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Research Article

THE COMBINED EFFECTS OF PH AND ACETONITRILE COMPOSITION ON THE SEPARATION OF TWO LINCOSAMIDE ANTIBIOTICS

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ABSTRACT

Objective: Reversed-phase chromatography has become the method of choice to separate compounds that are similar in structure to each other. Selectivity, resolution and retention time can be improved by controlling different variants. The choice of the mobile phase for the analysis is important to achieve the best chromatographic results. The retention time, shape and width of chromatographic peaks are dependent on the buffer pH and also on the amount of organic modifier in the mobile phase. The aim of this study is to separate two lincosamide antibiotics-clindamycin phosphate and lincomycin hydrochloride using different mobile phases.

Method: Each mobile phase was made up of a phosphate buffer and acetonitrile. The mobile phases had different buffer pHs and different percentage compositions of acetonitrile. Analysis was performed using nine different mobile phases to observe which of them gave the best results in terms of resolution, retention time, and peak shape.

Results: The best results were given when using a mobile phase having 30% acetonitrile with a buffer pH of 3.0.

Conclusion: This work shows the importance of adjusting the amount of the organic modifier in the mobile phase together with the buffer pH. The amount of organic modifier should not be too large as to cause loss of resolution between two neighboring peaks and not too small so as to result in too long of a retention time. The pH should be chosen to have the greatest percentage of ionized or unionized species of the analyte of interest present.

Keywords: Reversed-phase chromatography, Clindamycin, Lincomycin, Acetonitrile, pH.

INTRODUCTION

Owing to its versatility, specificity and sensitivity high-performance liquid chromatography (HPLC) has become the method of choice for separating and analyzing compounds in the pharmaceutical industry. Reversed- phase chromatography, which is the most commonly used type of liquid chromatography [1] can be used to distinguish between and quantify compounds that are structurally very similar to one another.

When selecting and establishing the ideal separation conditions, various parameters have to be taken into account. In the majority of practical scenarios, in method development, a single stationary phase is used, and optimization in selectivity, resolution and retention time is achieved by changing other variants [2]. Different mobile phase characteristics can affect chromatographic results. Among these are flow rate, type of buffer used, type of organic modifier used and pH of the mobile phase [3-5]. The retention of analyte peaks, as well as their shape and width, are all dependent on the pH of and the amount of organic modifier in the mobile phase [6]. When changing the mobile phase pH both the retention time and selectivity of the analytes of interest vary [7]. Ideally, results are favorable for both the retention time and selectivity. Shorter retention times result in quicker analyses, but retention times that are too short may lead to compromisation, lack of selectivity and loss in resolution between two or more neighboring peaks.

The work reported in this paper describes the effects of varying pH and quantity of organic modifier in the mobile phase when separating a mixture of clindamycin phosphate and lincomycin hydrochloride.

Clindamycin phosphate and lincomycin hydrochloride are two clinically widely used lincosamide antibiotics [8,9]. Lincomycin (Fig. 1) was the first lincosamide antibiotic to be produced after being isolated from Streptomyces lincolnensis [10]. The attachment of a chlorine group at the carbon-7 position to produce clindamycin increases the potency significantly by producing a more lipophilic compound [11]. A phosphate group attached to clindamycin produces clindamycin phosphate, a pro-drug with greater aqueous solubility and a marked decrease of pain upon injection [12,13].

Lincomycin has a pKa value of 7.6 [14] whilst clindamycin phosphate has two pKa values: pKa_1-0.96 and pKa_2-6.081 [15].

The Henderson-Hasslebalch equation:

$$pH = pKa + \log([A^-]/[HA]) \text{ for a weak acid and}$$

$$pH = pKb + \log([RNH_2]/[RNH_3^+]) \text{ for a weak base}$$

relates pH with the pKa of a weak acid and with the ratio of the concentrations of its protonated (HA) and unprotonated species (A^-) present and with the pKb of a weak base and the ratio of the concentrations of the base and its protonated species. These are all very important considerations when using this type of chromatography.

According to the Henderson-Hasslebalch equation, when the pH of the mobile phase is <7.6, lincomycin is increasingly more present in its protonated form (Fig. 1). At pH values above 7.6, lincomycin is increasingly more present in its unprotonated form.

