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Optimization of Phosphate Buffer Ph 4.4-Methanol as Mobile Phase for Analysis of Amoxicillin and Clavulanate Potassium Mixture in Tablets by High Performance Liquid Chromatography (HPLC)

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Abstract : The combination of amoxicillin dan clavulanate potassium is a combination of β-lactam antibiotic and β-lactamase inhibitor. The purpose of this experiment is to optimize the High-performance Liquid Chromatography (HPLC) condition using Shim-pack VP-ODS (4.6 mm x 25 cm) column of the determinitation of amoxicillin and clavulanate potassium mixture in tablets.

To get the optimum condition of analysis, the mobile phase and flow rate have been varied. Optimization result showed the best analysis condition was the mobile phase consisted of phosphate buffer pH 4.4-methanol 91:9 with 2.0 ml/minute flow rate.

The determination of calibration curve linearity showed the correlation coefficient, r = 0.9999 with the regression Y = 14997.26153X + 146176.518 for amoxicillin and r = 0.9999 with the regression Y = 17320.23929X + 68440.92704 for clavulanate potassium. The result of determination of amoxicillin and clavulanate mixture in four tablets dosages fulfilled the requirement of the thirtieth edition United States Pharmacopoeia.

In conclusion that this metohd fulfilled clauses of validation test method to the mixture of tablet showed percent recovery 99.09% (RSD/relative standard deviation = 0.21%) for amoxicillin and 99.71% (RSD = 0.98%) for clavulanate potassium. Limit of detection (LOD) and limit of quantitation (LOQ) of amoxicillin = 34.23 mcg/ml and 103.74 mcg/ml. Limit of detection (LOD) and limit of quantitation (LOQ) of clavulanate potassium = 8.83 mcg/ml and 26.75 mcg/ml.

Keywords: amoxicillin, clavulanate potassium, high performance liquid chromatography, mobile phase, flow rate, validation.

Introduction and Experiment

Combination of Amoxicillin and Clavulanate Potassium is an antibacterial combination consisting of the β-lactam derivatives and β-lactamase inhibitors. This combination is given in order to overcome the bacteria that can damage the β-lactam (β-lactamase-producing bacteria). Both of these compounds can be analyzed simultaneously using the method of High Performance Liquid Chromatography (HPLC). [1,2]

Optimization is an attempt to obtain a better separation, to obtain a faster analysis, to improve sensitivity and to save costs. Optimization can be performed on several variables such as mobile phase comparison, flow rate, the stationary phase or column. The simplest and most frequent optimization is the comparison to the mobile phase and flow rate.[3] Changing in the ratio of the mobile phase and flow rate can affect the analysis time, pressure, and efficiency of the column. [4,5,6]

According to USP XXX (2007), the mixture of amoxicillin tablet and clavulanate potassium can be determinate by High Performance Liquid Chromatography using 4 mm x 30 cm-L1 column (octadecyl silane) with a mobile phase of phosphate buffer pH 4.4-methanol (95:5), flow rate of 2.0 ml / min and a detection is done at a wavelength of 220 nm. [7]

Based on the above, authors are interested in optimizing the HPLC method with Shim-pack VP-ODS (4.6 mm x 25 cm) column. Optimization was done on comparison of the phosphate buffer pH 4.4 - methanol mobile phase and a flow rate. Then the selected comparison of the mobile phase and flow rate was used to establish the levels of amoxicillin and clavulanate potassium in tablets. Results obtained were compared with the requirements listed in the USP XXX (2007). [7]

To obtain validity of the methods used, the validation test of accuracy is performed as expressed as the percentage and precision test specified in the relative standard deviation (RSD) and then, determine the limit of detection and the limit of quantitation.[8]

Materials

The materials used were methanol, narium dihydrogen phosphate, 85% phosphoric acid. sodium hydroxide, *aquabidestilata* (PT Ikapharmindo Putramas), amoxicilllin trihydrate BPFI (PPOM Jakarta), raw potassium clavulanate (PT Meprofarm), generic tablets (PT Indofarma). Claneksi® tablets (PT Sanbe), Clavamox® tablets (PT Kalbe Farma) and Augmentin® tablets (PT Glaxo Smithkline Beecham).

