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Simultaneous determination of amoxycillin and clavulanic acid in pharmaceutical products by HPLC with β-cyclodextrin stationary phase

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Abstract

A simple, rapid and accurate method for simultaneous determination of amoxycillin and clavulanic acid using HPLC with β-cyclodextrin stationary phase was developed. It involves the use of tetraethylammonium acetate (TEAA) as an additive reagent, methanol-buffer solution (pH 4.5) (35:65; v/v) as the mobile phase, detection at 225 nm and chromatogram within 12 min. Linearity and precision of the internal standard method have been obtained. Recoveries ranged from 99.25 to 105.63% for amoxycillin in the synthetic mixture. For clavulanic acid it was from 99.50 to 101.64%. This method is convenient and reproducible for analyses of these two components in different dosage forms. © 1997 Elsevier Science B.V.

Keywords: HPLC assay; β-cyclodextrin; Amoxycillin; Clavulanic acid

1. Introduction

Amoxycillin combined with the novel β-lactamase inhibitor, clavulanic acid, is usually used as a new antibacterial formulation against various β-lactamase-producing bacteria [1,2]. Several methods were employed for the analyses of these two components including microbiological assay [3], enzymatic assay [4], ultraviolet spectrophotometry [5,6] or polarography [7] and these methods were generally not specific. The HPLC assay was first described by Foulstone [8] involving pretreatment of amoxycillin and clavulanic acid in an imidazole reaction. Consequently, Martin [9] used a pre-column method for assay of clavulanic acid in biological fluid after 1,2,4-triazole reaction. Other methods developed by Haginaka [10–13] using post-column techniques with processes involving alkaline degradation were frequently employed to determine clavulanic acid. Direct HPLC assay employing ion-interaction chromatography [14] or reverse-phase chromatography [15,16] for simultaneous determination of amoxycillin and clavulanic acid in pharmaceutical preparation has also been developed. In summary, these methods are carried out on either a poly(styrene-divinylbenzene) resin (PRP-1) or a conventional C18

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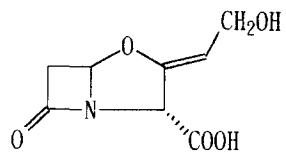
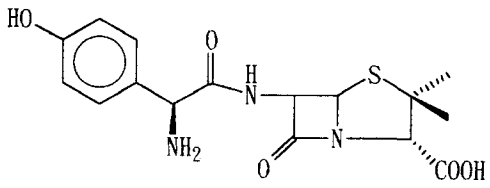
packing material column with different mobile phases.

The β-cyclodextrin (β-CyD) is an oligosaccharide of seven glucose units cyclized together to form a toroidal structure with a hydrophilic exterior face and a hydrophobic inner cavity. Armstrong [17] developed the first high efficiency bonded β-CyD phase on 5 μm silica gel as the packing material for HPLC. A wide variety of compounds have been separated by using the β-CyD bonded stationary phase via the formation of inclusion complexes [18–23]. In our previous paper, the separation of a series of cephalosporins by HPLC by β-CyD column has also been recently reported [24]. It is found that the β-lactam structural compounds can form inclusion complexes with β-CyD cavities. Clavulanic acid (I) contains a β-lactam ring fused with an oxazolidine ring and is structurally similar to amoxycillin (II) (Scheme 1).

Therefore, there has been considerable interest in the utilization of a β-CyD bonded phase to separate the mixtures of β-lactam antibiotic and β-lactamase inhibitor.



(I)



(II)

Scheme 1. The structure of clavulanic acid (I) and amoxycillin (II).

In this study, we describe a new HPLC method by using β-CyD stationary phase for the successful simultaneous separation of amoxycillin and clavulanic acid and for the quantitative analyses of both drugs in pharmaceutical preparations. In comparison with other methods, the advantages of the new HPLC method are discussed.

