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High Perfarmance Liquid Chromatographic Determination of Ascorbic Acid in Pharmaceutical Preparations, Fruit Juices and Human Serum

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ABSTRACT

A high performance liquid chromatographic method for determination of ascorbic acid is described. The analysis is achieved using cosmosil $5C_{18}$-MS-II column (250 mm x 4.6 mm i.d., 5 µm particle size) at room temperature. The mobile phase used was acetonitrile (ACN), dichloromethane (DCM), and 0.25% K_2HPO_4 solution in the ratio (90:5:5)(V:V:V). The flow rate is set to 1.0ml.min^{-1} with UV-detection at 246 nm. Beer's law is obeyed over the concentration range 0.05-30 µg.ml^{-1}. The method is accurate (relative error % is less than 0.12%), precise (RSD better than ±1.25%), successfully applied to the determination of ascorbic acidin its pharmaceutical preparations, fruit juices, and human serum.

Keywords: Ascorbic acid, HPLC, Cosmosil $5C_{18}$, Dichloromethane, Human serum.

تقدير حامض الاسكوربيك في المستحضرات الصيدلانية وعصائر الفاكهة ومصل دم الانسان باستخدام طريقة كروماتوغرافيا السائل عالي الاداء

الملخص

تم تقدير حامض الاسكوربيك باستخدام كروماتوغرافيا السائل عالي الاداء وذلك باستخدام عمود فصل نوع Cosmosil $5C_{18}$-MS-II column (250 mm x 4.6 mm i.d., 5 µm particle size) وبدرجة حرارة الغرفة، حيث كان الطور المتحرك اسيتونتريل: ثنائي كلورومیثان: 0.25% K_2HPO_4 وبنسبة (5:5:90) (V:V:V) وبسرعة جريان 1 مل. دقيقة$^{-1}$. باستخدام مطياف الاشعة فوق البنفسجية UV بوصفه مكشافا عند الطول الموجي 246 نانوميتر. تتبع الطريقة قانون بير لمدى من التراكيز من 0.05 الى 30 ميكروغرم مل$^{-1}$. كانت الطريقة ذات دقة جيدة (الخطأ النسبي افضل من 0.12 %) وذات توافق جيد (RSD أفضل من 1.25%±). تم تطبيق الطريقة المقترحة بنجاح لتقدير حامض الاسكوربيك في المستحضرات الصيدلانية و عصائر الفاكهة الطبيعية وفي مصل الانسان.

الكلمات الدالة: حامض الاسكوربيك، HPLC, Cosmosil $5C_{18}$، اسيتونتريل: ثنائي كلورومیثان، مصل الانسان.

INTRODUCTION

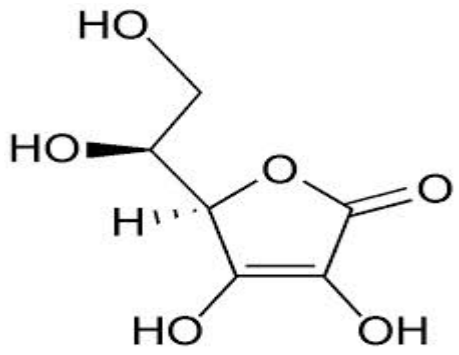
Water soluble and fat soluble vitamins are essential constituents of human bodies (Baiyi *et al*., 2008) because of that vitamins promote and regulate the essential biochemical reactions, and inspite of the fact that they do not provide energy themselves, they assist the convert of fat and carbohydrates into energy and assist in forming bone and tissue (Semih *et al*., 2008). Water soluble vitamins have limited retention in the body and need regular replacement (Azad *et al*., 2005).

As the lack of vitamin creates illness; vitamins in excess levels are also equally harmful to health; this clearly emphasizes the importance of having efficient assay methods to quantify

vitamins in biological samples, if vitamins are not excreted, toxic levels could occur (Ronald, 2008).

Ascorbic acid (Vitamin C) is one of water soluble vitamins, as protecting the body from infection and disease. However, illness or stress can substantially decrease the body's Vitamin C reserves, as also can smoking, drinking and some drugs such as aspirin (Jain, 2005).

Ascorbic acid is (R)-3,4-dihydroxy-5-((S)- 1,2-dihydroxyethyl)furan-2(5H)-one, or 2-Oxo-L-threo-hexono-1,4-lactone-2,3-enediol with the following chemical structure (Moffat, 2005).



M.w=176.13 g.mol^{-1}

A sensitive high performance liquid chromatographic method for determination of ascorbic acid in pharmaceutical preparation using a superspher RP-C_{18} (250 mm x 4.6 mm, 10μm) at 20ºC has been developed. The mobile phase used was acetic acid (1.5g in 500 ml) mixed with 1-hexanesulfonic acid sodium salt (pH 2.6), and the flow rate was 0.7 ml.min^{-1}with UV detection at 280 nm. (Snezana *et al*., 2011).

Vitamin C with some water and fat soluble vitamins has been determined simultaneously after several extraction procedures from tablets using 0.01% trifluoroacetic acid, (TFA) (pH 3.9) and methanol (20:80)(v:v) as a mobile phase. The separation was achieved at 30ºC with retention time less than 40 min on a Phenomenex Gemini C_{18} column (150 x 4.6 mm i.d., 3 μm) at 0.7 ml.min^{-1} flow rate and gradient elution of mobile phase. The detector was diode array at different wavelengths (Semahat *et al.*, 2011).

