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Short communication

Determination of azithromycin by ion-pair HPLC with UV detection

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ABSTRACT

An ion-pair reversed phase high performance liquid chromatographic method with UV detection was developed for the determination of azithromycin using sodium heptanesulfonate as an ion-pair reagent. The mobile phase consisted of a mixture of ammonium dihydrogen phosphate (0.045 M, pH 3.0 adjusted by phosphoric acid):acetonitrile 47:15 (v/v) and the concentration of sodium heptanesulfonate in the aqueous phase was 0.002 M. UV detection was performed at 210 nm. The chromatographic column was Dikma Technologies Diamonsil C_{18} column, 5 μm 150 mm × 4.6 mm, which was maintained at 25 °C. Applying the method to a stability study of azithromycin eye drops, it was found that the related substance could be detected and the profile of the AZM peak was symmetrical and the column efficiency was high. Accordingly, it is suitable for the routine analysis and stability testing of azithromycin preparations.

1. Introduction

Azithromycin (AZM) is a semisynthetic macrolide antibiotic with a 15-membered azalactone ring (as shown in Fig. 1). Like erythromycin, it appears to bind to the same receptor, 50 S ribosomal subunits of susceptible bacteria and suppresses protein synthesis. It is effective against a variety of Gram-positive and Gram-negative bacteria.

A large number of reports have been published about the determination of AZM. Several microbiological methods had been used for content assays, but these lacked specificity because they also detected the active metabolite of AZM. In order to overcome these problems, several HPLC methods [1–3] have been developed. An HPLC method with a diode-array detector for the determination of AZM and its impurities has been reported [1] but it had an asymmetric peak profile, indicating low column efficiency. Another similar method was reported by Zubata et al. [2] but it was not suitable for detecting substances related to raw AZM. Other methods, including electrochemical detection [4–9], fluorescence detection by pre-column derivatization [10–12] and liquid chromatography–mass spectrometry or LC–MS/MS [13–15] have been used to determine AZM in routine pharmaceutical dosage forms or biological matrices. Obviously, it would be a waste of LC–MS/MS if it was used for the routine quality control of AZM preparations. Moreover, pre-column derivatization is time-consuming due to the complex steps involved. Also, amperometric electrochemical detectors are not widely available in many laboratories. However, the USP method describes a high pH mobile phase (pH 11) as well as a specific column "Gamma-alumina" which is quite expensive and difficult to obtain in order to assay AZM using an amperometric electrochemical detector. There is a need for a convenient and effective method for the quality control of AZM. Therefore, in this paper, a new ion-pair HPLC method with UV detection was developed for the determination of AZM. It is a promising method which is precise, accurate, robust and practical, and is suitable for the routine quality control of AZM in pharmaceutical dosage forms.

2. Experimental

2.1. Chemicals and reagents

Standard AZM (99.2% of purity) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China, serial number 130352-200405). Acetonitrile (HPLC grade) was obtained from Concord (Tianjin Concord Technology Co. Ltd, Tianjin, China). The ion-pair reagent, sodium heptanesulfonate, and other chemicals including ammonium dihydrogen phosphate and phosphoric acid were obtained from Yuwang (Chemical Reagent Plant, Shan-dong, China). All other chemicals were of analytical grade. Water was purified by redistillation and passed through a 0.22 μm membrane filter before use.

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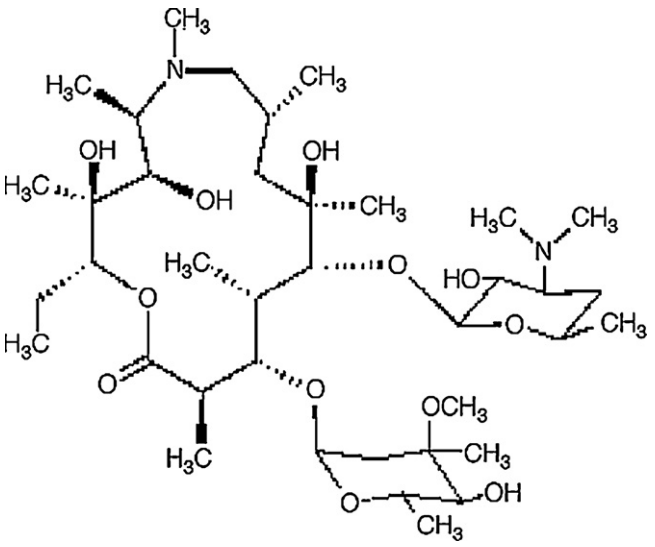

Fig. 1. Structure of azithromycin.