Since clindamycin phosphate has two pKa values, at pH values lower than 0.9, all of its oxygen atoms are protonated (Fig. 2). At pH values between 0.9 and 6.081 the ratio between the concentration of the species with one of the oxygens of its phosphate group protonated

and the concentration of the unprotonated species varies (Fig. 3). At values above 6.081, the species with all of its oxygens unprotonated predominates (Fig. 4).

When dealing with analytes, which can be ionized, control of pH is the key to avoid obtaining peaks, which are broad, split or non-symmetrical. This is because better peak shapes can be attained when the analyte of interest is present in a single form [16].

pH control is therefore of great importance when selecting chromatographic conditions in reversed-phase chromatography as it controls the ratio of the relative concentrations of the protonated and unprotonated species and hence the distribution between the non-polar stationary phase and the polar mobile phase which in turn influences retention times [17-19]. Most reversed-phase chromatographic methods operate at pH values lying in the range between 2 and 8 [20].

Among the different types of buffers used in the mobile phases for HPLC, are phosphate buffers [21]. Phosphate buffers are widely used and favored due to their high purity, low cost and ease to produce good chromatograms. Such buffers also operate best at useful pH ranges. Phosphoric acid having three ionizable groups has three pKa values-2.1, 7.2 and 12.3, respectively. When using these types of buffers, there are 3 different buffering ranges. These lie in the regions of around pH 1.1-3.1, pH 6.2-8.2 and pH 11.3-13.3 [22].

The most commonly used organic modifiers in reversed-phase chromatography are acetonitrile and methanol. Although being more expensive, HPLC-grade acetonitrile has lower absorbance than HPLC- grade methanol at the wavelength of interest for detection. Furthermore, when mixed with water there is a lower increase in pressure when using acetonitrile. It is for this reason that acetonitrile was used as the organic modifier in the mobile phase [23] used in this study.

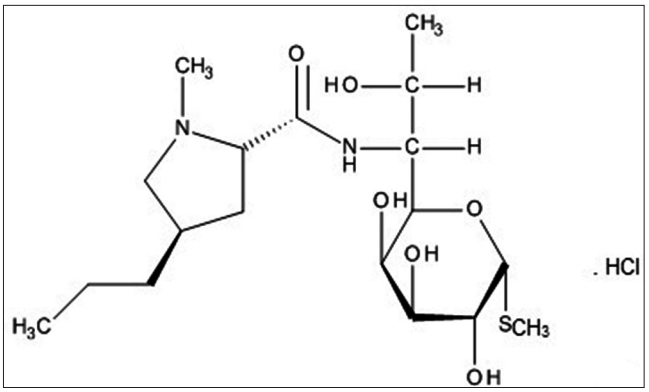

Fig. 1: Lincomycin hydrochloride at a pH <7.6

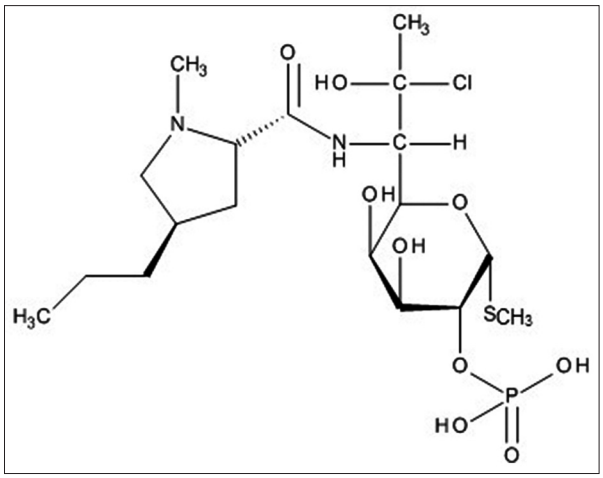

Fig. 2: Clindamycin phosphate at a pH lower than 0.9

MATERIALS AND METHODS

Sample preparation

Clindamycin phosphate and lincomycin hydrochloride standards were purchased from Sigma Aldrich (Steinheim, Germany). A 100 µg/mL solution of both clindamycin and lincomycin was prepared in HPLC-grade water (Fisher Chemical, Leicestershire, UK) and equal volumes of each were mixed together. These were stored until the time of analysis at 4°C in 100 mL amber-colored volumetric flasks, to be protected from light.