2. Experimental

2.1. Instrumentation

The liquid chromatographic system consists of a Waters Model 6000A pump connected to a U6K injector, a photodiode array detector model 990 set at 225 nm and a model 5200 printer/plotter (Waters Associates, Milford, MA). The separation was accomplished on a 5 μm bonded β-CyD column (Cyclobond I, 250 × 4.6 mm i.d.) purchased from Advanced Separation Technologies (Whippany, NJ). The quantification was based on integration of peak areas using a NEC model APC IV power mate 2 computing integrator (NECIS, MA). A model 112 pH meter (Photovolt, NY) was used to measure the pH values of the mobile phases. The data calculation was performed using Sigma plot software.

2.2. Materials

Amoxycillin sodium form was donated by Veterans Pharmaceutical Plant (Chung-Li, Taiwan). Amoxycillin trihydrate, cephaloridine and aspartame were purchased from Sigma (St. Louis, MO). Potassium clavulanate was obtained from Lek d.d. Ljubljana (Ljubljana, Slovenia). The HPLC solvents such as acetonitrile, methanol and glacial acetic acid were obtained from Merck (Frankfurt, Germany). Tetraethylammonium acetate (TEAA) was obtained from Aldrich (Milwaukee, WI). Distilled water was deionized twice before use.

Augmentin® tablets, injections and 100 ml syrups (Beecham, Jurong, Singapore) were purchased from a drug store. Each tablet (about 668 mg) was labeled as containing amoxycillin trihy-

drate (250 mg) and potassium clavulanate (125 mg). Each vial (730 mg) was labeled as containing sodium amoxycillin (500 mg) and potassium clavulanate (100 mg), while the syrup was labeled as containing per each 5 ml amoxycillin trihydrate (125 mg) and potassium clavulanate (31.25 mg).

2.3. Chromatographic conditions

The TEAA buffer solution was prepared with water and glacial acetic acid until the desired pH was obtained. The mobile phase was prepared by mixing methanol or acetonitrile with buffer solution and was degassed by bubbling helium through it for about 10 min. The mobile phase was filtered through a 0.45 μm filter (Millipore, Yonezawa, Japan) before use. The flow rate was adjusted to 0.8 ml min^{-1} and 20 μl sample solutions were injected. The attenuation unit for full-scale deflection was set at 0.1 ~ 1.2 mV.

2.4. Procedure for the standard curve

Amoxycillin trihydrate (200 mg), potassium clavulanate (200 mg), and cephaloridine (300 mg) were accurately weighed and each added to a 100 ml volumetric flask as the stock solution, respectively.

Two standard solutions were prepared by transferring quantitatively 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 ml amoxycillin or clavulanic acid stock solution into six 10 ml volumetric flasks. Then, 1 ml cephaloridine stock solution was added for each sample and diluted to the mark. After filtering, five replicate chromatograms from 20 μl per injection were recorded for each concentration. The final concentrations were equivalent to 1 ~ 10 μg ml^{-1} for amoxycillin and clavulanic acid, and 6 μg ml^{-1} for cephaloridine. A calibration graph of peak area ratio ($A_{cpd}/A_{I.S.}$) versus each component concentration was constructed.

2.5. Preparation and assay of the synthetic mixtures

Five synthetic mixtures were prepared by adding 125 mg potassium clavulanate to 125, 250, 375, 500 mg of amoxycillin trihydrate and a 250 mg amoxycillin sodium form, respectively. To each mixture, aspartame powder was added to make the amount of the blend equal 2.5 g.

Each synthetic mixture was weighed and suspended in a 100 ml volumetric flask with the mobile phase. After shaking and filtration, each 1 ml of filtrate and 1 ml cephaloridine stock solution were transferred to a 10 ml volumetric flask and diluted to the mark. The final injected amount ratios (m/m) of amoxycillin to clavulanic acid were equivalent to 1:1, 2:1, 3:1, 4:1 and 2:1 (for amoxycillin sodium form).