Determining Composition of Phosphate Buffer pH 4.4 Methanol Mobile Phase and Optimum Flow rate

Chromatographic conditions were varied to obtain the optimum results analysis. Chromatographic conditions that being varied was the composition of mobile phase and flow rate. The mobile phase composition of the phosphate buffer pH 4.4 solution and 98:2 methanol was varied at 96:4, 94:6, 92:8, 91:9 and 90:10. From the comparison of the selected mobile phase, the flow rate is determined at 1.0 ml / min. 1.2 ml / min. 1.4 ml / min. 1.5 ml / min. 1.6 ml / min. 1.8 ml / min and 2.0 ml / min.

Qualitative Analysis.

Qualitative analysis of amoxicillin and clavulanate potassium was performed by comparing the peaks that have nearly identical retention time from the HPLC chromatogram analysis of comparison standard solution of amoxicillin and clavulanate potassium with the sample solution at a wavelength of 220 nm.

Determination of Calibration Curve Linearity of Standard Comparative of Amoxicillin and Clavulanate Potassium

The main standard solution of amoxicillin and clavulanate potassium was respectively pipetted 1 ml and 0.5 ml, 2.5 ml and 1 ml, 5 ml and 2 ml, 7.5 ml and 3 ml, 10 ml and 4 ml, 12.5 ml and 5 ml. Next, put each solution into a 25 ml flask and diluted with solvent up to the mark line. amoxicillin solution concentration in a row was 100 ppm, 250 ppm, 500 ppm, 600 ppm, 750 ppm and 1250 ppm.

While the concentration of clavulanate potassium in a row was 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm.

Each solution was filtered through a 0.2 μm Cellulose Nitrate filter membrane and sonication for ± 20 minutes. Next, 100 μl referenced standard solution filtrate was injected into the HPLC system through a 20 μl loop injector.

Detection using a UV detector at a wavelength of 220 nm. Chromatograms were recorded and made the calibration curve of the peak area, then calculated the regression equations and correlation coefficients.

Determination of Amoxicillin and Clavulanate Potassium in the samples

Ten tablets that had been clean of film membranes were weighed and crushed homogeneously. Weighed powder was equivalent to 25 mg of Amoxicillin, put in a 50 ml flask and solvent was added to the line mark, shaken and then filtered. Solution was then filtered through a membrane filters PTFE 0,5 μm and cellulose nitrate membrane filters 0,45 μm and sonication for ± 20 minutes. Then a 100 μl solution were injected into the HPLC system through a 20 μl loop injector using isocratic elution system with mobile phase of phosphate buffer pH 4.4 - methanol (91:9) and flow rate of 2.0 ml / min. Detection using a UV detector at a wavelength of 220 nm. Chromatogram was recorded and logged the peak area. Levels were calculated by substituting the peak area into the regression equation ($y = ax + b$) obtained from the calibration curve.

Validation Methods

Validation parameters tested were accuracy, precision, limits of detection and quantitation limits.

Accuracy

Accuracy expressed as percent recovery (% recovery) using the standard addition method.[14]

Precision.

Method precision expressed by relative standard deviation/RSD) of the data series.[15]

Limits of detection and quantitation limits

Limits of detection and quantitation limits were calculated from the regression equation obtained in the calibration curve. [15]

RESULTS AND DISCUSSION

Identification of Amoxicillin and Clavulanate Potassium

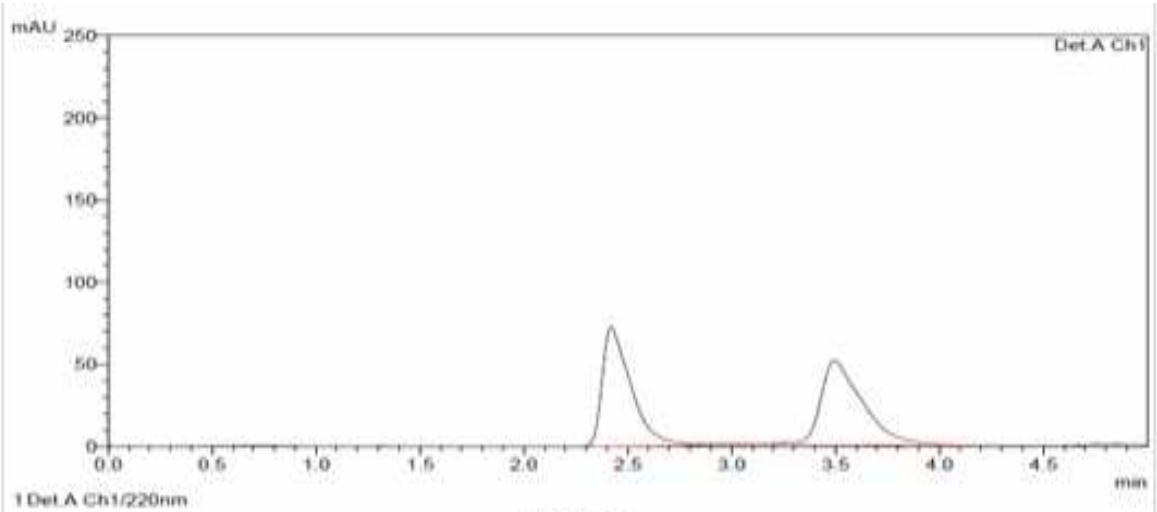
Identification is performed by spiking method with the addition of amoxicillin to the standard solution of amoxicillin and clavulanate potassium mixture that had been analyzed previously. Chromatograms results showed an increase in the area of amoxicillin. This showed the chromatogram area that being increased was amoxicillin while the chromatogram area that not being increased was clavulanate potassium. Chromatogram can be seen in Figures 1 and 2.

