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Validated HPLC method for assay of Xipamide, Triamterene and toxic impurity; Benzyl cyanide based on analytical Eco-scale assessment

Manal F. Mahrous*[a], Mokhtar .M. Mabrouk[b], Ahmed Habib[b],Mohammed E. Draz[a]

[a] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Delta University for Science and Technology, 35712, Gamasa, Egypt.*

[b] *Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Tanta University, Tanta,3111, Egypt*

Correspondence: Manal Fouad Mahrous; +201006839402; manalfouad220@gmail.com

ABSTRACT

Nowadays, green chemistry attracts the attention of analysts to develop methods that do not rely on hazardous chemicals, which negatively affect humans and the environment. Thus, an environmentally safe, sensitive, and selective HPLC technique was established for the analysis of commonly formulated water pills; Xipamide (XIP) and Triamterene (TRI), which are prescribed usually to treat hypertension in combination with the toxic impurity of TRI; Benzyl cyanide (BC). The assay of this water pills binary mixture was performed using several analytical techniques. Still, none of them paid attention to assay the related toxic impurity or evaluate the method's greenness. We utilized the HPLC C_8 column to resolve the ternary mixture using methanol and 50 mM phosphate buffer in the ratio of (50: 50 by volume, pH 4.5) as a mobile phase, delivered at 1.2 mL min^{-1} and 220 nm was selected for detection of the eluate. Calibration curves were constructed in the range of (1-100 µg mL^{-1}) for TRI and XIP, and (5-50 µg $mL^{-1)}$ for BC. Limit of detection was calculated to be 0.30 µg mL^{-1}, 0.45 µg mL^{-1}, 3.2 µg mL^{-1} for TRI, XIP and BC, respectively. The greenness profile of the suggested method was evaluated using the analytical eco-scale. Following the ICH requirements, the recommended method was validated and used to analyze the marketed formulations. The obtained results encourage the quality control laboratories to adopt the proposed approach routinely with the lowest harm to the environment.

Keywords:

Green assessment, HPLC, impurities, Xipamide, Triamterene, Benzyl cyanide

1. Introduction

Hypertension is a common chronic condition among seniors. It triggers several complications like coronary artery disease, stroke, and myocardial infarction, which may lead to sudden cardiac death. Thus, medical attention was paid to treating and controlling its adverse effects (Whalen, 2015). Diuretics are the first-line medication to control hypertension (Whalen, 2015). They are dispensed in several protocols, either alone or combined with other antihypertensives like sympatholytic agents, arteriolar vasodilators, blockers of angiotensin II receptors, ACE inhibitors, and calcium channel blockers, to manage the diseases (Laurence L. Brunton & Lazo, 2018). Patient compliance problems are usually associated with these protocols, so fixed-dose combinations (FDCs) - single finished pharmaceutical dosage forms containing two or more different drugs in a fixed ratio of doses - are considered a safe and effective alternative to satisfy patient compliance and enhance the quality of life (Bangalore,

Kamalakkannan, Parkar, & Messerli, 2007). One of these FDCs under study in this research is water pills like a combination of Xipamide (XIP) and Triamterene (TRI), which are used to treat water retention problems associated with heart failure, edema, liver cirrhosis, and kidney diseases or for management of hypertension (Berry, 2009). XIP; (4-Chloro-N-(2,6-dimethyl phenyl)-2-hydroxy-sulfamoylbenzamide) **(Fig.1a)** is a sulphonamide diuretic, which increases the water excretion through the kidney by inhibition of reabsorption of sodium in the distal tubule also it increases the excretion of potassium which may lead to hypokalaemia (Whalen, 2015). The patients can face this side effect by combining with TRI in FDCs (Hohenegger & Holzer, 1976). TRI (6-Phenylpteridine-2,4,7-triamine) **(Fig.1b)** is a potassium-sparing diuretic that acts by inhibiting the epithelial sodium transport at the late distal and collecting ducts of the kidney, which leads to the enhancement of sodium and water excretion (Whalen, 2015). Inhibition of sodium reabsorption in the collecting tubule leads to the prevention of potassium excretion in the urine and prevents hypokalaemia exerted by XIP (Whalen, 2015).