Mobile phases

Mobile phases were prepared using a combination of acetonitrile (Fisher Chemical, Leicestershire, UK) and phosphate buffer. The buffer solution was prepared by dissolving anhydrous extra pure di- sodium hydrogen phosphate (Scharlau, Sentmenat, Spain) in HPLC-grade water to make up a solution of 0.02 M. The pH was adjusted by adding HPLC-grade orthophosphoric acid (Fisher Chemical, Leicestershire, UK) dropwise.

A total of nine different mobile phases were used (Table 1). The composition of the mobile phases consisted of a combination of pH 2.0, 3.0 or 6.5 (higher pH values were not used due to the nature of the stationary phase) and a percentage acetonitrile composition of 20, 30 or 40%.

The particular pH values were selected for the analysis to ensure maximum buffering capacity, as it is best to use buffers within their

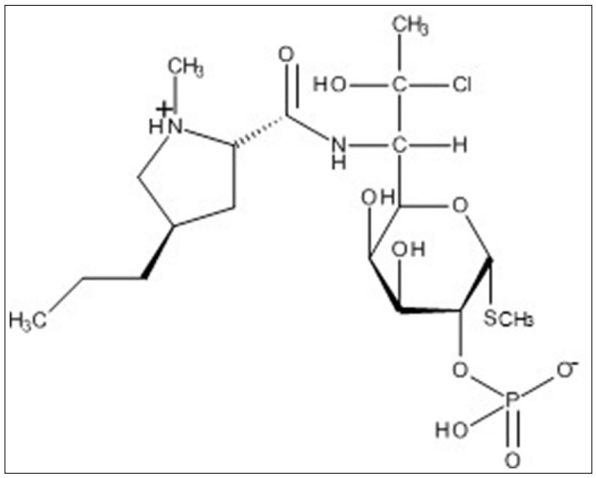

Fig. 3: Clindamycin phosphate at a pH value between 0.9 and 6.081

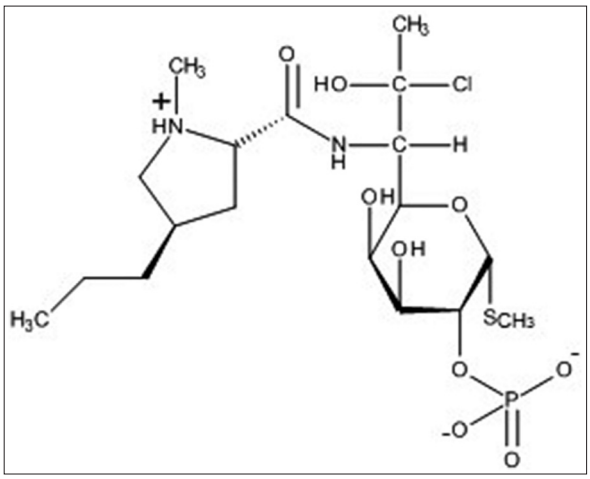

Fig. 4: Clindamycin phosphate at a pH value above 6.081

effective pH range by selecting a pH having a value of around ± 1 pH unit from the pKa of the buffer [24].

Instrumentation

Analysis was performed using an Agilent 1260 Infinity Series® liquid chromatographic system having a quaternary pump and multi-wavelength detector. Separation took place on an ACE® RP C-18 column (250 mm × 4.6 mm; particle size 5 µm) at a temperature of 25°C. Mobile phase flow rate was of 1 mL/minute. The UV/visible detector was set at 205 nm. Sample volumes (containing clindamycin phosphate and lincomycin hydrochloride) of 25 µl were injected. To assist with peak identification solutions containing pure clindamycin phosphate and lincomycin hydrochloride respectively were injected. Five replicate injections using each type of the nine mobile phases were carried out to ensure reproducibility. Run time was adjusted for each chromatogram according to the type of mobile phase used. Averages for the retention time, areas under the peak and area percentages were calculated for each mobile phase used (Figs. 5-13).

All pH measurements were done using a Mettler Toledo FiveGo™ pH meter. Prior to taking every reading, the pH meter was calibrated using standard Hanna® calibrator buffer solutions (at pH values of 4.01 and 7.01).