Analysis Composition oc Phosphate Buffer pH 4.4 - Methanol Mobile Phase and Optimum Flow rate

To determine whether the chromatographic conditions according to USP XXX (2007) can be done by separation mechanism of amoxicillin and clavulanate Potassium uses ODS column (octadecyl silane) based on the nature of the polarity of the two components. Judging from the structure, clavulanate potassium is more polar than that amoxicillin. clavulanate Potassium elute first from the column ODS rather than amoxicillin showed a good analysis had been performed. Next, the analyzes of the mixture of amoxicillin and standard clavulanate

potassium with HPLC using column Shim-pack VP-ODS and chromatographic conditions according to USP XXX (2007). [7] Chromatogram can be seen in Figure 3.

Chromatogram in Figure 3 showed the results of the analysis are quite good with a resolution of 6:38; theorytical plate 2669 for amoxicillin and 2501 for clavulanate potassium; retention time of 4.9 minutes for amoxicillin and 2.9 minutes for clavulanate potassium.

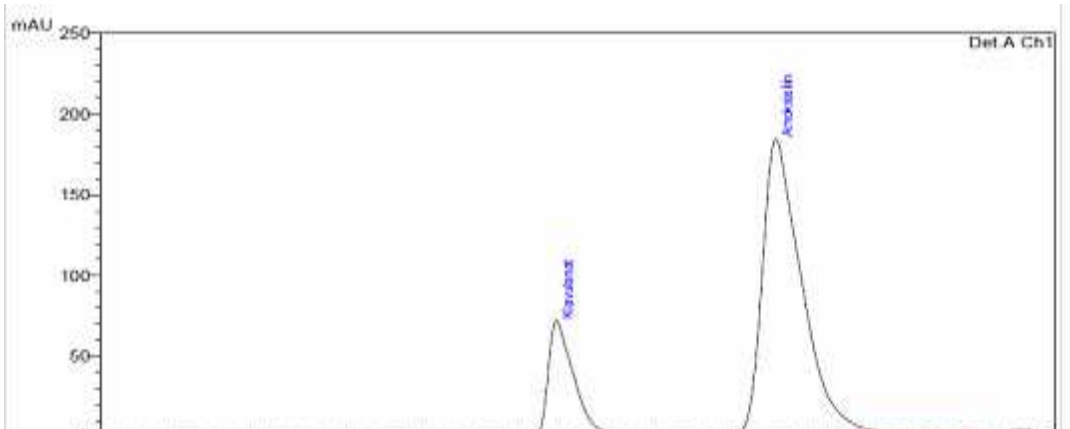


PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Resolution	HETP	Theoretical Plate#	Tailing Factor	Conc.
1	2.416	707797	72779	0.000	194.993	1282.095	2.060	0.000
2	3.401	728449	51595	3.242	198.261	1260.962	1.787	0.000

Figure 1. Chromatogram Identification of Amoxicillin and Clavulanate Potassium before spiking method (standards additions) amoxicilin

