Journal of Chromatographic Science 2012;50:102–107
doi:10.1093/chromsci/bmr035

Article

Determination of Trace Vitamin C by Ion-Pair HPLC with UV Detection in Calcium Gluconate and Vitamin C Compound Oral Solution

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A sensitive and specific reversed-phase high-performance liquid chromatographic method with ultraviolet detection was developed for the determination of vitamin C, using tetrabutylammonium hydroxide as an ion-pair reagent in a compound oral solution containing 100 mg/mL calcium gluconate and 1.25 mg/mL vitamin C. The aqueous phase contained 0.005 mol/L tetrabutylammonium hydroxide and the mobile phase consisted of a mixture of the aqueous phase–methanol (80:20, v/v, pH 6.0 adjusted by phosphoric acid). The linearity, sensitivity and specificity, accuracy, and stability of the procedure were evaluated. The calibration curves for vitamin C were linear in the range of 10.0–100.0 μg/mL. The percentage coefficient of variation of the quantitative analysis of the vitamin C in the products analysis was within 5%. The method was successfully applied to determine the stability of vitamin C in the compound oral solution. It was found that the vitamin C peak was symmetrical and the column efficiency was high. The method is simple and suitable for stability testing of a low concentration of vitamin C preparation.

Introduction

Vitamin C (also known as ascorbic acid) is a hydro-soluble vitamin. It is the least stable among the vitamins (1). It is easily oxidized, especially in aqueous solutions, and this process is greatly favored by the presence of oxygen, heavy metal ions, especially Cu^{2+}, Ag^{+}, and Fe^{3+}, and by alkaline pH, and high temperature (2).

The role of vitamin C in the human organism is to care and prevent several diseases, among them scorbute and the cold (3). It broadly participates in the body's metabolic processes, such as oxidation, reduction, and hydroxylation, and it plays an important role in the maintenance of normal physiological functions of the body. It is involved in wound healing, tyrosine metabolism, conversion of folic acid to folinic acid, carbohydrate metabolism, synthesis of lipids and protein, iron metabolism, resistance to infections, and cellular respiration (4). In addition, vitamin C plays an important role in the oxidative stress reactions (5). Lack of vitamin C may cause necrotic disease and reduce the capacity of the body's resistance.

In the Chinese Pharmacopoeia 2010, vitamin C preparations contain tablets, effervescent tablets, granules, effervescent granules, injection, etc.; there is no quality specification of oral solution yet. As is known, vitamin C was not stable in solution, but under the condition of an antioxidant and the protection of nitrogen, this problem can be solved. The oral solution is easy to distribute, and it is easy to take and absorb, especially for infants and the elderly. Traditionally, calcium gluconate and vitamin C are taken separately, which was not convenient for people. In this study, a new oral solution that has both calcium and vitamin C was investigated; it was convenient for people to get both of them by just a solution.

Because vitamin C has a strong reducibility, most quality monitoring criterions were carried out by using a titration assay. However, this method is not sensitive enough for low concentration vitamin C determinations; the determinate error of trace vitamin C is great (6). Various methods have been employed for the analysis of vitamin C.

Several methods, including electrochemical detection (7, 8), fluorimetric detection (9–11), flow injection method (12), capillary zone electrophoresis (13), and spectrophotometric (14) have been used to determine vitamin C content in pharmaceutical dosage forms or biological samples, but these methods are complicated, or the instruments used are not widely available in many laboratories. High-performance liquid chromatography (HPLC), with a different combination of pre- or post-column derivatization, was also used to quantify vitamin C (15), but these approaches require a derivatization step, which makes the method time consuming, and degradation of vitamin C can occur during the procedure. The United States Pharmacopeia (USP) describes an HPLC method as well as a specific column that contains packing L39, a hydrophilic poly hydroxylmethacrylae gel of totally porous spherical resin which is quite expensive and difficult to obtain in order to assay vitamin C.

Therefore, in this paper, a new ion-pair HPLC method with UV detection was developed for the determination of trace vitamin C in a compound oral solution containing 100 mg/mL calcium gluconate and 1.25 mg/mL vitamin C. It is a promising method, which is precise, accurate, convenient, and effective for the quality control of low concentration of vitamin C in preparations.