2.2. Chromatography

The HPLC system consisted of a PU-2080 pump, and a UV-2075 UV detector (Jasco, Japan). The chromatographic separation was performed on a Dikma Technologies Diamonsil C_{18} column, 5 μm 150 mm × 4.6 mm. The column was kept at 25 °C using a column heater (IC 2001 plus, TOHO CORP, Japan). The mobile phase was composed of a mixture ammonium dihydrogen phosphate (0.045 M):acetonitrile 47:15 (v/v) and the aqueous phase contained 0.002 M sodium heptanesulfonate. Before being mixed with the organic phase, the ammonium dihydrogen phosphate (0.045 M) was adjusted to pH 3.0 by phosphoric acid. The UV detector was operated at 210 nm and the volume of each injection was 20 μl.

2.3. Sample preparation and standard solutions

2.3.1. Preparation of AZM eye drops

We prepared AZM eye drops according to AZASITE (an AZM eye drop formulation prepared by InSite Vision Incorporated, USA). It was composed of AZM 1.0 g, and other excipients including citric acid, sodium citrate, the preservative benzalkonium bromide, and sodium chloride and sodium hydroxide to obtain a stable pH value for AZM, then diluted to 100 ml using water for injection. Before injection, the preparation was diluted 10-fold with purified water.

2.3.2. Standard solutions

Stock solutions of AZM were prepared, using 100 mg AZM made up to 10 ml in a volumetric flask using a solution of acetonitrile:water 50:50 (v/v). A series of solutions were prepared by further dilution of the stock solutions with mobile phase. The working standard, containing about 100 mg AZM (accurately weighed) was transferred to a 100 ml volumetric flask, followed by the addition of about 70 ml mobile phase and sonicated for 5 min, then made up to 100 ml with mobile phase.

3. Results and discussion

3.1. Validation

The validation was carried out by examining the linearity, limit of quantification, recovery, precision and stability of the method.

The linear range was determined by further dilution of stock solutions from 0.2 mg/ml to 2.0 mg/ml, to give a total of five different concentrations to construct the calibration curve. The linear regression equation was $y = 140{,}060x - 1402.6$ with a correlation coefficient $R = 1$. In the determination of the limit of related substances, where the concentration of AZM was extremely low, the linearity ranged from 10 μg/ml to 100 μg/ml and the regression equation was $y = 2692.2x - 1666.5$ with $R = 0.9997$. By steadily diluting the samples, the final limit of quantification was 3.0 μg/ml (S/N = 10).

Table 1
Recovery ($n = 3$) of three concentrations of simulated preparations.

Amount added (mg/ml)	Amount found (mg/ml)	Recovery (%)	R.S.D., $n = 3$
5.01	5.04	100.64	0.72%
10.03	10.12	100.94	0.17%
15.01	14.87	99.06	0.15%

A recovery study was performed by analysis of simulated preparations at three concentrations, namely 50%, 100% and 150% of the labeled amount of AZM preparations. The results of the recovery of the three concentrations of simulated AZM eye drop preparations are summarized in Table 1.

The precision of the method was determined by injecting the working standard six times in succession, and the R.S.D. of the six peak areas was calculated (R.S.D. = 0.31%, $n = 6$). Also, the content of six samples from the same batch, was determined by the external standard method (the concentration of the working standard was 0.98 mg/ml), and the R.S.D. of the relative content (%) was 0.42% ($n = 6$).

The stability of the sample solutions was tested by using the same working standard for 0 h, 2 h, 4 h, 6 h and 8 h with the same mobile phase. Between runs, solutions were stored at 4 °C. The areas of the five peaks were calculated with an R.S.D. of 0.35% ($n = 5$).