Table 1: Types of mobile phases used

Mobile phase number	pH	Percentage of acetonitrile used
1	2.0	20
2	2.0	30
3	2.0	40
4	3.0	20
5	3.0	30
6	3.0	40
7	6.5	20
8	6.5	30
9	6.5	40

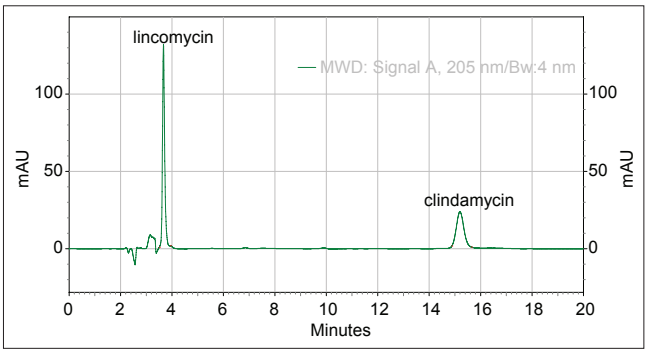

Fig. 5: Disodiumhydrogen phosphate (pH 2.0) and acetonitrile (80:20 v/v)

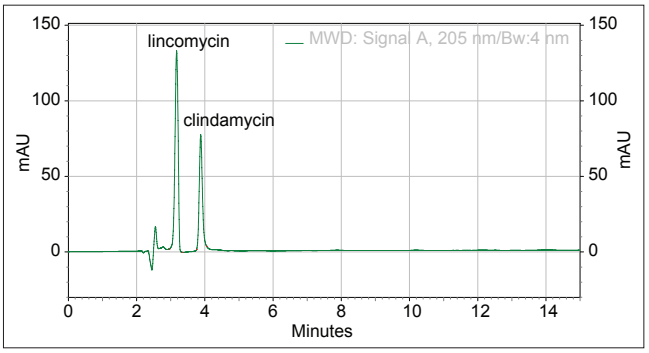

Fig. 6: Disodiumhydrogen phosphate (pH 2.0) and acetonitrile (70:30 v/v)

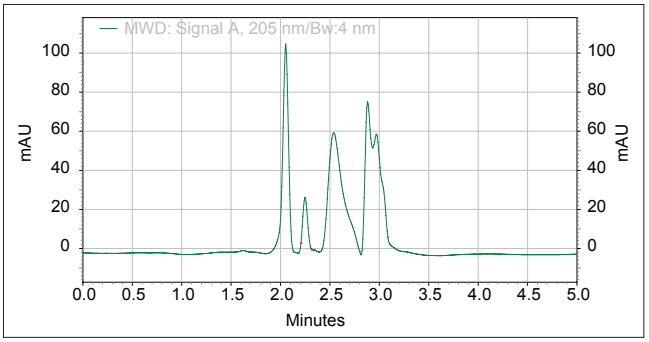

Fig. 7: Disodiumhydrogen phosphate (pH 2.0) and acetonitrile (60:40 v/v)

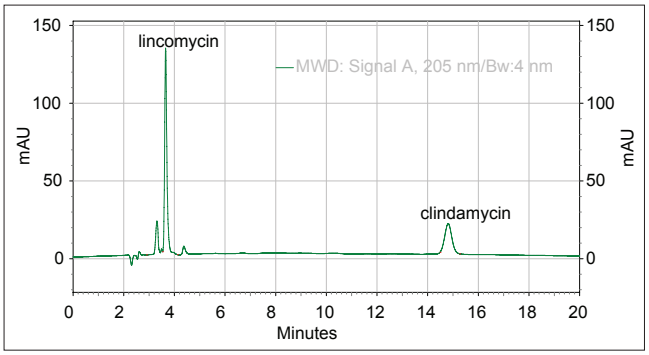

Fig. 8: Disodiumhydrogen phosphate (pH 3.0) and acetonitrile (80:20 v/v)

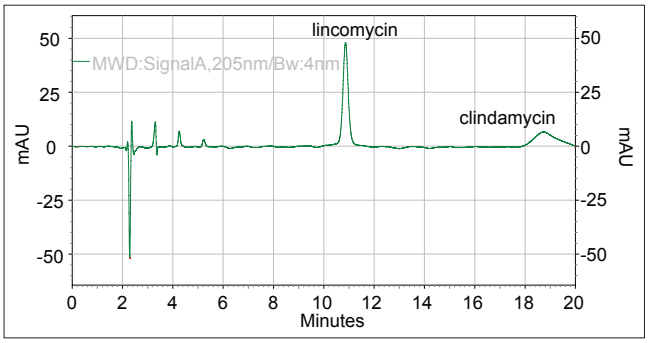

Fig. 9: Disodiumhydrogen phosphate (pH 6.5) and acetonitrile (80:20 v/v)

