Journal of Chromatographic Science, 2018, Vol. 56, No. 3, 209–215
doi: 10.1093/chromsci/bmx101
Advance Access Publication Date: 15 December 2017
Article

Article

Chromatographic Methods for Quantitative Determination of Ampicillin, Dicloxacillin and Their Impurity 6-Aminopenicillanic Acid

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Received 8 February 2016; Revised 1 April 2017; Editorial Decision 12 November 2017

Abstract

Two accurate, precise and sensitive high-performance thin layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) methods were developed for assay of ampicillin (AMP) and dicloxacillin (DX) in the presence of their impurity, 6-aminopenicillanic acid (APA). Method (A) is HPTLC method; using silica gel HPTLC F_{254} plates as a stationary phase with methanol: chloroform: acetic acid (1:9: 0.2, by volume) as a developing system. All the bands were scanned at 220 nm. Method (B) is reversed phase- HPLC which depended on isocratic elution using C_{18} column and mobile phase consisting of acetonitrile: water (60:40, *v/v*), pH adjusted to 4 with orthophosphoric acid, at a flow rate of 1 mL min^{-1} and ultraviolet detection at 240 nm. The proposed methods were validated as per ICH guidelines and their linearity was evident in the ranges of 0.5–2 μg $band^{-1}$, 0.4–2 μg $band^{-1}$ and 0.2–1.2 μg $band^{-1}$ for method (A) and 5–40 μg mL^{-1}, 5–40 μg mL^{-1} and 2–16 μg mL^{-1} for method (B) for AMP, DX and APA, respectively. The proposed methods were successfully used for assay of AMP and DX in pure form and in pharmaceutical formulation where no interference from the excipients was detected.

Introduction

Ampicillin (AMP) shown in Figure 1 is chemically known as (2 S, 5 R, 6 R)−6-([(2R)−2-amino-2-phenylacetyl] amino)−3, 3- dimethyl-7-oxo-4-thia-1-azabicyclo heptane-2-carboxylic acid. Dicloxacillin (DX) shown in Figure 1 is chemically known as (2 S, 5 R, 6 R)−6-{[3-(2, 6-dichlorophenyl)−5-methyl- oxazole-4-carbonyl] amino}−3, 3-dimethyl-7-oxo-4-thia-1-azabicyclo heptane-2-carboxylic acid. Both of them are used alone and in constant dose combinations as antimicrobial and antibacterial agents (1, 2). 6-aminopenicillanic acid (APA) shown in Figure 1, is chemically known as (2 S, 5 R, 6 R)−6-Amino-3, 3-dimethyl-7-oxo-4- thia-1-azabicyclo heptane-2- carboxylic acid.

AMP is a strong antibiotic with short-term stability in aqueous solution (3, 4). It is used clinically to treat a wide range of infections caused by bacteria (5–7). Using parenteral injection; AMP is distributed rapidly, leading to a high concentration of the drug in bile (8). From bile, it is excreted into the gut and it may cause changes in the normal intestinal micro-flora by decreasing the main flora and increasing the number of yeast also inducing a high risk of clostridium colitis (9).

DX (INN) is a strict spectrum antibiotic of the penicillin family. It is used in treating infections caused mainly by gram positive bacteria. It is similar to flucloxacillin and the two drugs can be used interchangeably, also DX can withstand acidity more than other

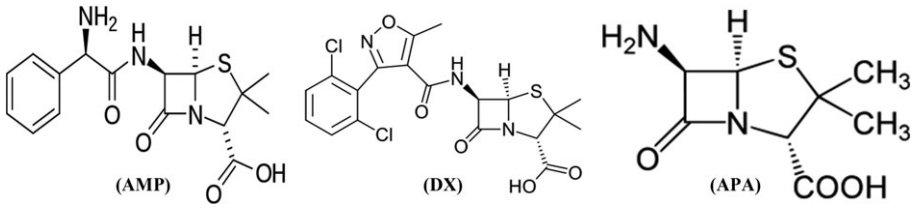

Figure 1. Chemical structures of AMP, DX and 6-APA.

penicillins so dicloxacilin usually is taken as oral drug as well as parental way (10).