Ascorbic acid has been determined in fresh fruits and human plasma using RP-HPLC with UV-detection technique. Chromatographic separation was achieved using a pre-packed Kromasil 100 C_{18} (25x0.46 mm, 5 μm) column and acetonitrile: water (60:40)(V:V) as a mobile phase at 0.75 ml.min^{-1} flow rate. The assay was linear over the concentration range of 0.5-100 μg.ml^{-1} with recovery ranging 99-100% inter and intra day (Muhammad *et al.*,2009).

Determination of ascorbic acid in local packed juices of Pakistan by HPLC using gradient pump system was developed, separation was carried out using C_{18} column and detection by UV detector. The retention time was within 1.63-1.65 minutes. Standard curves were linear over the concentration range of 0.1 to 2.5 mg.ml^{-1}. The extraction recovery was within 94 and 101%. (Shafqat *et al.*, 2012) .

Measurement of intracellular vitamin C levels in human lymphocytes by reverse phase high performance liquid chromatography using CSC-kromasil C_{18}, 5 μm, 25 cm × 4.6 mm column and detection by UV- detector at 245 nm was carried out. The mobile phase used was 0.2 M KH_2PO_4/H_3PO_4 (pH 3.0), containing 2 mM EDTA. The flow rate was 1 ml min^{-1}. The assay was linear over the concentration range from 1.25 to 100 μg/10 lymphocyte, LOD was 1.42 μg/10 lymphocyte (Emadi *et al.*, 2005).

A RP-HPLC method for simultaneous estimation of ascorbic acid and calcium pantothenate in a pharmaceutical multivitamin and multimineral unit dosage form has been developed. The

separation was achieved on a reverse phase C_{18} column (5 µm; 250x4.6 mm i.d.) with an isocratic mobile phase elution and a flow rate of 1.0 mL/min. The mobile phase was 0.1 % solution of triethylamine in water (pH 3.00 ± 0.05) (adjusted with ortho-phosphoric acid) and methanol in the ratio of 80:20 (v/v). Detection was performed with UV detector at 210 nm. The responses were linear in concentration range of 20-60 $\mu g.ml^{-1}$ for ascorbic acid and 2.5-7.5 $\mu g.ml^{-1}$ for calcium pantothenate. The RSD% ranged between 0.8 to 2.9 for ascorbic acid and between 2.8 to 3.3 for calcium pantothenate, and recovery % of the vitamins ranged between 98.0 to 99.7 for ascorbic acid and 98.1 to 98.7 for calcium pantothenate (Ramniwas *et al.*, 2011)

Determination of ascorbic acid in some vegetables was performed by derivative spectrophotometry using peak to peak amplitudes in second and third order derivative spectra of the extracts (Zeynep and Mamure, 2002).

A spectrophotometric method for the determination of vitamin C in various fruits and vegetable at Sylhet area has been reported, the method involves an oxidation of ascorbic acid by bromine water in presence of acetic acid and coupling with 2,4-dinitrophenyl hydrazine at 37˚C for three hours, the solution was treated with 85% H_2SO_4 to form acolor complex measured at 280 nm (Rahman *et al*., 2006).

The same principle was used for the determination of ascorbic acid in some fruits and vegetables at Koya-Area-kurdistan Region in Iraq (Qasim *et al.*, 2006).

A direct spectrophotometric determination of L-ascorbic acid in pharmaceutical preparations was developed using sodium oxalate (0.0056 mol/dm^3) with a molar absorptivity of $1.42x10^4$ $dm^3.mol^{-1}.cm^{-1}$ at 266 nm and a relative standard deviation better than ±0.81% (Amra *et al*., 2011).

The aim of this article is determination of ascorbic acid in different samples using HPLC method coupled with UV-detection.

EXPERIMENTAL

Apparatus

A (Shimadzue, LC-2010A, Japan) HPLC instrument was used with a pump (model LC-2010, high flow rate mode) and auto injector sampler. The detector was UV LC-2010 with D_2 lamp (800 mv minimum energy). Separation was achieved using a cosmosil $5C_{18}$-MS-II column (250 mm x 4.6 mm i.d., 5 µm particle size, Japan).

A Shimadzue UV-1650 PC double beam spectrophotometer with 1cm quartz cells have been used for scanning spectra.

pH measurement has been done by pH-meter (HANNA pH211, microprocessor pH meter, Mauritius), balance (Sartorins BL 210S, Germany) has been used for weight measurements.

MATERIALS

Working standards of ascorbic acid was provided from the biochemicals (BDH). Water was distilled and double filtered.

All chemicals and reagents are of analytical grade.

A 0.25% K_2HPO_4 solution was prepared by dissolving 0.25g of K_2HPO_4 (BUH) in 100 ml doubled filtred water.

Acetonitrile, methanol, ethanol, and dichloromethane (from Scharlan Company) are of HPLC grade.