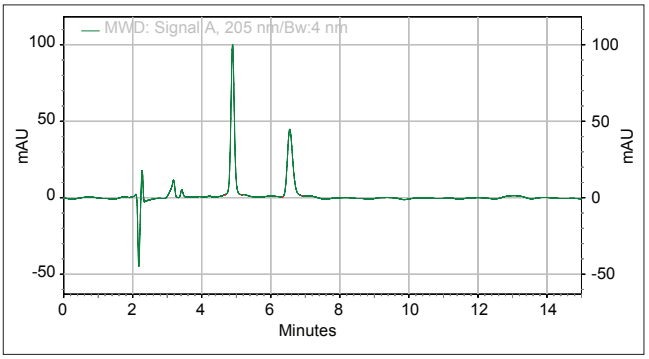

Fig. 10: Disodiumhydrogen phosphate (pH 6.5) and acetonitrile (70:30 v/v)

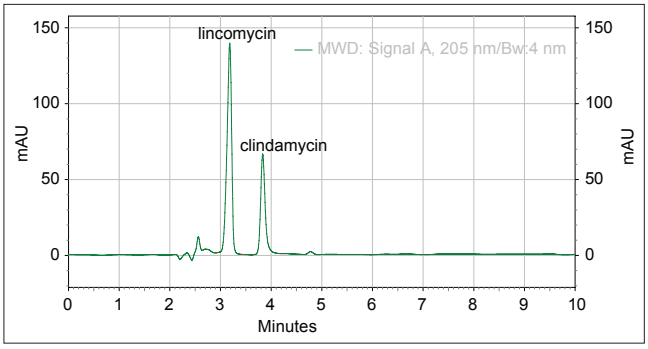

Fig. 11: Disodiumhydrogen phosphate (pH 3.0) and acetonitrile (70:30 v/v)

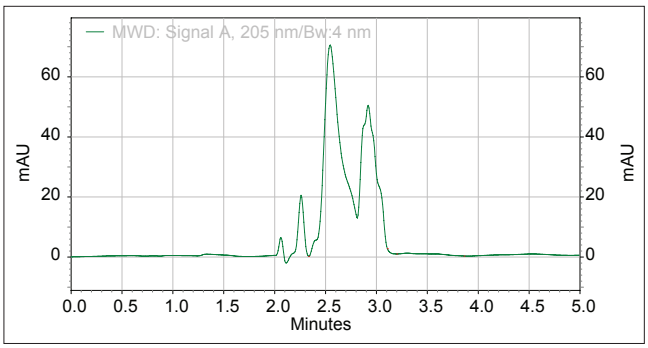

Fig. 12: Disodiumhydrogen phosphate (pH 3.0) and acetonitrile (60:40 v/v)

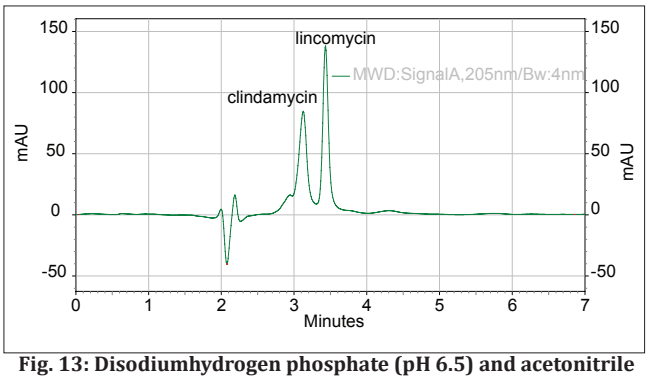

Fig. 13: Disodiumhydrogen phosphate (pH 6.5) and acetonitrile (60:40 v/v)

RESULTS AND DISCUSSION

When observing chromatograms produced, it could be noted that peaks given by lincomycin were higher than those given for clindamycin. Both drugs were present in solution in the same concentration (100 µg/ml). The reason for this is due to lincomycin having a higher absorptivity than clindamycin phosphate at the wavelength selected for detection [25].

