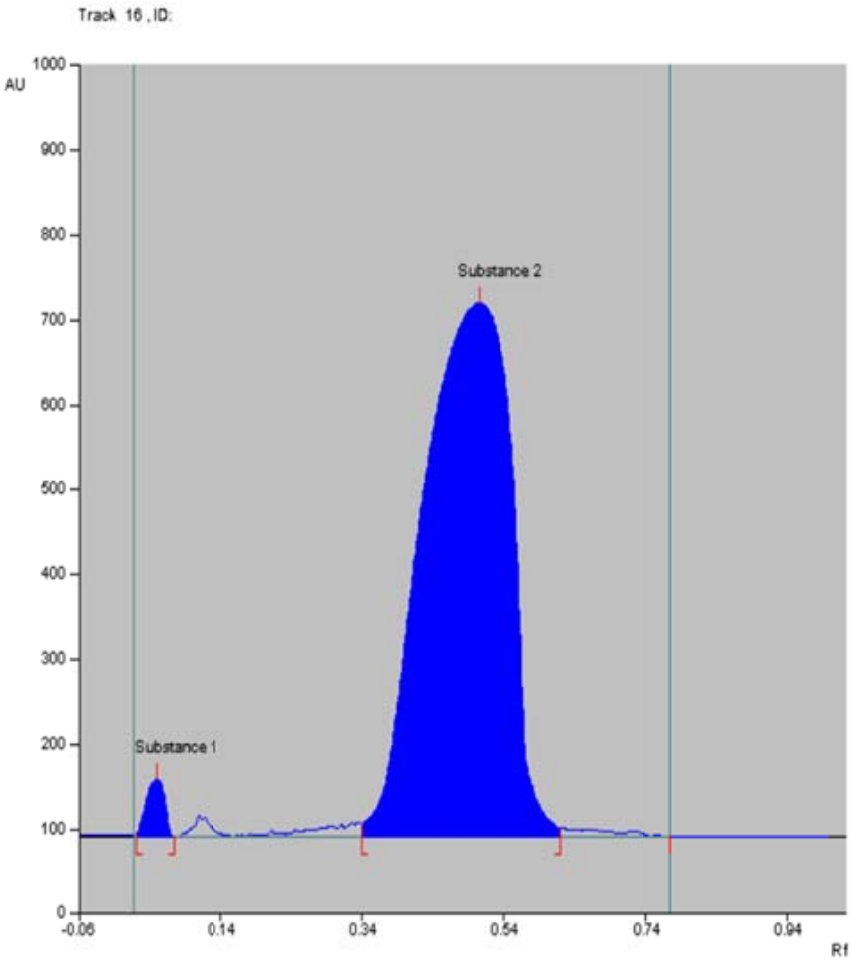

Fig. 3 2D chromatogram of a resolved mixture of ATR (*Rf* = 0.50) in concentration 10 µg/spot and its degradation product LDS (*Rf* = 0.06) using ethyl acetate: methanol: (7:3, *v*/*v*) measured at 282 nm.

compounds were used for HPLC and TLC respectively. The linearity of the calibration curves was validated by the high value of correlation coefficients. The analytical data of the calibration curves including standard deviation for the slope and intercept are summarized (Table 2). It showed good linearity in concentration of 1.0:8.0 µg/mL (r = 0.9995) and 2.0:18.0 µg/spot (r = 0.9990) for HPLC and TLC-densitometric methods, respectively.

5.5 Accuracy

The accuracy of the method was tested by analyzing different samples of ATR at various concentration levels in its pharmaceutical samples' solutions. The results were expressed as percent recoveries of the particular components in the samples. The results obtained including the mean of the recovery, standard deviation, interday and intraday precision, determined within the linearity range (using 3 different concentrations in triplicates for 3 days) are displayed (Table 2). A possible matrix effect was taken into consideration. No blank matrix being available, the method of standard addition was used to ensure accuracy of the proposed methods. The recovery experiments using standard addition method showed mean recoveries between 99.29 and 99.93, and 100.77 and 102.52%, for HPLC and TLC-densitometric methods, respectively, indicating good accuracy of the method.

5.6 Precision

Precision was estimated by repeatability. The repeatability of the method was assessed by analyzing

Table 2 Assay parameters and method validation obtained by applying HPLC and TLC-densitometric methods for the stability indicating determination of retardation factor atracurium besilate.