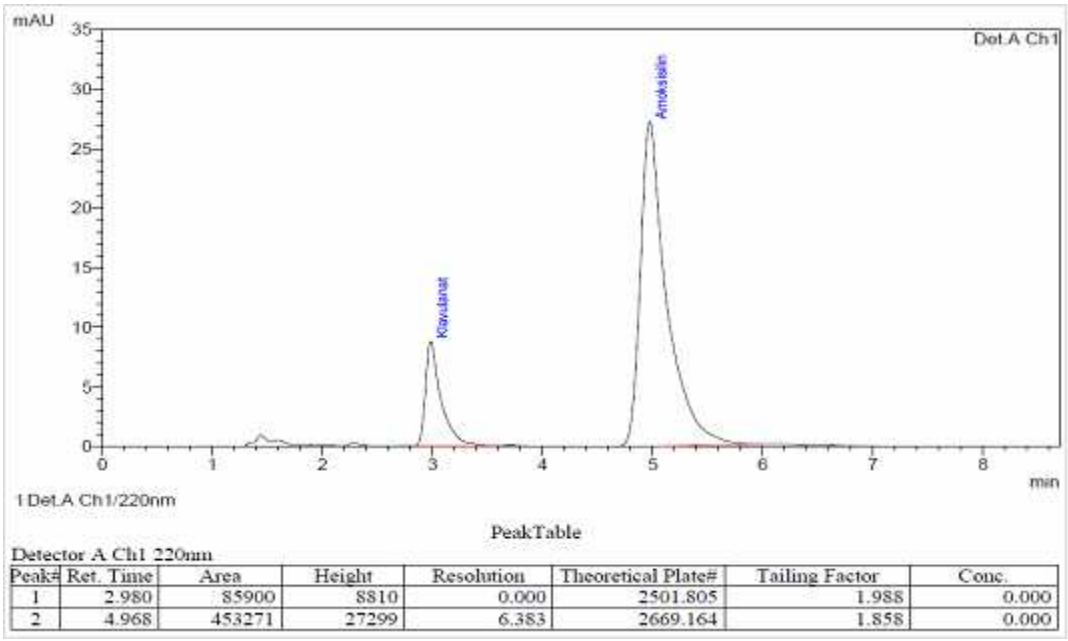


PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Resolution	HETP	Theoretical Plate#	Tailing Factor	Conc.
1	2.386	709840	73008	0.000	198.291	1260.772	2.058	0.000
2	3.536	2767703	185500	3.404	207.340	1205.748	1.791	0.000

Figure 2. Chromatogram Identification of Amoxicillin and Clavulanate Potassium after spiking method (standards additions) Amoxicillin.



PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Resolution	Theoretical Plate#	Tailing Factor	Conc.
1	2.980	85900	8810	0.000	2501.805	1.988	0.000
2	4.968	453271	27299	6.383	2669.164	1.858	0.000

Figure 3. Chromatogram analysis of the mixture of Amoxicillin and and Clavulanate Potassium using column Shim-pack VP-ODS. the Phosphate Buffer pH 4.4 solution and 95:5 methanol and flow rate 2 ml/min

To get the relatively shorter analysis time with theorytical plate and resolution which meets USP requirements, the comparison of mobile phase phosphate buffer pH 4.4-methanol had beed determined. Data analysis a of amoxicillin and standard clavulanate potassium mixture by HPLC using different mobile phase composition at a flow rate of 2 ml / min can be seen in Table 1.

Column efficiency of the HPLC can be seen from the theoretical plate parameters on each analyte peak and the separation power can be seen from the Resolution parameter. According to USP XXX, every peak from theoretical plate in assays amoxicillin and clavulanate potassium tablets simultaneously must be greater than 550 and resolution no smaller than 3.5. From Table 1, it can be seen that the ratio of phosphate buffer pH 4.4-methanol (90:10) gave a good column efficiency and meets the USP requirement. However the resulting separation is relatively poor which is 3414. Comparison (98:2) to (91:9) gave a good column efficiency and separation and meets the requirements of USP XXX (2007). but the required analysis time is much longer. According to research analysis was performed in a relatively shorter time which is 5 minutes.[9]

Therefore, the best mobile phase comparison phosphate buffer pH 4.4-methanol for analysis is (91:9) with a retention time of 3.9 minutes for amoxicillin and 2.6 minutes for clavulanate potassium; theoretical plate 1407 to 1346 for amoxicillin and clavulanate potassium; with a resolution of 3.88.

Table 1 show that the greater the concentration of methanol in mobile phase, the retention time of amoxicillin and clavulanate potassium is getting shorter. This is due to the strength of the solvent.*A* greater methanol concentrations will cause stronger nonpolar mobile phase, so on a reversed phase chromatography, elution process happen faster. Therefore, a shorter retention time.[6]

Next, from the selected mobile phase, had been determined the optimal flow rate. Data analysis of amoxicillin and standard clavulanate potassium mixture with HPLC at various flow rates with mobile phase of phosphate buffer pH 4.4-methanol (91:9) can be seen in Table 2. Just as in the determination of the comparison of mobile phase phosphate buffer pH 4.4 and methanol, parameters such as retention time, theoretical plate and resolution were the determent of the selection of the optimum flow rate.