In order to determine the limit of related substances, a raw materials destruction test was carried out. The test involved exposure to acid, base, H_2O_2 (oxidative medium) and heat. Fig. 2 shows the chromatograms of AZM raw materials after treatment with acid, base, heating and oxidation by H_2O_2. It can be seen that the related substances can be detected and separated effectively.

3.2. Establishment and optimization of the ion-pair HPLC method

AZM contains two nitrogen atoms (as shown in Fig. 1), which make it a weakly basic compound. There have always been problems in analyzing drugs like AZM because of their basic properties. These compounds will interact strongly with the polar ends of the HPLC column packing materials (for example, residual silanol groups), becoming adsorbed to the column and are not easily eluted by the mobile phase. Therefore, asymmetric peaks are often observed under these conditions. Moreover, the analysis of the drug is complicated, because azithromycin has only a weak UV absorbance in the wavelength range below 220 nm. Several chromatography systems were investigated by us to resolve these difficulties: acetonitrile was selected for the organic phase instead of methanol because of its higher eluting ability. The aqueous phase, involving KH_2PO_4–K_2HPO_4 or $NH_4H_2PO_4$ buffer solutions, was mixed with acetonitrile. Different pH values of the aqueous phase, ranging from 2.5 to 7.5, were investigated because increasing or decreasing the ionization of AZM by pH adjustment can change the column retention of AZM. A high pH will reduced the ionization of AZM and increase its retention time while a low pH will increase the ionization of AZM and shorten its retention time. However, irrespective of the mixture ratio and pH of the aqueous phase, the peak profile was not symmetric and the tailing factor was unacceptable. The use of organic modifiers, such as triethylamine, which usually improve the peak shape of compounds with amino groups, was not successful for azithromycin and increased the baseline noise; there-