Experimental

Chemicals and reagents

Standard vitamin C (99.2% of purity) was provided by the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China, serial number 100425–200301). Vitamin C raw material (99.0% of purity) was purchased from Tianjin Bei Fang Tian Yi Chemical Reagent Factory (Tianjin, China, serial number: 20070214). Calcium gluconate was purchased from Zhejiang Ruibang Pharmaceutical Co., Ltd. (Zhejiang, China, serial number 071229). Lactic acid was obtained from Tianjin Jingu Business Industrial Co., Ltd. (Tianjin, China). Calcium hydroxide and tetrabutylammonium hydroxide were obtained from Tianjin Huadong Chemical Reagent Factory (Tianjin, China). L-cysteine hydrochloride was obtained from Tianjin Kermel Chemical Reagent Co. Ltd. (Tianjin, China). Citric Acid was purchased from Tianjin Jinli Business Industrial Co. Ltd. (Tianjin, China). All the other

reagents were either of analytical or chromatographic grades. Water was purified by redistillation and passed through a 0.22 μm membrane filter before use.

Sample preparation and standard solutions

Preparation of calcium gluconate oral solution

Calcium gluconate is soluble in boiling water and slightly soluble in cold water. It can easily turn into calcium carbonate particles when in contact with carbon dioxide, which can accelerate the precipitation and affect the clarity of the solution.

The solution was prepared as follows: 10 g calcium gluconate was added to 90 mL boiled water and heated until it dissolved absolutely. Lactic acid and calcium hydroxide were then added, and the solution continued to be boiled for half an hour. The prepared solution was filtered using a 0.45 μm membrane filter and sterilized at 121°C for 1 h, and stood for 24 h before use.

Preparation of vitamin C oral solution

The vitamin C oral solution was composed of vitamin C and L-cysteine hydrochloride. 0.70 g L-cysteine hydrochloride was added to 8 mL purified water firstly, and then 0.14 g vitamin C was added. The concentration of vitamin C needed was 1.25 mg/mL. Vitamin C feeding in was a little higher, because the degradation of vitamin C can occur during the pH adjusting and sterilizing.

Preparation of the compound oral solution

The compound oral solution contained 100 mg/mL of calcium gluconate and 1.25 mg/mL of vitamin C. The calcium gluconate oral solution and the vitamin C oral solution, which was prepared as previously mentioned, was mixed together, and 0.10 g citric acid was added to the solution. Afterwards, the pH was adjusted to 6.0 with 0.1 mol/L sodium hydroxide solution, and purified water was added to 100 mL. The solution was then filtered with a 0.45 μm filter membrane, sealed, and sterilized at 100°C for 10 min. All the operation was under the protection of nitrogen.

Preparation of standard solutions

Standard stock solutions of vitamin C were prepared containing about 62.5 mg vitamin C, and were transferred to a 50-mL volumetric flask, followed by the addition of purified water and sonicated for 5 min. A series of solutions were prepared by further dilution of the stock solutions with purified water.

Chromatography

The HPLC system consisted of a P3000A pump and a UV3000 UV detector (Beijing Chuang Xin Tong Heng Science and Technology Co. Ltd.). The chromatographic separation was performed on a Venusil XBP C_{18} column (5 μm, 250 × 4.6 mm, Agela Technologies Inc.). The mobile phase was composed of a mixture of aqueous phase-methanol (80:20, v/v), and the aqueous phase contained 0.005 mol/L tetrabutylammonium hydroxide (pH 6.0, adjusted by phosphoric acid). The mobile phase was filtered through a 0.45 μm membrane filter and degassed ultrasonically before use. The UV detector was operated at 245 nm. Samples were introduced into the column through a manual injector equipped with a sample loop (20 μL). The flow rate was fixed at 0.9 mL/min at room temperature.

Method validation

The validation was carried out by examining the linearity, sensitivity and specificity, precision and accuracy, and stability of vitamin C and the compound oral solution.

