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Optimization and Evaluation of Column Efficiency in RP-HPLC Method for Pharmaceutical and Forensic Drug Analysis

تحسين وتقييم كفاءة عمود الفصل في طريقة الكروماتوجرافيا السائلة عالية الأداء ذات الطور المعكوس (RP-HPLC) لتحليل الأدوية الصيدلانية والجنائية

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Abstract

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) is an efficient and reliable technique employed in pharmaceutical and forensic analysis. The assay determination in pharmaceutical and certain molecular detection is performed using this technique. Performance of chromatographic columns play a central role in development of more efficient separation methods. It is one of the main component of system suitability testing in a chromatographic system. The column efficiency can be assessed by using various traditional methods and reproducible results can be evaluated using liquid chromatography We studied the column efficiency using RP-HPLC technique for pharmaceutical drug product Aceclofenac in a validated method. Two mobile phases combinations Acetonitrile: Methanol (80:20 v/v) and Acetonitrile: Methanol: Ammonia (225:50:1 v/v) were compared on C-8 Lichrospher RP-HPLC column for Aceclofenac and three tablet samples (30 ppm). The height equivalent to theoretical plate (HETP) referred as direct measure of column efficiency produced efficient results (530-545 and 190-230) for respective mobile phases. The lower HEPT is an indication of efficient elution in second mobile phase. The change in polarity as per pKa of the

Keywords: Forensic sciences, reversed-phase high-performance liquid chromatography (RP-HPLC), system suitability testing, Aceclofenac (ACE).

المستخلص

تُعدُ الكروماتوجرافيا السائلة عالية الأداء ذات الطور المعكوس (RP-HPLC) تقنية فعالة وموثوقة تُستخدم في التحليل الصيدلاني والتحليل الجنائي. يتم إجراء تحديد الاختبار في الأدوية واكتشاف جزيئات معينة باستخدام هذه التقنية. ويؤدي أداء الأعمدة الكروماتوجرافية دورًا رئيسًا في تطوير طرق فصل أكثر كفاءة. إنها أحد المكونات الرئيسة لاختبار صلاحية النظام في النظام الكروماتوجرافي. ويمكن تقييم كفاءة العمود باستخدام طرق تقليدية مختلفة؛ ويمكن تقييم النتائج القابلة للتكرار باستخدام الكروماتوجرافيا السائلة. درسنا تحديد كفاءة العمود باستخدام تقنية RP-HPLC لمستحضر دوائي صيدلاني وهو الأسيكلوفيناك بطريقة تم التأكد من صحتها. تمت مقارنة مجموعتين من الأطوار المتحركة أسيتونيتريل: ميثانول (80:20 v/v)، وأسيتونيتريل: ميثانول: أمونيا (225:50:1 v/v) على عمود RP-HPLC C-8 Lichrospher للأسيكلوفيناك وثلاث عينات أقراص (30 جزءاً في المليون). يعطي الارتفاع المكافئ للصفيحة النظرية (HETP) الذي يُشار إليه كمقياس مباشر لكفاءة العمود نتائج فعالة (530-545 و190-230) للأطوار المتحركة المعنية على التوالي. يشير انخفاض قيمة HETP إلى التصاق فعال في الطور المتحرك الثاني. قد يؤدي التغيير في القطبية وفقًا

الكلمات المفتاحية: علوم الأدلة الجنائية، كروماتوجرافيا السائل عالية الأداء بالطور العكسي (RP-HPLC)، اختبار صلاحية النظام، الأسيكلوفيناك (ACE).

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analyte may enhance elution and resolution. helpful in method development for pharmaceutical and forensic drug analysis using liquid chromatography.

لقيمة pKa للمحلل إلى تحسين الالتصاق والقدرة على الفصل. وهذا مفيد في تطوير طرق التحليل الصيدلاني والتحليل الجنائي للأدوية باستخدام الكروماتوجرافيا السائلة.