Recommended Procedure and Calibration Graph

A 20 µl of ascorbic acid solution in concentration range between (2.5 to 1500 $\mu g.ml^{-1}$) was injected under the optimum condition of analysis (acetonitrile : dichloromethane: 0.25% K_2HPO_4) (90:5:5)(V:V:V), the sample was isocratically eluted using 1.0 $ml.min^{-1}$ as a flow rate of mobile phase and separation was achaived using cosmosil $5C_{18}$-MS-II column (4.6 mm I.D. x 250 mm, 5 µm particle size) with UV detection at 246 nm. Fig. (1) shows the calibration graph of ascorbic acid

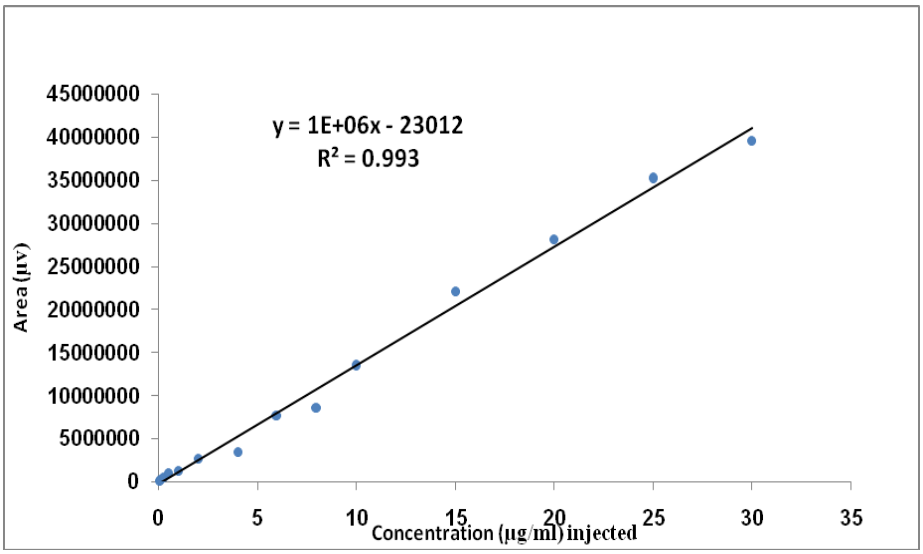


Fig. 1: Calibration graph of ascorbic acid

From Fig. (1), linear calibration graph is obtained between the area under the curve and the concentration over the range 0.05 -30 µg.ml^{-1} of ascorbic acid was obtained.
Table (1) shows the linear regression data of the calibration graph.

Table 1: Linear regression data of the calibration graph

Parameter	Data
Linearity range (µg.ml.$^{-1}$)	0.05 – 30
Slope	1x10^{6}
Intercept	-23012
Determination coefficient (R^2)	0.993
Correlation coefficient (r)	0.996

Optimization of condition

Selection of analytical wavelength

Absorption spectrum of 100 µg.ml^{-1} of ascorbic acid against blank solution (dissolution solvent) has been taken. Fig. (2) shows that ascorbic acid exhibit a maximum absorption intensity at 246 ±2 nm according to its dissolution solvent (Table 2).

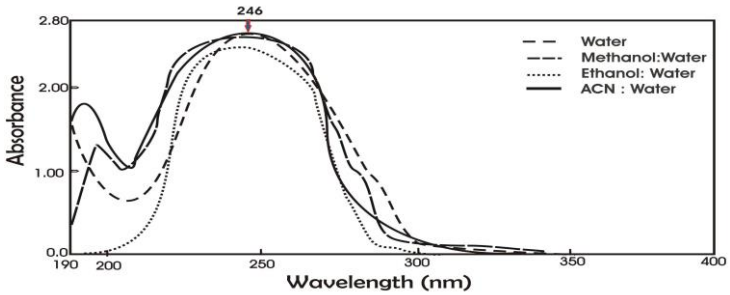


Fig. 2: Absorption spectrum of ascorbic acid in different dissolution solvents

Table 2 : Selection of dissolution solvent

No.	Solvent	λmax, nm	Abs.	Note
1	Water	246	2.61	Soluble
2	Methanol	-	-	Slightly soluble
3	Ethanol	-	-	Slightly soluble
4	ACN	-	-	Slightly soluble
5	Methanol: Water (1:1)	248	2.61	Soluble
6	Ethanol: Water (1:1)	244	1.72	Soluble
7	**ACN: Water (1:1)**	**246**	**2.67**	**Soluble**

Form Table (2) ACN:Water (1:1)(V:V) was selected as a dissolution solvent for ascorbic acid in all subsequent experiments.

Selection of the type and composition of mobile phase

Different solvents with different compositions have been used as mobile phases and were isocratically eluted with 1.0 ml.min^{-1} flow rate, chromatograms were followed at room temperature, and detected at 246 nm wavelength. Table (3) shows the results.

Table 3: Selection the type and composition of mobile phase

No.	Mobile phase	%	Retention time (min)	k`	Symmetry	Notes
1	Methanol	100%	2.80	0.1	0.55	Two peaks
2	Ethanol	100%				No peak High pressurs
3	ACN	100%				No peak
4	ACN	100%	2.73	0.25	0.77	Sharp peak
5	ACN:Water	90:10	2.58	2.55	0.37	Sharp peak
6		80:20	2.69	0.03	0.44	Sharp peak
7		70:30	2.70	0.09	0.45	Sharp peak
8		60:40	2.61	0.31	0.53	Sharp peak
9	ACN: Methanol	90:10	2.59	1.66	4.16	Two peaks
10		80:20	2.84	1.77	1.08	Two peaks
11		70:30	2.72	0.04	0.40	Two peaks
12		60:40	2.79	0.06	0.86	Two peaks
13	**ACN:DCM**	**90:10**	**2.45**	**1.71**	**1.12**	Sharp peak
14		80:20	2.71	0.04	0.25	Sharp peak
15		70:30	2.719	0.09	0.19	Sharp peak
16		60:40	2.61	0.29	0.49	Sharp peak

Table (3) shows that ACN: DCM (90:10) is the best mobile phase in which it give a symmetric chromatogram (0.9<symmetry<1.5) and an ideal capacity factor (k`) (accepted range 0.5<k`<20) (Danial *et al.,* 2010), ideal range (1<k`<5) (skoog *et al.,* 2004) therefore it is selected. Fig. (3) shows the chromatogram of the selected condition.