The pharmaceutical industry has been facing a challenge in analyzing the degradation products and the in-process impurities because they may induce many side effects, toxic concerns, or reduce the activity of the active ingredient (Ivanovic et al., 2007; Saad et al., 2022). we should routinely inspect the dosage form's stability to ensure the absence of hazardous impurities, which result from either the starting material or from degradation due to improper storage conditions. Hence, firm recommendations were issued by the International Council for Harmonization (ICH) regarding the concentrations of impurities in new pharmaceuticals that the drug impurities or degradation products should be subjected to quantitative and/or qualitative analysis, regardless of whether they reached the identification (concentration) threshold or not ("ICH Harmonized Tripartite Guideline," Validation of Analytical Procedures" ICH Harmonized Tripartite Guideline," Validation of Analytical Procedures," 2006). Moreover, impurities are frequently present in minimal concentrations and have structural similarities to the parent medication. Thus their detection is considered a challenge for the analyst (Sandor Gorge, n.d.). Accordingly, developing sensitive and selective analytical methods is a mandatory requirement. Chromatographic techniques are powerful analytical tools capable of separating complex mixtures with structural similarities like in-process impurities (Görög, 2006; Naguib, Abdelaleem, Draz, & Zaazaa, 2015; Naguib, Draz, & Abdallah, 2020). We directed the proposed work to develop a simple and eco-friendly HPLC method for the assay of co-formulated water pills, XIP, and TRI besides the toxic impurity; Benzyl cyanide (BC). BC (phenyl acetonitrile) **(Fig.1c)** is a specified and toxic impurity of TRI produced during synthesis and has a pharmacopeial limit of 50 ppm, as stated by British pharmacopeia (Her Majesty's, 2013). BC is a toxic compound whose toxicity results from its mode of metabolism through conversion to organic cyanide in vivo. In addition, it hinders the respiration of the mitochondria leading to death by reaction with the cytochrome oxidase (Egekeze & Oehme, 1980).

Xipamide and Triamterene were assayed in a binary mixture by several chromatographic(Abd El-Hay, Hashem, & Gouda, 2016; Ayad, Hosny, Ibrahim, El-Abassy, & Belal, 2020; Dadgar & Kelly, 1988; El-Kimary, 2016; El-Sayed & Abd El-Hay, 2016) and spectrophotometric(Elfiky, Victor, Badawey, & Abdelghany, 2021; Nassar, Attia, Mohamad, Said, & Abdel-Monem, 2017; Wagieh, Abbas, Abdelkawy, & Abdelrahman, 2010) methods**.** Our scientific literature research revealed that no approach for the simultaneous assay of the ternary mixture of XIP, TRI, and BC was presented at the time of conducting this research, which encourages the authors to carry out this study. Additionally, the interest of the analytical community is significantly given to applying green chemistry. Thus, several protocols were followed to decrease the use of hazardous chemicals that adversely affect human health and the environment by replacing such risky chemicals with more eco-friendly alternatives without affecting the efficiency of the analytical method. We evaluated the greenness of the proposed method using the analytical Eco-scale by collecting the total penalty points for the various analytical parameters.

2. Material and methods

2.1. Instruments

The HPLC instrument (Shimadzu SPD-20A, Kyoto, Japan) included a quaternary pump(G1310A), UV/VIS detector, column oven (CTO20A), and a degasser unit(DGU-207). A Shimadzu C8 analytical column(250 × 4.6 mm, 5 μm was used for the separation

2.2. Materials and reagents

2.2.1. Standard samples

XIP and TRI authentic samples with certified purity (99.62%) and (100.05 %) respectively were helpfully supplied by EIPICO Pharmaceutical Industries Company (10th Ramadan city, Egypt). BC with purity (98.00 %) and density (1.523) was purchased from Sigma-Aldrich (Steinheim, Germany).

2.2.2. Market samples

Epitens®tablets (Batch #54881) were produced by EIPICO Pharmaceutical Industries Company (10th Ramadan city, Egypt). It was claimed that each tablet contained 30 mg of TRI and 10 mg of XIP.