1. Introduction

Various technologies have been developed and used in pharmaceutical and forensic drug analysis. Chromatography is one of the most common technique in separation of target analytes. Various modifications of chromatography like gas chromatography (GC), paper chromatography, high-performance liquid chromatography (HPLC), and thin layer chromatography (TLC) are used for molecular detection. Samples like pharmaceutical products, drugs of abuse, hair, toxins, explosives, documents, fingerprints, gunshot residues, and inks can be analyzed and detected using chromatography [1]. Chromatography is one of the cheapest techniques that provides accurate results. Also, it is easy to operate, making it one of the most adaptable and abundant technique as well. Another modification of this technique is reversed-phase chromatography which is simple, versatile and efficient. RP-HPLC in analytical method development is important for analysis of drugs, proteins, peptides & pharmaceuticals etc [2]. Various hyphenated techniques related to liquid chromatography are used in forensic analysis such as HPLC coupled with Diode Array Detector (DAD) and HPLC-atmospheric pressure ionization mass spectrometry (HPLC–API-MS). HPLC–DAD is mostly used in identification of substances in forensic and toxicological scinces [3]. High-performance liquid chromatography (HPLC) has established itself as a cornerstone technique in analytical chemistry, especially in the field of pharmaceutical and forensic sciences. As most of the NPS are enantiomeric in nature i.e having a chiral center, so HPLC chiral columns play a vital role in their separation [28]. The efficiency of chromatographic columns is paramount in determining the resolution, selectivity, and overall performance of an HPLC system. Over the years, various methods have been developed and refined to evaluate column efficiency, offering invaluable insights into column performance and aiding in method optimization.

There is an increased demand for liquid chromatography coupled with ultra-sensitive detectors in identification of vast range of drugs of abuse in clinical and forensic toxicology [4]. The efficiency of liquid chromatography depends upon proper method selection, development and optimization. The results of Chromatographic analysis can be affected by various factors linked with chromatographic system. The official compendiums like European Pharmacopoeia and US Pharmacopoeia etc state various official requirements for system suitability testing and chromatographic efficiency [5]. The method selection and column efficiency determination in HPLC systems is a key factor in producing reliable results within a laboratory setup. The method parameters, like type of column, temperature, mobile phases etc play a crucial role in proper analyte elution. The International Conference on Harmonization (ICH) [6] explains application of certain standards for a method to be released for general use. In case of Liquid Chromatography, certain parameters like injection volume, rate of flow of mobile phase along with its composition, column temperature, wavelength and the manufacturer column lot need to be tested. For this purpose, column efficiency parameters of retention time (tR), theoretical plates number (N), Height equivalent

to theoretical plates (HETP), tailing factor (Tf) and resolution (Rs) are generally evaluated and often being used as system suitability requirements [7].

These methods provide valuable information about column packing quality, analyte retention mechanisms, and separation performance. The stability of HPLC column gives idea of feasibility of a method that can be applied for assay determination through meeting the acceptance criteria for authenticity of finished pharmaceutical product [8].

The reliable identification and quantification of pharmaceutical products, illicit drugs and toxic substances are pivotal for pharmaceutical drug development, criminal investigations and forensic toxicology. Many new psychoactive substances (NPS) and designer drugs like 25B-NBOMe, 4-CMC, mephedrone, Talbutal, JWH-016, JWH 369, Cocaethylene, fentanyl, Amphetamine and Propoxyphene etc have been identified in biological fluids & hair samples by HPLC-Q-TOF-MS [9, 10]. HPLC coupled with sensitive detectors is widely used in pharmaceutical, forensic drugs analysis and certain organic explosive compounds [11]. The determination of column efficiency is much effective and useful in various forensic and toxicological analyses of drugs of abuse and novel psychoactive substances (NPS). Mostly, such drugs are chiral and only one enantiomer is biologically active producing side effects [12, 13]. So enantioseparations proves to be quite obvious in forensic drug analysis. Such analysis is possible using chiral stationary phase (CSP) in Liquid Chromatography columns. The column efficiency in case of chiral columns is also much important in determining comparable performance of chiral columns for desired results in forensic analysis using HPLC technique [14]. Separation of synthetic chiral drugs such as methylenedioxymethamphetmaine (MDMA) and amphetamine have been studied using chiral stationary phase as Phenomenex LUX® AMP column [15]. HPLC has been effectively used in forensic drug and toxicological analysis producing higher resolution, better reproducibility and greater efficiency [16]. Many bath salts and NPS termed as legal highs like stimulants such as new amphetamine derivatives or cathinones which possess a chiral center are also analyzed & evaluated using chiral separation column [17]. It is also worth noting that proper selection of HPLC column is also a key factor in efficient method development for desired output. In most of cases, C-8, C-18 and C-4 column are preferred in HPLC analysis [18].