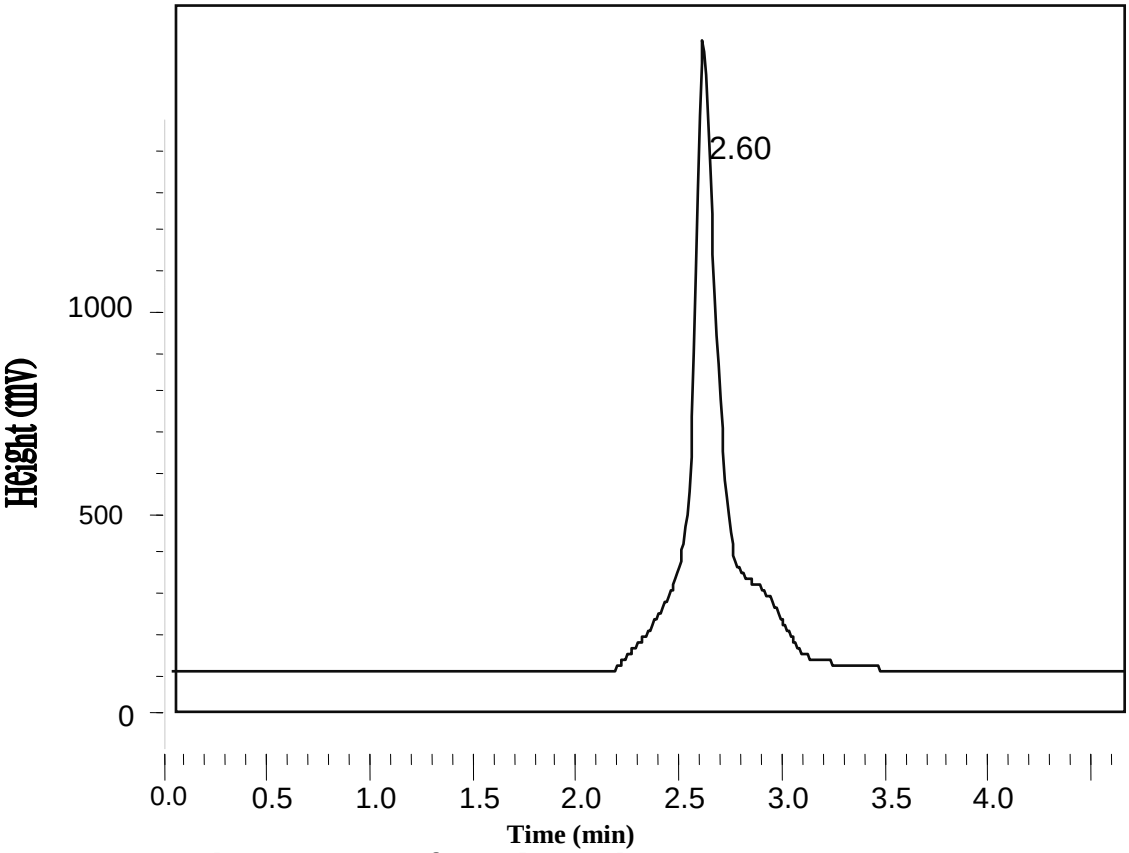


Fig. 3: Chromatogram of ascorbic acid using (ACN:DCM) (90:10) as a mobile phase

Selection of analysis media

Different weak acidic and basic medium have been used to enhance the symmetry and to get a specific chromatogram for ascorbic acid. Table (4) shows the effect of 1% of (KH_2 phthalate, KH_2 phosphate, ammonium acetate, sodium acetate, sodium bicarbonate, and K_2Hphosphate) on the symmetry and capacity factor of chromatogram and as a result on the retention time and the solution pH.

Table 4 : Selection of Analysis Media

No.	Mobile phase	%	pH	Retention time (min)	k`	Symmetry	Notes
1	ACN:DCM: KH_2 phthalate	90: 8: 2	4.1	2.71	1.7	3.81	Two peaks
2		90: 5: 5		2.69	7.5	0.38	Sharp peak
3		90: 2: 8		2.56	9.98		Two peaks
4	ACN:DCM: KH_2PO_4	90: 8: 2	5.1	2.51	1.28	0.65	Sharp peak
5		90: 5: 5		2.65	6.6	0.19	Tailing
6		90: 2: 8		2.66	11.8	0.36	Tailing
7		90: 0: 10		2.74	5.48	1.32	Two peaks
8	ACN:DCM: Ammonium acetate	90: 8: 2	6.7	2.47	29.0	0.88	Sharp peak
9		90: 5: 5		2.55	1.28	0.81	Sharp peak
10		90: 2: 8		2.61	6.80	1.32	Sharp peak
11		90: 0: 10		2.67	1.55	1.58	Tailing
12	ACN:DCM: sod. Acetate	90: 8: 2	7.8	2.48	0.35	1.14	Sharp peak
13		90: 5: 5		2.49	0.36	1.18	Sharp peak
14		90: 2: 8		2.57	0.12	0.59	Sharp peak
15	**ACN:DCM: K_2HPO_4**	90: 8: 2	**8.6**	2.54	0.13	0.22	Sharp peak
16		**90: 5: 5**		**2.53**	**1.27**	**1.28**	Sharp peak
17		90: 2: 8		2.61	1.90	0.21	Sharp peak
18		90: 0: 10		2.65	1.49	0.11	Sharp peak
19	ACN:DCM: Sod. Bicarbonate	90: 8: 2	10.1	2.72	0.74	3.7	Two peaks
20		90: 5: 5		2.64	1.93	0.32	Sharp peak
21		90: 2: 8		2.60	1.46	0.38	Tailing

From Table (4), ACN: DCM: K_2HPO_4 (90:5:5) give the best chromatogram. Fig. (4) shows the chromatogram of the selected condition.