2.6. Preparation and assay of the pharmaceutical samples

2.6.1. Augmentin® tablets

Ten tablets were weighed and ground to powder. Six samples from the powder were weighed as so that each contained about 250 mg amoxycillin trihydrate and 125 mg potassium clavulanate. Each sample was transferred to a 100 ml volumetric flask and diluted with mobile phase. From each filtrate a 3 ml sample and 2.5 µl cephaloridine stock solution were pipetted into a 25 ml volumetric flask, respectively. Then, 20 ml of the mixture with three replicates were injected into the HPLC. The unknown concentrations of each component were calculated by direct comparison with the standard curves.

2.6.2. Augmentin® injections

Each of three injection vials was weighed and dissolved in a 100 ml volumetric flask with mobile phase. The other procedures are the same as Section 2.6.1.

2.6.3. Augmentin® syrups

Each of the three 100 ml syrups was weighed and suspended in a 250 ml volumetric flask with mobile phase. After shaking, dilution and filtration, the 20 μl of aqueous mixture was injected into the HPLC.

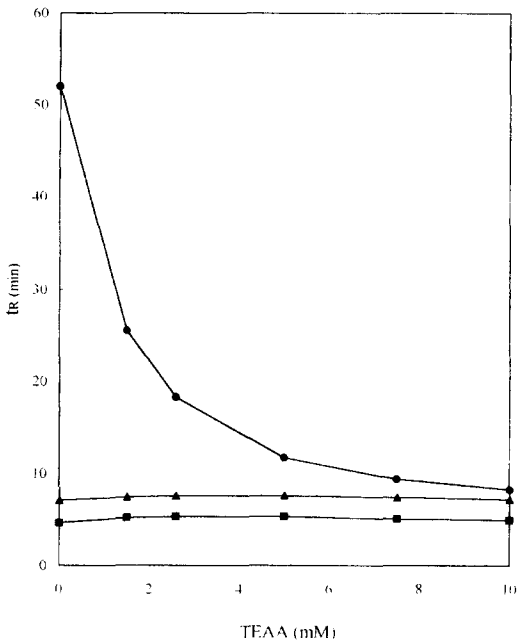


Fig. 1. Effect of TEAA on the retention time of amoxycillin (■), cephaloridin (▲) and clavulanic acid (●). Chromatographic condition: column, Cyclobond I, 250 × 4.6 mm i.d.; mobile phase, methanol–TEAA buffer (35:65; v/v), pH 4.5; column temperature 30°C; flow rate, 0.8 ml min^{-1}.

3. Results and discussion

In chromatographic studies, the added TEAA is an important factor which effects the separation of these β-lactam compounds. If there are no TEAA ions in the mobile phase, a longer retention and a worse peak shape of clavulanic acid will be observed. Fig. 1 shows the retention behavior of these compounds over a wide range of TEAA concentration from 0 to 10 mM. It is found that a relatively fast decrease in the retention of clavulanic acid occurs with increasing TEAA concentration. The results indicate that higher concentration of TEAA in the mobile phase saturates the β-CyD cavities. The saturation renders the strength of inclusion complexation between clavulanic acid and the β-CyD cavities weaker. It, therefore, causes clavulanic acid to have less retention time. Amoxycillin contains a hydrophilic α-amino group side chain that interacts weakly with β-CyD and is eluted rapidly. These phenomena were observed and discussed in a previous paper related to the separation of cephalosporins [24].