6-APA has a beta lactam nucleus and it is the major impurity of both of AMP and DX. APA has less pharmacological activity than any of the studied drugs (10).

Literature review revealed the analytical methods for the determination of AMP and DX, which include high-performance liquid chromatography (HPLC) method for determination of AMP and DX in plasma (11). HPLC–tandem mass spectrometry (HPLC–MS-MS) with electro spray ionization for determination of AMP and DX in human plasma, and another HPLC, LC–MS-MS for determination of AMP and DX in human plasma (12–14). Additionally, polarography (15) and ultraviolet (UV) spectrophotometry (16) were reported for the assay of AMP and DX but most of these techniques require expensive, complex procedures, instrumentation and cannot determine AMP, DX and APA in a ternary mixture (17). Another spectrophotometric methods were reported for simultaneous determination of AMP and DX in the presence of APA but these methods can determine AMP and DX only, however, cannot determine APA so still these spectrophotometric methods cannot determine all of AMP, DX and APA in a ternary mixture (18). In this paper, the development of these two chromatographic methods was established. These suggested methods have the capability to simultaneously separate and quantitate AMP, DX and APA which was a big challenge due to structure similarity between all of them. Determination of APA is very important as it has no pharmacological activity so decrease the efficacy and increase the toxicity of the formulation used by patients. The developed chromatographic methods are time saving, simple, accurate, economic, sensitive and reproducible and can be used in quality control laboratories.

Experimental

Instruments

A double beam UV-VIS spectrophotometer (SHIMADZU, Japan) model No. UV-1601 PC using1 cm path length quartz cell. The software was UVPC personal spectroscopy software version 3.7. Camag TLC scanner model 3 S/N 130319 using win-CATS software. The specifications used are 20 mm s^{-1} rate of scanning, 100 μm $step^{-1}$ resolutions of data, chromatogram coupled with integrated peak area as a result output, slit dimension: 0.3 × 6 mm, spraying speed 10 s μL^{-1}, band width: 6 mm, linomat IV with 100 μL syringe (Camag, Muttenz, Switzerland), high-performance thin layer chromatography (HPTLC) 20 cm × 20 cm aluminum plates coated with 0.25 mm silica gel 60 F_{254} (Merck, Germany) used due to its high ability of separation, resolution and rapid time of development compared to traditional TLC plates, also sonix TV ultrasonicator (USA) was used.

Shimadzu class-LC 10 AD liquid chromatograph with a UV-VIS spectrophotometer detector, a degasser (DGU-3 A) and a data processor (C-R4A) Shimadzu, Kyoto, Japan. The analytical column used was C_{18}-ODS a Phenomenex (Shimadzu, Kyoto, Japan), 25 cm × 4.6 mm I.D., 5 μm particle size protected by a C_{18} guard column.

Material and reagents

Pure samples

AMP, DX and 6-APA were purchased from SIGMA-ALDRICH, Talat Harb, Cairo, Egypt. Their purities were found to be 99.24%, 99.18% and 99.48%, respectively, according to the supplier analysis certificate.

Pharmaceutical dosage form

Cloxapen® capsules batch no. 2941060404830 are labeled to contain 250 mg AMP and 250 mg DX. It was manufactured by Misr Co. Pharmaceutical Industries, El Matareya, Cairo, Egypt.

Chemicals and reagents

All chemicals and solvents used in this work were of high-analytical grade. Methanol and chloroform HPLC grades were purchased from Sigma-Aldrich Chemie GmbH, Germany. However, glacial acetic acid was of analytical grade was purchased from (El-Nasr Pharmaceutical Chemicals Co., Abu- Zabaal, Cairo, Egypt) and deionized water was purchased from SEDICO Pharmaceuticals Co., Egypt.

Solutions

Stock standard solutions of AMP, DX and APA were dissolved in methanol in the concentration of 1 mg mL^{-1}. Working standard solutions of AMP, DX and APA were dissolved in methanol in the concentration of 100 μg mL^{-1}. Fresh preparations for all stock standard solutions prepared on the same day of analysis and kept in refrigerator to be used within 24 h.