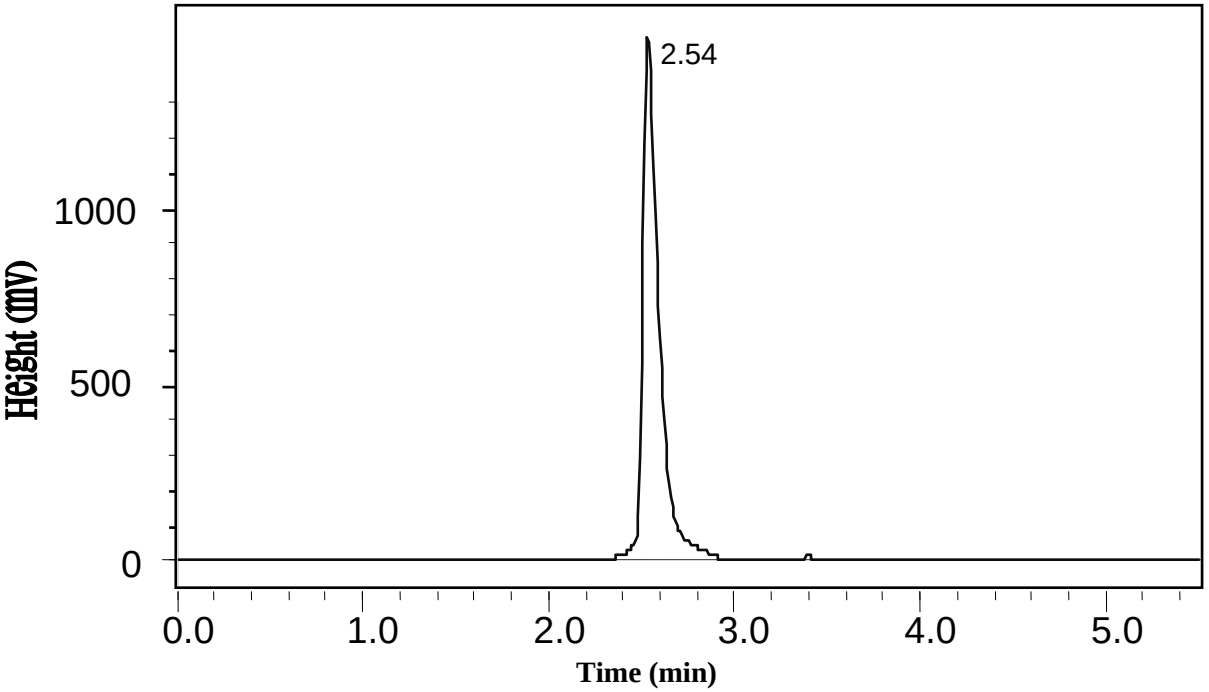


Fig. 4: Chromatogram of ascorbic acid using ACN: DCM: 1%K_2HPO_4 (90:5:5) as a selected medium for the analysis

Effect of K_2HPO_4 concentration

Different percentages 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, and 1.75 % of K_2HPO_4 solution have been prepared and mixed with the mobile phase in the order and ratio as ACN:DCM:K_2HPO_4 (90:5:5)(V:V:V), the results are listed in Table (5).

Table 5: Effect of K_2HPO_4 amount

No.	K_2HPO_4%	pH	Retention time (min)	k`	Symmetry	Notes
1	0.05	8.0	2.66	1.29	2.36	Two peaks
2	0.1	8.2	2.55	6.12	2.50	Tailing
3	**0.25**	**8.4**	**2.54**	**1.70**	**1.14**	Sharp peak
4	0.5	8.7	2.82	3.76	0.79	Sharp peak
5	1.0	8.8	2.64	1.49	0.39	Tailing
6	1.5	9.0	2.72	1.25	0.29	Sharp peak
7	1.75	9.2	2.85	12.4	2.18	Two peaks

Table (5) indicates that 0.25% of K_2HPO_4 as an analysis medium gives the best symmetry with the best capacity factor and it is followed in all subsequent experiments. Fig. (5) shows the chromatogram of the selected condition.