2.2.3 Chemicals and solvents

HPLC-grade acetonitrile and methanol were obtained from (Merck., Germany). Analytical-grade phosphoric acid, potassium dihydrogen phosphate, and sodium hydroxide were supplied by (El-NASR Pharmaceutical Chemical Co., Abu- Zabaal, Cairo, Egypt). Deionized water was used in the whole research. we used a 0.45 µm membrane filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) to filter all prepared chemicals and mobile phases

2.3. Standard solutions

TRI and XIP stock standard solutions (1000 µg mL^{-1}) were made by dissolving 100 mg of each into 100 mL of methanol, whereas BC stock standard solution (1000 µg mL^{-1}) was prepared by diluting 99 µL of pure BC (99.91 %, density 1.523) with methanol to 100 ml. The standard working solutions (100 µg mL^{-1}) for each drug were made by diluting the stock standard solution using the mobile phase. 6.8 gm KH_2PO_4 were dissolved in 1L of deionized water to prepare 0.05 M KH_2PO_4 then phosphoric acid was used to adjust the pH to 4.5.

2.4. Chromatographic conditions

The mobile phase was made by combining 0.05 M KH2PO4 at a pH of 4.5 and methanol (50: 50 by volume). A Shimadzu C8 analytical column (250 4.6 mm, 5 m) thermostated at 30°C was used to provide the mobile phase at a constant flow rate of 1.2 ml min^{-1}. The samples were filtered with a 0.45 µm syringe filter then 20 µL was injected. At a wavelength of 220 nm, the eluent could be observed.

2.5. Procedures

2.5.1. Construction of calibration curves

Aliquots corresponding to (0.01-1 mg) for TRI and XIP and (0.05-0.5 mg) for BC were transferred individually from their stock and working standard solutions into a distinct series of 10 mL volumetric flasks; then, we completed the volumes to 10 mL with the mobile phase. For each concentration, three injections of 20 µL were conducted. Peak areas vs. relative concentration were plotted to construct calibration graphs for each drug; then, the regression equations were developed.

2.5.1. Analysis of Epitens® tablets

Twenty Epitens® (Batch #54881) tablets were carefully weighed, ground, and blended, and a quantity equivalent to 100 mg of both TRI and XIP was separately transferred into two 100 mL volumetric flasks. Each drug's stock solution (1000 µg mL^{-1}) was prepared by ultrasonication dissolving the powder in 50 mL for 30 minutes, and then the solutions were cooled and filtered. Suitable dilutions using the mobile phase were performed to prepare their working solution (100 µg mL^{-1}). According to the labeled amount of drugs in the Epitens® tablet, a concentration of (60 µg mL^{-1}) of TRI and (20 µg mL^{-1}) of XIP was prepared and were injected six times, and we tracked the procedures under construction of calibration curve.

3. Results and discussion

The main goal of the presented work was to utilize the resolving power of the chromatographic technique to create and optimize a green and simple HPLC methodology for assay commonly prescribed co-formulated water pills XIP and TRI besides the toxic impurity of TRI, BC. The quality control laboratories urgently needed analytical methods to resolve the complex mixtures, especially those containing structurally related and toxic impurities. ICH suggested very constricted criteria for levels of impurities in the final pharmaceutical products (“ICH Harmonized Tripartite Guideline," Validation of Analytical Procedures" ICH Harmonized Tripartite Guideline," Validation of Analytical Procedures,” 2006). Even in minute quantities, these impurities threaten the safety and potency of pharmaceutical products, incredibly toxic.

In this respect, we established a selective, ecofriendly, and sensitive HPLC technique for the assay of the ternary mixture (Fig.1), which was not analyzed previously as cited in the literature. We followed the green chemistry guidelines in the whole steps of the research, and we applied the eco-scale assessment. Optimization and validation were performed for the developed HPLC method. It was successfully applied for the determination of XIP, TRI and BC in Epitens water pills and the results were of satisfactory accuracy and precision

3.1. Method development and optimization

Numerous chromatographic variables that affect the separation of the analyzed components were investigated and optimized, including the types and ratios of the aqueous and organic phases, the pH of the mobile phase, the scanning wavelength, and the temperature of the column, and the flow rate. All development and optimization trials on HPLC C_8 (250 × 4.6 mm, 5 µm) column were carried out. Concerning the aqueous phase, we tried water adjusted to pH 3 using phosphoric acid and methanol with different ratios. This led to the co-elution of TRI and BC peaks and the tailing of the XIP peak. Replacement of water with 50 mM KH_2PO_4 buffer modified to pH = 3 using phosphoric acid and methanol using the proportion (60: 40 by volume) led to enhancement of the resolution and shape of the studied compounds. However, the TRI peak was co-eluted with the solvent front.