Sample solutions

Ten capsules of the pharmaceutical formulation were weighed, powdered and mixed well. An accurately weighed aliquots equivalent to 100 mg of the pharmaceutical formulation was transferred into 100-mL volumetric flask and then 75 mL methanol was added. The prepared solution was sonicated for 10 min, cooled then completed to 100 mL with methanol. The solutions were filtered and diluted to obtain 100 μg mL^{-1} working solutions. When carrying out the standard addition technique, the powdered capsules and pure AMP and DX were mixed well together before doing the above mentioned steps.

Methods

For HPTLC method

Construction of calibration curves

Into a set of 10-mL volumetric flasks, different portions equivalent to 0.5–2 μg, 0.4–2 μg and 0.2–1.2 μg for AMP, DX and APA were

accurately taken from their standard stock solutions (1 mg mL^{-1}), and volume was completed with methanol. In all, 10 µL from each solution was applied to HPTLC plates (20 cm × 10 cm prewashed with methanol and dried at 60°C for 5 min before sample application) in the shape of bands (band length = 6 mm by a Camag Linomat IV applicator). The spaces between bands are 5 mm from each other and 10 mm apart from the bottom edge of the plate. Linear ascending development was done in a chromatographic chamber previously saturated with methanol: chloroform: acetic acid (1:9: 0.2, by volume) for 30 min. The migration distance was 100 mm from the lower edge of the plate, and the developed plates were air dried and scanned at 220 nm under the specified instrumental conditions. Calibration curves relating to the recorded integrated area under peak against the corresponding concentrations as µg band^{-1} were then constructed from which the regression equations were calculated.

Application to pharmaceutical formulation

The procedure previously illustrated under construction of calibration curve was followed on the prepared working solution (100 µg mL^{-1}) of AMP and DX then the concentrations of AMP and DX were computed from the corresponding regression equations.

For HPLC method

Construction of calibration curve

Accurate portions from AMP, DX and APA were individually transferred from their corresponding working standard solutions (100 µg mL^{-1}) into three separate sets of calibrated measuring flasks to prepare solutions having the concentration ranges of 5–40 µg mL^{-1}, 5–40 µg mL^{-1} and 2–16 µg mL^{-1} of AMP, DX and APA, respectively. Triplicate injections were done from each of the prepared solutions; the integrated peak area was used to construct the calibration curve for each analyte from which its regression equation was computed. Chromatographic separation was carried out in isocratic mode on a C_{18} column with a mobile phase consisting of acetonitrile: water (60:40, *v/v*), pH adjusted to 4 with orthophosphoric acid, delivered at a flow rate 1 mL min^{-1}. UV scanning at 240 nm and the injection volume was 20 µL at normal room temperature. The run time was 10 min and the integrated peak area was used to determine the studied analytes.

Application to pharmaceutical formulation

The steps mentioned under construction of calibration curve were done on the prepared working solution (100 µg mL^{-1}) of AMP and DX then the concentrations of AMP and DX were computed from the corresponding regression equations.

Results

HPTLC method

Planar chromatography with reproducible application of the samples and computed evaluation and assay of the developed chromatograms was perfect for purity control and qualitative drug testing. Also, HPTLC is one of the useful methods which does not need tedious clean-up, and by appropriate developing systems, all interfering agents will be omitted.

Upon applying the optimized conditions, the R_f values of the studied components were 0.28, 0.53 and 0.78 for AMP, DX, and APA, respectively. As can be seen in Figure 2A, the separated peaks are sharp, symmetric and well separated.

A linear relationship was established between the concentrations of (AMP, DX and APA) versus the integrated peaks area/10^4 in the range of 0.5–2 µg band^{-1}, 0.4–2 µg band^{-1} and 0.2–1.2 µg band^{-1} for AMP, DX and APA, respectively, and the regression equations were computed and found to be:

$A_1 = 0.2117\ C_1 + 0.1454$	$r_1 = 0.9998$	For AMP
$A_2 = 0.1937\ C_2 + 0.1844$	$r_2 = 0.9999$	For DX
$A_3 = 0.3016\ C_3 + 0.1101$	$r_3 = 0.9999$	For APA

Where A_1, A_2 and A_3 are the relative peaks area, C_1, C_2 and C_3 are the concentrations in µg band^{-1} and r_1, r_2 and r_3 are the correlation coefficients of AMP, DX and APA, respectively. Regression equation parameters are given in Table I.

