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RP-LC METHOD DEVELOPED FOR THE DETERMINATION OF ASCORBIC ACID IN VITAMIN C SYRUP PREPARATION

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ABSTRACT: Ascorbic acid is one of the essential and most vital medicines needed to maintain healthy living. Due to its non-availability through endogenous biosynthesis in humans, it is arguably one of the most consumed Over-The-Counter drugs. Thus, a simple, precise, low-cost and accurate reversed-phase liquid chromatographic (RP-LC) method was developed and optimized to analyse for the percentage content of ascorbic acid in different brands of Vitamin C syrups. The chromatographic separation carried out on an ODS (C18) Ultra sphere column, 5µm (25cm x 2mm), was achieved with HPLC water and methanol as the mobile phase, pumped at a flow rate of 1ml/min and using an ultraviolet detector for detection. The calibration curve was linear over the range of 10-100µg/ml and the method was found to be specific as no interference peaks of impurities and excipients were observed. The method developed was used to assess the quality of fifty-five samples of different types of Vitamin C syrup. Among the samples assessed for their percentage content of ascorbic acid, 18.2% fell below B.P official standard, 7.3% fell within range and 74.6% of the total samples analyzed were above the specified range. We have found this method to be simple, rapid and effective for analysis of ascorbic acid in Vitamin C syrups.

INTRODUCTION: Vitamin C [Ascorbic acid (AA)] remains a popular essential drug in the pharmaceutical world. Primates and several other mammals including human are not able to synthesize ascorbic acid, [1] and food substances, fruits and vegetables are the only natural way through which ascorbic acid is introduced into the human body. 45% of children worldwide are under-nourished.

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However, it has been suggested that daily uptake of Vitamin C has to be within units of grams of AA to reduce the incidence of colds and other diseases. Although the Recommended Dietary Allowances (RDA) for Vitamin C depends on age and gender with other factors such as pregnancy and illnesses, nevertheless, the RDA is 75 and 90mg/day for adult normal women and men, respectively [2, 3]. But the content of ascorbic acid in food can be affected by many factors such as processing, storage and climatic conditions.

Hence, the need to supplement daily intake with exogenous sources such as pharmaceutically prepared Vitamin C formulations. Vitamin C is required for the proper development and function

of many parts of the body. It also plays an important role in maintaining proper immune function and help with increasing the absorption of iron from food to correct protein imbalance in certain newborns (tyrosinmia). Vitamin C can also help to lower the risk of getting cancer. As a result, Vitamin C preparations is one of the frequently consumed pharmaceuticals which necessitates that such product ought to be of good quality and the percentage content should be according to official specification.

Various types of analytical methods have been reported in journals for the determination of AA in many dosage forms.[4-10] The most common and simple method is a certain redox titrimetric reaction method based on the reduction of the blue dye reagent 2,6-dichlorophenolindophenol by Vitamin C in which the endpoint of the reaction is usually indicated as a pink colour.[11-13] However, this method is not without limitation.[14] Some High Performance Liquid Chromatographic (HPLC) methods were widely utilized in the determination of AA but some of them are time consuming, poorly reproducible and somehow cumbersome.[15-19]

Thus, a simple and efficient HPLC method have been developed and validated in our laboratory and applied for the assessment of the quality of Vitamin C syrups sold in pharmacies within the Abuja metropolis, by determining the percentage contents of the ascorbic acid.

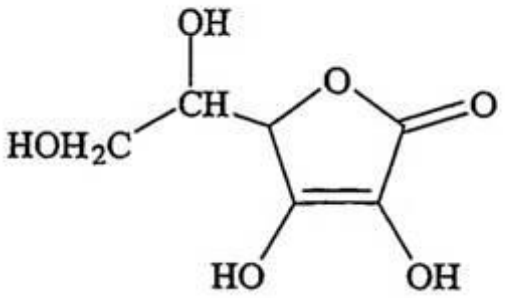

FIG.1: CHEMICAL STRUCTURE OF ASCORBIC ACID

MATERIALS AND METHODS:

Chemicals and Reagents:

L-Ascorbic Acid was obtained from Sigma Chemical Company. HPLC grade Methanol from Sigma Aldrich. Vitamin C syrup formulations were obtained commercially from various pharmacies in Abuja. All other reagents were of analytical grade.