When using HPLC, working at high pH values can result in the solubilization of the silica support. This in turn damages the column and reduces its lifetime [26]. Since it is not advisable to conduct HPLC analysis at relatively alkaline pHs [27], lincomycin could only be mainly observed in its protonated form (at pH values lower than its pKa value). When analysis was conducted at pH values of 2.5 and 3.0, the first oxygen of the phosphate group of clindamycin was present in its unprotonated form (as the pH value was higher than the pKa_1 value) whilst the second oxygen on the phosphate group was present in its protonated form (pH lower than pKa_2). At a pH of 6.5 both oxygens of the phosphate group were unprotonated.

The higher the percentage of acetonitrile in the mobile phase, the shorter is the retention time [28]. At pH values of 2.0 and 3.0 there was a marked decrease in the retention time of clindamycin when more acetonitrile was used (Figs. 5-8). This shows that when the amount of solvent in the mobile phase is increased, there is less interaction of relatively polar compounds such as clindamycin with the stationary phase resulting in quicker elution probably due to a change in partition coefficient favouring more the mobile phase containing higher amounts of organic modifier.

At low pH values, the retention time of relatively less polar compounds such as lincomycin was unaffected. At a higher pH value of 6.5, however, increasing the acetonitrile percentage by 10% also affected the retention time of lincomycin with lincomycin eluting at a later time (Figs. 9 and 10).

At an increased composition of acetonitrile at pH 6.5, clindamycin eluted before lincomycin. This is because at pH 6.5 (which is above the two pKa values of clindamycin phosphate), clindamycin phosphate is present in its fully ionized form with both H's of the phosphate group dissociated. This makes it relatively more polar, thus having less of an affinity for the stationary phase andeluting quicker. This is not observed in lower acetonitrile concentrations where lincomycin elutes first indicating that more acetonitrile would be needed to elute clindamycin before lincomycin at this pH.

Increasing the amount of acetonitrile produces sharper peaks (Figs. 6, 10 and 11).

Larger volumes of acetonitrile can also result in a decrease in resolution between two neighboring peaks. This could be clearly seen at pH values of 2.0 and 3.0 when the acetonitrile composition was adjusted to 40% (Figs. 7 and 12).

More than two peaks could be observed at one instance (Fig. 7) and the peaks produced were not symmetrical and were split. This is probably due to the reduced buffering capacity of the phosphate buffer at high organic modifier compositions. The use of a buffer having a stronger concentration would probably counteract such an occurrence, especially at lower pH values. When using 40% acetonitrile at pH 6.5 better resolution of the two more symmetrical peaks was attained with less splitting when compared with the other pH values (Fig. 13).

CONCLUSION

This work shows how changing two important parameters such as pH of the buffer and percentage of acetonitrile in the mobile phase can affect separation and chromatographic results. In reversed- phase chromatography, buffer pH and amount of organic modifier in the mobile phase are two very important parameters that govern chromatographic results [29-33]. Effective and efficient separation of lincomycin hydrochloride and clindamycin phosphate can be achieved if the mobile phase is made up of the appropriate blend of polar and non- polar or less polar components as this modifies the distribution coefficient (K_d) for the partitioning of components between the stationary C_{18} phase and the mobile phase made up of acetonitrile and phosphate buffer.

The pH of the mobile phase is important when it comes to selectivity, efficiency and peak shape. The most appropriate pH must be chosen according to the type of buffer being used and also according to the compound's pKa values. pH affects efficacy and efficiency of separation as it alters the relative amounts of non-ionized to ionized species in the mobile phase which in turn influences distribution of the non-ionized between the two phases.

For adequate retention and resolution, the amount of organic modifier in the mobile phase has to be adjusted simultaneously with the pH. To achieve adequate separation and resolution, buffer strength and concentration must be controlled in order to attain the maximum buffering capacity especially at high compositions of organic modifier.

The amount of organic modifier must not be too low to avoid having retention times that are too long. However, it must not be increased to such an extent that the resolution between two or more peaks is decreased, and the buffering capacity altered.

The best separation of lincomycin hydrochloride and clindamycin phosphate with respect to retention times and areas of peaks was achieved when using a mobile phase of disodium hydrogen phosphate buffer and acetonitrile in the ratio of 70:30 (v/v) at a pH of 2 or 3.

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