Table 1. Data analysis of Amoxicillin and Standard Clavulanate Potassium Mixture by HPLC Using Various Mobile Phase composition at a flow rate of 2 ml / min.

Mobile phase comparison		Retention time		Peak area		Theoretical Plate		Resolution
Phosphate Buffer pH 4.4 (%)	Methanol (%)	Clavulanate Potassium	Amoxicillin	Clavulanate Potassium	Amoxicillin	Clavulanate Potassium	Amoxicillin	
98	2	4 .633	9 .193	643 071	2741999	1467.550	1576.913	6 .469
96	4	3 .555	6 .446	667 042	2712624	1427.275	1431.872	5 .467
94	6	3 .015	5 .009	666 382	2706867	1392.643	1384.399	4 .628
92	8	2 .780	4 .429	679 084	2718066	1374.784	1247.684	4 .114
91	9	2 .586	3 .948	666 399	2736978	1346.643	1407.457	3 .877
90	10	2 .386	3 .536	680 856	2767703	1278.310	1205.748	3 .414

Table 2: Data Analysis of Amoxicillin and standard Clavulanate Potassium mixture by using various flow rate with mobile phase composition of Phosphate Buffer pH 4.4 and methanol.

Flow rate ml/menit	Pressure(kgf/cm2	Retention time		Peak Area		Theoretical Plate		Resolution
		Clavulanate Potasium	Amoxi cillin	Clavulanate Potasium	Amoxicillin	Clavulanate Potasium	Amoxicillin	
1.0	86	4.884	7.189	1219086	5170048	2071.063	2056.827	4.336
1.2	103	4.100	6.056	1052449	4364153	1949.893	1918.809	4.231
1.4	120	3.569	5.334	930630	3786725	1777.312	1835.448	4.218
1.5	126	3.326	4.876	844967	3521627	1828.749	1790.602	4.015
1.6	135	3.121	4.660	820520	3308022	1711.081	1636.786	4.038
1.8	150	2.771	4.164	743499	2935505	1594.399	1517.399	3.951
2.0	168	2.586	3.948	666399	2736978	1346.643	1407.457	3.877

The result showed that the best flow rate for analysis was 2 ml / min with a retention time of 3.9 minutes for amoxicillin and 2.6 minutes for clavulanate potassium; theoretical plate 1407 for amoxicillin and 1346 for clavulanate potassium; with a resolution of 3.88.

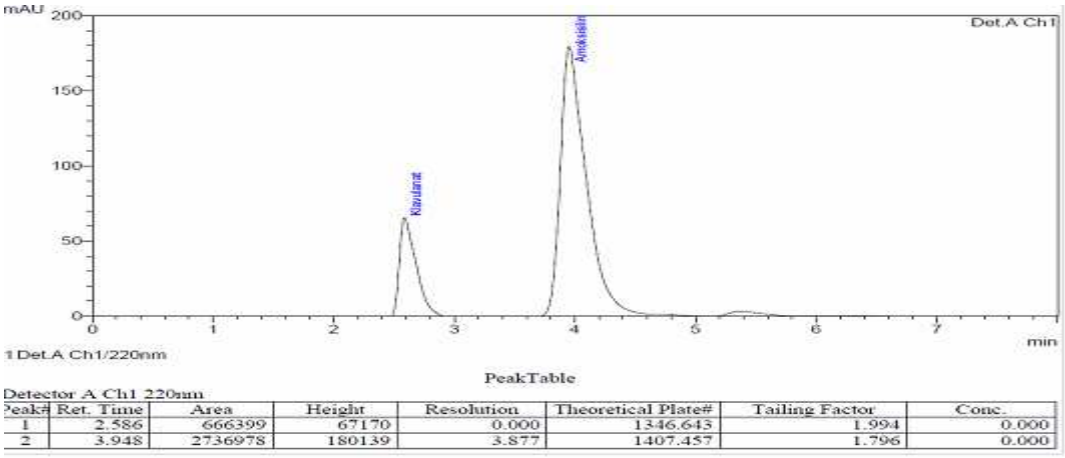
Chromatogram of the optimization results of HPLC method is carried out on a mixture of amoxicillin and standard clavulanate potassium with mobile phase phosphate buffer pH 4.4-methanol (91:9) and a flow rate of 2 ml / min can be seen in Figure 4.