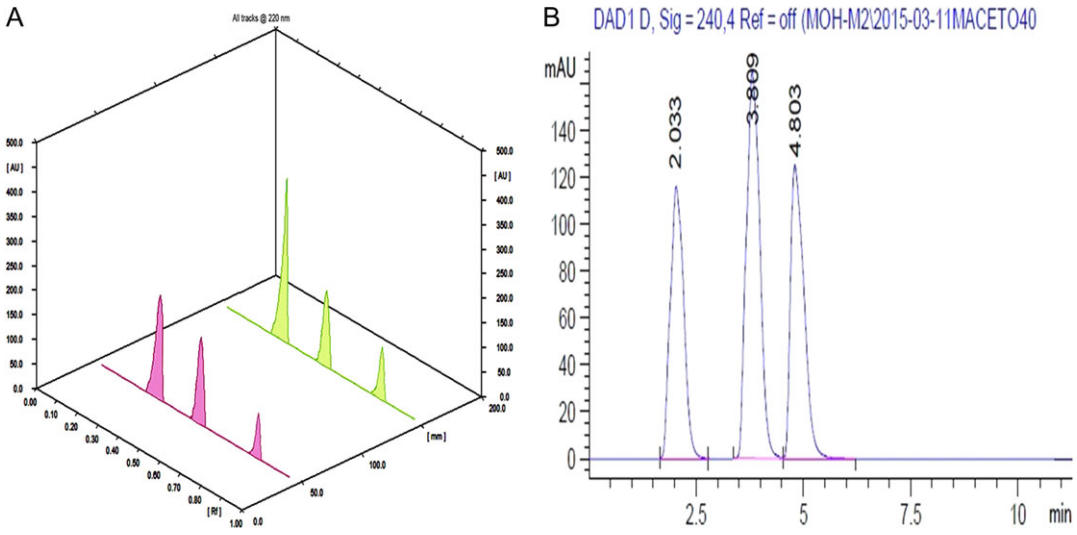

Figure 2. (A) HPTLC-densitogram of a resolved mixture of AMP (R_f = 0.28), DX (R_f = 0.53) and 6-APA (R_f = 0.78) using methanol: chloroform: glacial acetic acid (1:9: 0.2, by volume) as a developing system, (B) reversed phase- HPLC (RP-HPLC) chromatogram of a resolved mixture of AMP, DX and 6-APA using acetonitrile: water (60:40, *v/v*), pH adjusted to 4 with orthophosphoric acid.

RP-HPLC method

After method optimization, chromatographic separation of the three components was achieved using C_{18} column with acetonitrile: water (60:40, *v/v*), pH adjusted to 4 with orthophosphoric acid, at flow rate of 1 mL min^{-1} with UV detection at 240 nm. The retention time values were found to be 2.033 min, 3.809 min and 4.803 min for AMP, DX and APA, respectively, as shown in Figure 2B. Calibration graphs were constructed by plotting the relative peak area (using 10 μg mL^{-1} of each component as external standard solution) versus the corresponding concentrations of each component and the regression equations were then computed and found to be:

$A_1 = 0.0831\ C_1 + 0.0042$	$r_1 = 0.9999$	For AMP
$A_2 = 0.1212\ C_2 - 0.0127$	$r_2 = 0.9999$	For DX
$A_3 = 0.27141\ C_3 + 0.0055$	$r_3 = 0.9998$	For APA

Where A_1, A_2 and A_3 are the relative peaks area, C_1, C_2 and C_3 are the concentrations in μg $band^{-1}$ and r_1, r_2 and r_3 are the correlation coefficients of AMP, DX and APA, respectively. Regression equation parameters are listed in Table I.

The validity of the proposed methods for analysis of AMP and DX was studied by assaying Cloxapen® capsules (Table II). It was further validated by applying standard addition technique, which proved that excipients do not cause any interference (Table II).