To enhance the resolution of the studied compounds, acetonitrile was added in conjunction with methanol and phosphate buffer to the mobile phase with different ratios. It shortens the analysis time but inversely worsens the shapes of the separated peaks. So, we discarded acetonitrile's inclusion, which augmented the greenness of the resultant mobile phase. Eventually, increasing the ratio of the methanol by 10 % by using 50 mM phosphate buffer and methanol in a percentage (50: 50 by volume) resulted in enhancing the resolution and symmetry of the separated compounds. Boosting the methanol proportion by over 50% led to a resolution decrease. Regarding the mobile phase delivery through the column, we investigated different flow rates like 0.8, 1 and 1.2 mL min^{-1}; good ƸSeparation with a minimum run time (8 min) was obtained using a flow rate of 1.2 ml min^{-1}. A pH study was performed by changing the pH of the mobile phase in the range (2 - 7) while the resolution and symmetry of the separated peaks were investigated. Increasing the pH value above five led to poor separation and symmetry of all studied compounds, where at PH 6 and 7, TRI and XIP peaks were co- eluted as one forked peak and BC peak was broadened. Hence, the optimum pH value-enhancing symmetry and resolution was 4.5, as shown in (Fig.2). Different column temperatures ranging from (25 – to 40ºC) were studied, where keeping the column oven thermostated at 30ºC enhances the stability of the chromatographic system and yields repeatable peak areas and retention times. Additionally, increasing the column temperature above 30 ºC deteriorates the resolutions between the separated components (Fig. 2). Scanning the effluent at various wavelengths (220, 230, 240, and 254 nm) was used to study the effect of scanning wavelength on the sensitivity of the proposed method, with scanning at 220 nm resulting in the maximum sensitivity with the minor baseline noise. (Fig. 3) illustrated the representative chromatogram of the investigated mixture utilizing the optimized chromatographic conditions. The calibration graphs for each compound in the ternary mixture were plotted using the peak areas and related concentrations then regression equations were derived. The obtained regression equations were:

$Y = 0.9939 X + 1.389$ (For TRI) $r = 0.9996$

$Y = 0.0213X + 0.4104$ (For XIP) $r = 0.9998$

$Y = 0.100 X + 0.4047$ (For BC) $r = 0.9996$

Y refers to the integrated peak area, X stands for the concentration in µg ml^{-1}, and the correlation coefficient is r.

3.2. Validation of HPLC method

Based on ICH guidelines, the proposed method was tested, and its validity was checked for the intended purpose ("ICH Harmonized Tripartite Guideline," Validation of Analytical Procedures" ICH Harmonized Tripartite Guideline," Validation of Analytical Procedures," 2006). Different parameters were investigated, such as:

3.2.1 linearity

we assessed the method's linearity by computing the peak areas for each component at various concentration levels, then constructing calibration curves and deducing regression equations. Correlation coefficients were calculated, and their values approached unity, as evident in (Table 1).

3.2.2 range

The studied drugs showed a wide linearity range – the span between the high and low levels of the studied drug as shown in (Table 1).

3.2.3 Accuracy

It was assessed as percent recoveries of pure samples assayed at various concentration levels within the linearity range employing the previously derived regression equation. We obtained satisfactory results, as evident in (Table 1).

3.2.4 Specificity

The devised HPLC technique could efficiently separate the investigated ternary mixture within 8 min with retention times of 3.8 min, 6.2 min, and 8 min for TRI, BC, and XIP, respectively, as indicated in (Fig. 3).

3.2.5 Precision

Repeatability and intermediate precision were evaluated to check the precision. Three different concentrations of the pure sample (60, 80, and 100 µg ml^{-1} for TRI), (20, 80, and 100 µg ml^{-1} for XIP), and (30, 40, and 50 µg ml^{-1} for BC) were analyzed thrice in the same day to determine the repeatability. Intermediate precision was estimated by recurring the analysis of the previous concentrations over three days consecutively. The results were satisfactory, with an acceptable standard deviation (SD) as shown in (Table1).

3.2.6 LOD and LOQ.

We estimated LOD and LOQ values concerning ICH recommendations ("ICH Harmonized Tripartite Guideline," Validation of Analytical Procedures" ICH Harmonized Tripartite Guideline," Validation of Analytical Procedures," 2006). Relatively low values assured the high sensitivity of the devised method. The values of LOD and LOQ are listed in (Table 1).