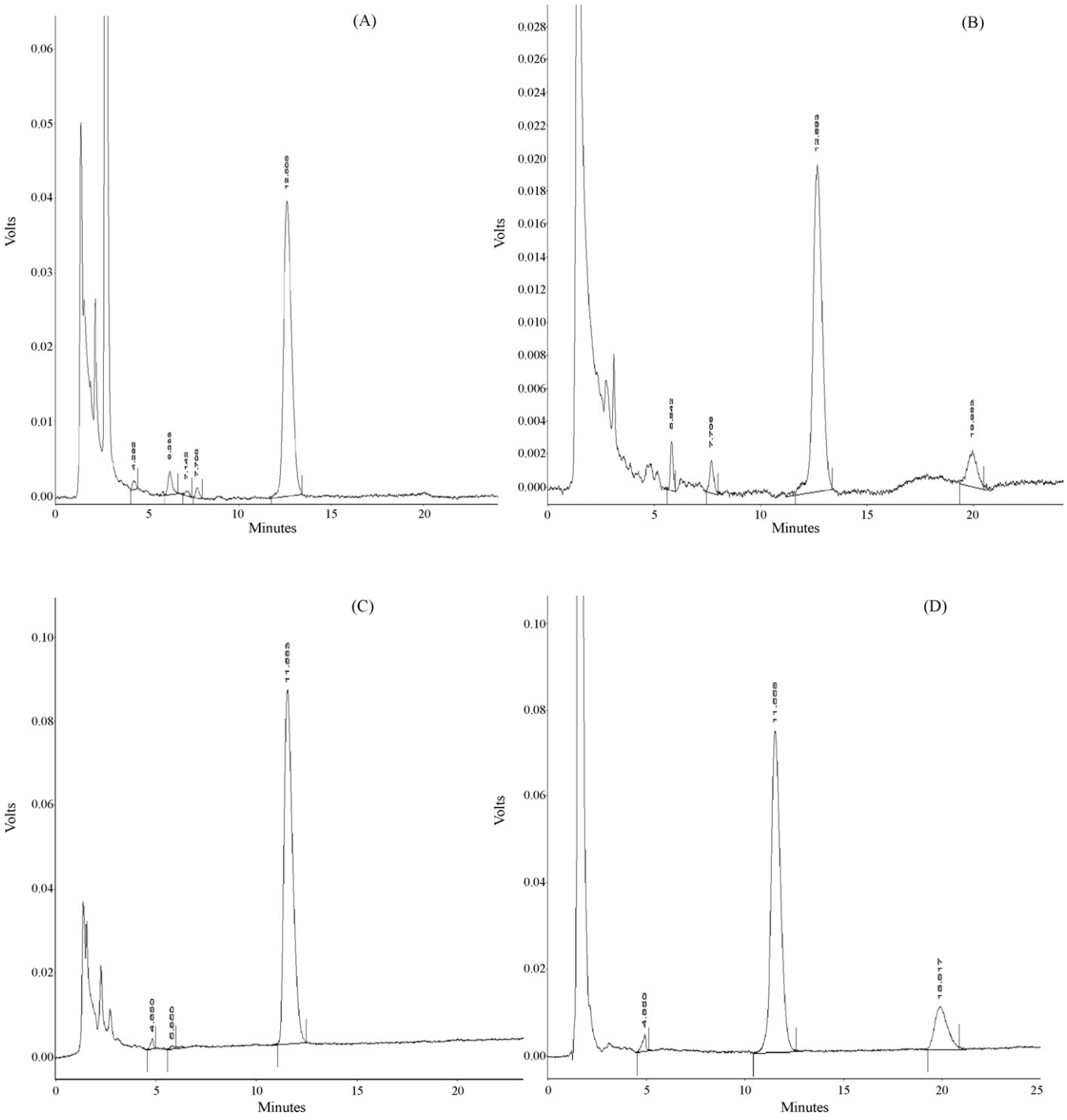

Fig. 2. Representative chromatograms of AZM raw material after treatment with 1 M hydrochloride (A); heat (B); 1 M sodium hydroxide (C); 10% H_2O_2 (D). The mobile phase was a mixture of ammonium dihydrogen phosphate (0.045 M, pH 3.0 adjusted by phosphoric acid):acetonitrile 47:15 (v/v) and the concentration of sodium heptanesulfonate in the aqueous phase was 0.002 M. The chromatographic column was a Dikma Technologies Diamonsil C_{18} column, 5 μm 150 mm × 4.6 mm, which was maintained at 25 °C. The AZM raw material was dissolved to give a concentration of about 1 mg/ml and passed through a 0.45 μm membrane before injection.

fore, they were not used. It appears that traditional HPLC is unable to improve the AZM peak profile. In addition, the column efficiency was extremely low because of the peak asymmetry (number of theoretical plates $n < 1000$). Although Zubata et al. [2] reported a new HPLC method for AZM quantitation, the method was not suitable for detecting substances related to AZM. In that report, the retention time was about 5 min which was so short that the related substances cannot be separated. In addition, some related substances with retention times a little longer than AZM were not detected chromatographically.

However, with the addition of the ion-pair reagent, sodium heptanesulfonate, the chromatography conditions improved markedly. Because of the presence of the two nitrogen atoms, AZM is basic, and provides lone-pair electrons. Therefore, under acidic conditions (pH value of the mobile phase was 3.0), it will combine with H^+ based on the attraction between the positive and negative charges, changing AZM to the H^+-conjugate. Subsequently, in the mobile phase, the H^+-conjugate of AZM binds to the negative group of sodium heptanesulfonate and is converted to a neutral molecule. So, compared with the ionic form of AZM, the neutral complex results in enhanced