Linearity

The linearity of the assayed method was evaluated by crossing the range of the analytical procedure. It was evaluated by analyzing each solution spiked with a known amount of the analytes at low, medium, and high concentrations. Stock standard solutions of vitamin C were diluted with the purified water to concentration ranges of 10.0−100.0 μg/mL. Aliquots (20 μL) were analyzed by HPLC as described previously. Triplicate injections were made for each concentration. For each spiked sample was obtained the corresponding chromatogram and constructed a graphical plotting the means of areas against their concentrations. The linearity of the standard curve was evaluated using a linear regression analysis (16).

Sensitivity and specificity

The lowest limit of detection (LOD) was defined as the amount that could be detected with a signal-to-noise ratio of 3 (S/N = 3). It was determined by injecting ($n = 5$) solutions of vitamin C of known concentrations (50 μg/mL), and by lowering the concentration of the samples until the detection of the peak was three times the height of the baseline noise. The corresponding concentration was considered as being the minimal concentration detectable by HPLC. The lowest limit of quantitation (LLOQ) was determined as the minimum concentration that could be accurately and precisely quantified. It was determined by multiplying 10× the height of the baseline noise.

Accuracy and precision

The accuracy of the assay method was evaluated by performing replicate analysis against a calibration curve and calculating the mean percent differences between the theoretical values and the measured values (16). The accuracy was evaluated by HPLC in triplicate using three concentration levels: 50%, 100%, and 150% of the analytical method concentration.

The precision of the method indicates the degree of dispersion within a series of determinations on the same sample. The precision of the method was determined by the external standard method (the concentration of the working standard was 50 μg/mL), and the relative standard deviation (RSD) was calculated. The intra-day precision was evaluated by analysis of three different concentrations: 50, 100, and 150% of the analytical concentration in triplicate on the same day. The inter-day precision studies were performed by repeating the studies on three consecutive days. The data were evaluated as RSD% for the determinations.

Stability test

The stability of the vitamin C content was determined after storage in a refrigerator maintained at 4°C, and at room temperature (25°C). All samples were kept in light protected containers. The stability of the vitamin C was tested for 0 min,

30 min, 1 h, 2 h, 3 h, and 6 h using the HPLC method. A loss exceeding 3% of the initial content is considered to indicate instability of vitamin C (18).

A raw materials destruction test, involving exposure to 1 mol/L hydrochloride acid, 1 mol/L sodium hydroxide, 10% H_2O_2, and light (5000 Lx), was also carried out in the experiment.

Stability of the compound oral solution

Three batches of the compound solution were prepared and sealed in the brown bottles (nitrogen was filled in the vacant space). The solutions were stored at 40°C in an accelerated test and 25°C for long term test, respectively. The stability of the vitamin C was tested for 0, 0.5, 1, 2, 3, and 6 months using this HPLC method.

Results and Discussion

Method development and optimization

Vitamin C contains hydroxyls, which make it an acidic compound. It interacts weakly with the apolar stationary phase of the HPLC column, hardly adsorbs to the column, and is easily eluted by the mobile phase. Several chromatography systems were investigated in this study to resolve this problem: Methanol was selected for the organic phase, the aqueous phase, involving NH_4AC–HAC buffer solutions, was mixed with methanol. Different proportions of the aqueous phase and the organic phase, ranging from 10:90 to 90:10, were investigated; moreover, different pH values of the aqueous phase, ranging from 2.5 to 7.5, were also investigated. However, irrespective of the mixture ratio and the pH of the aqueous phase, none of them can separate vitamin C well. Vitamin C has no retention in the column.

With the addition of the ion-pair reagent, tetrabutylammonium hydroxide, the chromatography conditions improved markedly. Because of the presence of the enol form structure, vitamin C is regarded as an acid. Therefore, under neutral and basic mobile phase conditions, vitamin C will combine with OH− based on the attraction between the positive and negative charges, changing vitamin C to the OH− conjugate. Subsequently, in the mobile phase, the OH− conjugate of vitamin C binds to the positive group of tetrabutylammonium hydroxide and is converted to a neutral molecule. Compared with the ionic form of vitamin C in acidic conditions, the neutral complex enhanced the retention ability of vitamin C on the C_{18} column. Because the neutral complex has a weaker polarity than ionized vitamin C, the compounds with a higher polarity to the column can be retained or adsorbed effectively.