3.2.7 Robustness

The analytical method can withstand the small and willful change in method parameters indicating its reliability (Wells & Dantus, 2004). we assessed method robustness by provoking a minor change in the organic strength (± 1 %), flow rate (±0.05ml min^{-1}), and wavelength (± 1 nm). However, the retention time, peak area, peak symmetry, or selectivity factor values did not show significant changes, which assured that the proposed method was robust (Table 2).

3.2.8 System suitability

Different chromatographic parameters were investigated like symmetry factor (Ta), resolution (Rs), selectivity factor (Ka), column efficiency (N), and others. The obtained results were excellent and compatible with USP reference values, as shown in (Table 3) (Pharmacopeia, 2014).

3.3 Ecological evaluation of the proposed method

The analytical community is always looking for ways to evaluate the environmental impact of new methodologies. These trends significantly protect the environment from hazardous chemicals and provide a healthy ecosystem for animals and humans. The greenness profile of the new approach was evaluated and compared to the HPLC method previously described(Abd El-Hay et al., 2016) utilizing the analytical Eco-Scale, outlined by the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), which is a simple and direct semi-quantitative assessment method (Mohamed & Lamie, 2016). we performed the analytical Eco-Scale evaluation by collecting the total penalty points assigned for different parameters like chemicals consumption, accumulation, degree of their corrosiveness, waste production, and energy consumption. The score was calculated by subtracting the total penalty points from 100. The analytical method was considered green if its eco-scale score equals 100, excellent if more than 75, fair if less than 50, and deficient if <50 (Gałuszka, Migaszewski, Konieczka, & Namieśnik, 2012). The Eco-Scale was evaluated for the devised method and compared to the published one (Table 4). The developed method had high score than the reported one, which implies that it is greener than the published method (Abd El-Hay et al., 2016).

3.4 Comparative analysis to the published method

The proposed method's results were compared statistically to the previously published HPLC method for Epitens® tablets (Abd El-Hay et al., 2016), utilizing a 95 percent confidence level variance ratio F-test and Student's t-test. The suggested method's precision and accuracy are suitable, as evidenced by computed t and F values that are lower than the tabulated ones, and the results are displayed in (Table 5).

4. Conclusion

The suggested HPLC method provides a green, sensitive, and selective analytical tool for analyzing XIP, TRI, and the toxic impurity of TRI; BC in a short analysis time with reasonable accuracy and precision. Moreover, the greenness of the suggested method was evaluated and compared with the reported method. The results showed that the recommended method had a higher Eco-value than the reported one. These findings encourage the analysts to implement the suggested method routinely in quality control laboratories.

Disclosure

Authors have no conflict of interest**.**

Table (1): Regression and validation parameters results of the suggested HPLC method for the assay of XIP, TRI, and BC.

Parameters	TRI	XIP	BC
Range (µg ml^{-1})	1-100	1-100	5-50
Slope	0.9939	0.0213	0.9874
Intercept	1.3895	0.4104	0.4074
Correlation coefficient	0.9996	0.9998	0.9996
Accuracy (%) ± SD	99.70 ±1.2	99.56±1.10	100.48±1.3
Precision			
Repeatability (RSD %)[a]*	0.74	0.18	0.46
Intermediate precision (RSD%)[b]*	1.24	0.64	0.95
LOD[c]* (µg/mL)	0.30	0.45	3.20
LOQ[c]* (µg/mL)	1.00	1.00	5.00

a*. The repeatability (n=3), an average of three various concentrations iterated thrice through a day.
b*. The intermediate precision (n=3), an average of three various concentrations iterated thrice in three consecutive days.
[c]*. Detection and quantitation limits are calculated as : LOD = (SD of the response/slope) × 3.3; LOQ = (SD of the response/slope) × 10.

Table (2): Robustness of the developed HPLC method by inducing minor modification in the chromatographic parameters.