Instrument and chromatographic system:

A High Performance Liquid Chromatographic (HPLC) system (Agilent 1100series) with a G1322A Degasser, G1311A Quart pump and a G1314A VWD detector was used for analysis. Injection was done manually by a Rheodyne model 7725 valve (Cotati, California, U.S.A) fitted with a 20ul loop. The data was recorded using Agilent ChemStation software. Separation was carried out at 25^{o}C (oven set temperature) with an ODS (C_{18}) Ultrasphere 5µm, (2 x25) cm. Analysis was performed by isocratic elution with a flow rate of 1.0ml/min.

Sampling of Vitamin C Syrups:

55 Vitamin C syrup samples were purchased from the pharmacies and patent medicine stores from the metropolis and other satellite towns within Abuja namely: Wuse, Garki, Asokoro, Maitama, Kubwa, Karmo, Idu and Gwagwalada. These outlets were divided into groups such as Category A (pharmacies with air-conditioning system, fan, stable electricity supply and conducive environments), Category B (pharmacies with air-conditioning system or fans with relatively stable electricity supply and fair dispensing area), Category C (No stable electricity supply, with air-conditioning system or fan with poor dispensing area).

Preparation of Standard Solutions:

25mg Ascorbic Acid (AA) reference standard was carefully weighed and dissolved in 10ml HPLC water in a 25ml volumetric flask to prepare standard stock solution of 1mg/ml concentration. From the standard stock solution, concentration ranges of 10µg/ml to 100µg/ml were prepared for the calibration curve.

Preparation of Sample Solutions:

All the samples of Vitamin C syrup obtained had dosage strength of 100mg/5ml of AA. For each sample, 1ml of the syrup was taken after shaking the content of the bottle and dissolved in 25ml of HPLC water using a

standard volumetric flask and was diluted to a final concentration of 16µg/ml. 20µl of each sample was injected.

All samples including the standard were protected from light.

Method Development and Validation:
Validation of the method was performed according to the requirements of International Conference on Harmonization (ICH) guideline.[20] Validation of the HPLC method was carried out with the following parameters.

Linearity:
1mg/ml of ascorbic acid reference standard was used as stock solution for the preparation of subsequent aliquots of 10, 20, 40, 60, 80 and 100µg/ml by serial dilution. 20µl of each aliquot was injected into the LC system. All determinations were carried out in triplicates for each concentration. The calibration curve of peak area (*vs*) concentration was plotted and correlation coefficient with regression line equation was determined. The results for the linearity study are given below.

Accuracy:
The accuracy of the method is the closeness of the measured value to the true value for the sample. Accuracy was assessed as the percent relative error and mean % recovery. The solutions of 10, 40 & 100 µg/ml standard ascorbic acid were prepared accurately. Individual concentrations injected in triplicates and the accuracy of the method was checked by determining recovery values. Accuracy was calculated for three runs of each solution.

Precision:
The precision was determined by measuring three samples probes under the same experimental conditions. To calculate precision, within-day and day to day tests were performed and the results were expressed as relative standard deviation (RSD %). Limits of detection (LOD) and Limits of Quantification (LOQ). LOQ was defined as the lowest concentration that could be detected with acceptable accuracy and precision. The limits of detection and quantification were determined by serial dilutions of known concentrations of ascorbic acid solutions in order to obtain signal/noise ratio of ≈ 3:1 for LOD and ≈ 10:1 for LOQ. Reproducibility was estimated by assaying six replicate solutions on day 1 and day 2.

RESULTS:
Method Validation:
Both within-day and day to day runs for the AA standard is shown below in **Tables 1** and **2**. The coefficients of variance for the AA concentrations of 10 20, 40, 60, 80 and 100µg/ml, were 3.58, 1.26, 0.67, 1.53, 3.78, 2.48% for within runs and 2.30, 1.98, 2.88, 5.61, 1.74 and 1.59% for day to day runs respectively.

TABLE 1: WITHIN–DAY RUNS

Concentration (µg/ml)	Ave. Peak Area (X)	SD (±)	SD/ X	C.V (%)
10	1170.376	41.865	0.0358	3.58
20	2449.635	31.71	0.0113	1.26
40	4852.931	32.722	0.0067	0.67
60	6803.732	104.36	0.0153	1.53
80	9354.932	354.31	0.0378	3.78
100	11882.96	294.79	0.0248	2.48

TABLE 2: DAY TO DAY RUNS

Concentration (µg/ml)	Ave. Peak Area (X)	SD (±)	SD/ X	C.V (%)
10	1089.649	25.062	0.023	2.3
20	2347.078	46.537	0.0198	1.98
40	4728.394	136.15	0.0288	2.88
60	7191.554	403.21	0.0561	5.61
80	9148.200	158.91	0.0174	1.74
100	11304.120	179.58	0.0159	1.59

Standard Deviation – SD
Coefficient of Variation – C.V

Calibration Curve for AA Standard:
There was a linear relationship between the peak areas of AA and concentration of AA over the range of 10µg/ml to 100µg/ml as shown below in **Fig. 2**. The regression equation obtained for the calibration curves was Y= 107.35x + 275.62 with a correlation coefficient R^2 of 0.9987.