Both of the proposed methods can accurately determine the ternary mixture of AMP, DX and their impurity APA in a short time with high selectivity and precision.

Application to pharmaceutical formulation

The proposed chromatographic methods were used for the determination of AMP and DX in its pharmaceutical formulation; Cloxapen® capsules. The results are illustrated in Table II. Standard addition technique was also successfully done as illustrated in Table II. The acceptable percentage recoveries values prove the suitability of the proposed methods for the usual determination of these components in their pharmaceutical formulation.

Statistical analysis

Statistical comparison was established between results obtained by the proposed methods for determination of pure samples of AMP and DX and those obtained by reference HPLC method (12).The values showed no significant difference between them Table III.

Discussion

For HPTLC method development and optimization

For application of the method, the conditions were optimized to get the best resolution and efficiency.

Several trial experiments were performed in order to select a suitable mobile phase for optimum separation of the three components. Several solvent systems were tried e.g. methanol–chloroform (6:4, *v/v*), chloroform–methanol (8:2, *v/v*) but the polarity was unsuitable to separate AMP from DX specially due to structure similarity, so tried addition of some acidity by using glacial acetic acid in a system composed from methanol–chloroform–acetic acid (8:2:0.3, by volume)

Table I Results of Validation Parameters' assay of the proposed HPTLC and HPLC methods for the determination of AMP and DX in the presence of their major impurity 6-APA

Parameter	HPTLC method			HPLC method		
	AMP	DX	APA	AMP	DX	APA
Range	0.5–2 μg $band^{-1}$	0.4–2 μg $band^{-1}$	0.2–1.2 μg $band^{-1}$	5–40 μ mL^{-1}	5–40 μ mL^{-1}	2–16 μ mL^{-1}
Slope	0. 2117	0.1937	0.3016	0.0831	0.1212	0.2714
Intercept	0.1454	0.1844	0.1101	0.0042	0.0127	0.0055
Correlation coefficient (*r*)	0.9998	0.9999	0.9999	0.9999	0.9999	0.9998
Accuracy (mean ± SD)	100.33 ± 0.91	100.82 ± 1.00	100.42 ± 1.10	100.36 ± 0.94	100.22 ± 0.82	99.98 ± 1.11
Repeatability (RSD%)	0.367	0.436	0.482	0.530	0.486	0.528
Intermediate precision (RSD%)	0.857	0.766	0.902	0.806	0.703	0.925
LOD	0.16	0.13	0.05	1.53	1.56	0.53
LOQ	0.47	0.38	0.17	4.58	4.75	1.60

[a]The intraday precision ($n = 3$), average of three different concentrations repeated three times within 1 day (19).
[b]The interday precision ($n = 3$), average of three different concentrations repeated three times on 5 successive days (19).

Table II. Determination of AMP and DX in Their Pharmaceutical Formulation (Cloxapen® Capsules Batch No. 2941060404830) by the Proposed HPTLC and HPLC Methods and Application of the Standard Addition Technique

HPTLC method								HPLC method							
Taken (μg $band^{-1}$)		Found%*		Pure added (μg $band^{-1}$)		Recovery%		Taken (μg mL^{-1})		Found%*		Pure added (μg mL^{-1})		Recovery%	
AMP	DX	AMP	DX	AMP	DX	AMP	DX	AMP	DX	AMP	DX	AMP	DX	AMP	DX
1	1	99.95	99.43	0.3	0.3	100.60	99.87	10	10	99.76	98.20	5	5	100.38	100.07
				0.7	0.7	98.81	99.99					7	10	99.30	98.11
				1.0	1.0	100.76	98.32					10	15	98.26	99.04
Mean ± SD						100.06 ± 1.08	99.40 ± 0.93	Mean ± SD						98.86 ± 0.91	99.07 ± 0.98

*Average of 6 determinations.