Drug	Parameters		Ta	Ka	Rs	%Assay
TRI	Wavelength	220+1nm	1.65	0.51	6.10	100.20
		220-1nm	1.65	0.53	6.59	99.87
	Methanol composition	50+1%	1.63	0.52	6.01	100.11
		50-1%	1.64	0.51	6.02	99.88
	Flow rate	1.2+0.05ml/min	1.69	0.54	6.54	99.81
		1.2-0.05ml/min	1.63	0.55	6.71	100.32
BC	Wavelength	220+1nm	1.35	1.41	3.52	98.02
		220-1nm	1.37	1.40	3.61	98.03
	Methanol composition	50+1%	1.36	1.43	3.45	99.22
		50-1%	1.34	1.40	3.52	99.34
	Flow rate	1.2+0.05ml/min	1.31	1.45	3.55	98.01
		1.2-0.05ml/min	1.32	1.41	3.48	98.04
XIP	Wavelength	220+1nm	1.20	2.21	--	98.53
		220-1nm	1.21	2.20	--	98.55
	Methanol composition	50+1%	1.23	2.23	--	98.41
		50-1%	1.22	2.25	--	98.43
	Flow rate	1.2+0.05ml/min	1.27	2.26	--	98.35
		1.2-0.05ml/min	1.25	2.24	--	98.34

Table (3): System suitability parameters of the suggested HPLC Method according to USP.

Parameter	TRI	BC	XIP	Reference value
Resolution (Rs)	6.03	3.50		> 1.5
Tailing factor(Ta)	1.65	1.33	1.28	T = 1 for a typical symmetric peak
α (relative retention time)	2.80	1.50		> 1
Ka (column capacity)	0.51	1.40	2.20	1-10
N (column efficiency)	2355	2785	3458	Increases with the efficiency of the separation > 2000
HETP = L (length of column in cm)/N	1.06×10^{-2}	7.2×10^{-3}	8.9×10^{-3}	The smaller the value, the higher the column efficiency

Table (4): Analytical eco-scale assessment for evaluation of the proposed HPLC method greenness by calculating the total Penalty points.

Reagent/instruments	Proposed HPLC method	Reported HPLC method(Abd El-Hay et al., 2016)
Methanol	8	----
Pot dihydrogen phosphate	0	0
Acetonitrile	----	12
phosphoric acid	8	16
HPLC	0	0
Occupational hazard	0	0
Waste	5	5
Total penalty points	21	33
Analytical eco-scale total score	79	67

Table (5): Statistical comparison between the proposed HPLC method and reported HPLC method results on Epitens®.

Parameters		Proposed HPLC method		Reported HPLC methods (Abd El-Hay et al., 2016)	
		TRI	**XIP**	**TRI**	**XIP**
Epitens® tablet	**Mean[a]**	98.23	97.04	99.60	99.12
	SD	0.57	1.5	0.70	1.9
	n	6	6	6	6
	Student t-test	1.53(1.83)[b]	1.62(1.83)*	--	--
	F value	1.9 (5.1)[b]	1.5 (5.05)*	--	--

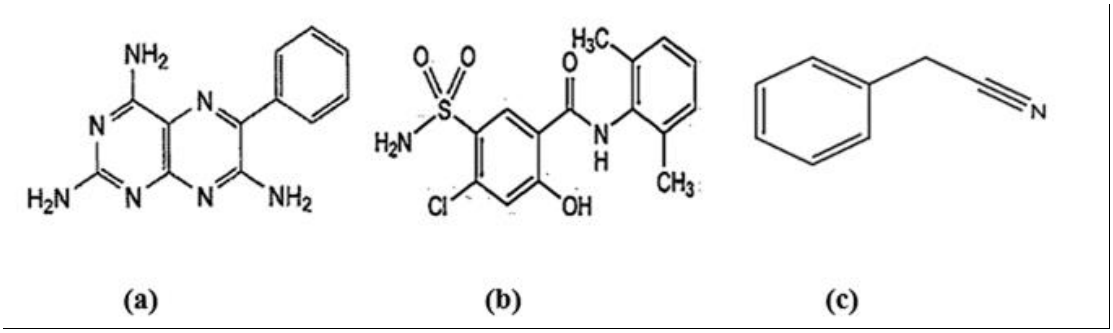

Figure (1): Chemical structure of Triamterene (a), Xipamide (b) and Benzyl cyanide (c).