Understanding and optimizing column efficiency in RP-HPLC can enhance method robustness, sensitivity, and reproducibility, thereby improving the reliability and validity of analytical results. Additionally, it can facilitate the development of efficient chromatographic methods for complex sample matrices commonly encountered in pharmaceutical and forensic laboratories. Some Important Parameters of HPLC performance are [19]:

- System Resolution

Resolution of a column is determined by the ability of column to separate peaks. Efficiency, selectivity and retention of peaks can increase resolution of column. The resolution of HPLC system is calculated by:

Equation 1: $R=tR2 - tR1/0.5(Wb1 + Wb2)$

where tR1 and tR2, are the retention times of the two components and Wb1 and Wb2 are the corresponding peak widths [20].

- Asymmetry Factor or Tailing Factor

The Tailing Factor is calculated by finding the distance from peak front edge to the back edge, di-

vided by the distance of front edge to the centerline, with all distances measured at 5% of the maximum peak height. Peak Tailing (T) can be expressed as

Equation 2: $T = (\chi + y) / 2\chi$ [21]

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- Column Efficiency

The column efficiency can be calculated by finding N (number of theoretical plates) as

Equation 3: $N = 5.54 (t_R / W_{0.5})^2$,

where t_R represents retention time and $W_{0.5}$ corresponds to peak width at half-height or W is the width at the base of the peak or.

***Equation 4*:** $N = 16 (\frac{tR}{W})^2$

The height equivalent to one theoretical plate is calculated by:

Equation 5: $h = L / N$,

where L is the length of the column & N is number of theoretical plates [21].

Traditional Methods for Column Efficiency Determination

Following four traditional methods are used for determining N as potential factor in column efficiency and performance [22]:

- Tangent Line Method

This method is used for determination of efficiency corresponding to peak width. The distance between inflection point at right baseline of the peak and tangent line to left peak point is calculated and represented by Equation 4. This method is commonly applied in United States Pharmacopeia (USP). The smaller is the N values the larger is the peak overlap.

- Half Peak Method (Half Peak Height)

This method is usually used by British Pharmacopeia (BP), German Pharmacopeia), and European Pharmacopeia (EP). The peak width is determined easily by hand from W0.5 (width at half peak height). It is calculated by Equation 3. The the factor 5.55 was converted to 5.54 by the Japanese Pharmacopoeia (JP).

- Exponentially Modified Gaussian (EMG Method):

This method is used unless the peaks are completely resolved. The asymmetric peak parameters and 10% peak width (W0.1) are used in this method and calculated by:

Equation 6: $N = 41.7 \frac{\left(\frac{tr}{W0.1}\right)2}{\frac{b0.1}{a0.1}+1.25}$

where a0.1 is first half width of peak at 10 % height and b0.1 is second half width of peak at 10 %.

- Area Height Method (AHM)

The peak area and peak height are used for determination of width providing reproducible and accuracy in results even for distorted peaks and is calculated by:

Equation 7: $N = 2\pi (tr. H/A)^2$

where A is Area and H as height of peak

2. Materials and Methods

Aceclofenac (ACE) chemically a derivative of phenyl acetic acid [2-(2',6'-dichlorophenylamino) phenyl] acetoxyacetic acid) [22]. It is an NSAID and