Furthermore, from the comparison of mobile phase and selected flow rate, conducted an analysis of the sample tablets Clavamox®. .

Chromatogram analysis of amoxicillin and clavulanate potassium mixtures from Clavamox® tablets with mobile phase phosphate buffer pH 4.4-methanol (91:9) and a flow rate of 2 ml /min can be seen in Figure 5.

Figure 5 shows the optimization HPLC with mobile phase phosphate buffer pH 4.4-methanol (91:9) and a flow rate of 2 ml / min give the similar optimal results to the reference standard with a retention time of 3.9 minutes for amoxicillin and 2.6 minutes for clavulanate potassium; theoretical plate 1888 for amoxicillin and 1691 for clavulanate potassium; with a resolution of 4:34.

From the chromatograms, all tablets that were analyzed, was obtained retention time with a difference not exceed than 5% of the Amoxicillin and standards clavulanate potassium is 3.9 minutes for amoxicillin and 2.6 minutes for clavulanate potassium. This means that the sample used in this study contains amoxicillin and clavulanate potassium.[10].

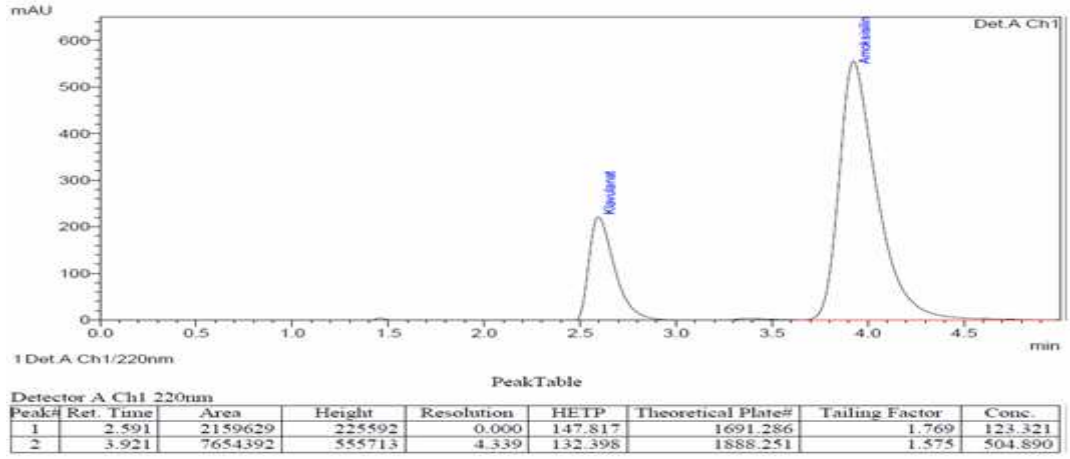


PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Resolution	Theoretical Plate#	Tailing Factor	Conc.
1	2.586	666399	67170	0.000	1346.643	1.994	0.000
2	3.948	2736978	180139	3.877	1407.457	1.796	0.000

Figure 4. Chromatogram of the optimization results of HPLC method on a mixture of Amoxicillin and standard Clavulanate Potassium with mobile phase Phosphate Buffer pH 4.4-methanol (91:9) and a flow rate of 2 ml/min.



PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Resolution	HETP	Theoretical Plate#	Tailing Factor	Conc.
1	2.591	2159629	225592	0.000	147.817	1691.286	1.769	123.321
2	3.921	7654392	555713	4.339	132.398	1888.251	1.575	504.890

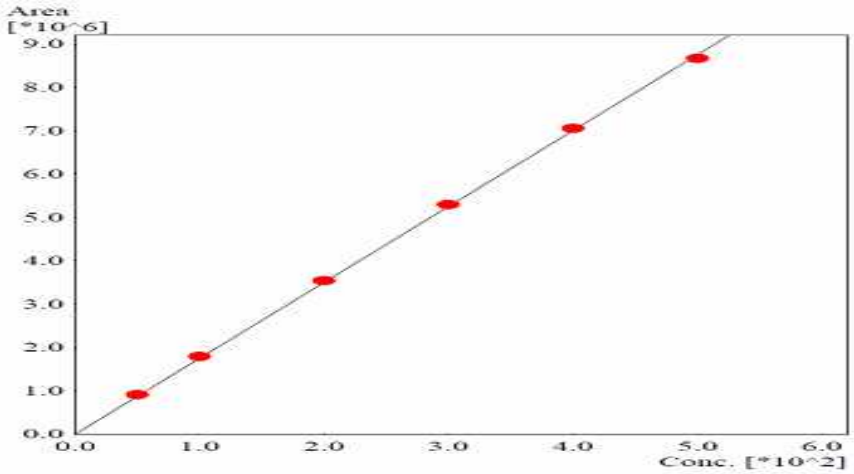
Figure 5 . Chromatogram analysis of Amoxicillin and clavulanate potassium mixtures Clavamox ® tablets with mobile phase Phosphate Buffer pH 4.4-methanol (91:9) and a flow rate of 2 ml/min.