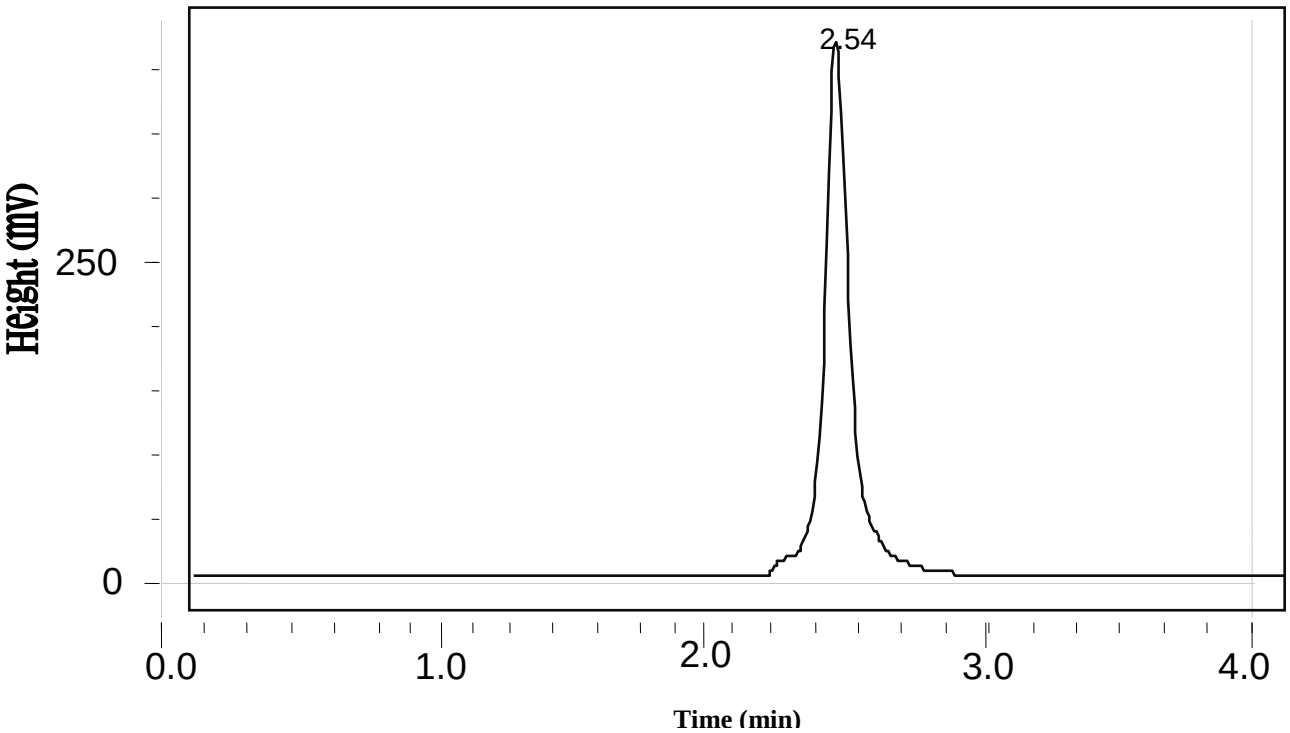


Fig. 5 : Chromatogram of ascorbic acid in the presence of 0.25% K_2HPO_4 and ACN: DCM in (90:5:5)(V:V:V) ratio

Effect of flow rate

Between 0.25 to 2 ml.min.$^{-1}$ flow rate has been followed. Table (6) shows that 1.0 ml.min.$^{-1}$ give a good result in spite of that higher flow rate decrease the analysis time but it also increase the pressure, 1.0 ml.min.$^{-1}$ was used in all above and subsequent experiments.

Table 6 : Effect of flow rate

No.	Flow rate (ml.min^{-1})	Retention time (min)	k`	Symmetry	Pressure (MPa)	Notes
1	0.25	8.74	6.21	2.14	4.5	Two peaks
2	0.5	4.33	6.34	1.80	4.8	Sharp peak
3	0.75	2.85	4.30	2.18	5.0	Sharp peak
4	**1.0**	**2.53**	**1.53**	**1.30**	**7.0**	**Sharp peak**
5	1.25	1.76	1.33	2.0	13.5	Sharp peak
6	1.5	1.47	2.49	1.37	15.0	Sharp peak
7	1.75	1.26	0.47	0.67	16.3	Sharp peak
8	2.0	1.11	0.53	0.61	17.0	Sharp peak

Effect of temperature

Effect of different temperatures 12, 25, 30, and 35 ºC on the retention time, capacity factor and symmetry has been followed. Table (7) shows the results.

Table 7: Effect of temperature

No.	Temperature(C°)	Retention time (min)	k`	Symmetry
1	12	2.58	0.83	3.00
2	**25**	**2.58**	**1.90**	**1.60**
3	30	2.58	0.99	1.68
4	35	2.57	0.99	2.44

From Table (7) the capacity factor and symmetry of chromatogram at room temperature was within the ideal range, therefore, analysis at room temperature has been selected.

The optimum chromatographic conditions

The dissolution solvent was ACN: Water **(**1:1), the mobile phase was (90:5:5) (CAN: DCM:0.25% K_2HPO_4). The flow rate was set to 1.0 ml.min.$^{-1}$ and the detection was carried out at 246 nm at room temperature.

Accuracy and precision

Table (8) shows the accuracy and precision of three different concentrations of ascorbic acid (2, 10, and 15 µg.ml^{-1}) in the form of recovery %, relative error RE %, and relative standard deviation RSD %.

Table 8: Accuracy and precision of the calibration graph

Ascorbic acid(µg.ml^{-1})	Recovery, %*	Relative error,% *	RSD,% *
2	99.94	- 0.06	±1.33
10	99.85	- 0.15	±1.17
15	99.86	- 0.14	±1.27
Average	**99.88**	**- 0.12**	**±1.25**

*****Average of five determinations

Table (8) shows that the proposed method provides a good accuracy (average R.E.% is 0.12) and a good precision (average of RSD% is better than ±1.25).

Effect of ingredients

The recovery % of 10 µg.ml^{-1} ascorbic acid in the presence of different amounts (10, 20. and 30 µg.ml^{-1}) of eight expected ingredients has been followed under the optimum analysis conditions. Table (9) shows the results.