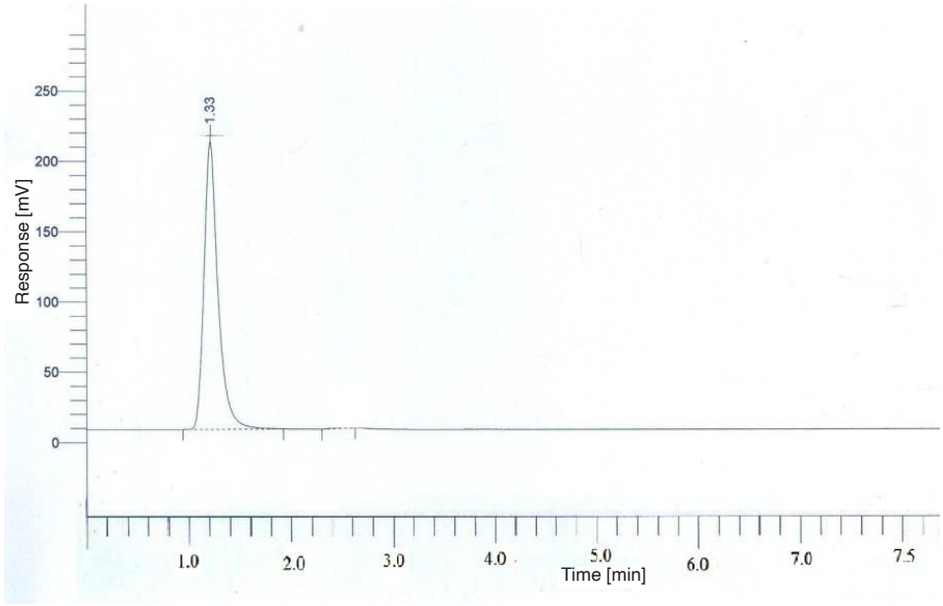

Figure 1- *ACE standard Chromatogram (30 ppm) in MP-A*

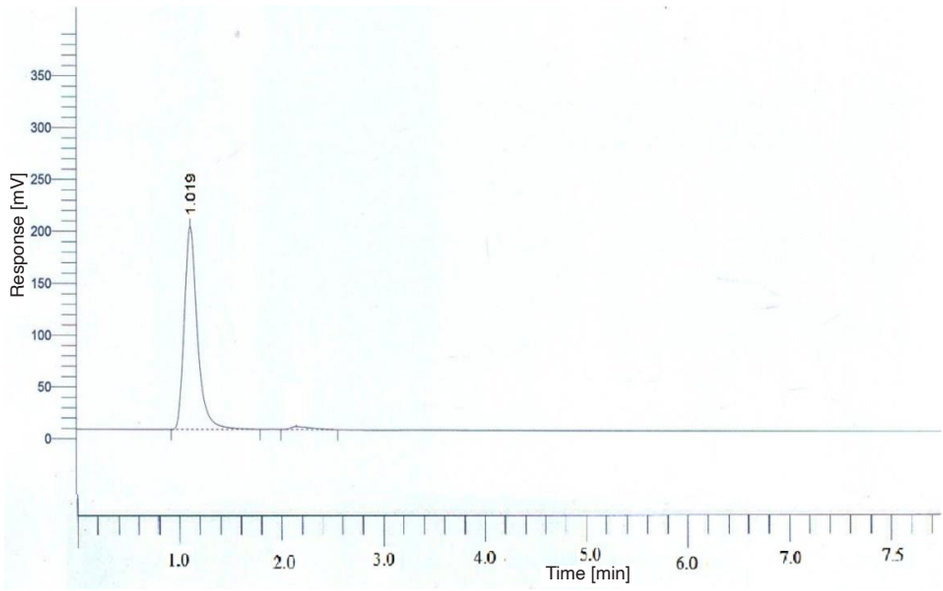

Figure 2- *ACE standard Chromatogram (30 ppm) in MP-B*

COX inhibitor commonly used for treatment of rheumatoid arthritis and other joint ailments [23]. The current study was performed on ACE active pharmaceutical ingredient and commercial tablet samples for comparative evaluation of some of the above parameters. The RP-HPLC technique in isocratic mode with two novel mobile phases MP-A (Acetonitrile and Methanol mixed in v/v ratio of 80:20 & MP-B (Acetonitrile, Methanol and NH3 as 225:50:1 v/v) was used. All the solvents used were of HPLC grade. An already validated method was used in the study for determination of column efficiency [24]. The standard along with certificate of analysis was gifted by the renowned pharmaceutical firm in Lahore, Pakistan on request for the research purpose.