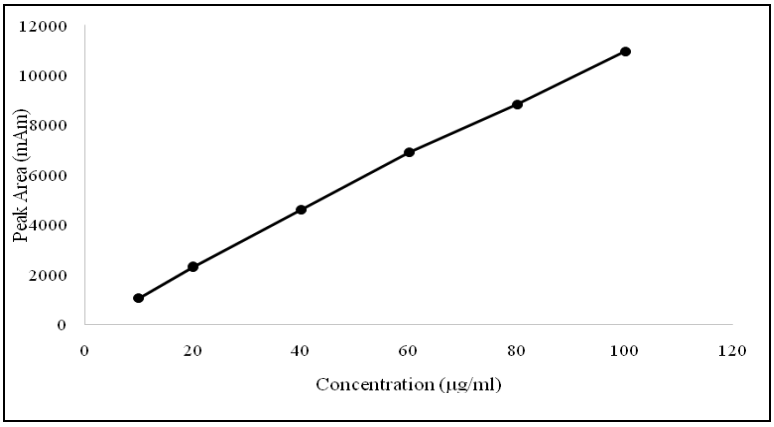

FIG.2: CALIBRATION CURVE OF ASCORBIC ACID STANDARD

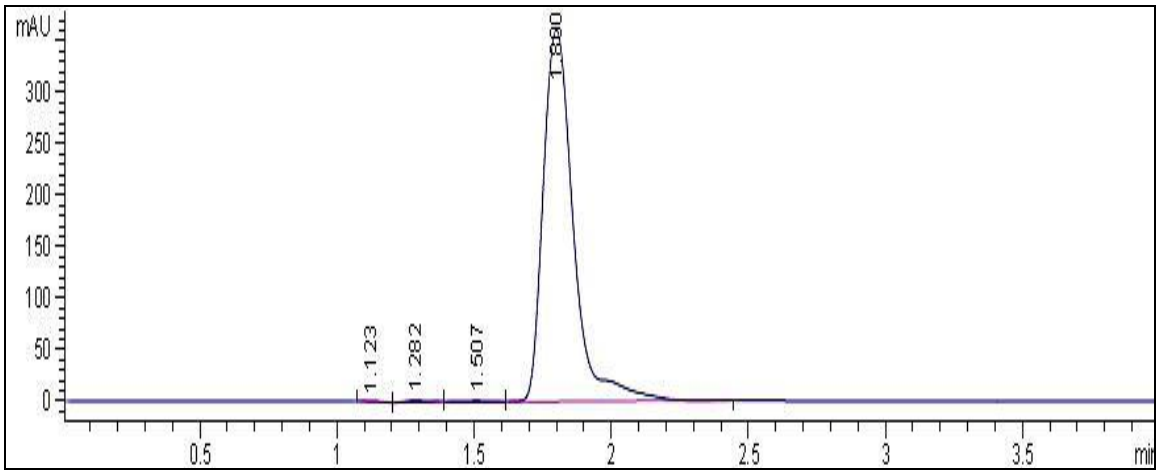

FIG. 3: HPLC CHROMATOGRAM OF ASCORBIC ACID STANDARD

DISCUSSION: The HPLC method used for the assessment of the ascorbic acid in Vitamin C syrups was developed in the Department of Medicinal Chemistry and Quality Control laboratories of the National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja. From the calibration readings, a plot of peak-area against concentration (µg/ml) gave a straight line curve, which obeyed Beer Lambert's law. [21] The correlation co-efficient was 0.9987 (**Fig. 2**), the within day and day to day runs was calculated as shown in **Tables 1** and **2**. The developed and validated method has been demonstrated to be rapid (**Fig. 3)**, accurate and concise. This is a departure from series of complicated, time-consuming, out-dated and sometimes inaccurate analytical procedures that are often used to access ascorbic acid content.

Thus, this method was used in the assessment of ascorbic acid in the fifty-five bottles of fourteen brands of vitamin C syrups. The results from the experiment data showed a high confidence limit of 95%. The fourteen brands were coded thus, as seen in the **Table 3**: PD04, PZ09, LP05, KP06, SK07, TOP08, CEV, Tu011, VI, VIT, AC01, MN03, ER01 and ES02

Percentage content of AA in the various samples of Vitamin C syrups:
The % content of ascorbic acid in the various preparations of Vitamin C syrup is shown in **Table 3**.