The retention behaviors of these analytes with the same mobile phase (MeOH–5 mM TEAA; 35:65, v/v) at different pH values are indicated in Fig. 2. The retention times of amoxycillin and cephaloridin have no significant changes for pH 3.6 ~ 7.0, while clavulanic acid shows lower retention with increasing pH. This can be explained by considering the effect of pH on the affinity of the clavulanic acid to the hydroxyl group of β-CyD. It had been reported that the OH^- ion exhibited a higher hydrogen bonding ability to the hydroxy groups of β-CyD [25]. Therefore, the increase of OH^- ion concentrations by increasing pH will compete with the clavulanic acid to interact with the hydroxy group of β-CyD. As a result, a decreased retention time accompanying the increase of pH is observed. However, amoxycillin, clavulanic acid and the internal standard,

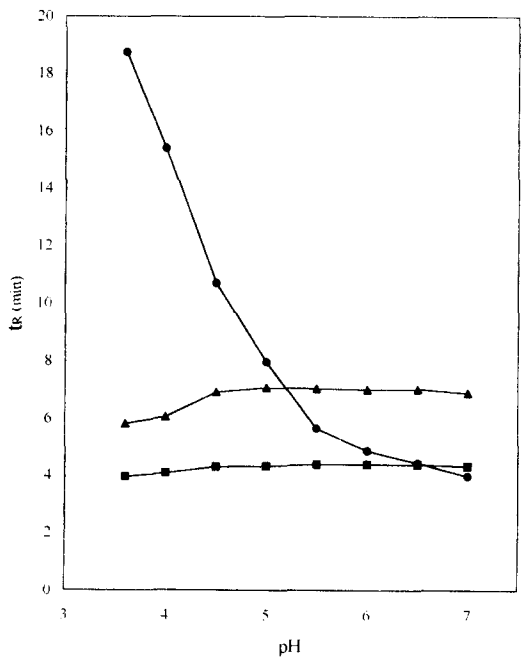


Fig. 2. Effect of pH on the retention time of amoxycillin (■), cephaloridin (▲) and clavulanic acid (●). Chromatographic condition: column, Cyclobond I, 250 × 4.6 mm i.d.; mobile phase, methanol–TEAA buffer (5 mM) (35:65; v/v); column temperature 30°C; flow rate, 0.8 ml min^{-1}.