The registered commercial tablet samples (Acenac, Alkeris and Airtal) of the drug were purchased from local market.

2.1. Instrumentation & Chromatographic Conditions

A Perkin Elmer Series 200 RP-HPLC System consisted of Series 200 Micro Pump in binary high pressure mode with flow rate at 1.0 ml/min. Series 200 Auto sampler was used for sample injection with injection volume of 20 mL. The column temperature was maintained at 45oC by series 200 oven. Series 200 PDA (Photo Diode Array Detector) was set at 273 nm for wavelength detection. The HPLC system was operated in isocratic reverse phase mode with Merck Millipore Lichrospher® C-8 column (5 mm) (4.6 x 150 mm) column fitted with Diode Array Detector (DAD).

2.2. Standard & Sample Preparation

A standard methanolic stock solution of 250 ppm was prepared by adding 25 mg of Aceclofenac (ACE) in 100 mL of methanol. Working standard solution having concentration of 30 mg/mL was prepared by stock solution dilution. Similarly, tablet powder (equivalent to 25 mg ACE) was dissolved in 100 mL methanol to prepare a solution of 250 ppm and sample solution of 30 ppm was obtained

Table 1- *Column efficiency Parameters for Standard and Sample using MP-A*

	Mobile Phase-A							
Sample	**Retention Time (min)**	**Peak Height (h) mm**	**Half Peak Width ($w_{50\%}$) mm**	**Peak Width at 10% ($w_{10\%}$) mm**	**Width at Base Line mm**	**a (0.1) mm**	**b (0.1) mm**	**Peak Area mm**
ACE Standard	1.32	50.5	6	10	10	5.8	6.5	303
Acenac Tablet	1.33	54	6	10	10	6.4	5	324
Alkeris Tablet	1.32	49	6.5	10	10	5	5	318
Airtal Tablet	1.31	49	6	10	10	5	5	294

Table 2- *Column efficiency Parameters for Standard and Sample using MP-B*

	Mobile Phase-B							
Sample	**Retention Time (min)**	**Peak Height (h) mm**	**Half Peak Width ($w_{50\%}$) mm**	**Peak Width at 10% ($w_{10\%}$) mm**	**Width at Base Line mm**	**a (0.1) mm**	**b (0.1) mm**	**Peak Area mm**
ACE Standard	1.017	64.8	2.5	4.6	4.6	2.1	3.0	162
Acenac Tablet	1.019	67.5	2.5	5.0	5.0	2.0	3.0	168.75
Alkeris Tablet	1.023	65.0	2.5	5.0	4.8	2.0	3.0	162.5
Airtal Tablet	1.020	62.0	2.5	5.0	4.6	2.1	2.8	155

by further dilution. Automated injector was used to inject 20 μL of this sample was injected for HPLC analysis.

2.3. Data Evaluation

RP-HPLC data generated for standard and samples through TotalChrom® software. Scientific validated scale was used for determination of peak height, width at 10% from base line (both a0.1and b0.1), half peak width and peak area. Other parameters like the number of theoretical plates, tailing factor, peak asymmetry factor and HETP were determined for the C-8 column using both of the mobile phases. Traditional techniques like TLM (Tangent line method), HPM (half peak method), AHM (area height method) and EMG (Exponentially Modified Gaussian) method were used for theoretical plate caluclation.

The theoretical plate number (N) was estimated by the equation $N = 16$ tR/w [21], where tR represents retention time and peak width as W. Height Equivalent to Theoretical Plate represented as H or HETP is another parameter alongside Peak Asymmetry Factor and Tailing Factor to evaluate column performance [24].

3. Results and Discussion

The efficiency of column in a particular liquid chromatographic condition is important for method development and analyte resolution. The polarity of mobile phase plays a crucial role in resolution and system efficiency. In this study, the optimization of method involved comparing both mobile phases i.e. MP-A and MP-B with later being slightly polar due to addition of NH3. Various parameters like half peak width, peak height, width from baseline at 10% and peak area were calculated and the results are presented in Table-1-2. Furthermore, data obtained for these parameters were used for calculation of No. of theoretical plates by above mentioned methods. Other parameters like peak tailing factor, HETP and asymmetry factor were also calculated.