Table 9 : Effect of ingredients

Ingredients	Recovery % of 10µg ascorbic acid / µg ingredient		
	10	**20**	**30**
Paracetamol	318	-	-
Lactose	116.5	128	140
Sucrose	112	140	177
Starch	118	126	133
$CaSO_4$	104	112	130
Glucose	103	107	112

Citric acid	99.7	103	110
Tartaric acid	98.7	105	106

Table (9) shows that paracetamol, and lactose, are seriously and positivly interfered in the analysis of ascorbic acid, sucrose and starch are seriously and positively interfered, while (calcium sulphate and glucose), citric acid and tartaric acid do not interfere to about same, doublicate, and triplicate amount of ascorbic acid, respectively.

Limit of detection and limit of quantification

Table (10) shows that the limit of detection of the method is 0.071 µg.ml^{-1} and the limit of quantification is 0.236 µg.ml^{-1}.

Table 10: Limit of detection and limitof quantification

No.	Area	$(xi - \bar{x})$	$(xi - \bar{x})^2$
1	154947	2137	4.57E+06
2	136969	-15841	2.51E+08
3	138153	-14657	2.15E+08
4	127593	-25217	6.36E+08
5	182122	29312	8.59E+08
6	131779	-21031	4.42E+08
7	144039	-8771	7.69E+07
8	163509	10699	1.14E+08
9	196176	43366	1.88E+09
	Mean ($\bar{x}$) = 152810		$\Sigma(xi - \bar{x})^2$ = 4.48E+09
	S = 23664.32		
	LOD*= 0.071 µg.ml^{-1}		
	LOQ= 0.236 µg.ml^{-1}**		

* Limit of detection = 3 S / Slope

** Limit of quantification = 10 S/ slope

Application of the method

Pharmaceutical preparations

Using the proposed chromatographic method, assay of ascorbic acid in its pharmaceutical preparations, (1000 mg ascorbic acid/ tablet Quvit C,UK), and (500 mg ascorbic acid / tablet, AL-Shahba, Syria) has been followed by dissolving a weight of tablets powder equivalent to 0.025 g ascorbic acid in 50ml of ACN:Water (1:1)(V:V), further preparations were followed to produce diluted solution.

A 20 µl of sample solution was injected under the optimum analysis conditions. The results are shown in Table (11).

Table 11: Application to pharmaceutical preparation

Pharmaceutical preparations	Ascorbic acid ($\mu g.ml^{-1}$)	Area (µV)		Retention time(min)		Recovery, %
		Tablet	Standard	Tablet	Standard	
QuVit C QUEST – UK (tablet)	10	13095911	13405133	2.63	2.52	97.6
	15	21210009	22311644	2.63	2.50	95
	20	30744716	29124982	2.60	2.50	105.5
	25	35745445	35160565	2.58	2.52	101.6
Cetavit ALSHAHBA LABS. SYRIA (tablet)	5	4985804	5142565	2.67	2.54	97.0
	10	13122076	13405133	2.63	2.52	97.8
	15	21303224	22311644	2.65	2.50	95.4
	20	27726552	29124982	2.66	2.50	95.1

Fruit Juices

Ascorbic acid from fresh fruits sources was estimated using the present method. 50 g of each fruit (Lemon, Orange, Alink) was squeezed by juice extractor, centrifuged at 3000 $cyc.min^{-1}$for 10 min. and filtered by syringe filter (0.45 µm).

A 20 µl of each juice were injected to HPLC column under the optimum analysis condition Table (12) shows the results of Iraqi and Turkish fruits.

Table 12: Application to fruits juice

Fruit		Retention time(min)	Area (µV)	Amount (found)	
				mg/50g	mg/1g
Iraqi	Lemon	2.60	33805315	24	0.48
	Orange	2.57	30568236	23	0.46
	Alink	2.58	11529423	9	0.18
Turkish	Lemon	2.60	24121292	18.5	0.37
	Orange	2.58	23830513	18	0.36

From Table (12) lemon content of ascorbic acid of Iraqi fruits is the higher content, while Iraqi alank content is the lowest. Fig. (12) shows the chromatogram of Iraqi lemon, orange and alink.

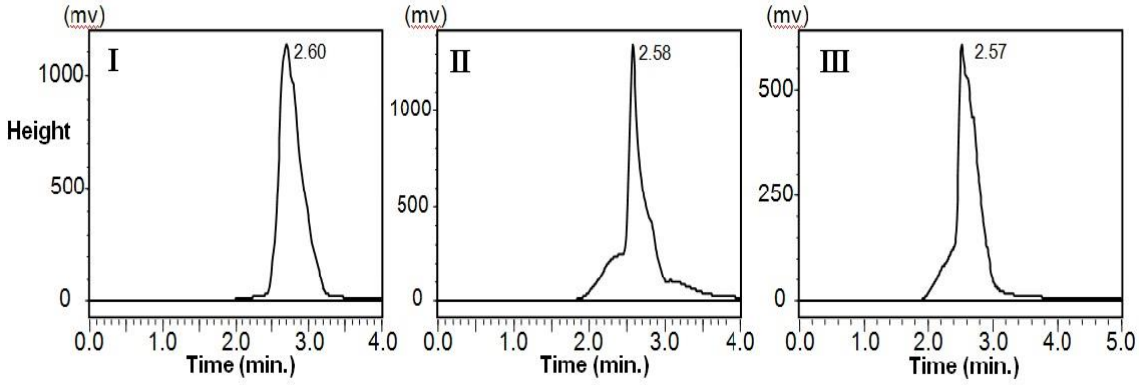


Fig. 5 : Chromatogram of fruit juices: (I) lemon, (II) orange, (III)alink.