TABLE 3: PERCENTAGE CONTENT OF AA IN THE VARIOUS SAMPLES OF VITAMIN C SYRUPS

S/N	Sample Code	Average Peak Area	Cal. Conc. ± S.D (µg/ml)	% Content
1	PD04.BN	1534.81	11.73 ± 0.10	73.31
2	PD04.DOR	2188.95	17.82 ± 0.03	111.40
3	PD04.DIL	2205.41	17.98 ± 0.05	112.35
4	PZ09.VAM	2507.55	20.79 ± 1.09	129.94
5	PZ09.DOR	2686.39	22.46 ± 0.27	140.36
6	PZ09.SG	2633.45	21.96 ± 0.35	137.27
7	LP05.MAL	2583.13	21.50 ± 0.12	134.35
8	LP05.AM	2353.31	19.40 ± 1.11	121.25
9	KP06.AT	657.75	3.56 ± 0.08	22.25
10	KP06.JAL	1056.22	7.27 ± 1.90	45.44
11	SK07.JAL	2278.24	18.66 ± 0.02	116.59
12	SK07.SKY	1181.66	8.44 ± 0.10	52.75
13	TOP08.DIL	2246.63	18.36 ± 0.35	114.75
14	CEV.PAT	2146.72	17.43 ± 0.02	108.94
15	TU011.RA	1393.77	10.42 ± 0.48	65.10
16	TU011.AT	907.51	5.89 ± 0.22	36.79
17	VI.PAT	740.75	4.33 ± 0.16	27.08
18	VIT.DOR	1728.71	13.54 ± 0.45	84.60
19	AC01.OD	3601.87	30.99 ± 0.13	193.66
20	MN03.OS	4455.34	38.94 ± 0.23	81.12
21	MN03.FK	7255.34	65.02 ± 0.32	135.45
22	MN03.MX	6056.45	53.85 ± 0.45	112.19
23	MN03.AT	7164.51	64.17 ± 0.05	133.69
24	MN03.MC	5430.24	48.02 ± 0.77	100.04
25	MN03.MY	6253.16	55.68 ± 0.54	116.01
26	MN03.JAL	6366.32	56.74 ± 1.30	118.20
27	MN03.AM	6647.69	59.36 ± 0.07	123.66
28	MN03.MAL	6342.66	56.52 ± 0.43	117.74
29	ER01.OS	1373.77	10.23 ± 0.05	63.94
30	ER01.GC	3238.99	27.60 ± 0.45	172.53
31	ER01.SG	2322.40	19.07 ± 0.04	119.17
32	ER01.SA	3190.86	27.16 ± 1.48	169.73
33	ER01.RA	2875.73	24.22 ± 0.30	151.38
34	ER01.BN	2819.97	23.70 ± 0.00	148.13
35	ER01.VA	2906.87	24.51 ± 0.37	153.19
36	ER01.SK	3328.74	28.44 ± 0.15	177.75
37	ER01.JAL	2425.36	20.03 ± 0.19	125.16
38	ER01.PA	2053.39	16.56 ± 0.40	103.50
39	ER01.AT	2428.61	20.06 ± 0.17	125.35
40	ER01.DO	3152.02	26.79 ± 0.15	167.47
41	ER01.DIL	2936.82	24.79 ± 0.11	154.94
42	ER01.DX	3112.98	26.43 ± 0.25	165.19
43	ER01.MC	1979.98	15.88 ± 0.23	99.23
44	ER01.MA	2065.33	16.67 ± 0.07	104.20
45	ER01.MX	2626.43	21.90 ± 0.04	136.87
46	ER01.AM	2915.82	24.59 ± 0.40	153.71
47	ES02.RA	2846.08	23.94 ± 0.05	149.65
48	ES02.FS	2851.41	23.99 ± 0.26	149.96
49	ES02.GH	2222.41	18.13 ± 0.22	113.34
50	ES02.DIL	2548.76	21.18 ± 0.14	132.34
51	ES02.MA	2481.55	20.55 ± 0.08	128.43
52	ES02.DOR	2382.11	19.62 ± 0.07	122.64
53	ES02.MY	2859.52	24.07 ± 0.02	150.44
54	ES02.SKY	3070.17	26.03 ± 0.61	162.70
55	ES02.JAL	2654.63	22.16 ± 0.01	138.51

According to the British Pharmacopoeia specification for ascorbic acid syrup, it should contain not less than 95% and not more than 105% of ascorbic acid [22].