Table III. Statistical Comparison of the Results Obtained by the Proposed HPTLC and HPLC Methods and the Reported Method for Determination of Pure AMP and DX in the Presence of Their Major Impurity 6-APA

Parameter	HPTLC method		HPLC method		Reported method (14)	
	AMP	DX	AMP	DX	AMP	DX
Mean	100.33	100.83	100.26	100.18	99.78	99.73
SD	0.914	1.003	1.031	0.880	1.392	1.347
Variance	0.835	1.006	1.062	0.774	1.938	1.814
N*	7	7	7	7	7	7
Student's *t*-test**(2.45)**	0.885	1.733	0.742	0.752	–	–
F*-test*(4.28)**	2.320	1.803	1.825	2.343	–	–

*N is the number of tested samples.

**Figures in parenthesis are the corresponding tabulated values at $P = 0.05$.

but no good resolution was obtained from the latter as high acidity made some tailing for AMP. Complete separation of AMP, DX and APA and avoidance of tailing was achieved by using methanol: chloroform: glacial acetic acid (1:9: 0.2 by volume) as a developing system where the acidity was suitable for separation of AMP from DX without any tailing for each of them.

The saturation time required before development is important to achieve a homogenous atmosphere, thus, minimizing the evaporation of the solvent from HPTLC plate during the development. The saturation time of the developing system was optimized and found to be 30 min.

Different scanning wavelengths (210 nm, 220 nm, 230 nm, 251 nm, 269 nm and 254 nm) were tried in order to enhance the sensitivity of the method; scanning at 220 nm gave the best sensitivity for all the studied components as they all has high absorbance at 220 nm.

For RP-HPLC method development and optimization

Many important factors affecting chromatographic separation were studied and optimized like the type of organic modifier (methanol and acetonitrile) and its ratio (25:75% of the mobile phase), flow rate (0.8:1.5 mL min^{-1}) and scanning wavelength (210 nm, 220 nm, 230 nm, 269 nm and 254 nm). Different mobile phases were tried to obtain the best chromatographic separation between the studied drugs such as methanol: water (40:60 and 80:20, *v/v*), methanol: 0.1% acetic acid solution (80:20, *v/v*) but using methanol cause some noise and do not gave the desired separation specially between AMP and DX, so tried acetonitrile: water (60:40, *v/v*) which gave good separation but there was a problem in peaks symmetry which eliminated by adjusting pH to 4 with orthophosphoric acid. Using the last system gave the best chromatographic resolution with sharp symmetric peaks. Effect of scanning wavelength on the sensitivity of the method was studied by testing different scanning wavelengths (210 nm, 220 nm, 240 nm, 254 nm and 269 nm) where scanning at 240 nm gave the lowest limits of detection and quantitation (LOD and LOQ) values. Additionally, the effect of the flow rate of mobile phase was tried and a flow rate of 1 mL min^{-1} gave good resolution and rapid analysis. The first analytical column tried was C_8 but no acceptable separation obtained due to unsuitable polarity so tried C_{18}-ODS analytical column which gave good separation and resolution.

Method validation

Method validation was carried out following ICH guidelines (19).

Linearity

Using optimum chromatographic conditions; linearity of developed methods was assessed by measuring the integrated peak area for many concentrations from AMP, DX and APA and then plotting the calibration curves relating the peak area versus the corresponding concentrations from which the regression equations were calculated. Parameters of regression equation and linearity ranges are listed in Table I.

Accuracy

It was expressed as the percentage recoveries of pure components, it was validated by performing recovery studies at three levels (80%, 100% and 120% addition) and the mean recovery percent was then calculated. Acceptable recovery percents were obtained and are illustrated in Table I.

Accuracy of the method was calculated by applying the standard addition technique on pharmaceutical formulation where good recoveries were obtained revealing no interference from excipients, as given in Table II.

Precision

It was studied regarding both repeatability and intermediate precision as follows.

Repeatability

Three different concentrations of pure components in triplicate on the same day of (0.5 µg, 1 µg and 1.2 µg $band^{-1}$) of AMP, DX and APA for HPTLC method, and (5 µg mL^{-1}, 10 µg mL^{-1} and 15 µg mL^{-1} of AMP, DX, and APA for HPLC method were analyzed three times intra-daily using the proposed method. Good RSD% values were obtained proving the repeatability of the methods as given in Table I.