Calibration Curve Linearity of Standard Comparative of Amoxicillin and Clavulanate Potassium

Injection a mixture solution of amoxicillin and standard clavulanate potassium for the calibration curve performed simultaneously. The calibration curve of standard clavulanate potassium with concentration range of 50 ppm to 500 ppm and amoxicillin 100 ppm up to 1250 ppm.

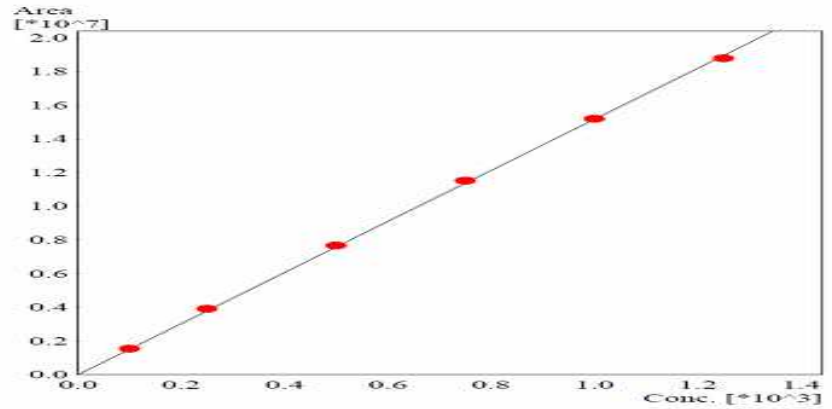
Chromatogram calibration of Amoxicillin and Clavulanate Potassium mixture can be seen in Figure 6 and 7. Calibration curve shows the linear relationship between peak area and concentration with correlation coefficients. R = 0.9999 for amoxicillin and clavulanate potassium. The correlation coefficient is comply with the requirements of greater than 0.999. [12]

Based on the value of r is close to 1 means that there is a linear relationship between peak area and concentration.[13] Therefore amoxicillin and clavulanate potassium concentration in the sample can be calculated by the regression equation with the peak area substituting Y. ([14]



#	Conc. (Ratio)	MeanArea	Area
1	50.000	909850.6	909851
2	100.000	1793924.4	1793924
3	200.000	3539507.9	3539508
4	300.000	5294127.2	5294127
5	400.000	7053113.8	7053114
6	500.000	8666489.5	8666489

Figure 6. The calibration curve of standard clavulanate potassium by HPLC potassium using column Shim-pack VP-ODS (4.6 x 250mm). Mobile Phase Phosphate Buffer pH 4.4 solution and 91:9 methanol and flow rate 2.0 ml/min.



#	Conc. (Ratio)	MeanArea	Area
1	100.000	1551848.7	1551849
2	250.000	3912796.5	3912796
3	500.000	7669875.7	7669876
4	750.000	11516484.3	11516484
5	1000.000	15186946.9	15186947
6	1250.000	18778567.2	18778567

Figure 7. The calibration curve of standard amoxicillin by HPLC potassium using column Shim-pack VP-ODS (4.6 x 250mm). Mobile Phase Phosphate Buffer.
pH 4.4 solution and 91:9 methanol and flow rate 2.0 ml/min

Quantitative analysis is determined by the peak areas as the obtained chromatograms are not symmetrical. The measurement of peak areas are not much affected by the chromatographic condition compare to the peak's high excluding the flow rate. Therefore, the measurement of the peak areas is the best choices in the quantitative analysis of HPLC. [11].