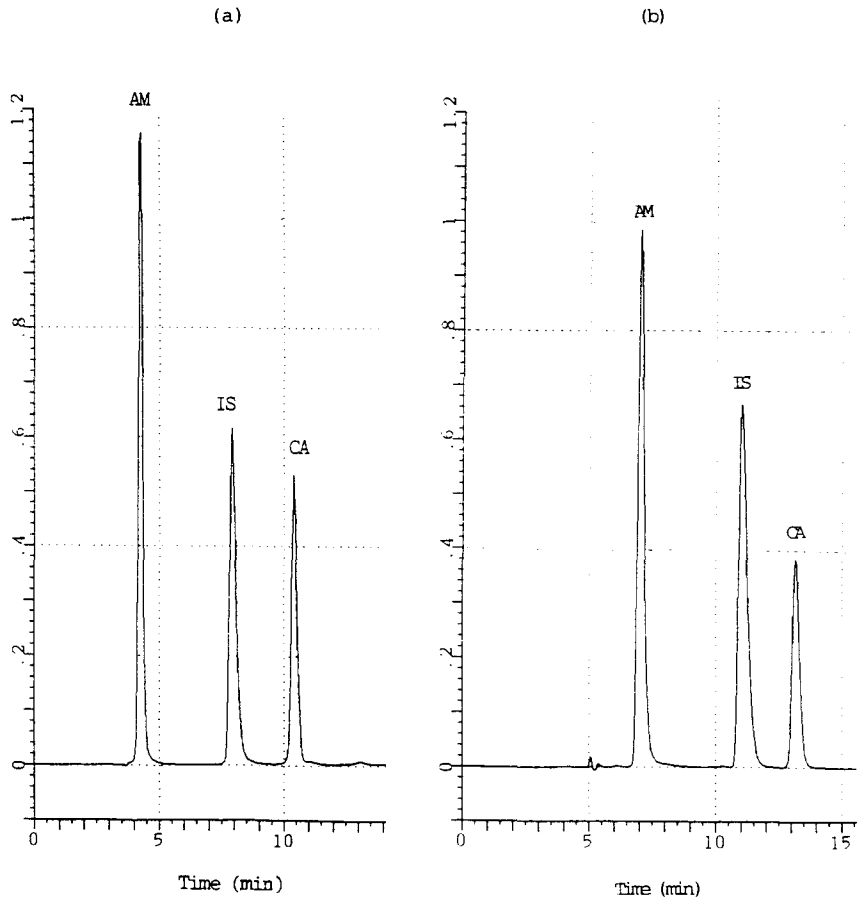


Fig. 3. Chromatogram of amoxycillin (AM), cephaloridin (IS), and clavulanic acid (CA) in pharmaceutical preparations. (a) The column (Cyclobond I, 250 × 4.6 mm i.d.) was eluted with methanol–TEAA buffer (pH 4.5; 5 mM) (35:65; v/v), 0.8 ml min^{-1} and monitored at 225 nm. (b) The same condition as (a) except using acetonitrile–TEAA buffer (pH 5; 4 mM) (10:90; v/v).

cephaloridine, were completely separated within 12 min using the mobile phase of methanol–TEAA (pH 4.5; 5 mM; 35:65, v/v) and a β-CyD bonded column. Another mobile phase, acetonitrile–TEAA (pH 5.0; 4 mM; 10:90, v/v), could be selected for chromatography when the organic solvent was changed. The results are shown in Fig. 3.

For comparison, the currently proposed and three previously published procedures were employed for simultaneous separation and quantification of amoxycillin and clavulanic acid. Table 1 summarizes the results. Due to different chromatographic mechanism, these two analytes have opposite elution orders on C18 and β-CyD stationary phases. As for the Salto method [14], although the same elution order was observed as the proposed method, the retention time of clavulanic acid was increased with increasing counter ion, TBAB, in the mobile phase. This phenomenon was contrary to the results of the proposed method in which the retention of clavulanic acid decreased with increasing concentration of TEAA. On the other hand, in spite of using the different column, pH, mobile composition and flow rate, the proposed method gives a sharp and symmetric peak separation with a resolution as good as USP XXII does. Thus, we considered that the proposed method is an alternate method for the determination of amoxycillin and clavulanic acid in pharmaceutical preparations.

Table 1
Comparison of HPLC procedures for the simultaneous determination of amoxycillin (AM) and clavulanic acid (CA)

Procedure	Abounassif [15]	USP XXII [16]	Salto [14]	The currently proposed method
Stationary phase				
Column	μ-Bondapak C18	C18	PRP-1	Cyclobond I
Size	30 cm × 4 mm	30 cm × 4 mm	15 cm × 4.1 mm	25 cm × 4.6 mm
Particles (μm)	10	3 ~ 10	10	5
Mobile phase (%v/v)				
Acetonitrile	0	0	10	0
Methanol	15	5	0	35
Aqueous buffer	1 (0.2 M PBS)	95 (0.5 M PBS)	90 (1 mM PBS + 3 mM TBAB)	65 (5 mM TEAA)
H_2O	84	0	0	0
pH	6	4.4	6 ~ 7	4.5
Flow rate (ml min^{-1})	1.0	2.0	1.0	0.8
Characteristics				
Loop volume (μl)	20	20	10	20
Wavelength (nm)	235	220	230	225
Chromatography mechanism	Reverse-phase	Reverse-phase	Ion-interaction	Inclusion-complexation
Retention time (min)				
AM	5.3	6.0	2.1	4.3
CA	2.3	4.5	2.8	10.4

Simultaneous quantification of amoxycillin and clavulanic acid could be achieved when the internal standard was included. The calibration curve data were determined by injection of a series of standard solutions at six levels. The peak area ratio ($A_{cpd}/A_{I.S}$) relative to each component plotted against its consecutive concentration are shown in Table 2. It gave a straight line according to the following equation: $Y = 0.1244X + 0.0948$ for amoxycillin; $Y = 0.1118X - 0.0466$ for clavulanic acid. The correlative coefficient values (r) yielded by regression analyses corresponding very well for both ingredients.