Item	HPLC method	TLC-densitometric method
Retention time	19.33 ± 0.02	-
Retardation factor	-	0.34 ± 0.02
Wavelength of detection	280	282
LOD	0.0539 μg/mL	0.2946 μg/mL
LOQ	0.1635 μg/mL	0.8929 μg/mL
Range of linearity	1.0:8.0 μg/mg	2.0:18.0 μg/spot
Regression equation	$y = 0.197x + 0.005$	$y = 0.098x + 0.218$
Correlation coefficient (r)	0.9995	0.9990
Slope	0.197	0.098
Standard error of the slope	0.0109	0.0096
Intercept	0.005	0.218
Standard error of the intercept	0.000099	0.0475
Accuracy	99.61 ± 0.322	101.64 ± 0.875
Specificity	100.41 ± 0.696	100.37 ± 1.567
Intraday precision[a]	100.68 ± 0.213	100.83 ± 0.424
Interday precision[b]	98.92 ± 1.232	99.22 ± 0.963
Drug in dosage form	100.44 ± 0.651	100.33 ± 0.420

[a] The intraday (n = 3), average of three concentrations (2.0, 4.0, 6.0 μg/mL) (2.0, 4.0, 6.0 μg/spot), in case of HPLC and TLC-densitometric methods, respectively, repeated three times within the day.

[b] The interday (n = 3), average of three concentrations (2.0, 4.0, 6.0 μg/mL) (2.0, 4.0, 6.0 μg/spot), in case of HPLC and TLC-densitometric methods, respectively, repeated three times in three successive days.

a sample containing 4 μg/mL and 4 μg/spot of ATR (n = 6), for HPLC and TLC-densitometric methods, respectively. The values of the precision (RSD) were 0.21 and 0.42%, for HPLC and TLC-densitometric methods, respectively.

5.7 Specificity

5.7.1 HPLC Method

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences. In the present work, the chromatograms of the samples were checked for the appearance of any extra peaks. No chromatographic interference from any of the excipients was found at the retention times of ATR and LDS (Fig. 2). In addition, the chromatogram of each drug in the sample solution is identical to that received by the standard solutions at the wavelength applied. These results demonstrate that there was no interference from other materials in the pharmaceutical formulation and therefore confirm the specificity of the method.

5.7.2 TLC-Densitometric Method

The specificity of the method was ascertained by comparing laboratory prepared mixture and sample. The spots for ATR and LDS in sample were confirmed by comparing the *Rf* and spectra of the sample with that of standard. Moreover, no other peaks appeared besides the two compounds (Fig. 3).

5.8 Robustness

Robustness is a measure of the method ability to remain unaffected by small variations in the method conditions and is an indication of the method reliability.

5.8.1 HPLC Method

Robustness was performed by deliberately changing the chromatographic conditions. The most important parameter to be studied was the resolution factor between the two peaks of ATR and LDS. The flow rate of the mobile phase was changed from 1.0 to 0.8 and 1.2 mL/min, where resolution factors obtained were 35.29, 35.35 and 35.24, respectively. Values of

Table 3 Statistical analysis of the results obtained by determination of atracurium besilate ampoules by the proposed HPLC and TLC-densitometric methods.

Item	HPLC method	TLC-densitometric method
Mean	100.44	100.33
SD	0.651	0.420
RSD	0.648	0.419
Variance	0.424	0.176
n	3	3
Student's *t*-test	1.706 (2.132)[a]	
F value	17.125 (19)[a]	

[a] Figures in parentheses are the corresponding theoretical *t* and *F* values at $P = 0.05$.

pH of the phosphate buffer were varied from 3.1 to 3.0 and 3.2, where resolution factors obtained were 35.29, 35.22 and 35.39, respectively. Finally, concentration of buffer was varied from 75 to 74 and 76 mM, where resolution factors obtained were 35.29, 35.23 and 35.25, respectively. As can be seen from these results, good values of the resolution factor were obtained for all these variations, indicating good robustness of the proposed LC method.

5.8.2 TLC-Densitometric Method

For robustness, different parameters, including developing solvent composition, chamber saturation, and time from spotting to chromatography and from chromatography to scanning were studied. Minor changes in the ratios of the developing solvent components, up to ±1% and in the time for chamber saturation by ±5 min caused no significant changes in *Rf* or peak areas. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 10, 20, 30, 40, 50 and 60 min. These modified parameters afforded good resolution between ATR and LDS spots and the method was found to be robust.

5.9 Limit of Detection and Limit of Quantification

Limit of detection (LOD), which represents the concentration of analyte at S/N ratio of 3 and limit of quantification (LOQ) at S/N ratio of 10, were determined for the proposed method and results are given in Table 2. LOD and LOQ were computed based on the standard deviation of the response and the slope.

5.10 HPLC versus TLC

Three different Atracurium Besilate samples were determined by HPLC and TLC-densitometric methods. Each sample was analyzed in duplicate. A paired *t*-test and *F* ratio were applied. The obtained values of *t* and *F* are lower than the tabulated ones, which leads to the conclusion that there is no significant difference between the suggested methods. The results of paired *t*-test and *F* ratio are given in Table 3.

6. Conclusions

The proposed HPLC and TLC-densitometric methods provide simple, accurate, selective and reproducible quantitative analysis for determination of Atracurium Besilate in pure form, ampoules and laboratory prepared mixtures with its major degradant (Laudanosine). Both methods were validated as per ICH guidelines. The two proposed methods are suitable for the quality control determination of the pharmaceutical dosage form in ordinary laboratories.

Conflicts of Interest

I declare that I have no funding or no conflict of interest with any person or organization.

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