Intermediate precision

The previous steps were repeated inter-daily on 5 consecutive days for the analysis of the three chosen concentrations. Good RSD% values were obtained as given in Table I.

Specificity

The specificity of the methods was proven by good separation of the three studied components, Figure 2. The specificity of methods was evaluated by how specifically and accurately the analyte of interest is determined in the presence of other components (e.g. co-formulated drugs, impurities, excipients, etc.), Table II.

Table IV. Experimental Results of Robustness Testing for Determination of AMP, DX and APA by the Proposed HPTLC Method

Parameters (%RSD)	AMP, HPTLC method RSD (R_f)	DX, HPTLC method RSD (R_f)	APA, HPTLC method RSD (R_f)
Methanol (1 mL ± 1%)	0.49	0.63	0.59
Chloroform (9 mL ± 1%)	0.35	0.29	0.39

Table V. Experimental Results of Robustness Testing for Determination of AMP, DX and APA by the Proposed HPLC Method

Parameters (%RSD)	AMP, HPLC method RSD (AUC)	DIC, HPLC method RSD (AUC)	APA, HPLC method RSD (AUC)
Acetonitrile/buffer (63:73, *v/v*)	1.42	1.37	1.32
Acetonitrile /buffer (20:80, *v/v*)	0.85	0.73	0.74
Flow rate (1.5 mL min^{-1})	0.72	0.67	0.65
Flow rate (0.8 mL min^{-1})	0.58	0.59	0.62
pH (4 + 0.1)	0.95	0.87	0.93
pH (4 – 0.1)	0.52	0.63	0.58
Separation time (+5%)	0.94	0.82	0.61
Separation time (−5%)	0.46	0.39	0.31

LOD and LOQ

According to the HPLC method, the process based on the standard deviation of the response and the slope was used for the determination of the detection and quantitation limits, Table I.

$$LOD = 3.3 \times SD / slope \qquad LOQ = 10 \times SD / slope \quad (19)$$

LOD and LOQ were calculated using a noninstrumental visual method according to the ICH recommendations (19), in case of HPTLC method.

Low values of both LOQ and LOD indicate the high sensitivity of the developed methods, Table I.

Robustness

The proposed methods were found to be robust. Very small changes in the studied chromatographic conditions (e.g. change in methanol and chloroform amount ±1%) in case of HPTLC method or (change in the amount of the organic modifier ± 1%, flow rate ± 0.05 mL min^{-1}, pH ± 0.1 and separation time ± 5%) for HPLC method did not result in a significant change in R_f values, R_t values, peak area or symmetry of the peaks; Tables IV and V.

System suitability

Any analytical procedures must be evaluated by system suitability as ICH guidelines consider it an integral part of different analytical techniques. System suitability tests were done for all the proposed methods to prove that they perform in good manner. Parameters were calculated where acceptable results were obtained: tailing factor (1.30, 1.23 and 1.37), capacity factor (*K'*) (2.30, 5.30 and 8.00) for AMP, DX and APA, respectively, resolution (R_s) (2.39 and 1.93) and selectivity factor (α) (2.30 and 1.51) for HPTLC method. Also parameters were calculated where good results were obtained: tailing factor (0.92, 0.86 and 0.80), capacity factor (*K'*) (1.03, 2.81 and 3.80) for AMP, DX and APA, respectively, resolution (R_s) (2.40 and 1.53) and selectivity factor (α) (2.80 and 1.36) for RP-HPLC method.

Conclusion

The proposed HPTLC and RP-HPLC methods are precise, accurate and were validated as per ICH guidelines. The advantage of the developed HPTLC method is that many samples can be run once using a small amount of mobile phase and can be used for the determination of AMP and DX with high sensitivity in the presence of their major impurity, while HPLC method introduces the desired reproducibility and accuracy. Owing to nonpharmacological and allergic effect of APA, these suggested methods have a valuable importance to determine the amount of APA to avoid its toxicity and the purity of AMP and DX to maximize their efficacy and biological activity. On the other hand, application of these methods to a pharmaceutical preparation proved that excipients do not interfere with the assay of the studied components and hence it can be used for their determination either in pharmaceutical formulation or in bulk powder.

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