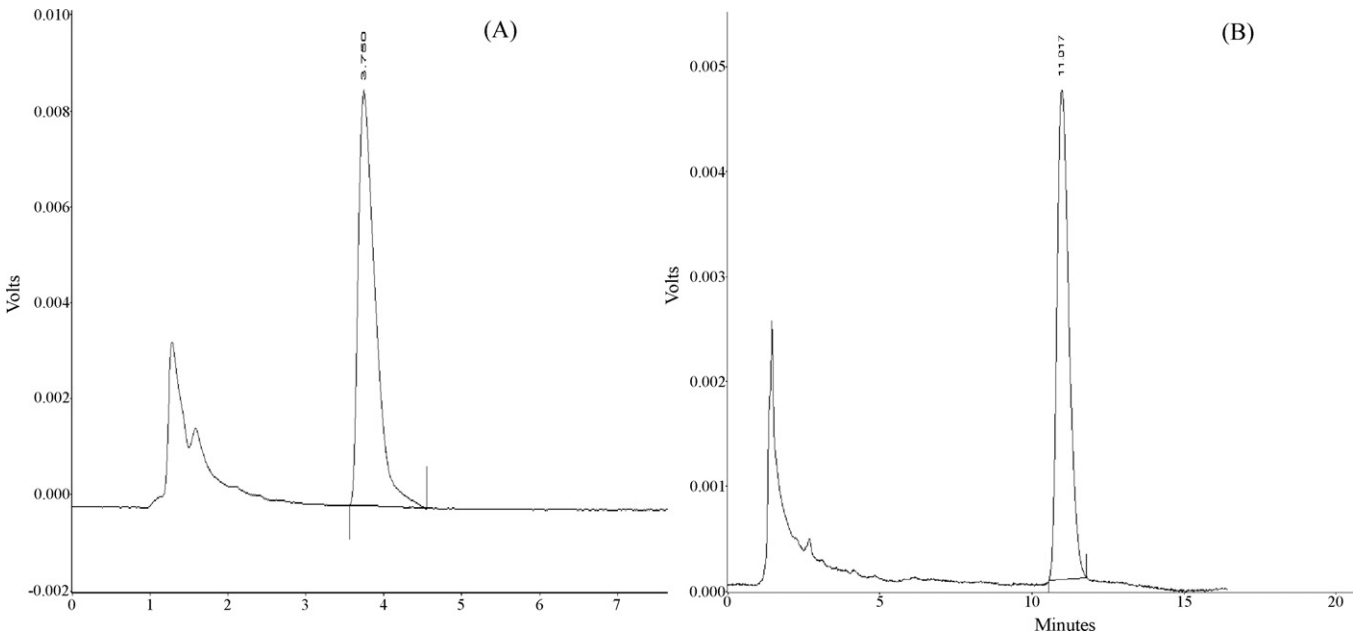

Fig. 3. Chromatograms of the same AZM working standard solution (0.98 mg/ml) with (B) and without (A) the ion-pair reagent, sodium heptanesulfonate. The mobile phase consisted of a mixture of ammonium dihydrogen phosphate (0.045 M, pH 3.0 adjusted by phosphoric acid):acetonitrile 47:15 (v/v) and the concentration of sodium heptanesulfonate was 0.002 M in the aqueous phase. Chromatography was carried out on a Dikma Technologies Diamonsil C_{18} column, 5 μm 150 mm × 4.6 mm, maintained at 25 °C.

retention ability of AZM on the C_{18} column, since the neutral complex has a weaker polarity than ionized AZM, and compounds with a similar polarity to the column are retained or adsorbed effectively. In addition, because of the H^+-conjugate and the neutral complex, the two nitrogen atoms are masked. Consequently, there is no nitrogen adsorption to the column and, therefore, the peak tailing is dramatically improved. Taking account of all these factors, our chromatography system was developed and optimized.

The mobile phase was a mixture of ammonium dihydrogen phosphate (0.045 M):acetonitrile 47:15 (v/v). In order to form the H^+-conjugate, however, the ammonium dihydrogen phosphate solution was adjusted to pH 3.0 with phosphoric acid, for a low pH value means a high concentration of H^+, and this can be obtained by the combination of acid and alkali. But, too low a pH will shorten the working life of the column. Accordingly, the pH value of aqueous phase was adjusted to 3.0. The concentration of sodium heptanesulfonate in the aqueous phase played a significant role in this chromatographic system, because formation of the neutral complex depends on the amount of sodium heptanesulfonate. A series of concentrations of sodium heptanesulfonate were evaluated, ranging from 0.002 M to 0.005 M. Increasing the concentration of sodium heptanesulfonate did not improve the peak shape obviously. However, as the concentration increased, the retention time was increased at the same mixing ratio of the mobile phase. Considering the analytical time, the concentration of sodium heptanesulfonate in the aqueous phase was set at 0.002 M. We also found that fluctuations in column temperature markedly affected the retention time and, so, the column temperature was maintained at a constant level, 25 °C.