Recovery studies were carried out by adding known amounts of amoxycillin sodium or trihydrate to clavulanic acid ratio of 1:1, 2:1, 3:1 and 4:1, respectively, in the synthetic mixtures. The accuracy and precision of different injections ($n = 5$) within a day are summarized in Table 3. In all cases the recoveries of amoxycillin ranged from 99.25 to 105.63%, while those of clavulanic acid were between 99.54 and 101.64%. The coefficients of variation were less than 1.89 and 2.01% for amoxycillin and clavulanic acid, respectively. It indicates that the proposed method has good reproducibility for determination of these two chemicals simultaneously.

Table 2
Calibration data for the standard curve of the peak area ratio of amoxycillin (AM) and clavulanic acid (CA) vs. the internal standard

Compound	Concentration (μg ml^{-1})	Correlation coefficient ($r \pm$ S.D.)[a]	Slope	Intercept
AM	1 ~ 10	0.9986 ± 0.0217	0.1244	0.0948
CA	1 ~ 10	0.9962 ± 0.0583	0.1118	−0.0466

[a] Results are the mean of five replicate analyses.

Table 3
Determination of amoxycillin (AM) and clavulanic acid (CA) in the known amount of synthetic mixtures

Number	Prepared mixture	Added (mg)	Found ± S.D.[a] (mg)	Recovery (%)	CV (%)
1	AM[b]	250	264.08 ± 3.74	105.63	1.44
	CA	125	126.35 ± 1.14	101.08	0.90
2	AM	125	125.79 ± 1.87	100.63	1.49
	CA	125	126.74 ± 0.15	101.39	0.12
3	AM	125	248.75 ± 3.33	99.50	1.34
	CA	250	124.69 ± 0.66	99.75	1.53
4	AM	375	372.19 ± 5.92	99.25	1.59
	CA	125	124.43 ± 2.50	99.54	2.01
5	AM	500	506.35 ± 9.57	101.27	1.89
	CA	125	127.05 ± 2.35	101.64	1.85

[a] Results are the mean of five replicate analyses.
[b] Amoxycillin in sodium form.

Table 4
Simultaneous determination of amoxycillin (AM) and clavulanic acid (CA) in pharmaceutical preparations

Preparation	Claimed amount (mg) per unit	Assay amount[a] (mg) per unit	Assay amount (%)	
			$\bar{x}$	±S.D.
Augmentin® tablets	AM 250	258.74	103.50	1.47
	CA 125	127.11	101.69	0.51
Augmentin® injections	AM 500	535.85	107.17	2.21
	CA 100	99.74	99.74	0.94
Augmentin® syrups[b]	AM 125	129.67	103.74	0.98
	CA 31.25	30.82	98.62	0.75

[a] Results are the mean of of 6 tablets, 3 injections, and 3 syrups with three replicate analyses for each pharmaceutical preparation.
[b] Each 5 ml labeled to contain the amount shown.

The method can be applied for detection and quantification of commercial products. Three types of Augmentin® were assayed as showed in Table 4. The results show variations between the amount determined in this study and the amount claimed by the manufacturer. In terms of % per unit, they were between 103.50 and 107.17% of the claimed values for amoxycillin. Similar results for potassium clavulanate were obtained between 98.62 and 101.69% of the manufacturer's claim. Nevertheless, the quantities determined in this study are within the range of the USP XXII standard.

4. Conclusion

This paper describes a new liquid chromatographic analysis of amoxycillin and clavulanic acid using special β-cyclodextrin as stationary phase. The procedure is simple, rapid, and reproducible.

It may represent an alternate method for analysis of these two components in pharmaceutical forms. It is also planned to apply this method in biological fluid studies.

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