The retention time for ACE standard and tablet samples peaks were detected at 1.30 + 0.01 with MP-A and 1.01 + 0.01 with MP-B. The peaks were sharper in ammoniated mobile phase with half peak width (w50%), base line widths (a(0.1), b(0.1)) and peak area being lesser than that in other mobile phase (Table 1-2). Other factors like plates count, peak asymmetry factor (As) and tailing factor (Tf) were calculated for both mobile phases. It was observed that higher number of theoretical plates were obtained using MP-B as compared to MP-A (Table 3-4). This observation lead to the fact that the column showed efficient behavior towards resolution of target analyte under same set of conditions with better performance in MP-B. Tailing Factor (Tf, a USP coefficient of the peak symmetry)

Table 3- *HETP Calculation for Standard and Sample Using MP-A*

Samples	HETP Determination						
	No. of Theoretical Plates				Peak Asymmetry Factor A_s	Tailing Factor T_f	HETP
	TLM	HPM	AHM	EMG Method			
ACE	0.28	0.27	0.30	0.31	1.12	35.67	536.42
Acenac Tablet	0.28	0.27	0.31	0.36	0.78	36.48	530.78
Alkeris Tablet	0.28	0.22	0.26	0.32	1.00	25	535.61
Airtal Tablet	0.27	0.26	0.30	0.32	1.00	25	542.15

Table 4- *HETP Calculation for Standard and Sample Using MP-B*

Samples	HETP Determination						
	No. of Theoretical Plates				Peak Asymmetry Factor A_s	Tailing Factor T_f	HETP
	TLM	HPM	AHM	EMG Method			
ACE Standard	0.78	0.92	1.04	0.76	1.43	5.35	191.79
Acenac Tablet	0.66	0.92	1.04	0.63	1.50	5.00	225.71
Alkeris Tablet	0.72	0.92	1.05	0.63	1.50	5.00	206.39
Airtal Tablet	0.78	0.92	1.05	0.67	1.33	5.14	190.67

which was determined as lesser in magnitude in MP-B than MP-A indicating a sharper resolution of analyte.

The increased efficiency is also attributed to the fact that raising pH greater than the analyte (ACE) i.e. pKa >4.7 [27] leading to enhanced solubility of the target drug in the mobile phase. This resulted in a sharper peak. better resolution and reproducibility of the analyte.

4. Conclusion

Column efficiency parameters are optimized based on the specific requirements of analysis. The C-8 Lichrospher Column produced efficient results in the analytical method with a slight change in polarity of mobile phase. Addition of NH3 in Acetonitrile and methanol mixture makes it more polar than Acetonitrile methanol mixture. This causes the efficient elution of target analyte. Our comprehensive study demonstrated that meticulous selection of proper stationary phase, mobile phase composition and specified method parameters can significantly enhance column performance. In pharmaceutical and forensic drugs analysis, the optimization of method in a chromatographic system is important for proper resolution & detection. Furthermore, optimizing certain parameters like resolution, tailing factor and plate count, peak symmetry, retention time, and overall separation efficiency, are crucial for the precise identification and quantification of pharmaceutical compounds and forensic substances. The study underscored the importance of the utilization and implementation of column efficiency parameters as an integral component of standardized drug testing both in pharmaceutical and forensic sciences. As most of the NPS are enantiomeric in nature i.e having a chiral center, so HPLC chiral columns play a vital role in their separation [28].

The column efficiency thus needs to be determined while developing a method for their analysis. As far as the detection and quantification of illegal drugs, toxins, poisons, pesticides and other important analytes are concerned, use of state of the art chromatographic techniques with highly sensitive detectors should be evaluated using system suitability tests for forensic reporting in court of law. Future research should focus on integrating advanced stationary phases and technologies like ultrahigh-pressure systems and sophisticated data generation software to further elevate the capabilities of RP-HPLC in complex analytical applications.

Conflict of interest

The authors declare no conflicts of interest.

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