Human serum

Sample preparation:

A 5 ml of blood of healthy volunteers has been collected in plain tubes, placed at 37ºC water bath for 20 min, and centerfugate for another 10 min at 4000 $cyc.min^{-1}$. Protein was precipitate using 10% trichloroacetic acid in the ratio of 1:1 and separated at 4000 $cyc.min^{-1}$ for 10 min (Stanley *et al*., 1979).

A 20 µl of the filtrate has been injected to HPLC column. The area under the chromatogram has been compared with the area of calibration graph. The results are listed in Table (13).

Table 13: Application to human serum

No. of case	Sex	Retention time(min)	Area (µv)	Amount		
				Found ($\mu g.ml^{-1}$)	Found x dilution factor ($\mu g.ml^{-1}$)	Found ($mg.dl^{-1}$)
1	Male	2.52	6471547	6.50	13	1.3
2	Male	2.49	7707642	7.70	15.4	1.54
3	Male	2.49	5525439	5.54	11.1	1.11
4	Male	2.50	6811515	6.80	13.6	1.36
5	Female	2.50	9765198	9.78	19.5	1.95

From Table (13) the serum content of ascorbic acid of taken samples were between 6.5 to 9.78 $\mu g.ml^{-1}$ within the normal range, normal range is (0.6 – 2.0) $mg.dl^{-1}$(Mayo, 2014).

Fig. (6) shows the chromatogram of human serum as it is and after two dilution with ascorbic acid (1:1) in first case and (1:2) in second case.

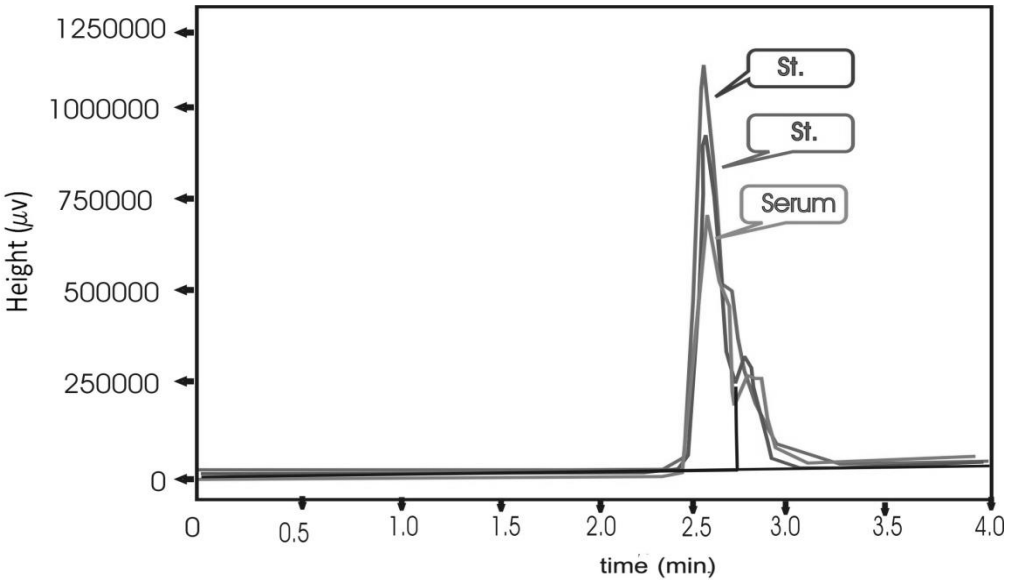


Fig. 6: Chromatogram of human serum

In Fig. (6) the retention time of all samples were 2.52, there is no shift in chromategram, and there is another peak at 2.77 which appear in all chromatograms of serum.

Comparison of the method

Comparison of the present method with other HPLC methods used for the determination of ascorbic acid, has been listed in Table (14), which indicate a wide range of applications of the present method.

Table 14: Comparison of the method

Parameter	Present method	Litruture method (Snezana *et al.*, 2011)	Litruture method (Shafqat *et al.*, 2012)
Column	Cosmosil $5C_{18}$-MS-II (250 mm x 4.6 mm i.d., 5µm particle size)	Superspher RP-C_{18} (250 mm x 4.6 mm i.d., 10µm)	Intersil ODS-3 C_{18} GL Sciences Inc., (250 mm x 4.6 mm i.d., 5 µm)

Mobile phase	Acetonitrile, dichloromethane, and 0.25% K_2HPO_4 solution (90:5:5) (V:V:V).	Acetic acid (1.5g in 500 ml) mixed with 1-hexanesulfonic acid sodium salt (pH 2.6)	Methanol with buffer (KH_2PO_4) at pH 3.0 (20:80) (V:V)
Ttemperature	Room temperature	20ºC	Room temperature
Detection	UV-detection at 246 nm	UV- detection at 280 nm	UV- visible detection at 240 nm
Flow rate (ml.min^{-1})	1.0	0.7	1.0
Linearity (µg.ml^{-1})	0.05-30	0.15 – 0.25	0.1 to 2.5
LOD (µgml^{-1})	0.071	1.95	0.05
LOQ (µgml^{-1})	0.236	6.5	0.1
RSD %	1.25	-	-
Application	Pharmaceutical Preparations, fruit Juices and human serum	Pharmaceutical Preparations	Packed juices

CONCLUSION

A simple, precise, and accurate HPLC method for determination of ascorbic acid in different samples (pharmaceutical preparations, fruit juices and human serum) using the proposed procedure without adjustment of pH and temperature control has been established.

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