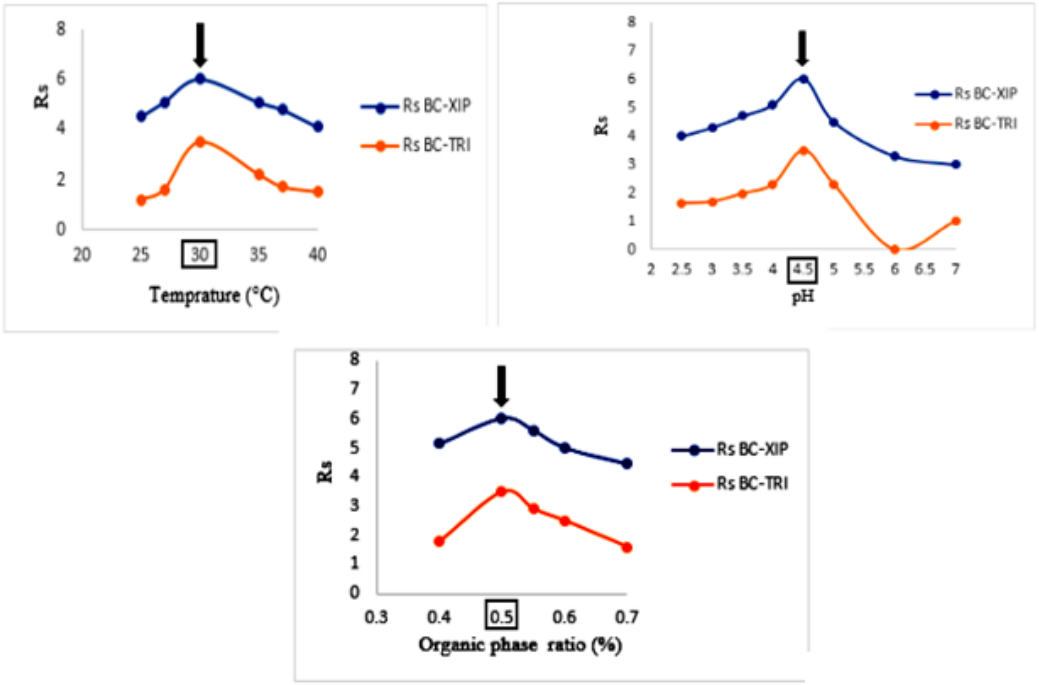

Figure (2): The effect of different optimization parameters (Temperature, pH, ratio of organic phase) on the resolution of separated peaks.

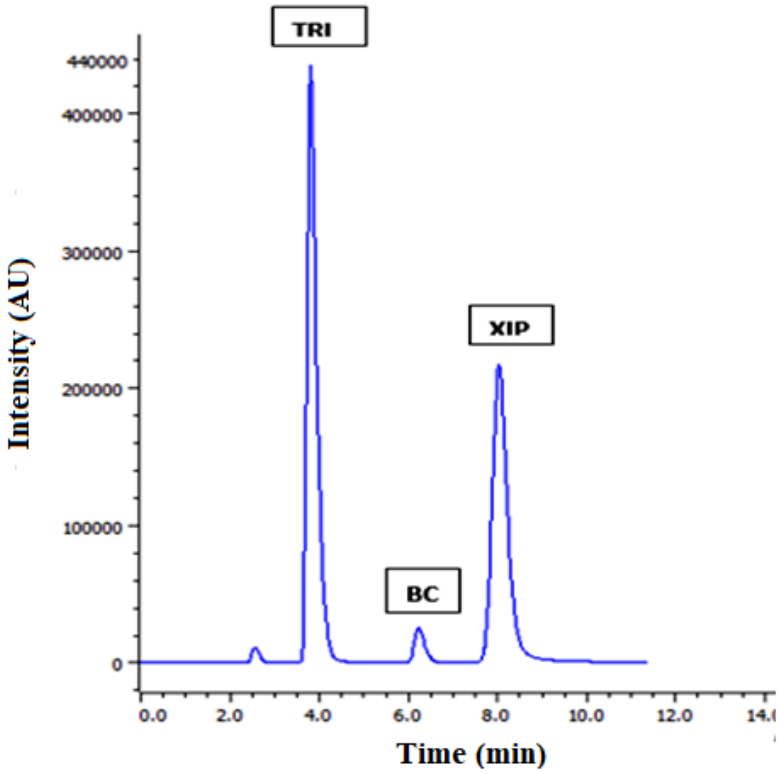

Figure (3): A chromatogram of a 20-µL injection of a mixture consisting of XIP and TRI (100 µg mL^{-1}) and BC (50 µg mL^{-1}) using a 0.05 mol L^{-1} pot. dihydrogen phosphate buffer (pH 4.5) and methanol with the ratio of (50:50) as the mobile phase at wavelength 220 nm using a flow rate of 1.2 ml min^{-1}.

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