Chromatogram of injection results of a mixture solution of amoxicillin and standards clavulanate potassium in producing of calibration curves, shows a peak that extends to the rear (tailings). Parameters that can be used as an indicator of not symmetric peak is tailing factor. Tailing factor of chromatograms injection of amoxicillin and standard clavulanate potassium for producing calibration curve obtained ranged from 1.729 to 1.475 for amoxicillin and 1.859 up to 1.929 for clavulanate potassium. The analysis results are acceptable because tailing factor smaller than 2. [13]

Analysis Result of Amoxicillin and Clavulanate Potassium Mixture in Tablets.

Assay results of amoxicillin and clavulanate potassium in a variety of tablets on the market using statistical calculations can be seen in Table 3.

Amoxicillin and clavulanate Potassium levels in the tablet which were determined based on the overall area meets USP XXX requirements (2007). The amoxicillin and clavulanate potassium contain not less than 90.0% and not more than 120.0% of the amount listed on the label.

Table 3. Assay results of Amoxicillin and Clavulanate Potassium in a variety of tablets.

No	Name	Clavulanate potasium (%)	Amoxicillin (%)
1	Generic Tablet (PT Indofarma)	93.5245 ± 2.5351	100.5742 ± 3.0142
2	Claneksi® Tablet (PT Sanbe)	92.8401 ± 2.5458	100.6710 ± 2.5007
3	Clavamox® Tablet (PT Kalbe Farma)	91.3741 ± 1.2008	112.1678 ± 2.2739
4	Augmentin® Tablet (PT Glaxo Smithkline Beecham)	97.1150 ± 2.5457	107.7703 ± 2.5001

Results of the Validation test

Validation parameters tested were accuracy, precision, limits of detection and quantitation limits. Accuracy method expressed as percentage recovery which determined by using standard addition method. Precision is expressed in relative standard deviation. Data accuracy and precision test results can be seen in Table 4 and 5.

Tables 4 and 5 show that the average percent recoveries obtained are qualified for the accuracy validation of analytical procedures because the average is between 98-102% range which is 99.09% for amoxicillin and 99.71% for clavulanate Potassium. Relative standard deviation obtained are qualified precision for validation of analytical procedures because less than 2% which is 0.21% for amoxicillin and 0.98% for clavulanate Potassium.[14]

The detection limits and quantitation limits of amoxicillin analysis are respectively 34.23 mcg / ml and 103.74 mcg / ml. While The detection limits and quantitation limits of clavulanate potassium analysis are respectively 8.83 mcg / ml and 26.75 mcg/ml.

This shows that the work concentration of amoxicillin (500 mcg / ml) and clavulanate potassium (125 mcg / ml) can be detected and quantified by the HPLC method used.

From the above discussion. It can be said that analytical procedures that had been done in this study is valid and can be used to assay the mixture of amoxicillin and clavulanate potassium in tablets as it has met the requirements of method validation.

Table 4. Data accuracy and precision test results of Amoxicillin using added standard analyte

No..	Added Analyte (ug / ml)	Peak area	Recovery (%)
1	252	11438921	99.1262
2	244	11453309	98.7748
3	248	11377394	99.0707
4	254	11476636	99.3357
5	254	11461869	98.9480
6	256	11505171	99.3029
The mean recovery			99.0930
Standard deviation (SD)			0.2130
Relative standard deviation (RSD)			0.2149

Table 5. Data accuracy and precision test results of Amoxicillin and Clavulanate potassium added standard analyte.

No..	Analyte is added (Ug / ml)	Peak area	Recovery (%)
1	68	3614832	98.4162
2	66	3605623	100.5929
3	62	3536457	100.6418
4	70	3668019	99.9910
5	72	3685364	98.6044
6	72	3703305	100.0430
The mean recovery			99.7148
Standard deviation (SD)			0.9731
Relative standard deviation (RSD)			0.9759

Conclusion

The optimization results obtained that the comparison of mobile phase pH 4.4 phosphate buffer-methanol (91:9) with a flow rate of 2 ml/min can produce good separation with retention time 3.9 min for amoxicillin and 2.6 min for clavulanate potassium; theoretical plate 1346 for amoxicillin and 1407 for clavulanate potassium with a resolution of 3.88. The assay of amoxicillin and clavulanate potassium mixture were analyzed in 4 dosage tablets with obtained optimum HPLC conditions complies with the requirements of USP XXX (2007).

The conclusion of this method that validation test method results obtained percent recovery 99.09% for amoxicillin (RSD = 0.21%) and 99.71% for clavulanate potassium, (RSD = 0.98%). Thus this method can be used to assay mixture of amoxicillin and clavulanate potassium in a tablet.

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