From the fifty-five bottles of Vitamin C syrups assessed, ER01 brand was the most widely distributed among the samples, being found in about 85% of the pharmacies assessed. Among the ER01 brand, 78.7% of the samples contained contents of ascorbic acid above the specified range, 16.7% had theirs within the BP specified range, and 5.6% below the range. The second widely distributed Vitamin C syrup brand is ES02 samples, all the samples of this brand contained amount of AA above the official specified range. MN03 brand had a similar distribution pattern as the ES02 brand. However, 11.1% of the MN03 samples contained AA below the specified range while 77.8% contained AA above the specified range, whereas only 11.1% had contents of AA within the B.P. specification of ascorbic acid in vitamin C syrup. Overages in the preparation of vitamins and multivitamin products are a common phenomenon in pharmaceutical manufacturing and this is often used to forestall the effect of packaging on degradation of sensitive vitamins. Thus, it was not surprised that much overages was observed in this analysis. However, the high percentage of these overages is of concern as there ought to be a regulatory guideline in this regard.

On the other hand, considering that both particular brands are from reputable pharmaceutical companies, and are highly sorted-after, presence of samples whose ascorbic acid content fell below the B.P standard could have been as a result of substandard product prepared by some unknown perpetrators of substandard drug products or the sample might have degraded. It could as well be due to manufacturing error during processing or error in weighing. It was once noted by one of the managing directors of a pharmaceutical industry that these fakers do go for successful products that bring succor to the patients.

Out of the remaining eleven brands of vitamin C syrup only KP06 and Tu011 were found in two pharmacies which suggest that they are relatively not common within Abuja. According to the test result, these brands failed woefully in the assessment as regards the percentage content test, with 22.3% AA in KP06.AT, 45.4% for KP06.JAL, while Tu011.RA was 65.10% and Tu011.AT was 36.79% for the two samples of the two brands obtained respectively, although they satisfied the criteria for physical assessment. The fact that Vitamin C syrup preparations are among the fast-selling over-the-counter (OTC) drugs could have been a motivation for the perpetrators of fake and substandard drugs.

The pharmacies where the vitamin C syrups were purchased were categorized into three: A, B, and C. Category A Pharmacies were: MAL; DOR, FSH and GHA, Category B were DIL, MY, JAL, SKY, SG and VAM, and Category C were BN, RA, MX, GC, SA, DX, MC, AM, AT, FK and PAT. Category A pharmacies had fans and air conditioners; good dispensing area and stable electricity supply, while category B had fans and air conditioner, but had fairly stable electricity supply with relatively fair dispensing area. Category C had small dispensing areas, fan only, epileptic electricity supply and hot humid environment.

From the result, it could be seen that the failure rate in the assessment is highest in category C followed by B and one case of failure in A. This could be attributed to the difference in the dispensing and stocking environment of the three categories of pharmacies considering the instability rate of AA under hot humid environment. During this study, there was a demonstration in which two samples of the selected Vitamin C syrup, ER01 and ES02 brands were assessed and found to pass the % content test of AA. They were then kept on the open shelf in our laboratory in NIPRD for six months after which they were assessed again and found to have % content of 74.75% AA for ES02 and 77.5% of AA for ER01.

This clearly demonstrated the unstable nature of AA, as the laboratory where the two bottles were kept has air conditioner that is switched-on for about 8 hours in a day. Vitamin C as an antioxidant substance is well known to be unstable under certain atmospheric or storage conditions especially in the presence of oxygen. If these two products

were kept in a room without air conditioner, the percentage content would have probably been much lower than that.

It was also observed that 50% of samples that did not meet the specified standard were selected from the category C pharmacy. And this could be attributed to the overages of the content of AA. Also, the satisfactory rate noted in some brands could be attributed to the stocking pattern of most of the pharmacies with not more than three brands of Vitamin C syrups, which allowed for rapid replacement of dispensed ones from the shelves. The container type did not seem to matter as all the Vitamin C syrups in plastic bottles with screw cap did not fall below the B.P standard in terms of AA content; the plastic container samples formed 30.76% of the total samples. However, it may also be part of the reasons for the much observed general overages (74.6%) among the samples since it has been reported that oxygen readily passes through plastics.

CONCLUSION: The HPLC method developed was simple, reproducible, accurate and sensitive and can be applied in the quality control determination of the ascorbic acid in Vitamin C syrups. Quality control and establishment of standard permissible overages for vitamin products are essential for the efficacy and safety of pharmaceutical products. And proper enforcement of quality by the regulatory agencies is of utmost importance if adequate health delivery is to be ensured to the populace.

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