Fig. 3 shows the same working standard solution injected into the identical chromatography system except that A is without sodium heptanesulfonate and B is with it. The retention time of

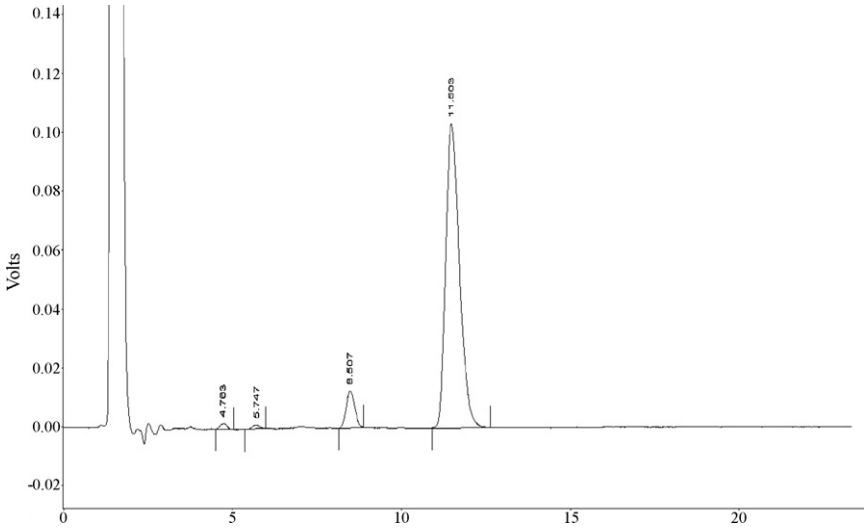

Fig. 4. Representative chromatogram of one batch of AZM eye drops stored under standard storage conditions (4 °C) for 12 months. The peak before the AZM main peak is benzalkonium bromide.

Table 2
Stability data for one batch of AZM eye drops.

Month	Content (%)		Limit of related substances (%)	
	4 °C	25 °C	4 °C	25 °C
0	101.15	101.15	0.44	0.44
1		100.51		0.66
2		99.94		0.78
3	99.95	96.05	0.54	1.72
6	98.45	92.91	0.64	3.68
9	97.59		0.70	
12	96.95		0.96	

A was less than 5 min, whereas B was about 11 min. This indicates that the ion-pair reagent does indeed interact with AZM due to the increase in retention time and the peak profile is clearly improved.

3.3. Application of the method

The developed method was applied to the determination of the stability of AZM eye drops. The content of AZM eye drops was reduced by about 15% over 10 days in accelerated testing at a temperature of 40 °C, and by 35% under 60 °C after a storage period of 10 days. Therefore, AZM eye drops can be considered to be a heat-sensitive preparation. Three batches of AZM eye drops were prepared and stored at 25 °C for accelerated testing and at 4 °C for storage conditions testing, respectively. The stability data of one batch are summarized in Table 2. After 6 months under accelerated conditions, the AZM content of the eye drops was still above 90%, and the limit of related substances was below 5%. Consequently, the AZM eye drops were stable. In our experiment, the limit of related substances was tested by self-control method. In this, a high concentration AZM sample solution (test solution) was diluted 50-fold (control solution), and then the limit of related substances was calculated as the ratio of the summed peak areas of the related substances from the test solution to the peak area of AZM from the control solution. Fig. 4 shows a representative chromatogram of one batch of eye drops stored under standard storage conditions for 12 months. It can be seen that related substances are present and can be completely separated.

4. Conclusion

A new ion-pair HPLC method for the determination of AZM was developed and successfully applied to the stability study of AZM eye drops. This new method is accurate, precise and very practical for the routine determination of AZM preparations. The AZM eye drops were found to be stable in an accelerated test (25 °C) and a storage test (4 °C).

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