The concentration of tetrabutylammonium hydroxide in the aqueous phase played a significant role in this chromatographic system, because the formation of the neutral complex depends on the amount of tetrabutylammonium hydroxide. A series of concentrations of tetrabutylammonium hydroxide, ranging from 0.002 mol/L to 0.020 mol/L, were evaluated; as the concentration of tetrabutylammonium hydroxide increased, the retention time increased at the same mixing ratio of the mobile phase. Considering the analytical time, the concentration of tetrabutylammonium hydroxide in the aqueous phase was set at 0.005 mol/L.

The mobile phase was a mixture of aqueous phase-methanol (80:20, v/v). The addition of tetrabutylammonium hydroxide increased the pH above 8.0. In order to extend the working life of the column, the pH value of mobile phase was adjusted to 6.0. Figure 1 shows the same working standard solution injected into the identical chromatography system (A is without tetrabutylammonium hydroxide and B is with it). A is almost no retention in the column, whereas B was about 8.2 min. This indicates that the ion-pair reagent does interact with vitamin C due to the increase in the retention time, and

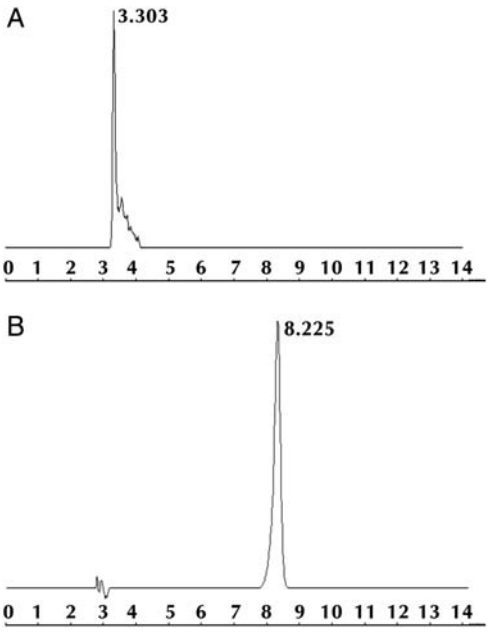

Figure 1. HPLC chromatograms of vitamin C. Vitamin C was the same working standard solution and injected into the identical chromatography system, except that A is without tetrabutylammonium hydroxide, and B is with it.

Table I
Result of Recovery for VC assay ($n = 3$)

Administered Concentration (μg/mL)	Measured Concentration (μg/mL)	Recovery (%)	Mean (%)	RSD (%)
26.24	26.27	100.11		
25.04	24.78	98.96		
27.68	27.59	99.67		
50.56	51.24	101.34		
50.48	50.17	99.39	99.72	0.97
50.72	49.83	98.25		
75.92	75.11	98.93		
75.36	75.94	100.77		
75.84	75.91	100.09		

Table II
Intra- and Inter-Day Precision of Method for Determination of VC ($n = 3$)

Theoretical Concentration (μg/mL)	Intra-day measured concentration (μg/mL)		Intra-day measured concentration (μg/mL)	
	Mean*	RSD%	Mean*	RSD%
26.16	25.09	1.25	25.95	1.03
50.88	50.58	1.04	51.14	1.55
75.68	73.55	1.51	75.05	1.37

*Mean and RSD% of three determinations.

Table III
Stability of VC Samples ($n = 3$)

Time	Concentration ± SD (%)					
	4°C	25°C	1 mol/L hydrochloride	1 mol/L sodium hydroxide	H_2O_2	Light (5000 Lx)
0 min*	99.87 ± 2.31	99.87 ± 2.63	101.87 ± 2.05	99.63 ± 2.15	97.69 ± 1.38	98.07 ± 1.63
30 min	99.76 ± 2.17	98.46 ± 2.55	73.12 ± 8.73	65.74 ± 3.67	45.46 ± 3.75	75.39 ± 4.44
1 h	99.24 ± 1.73	97.28 ± 1.88	56.33 ± 6.81	47.35 ± 4.83	11.22 ± 1.61	47.28 ± 6.98
2 h	98.96 ± 2.35	94.76 ± 2.82	21.24 ± 4.36	16.41 ± 2.21	–†	24.73 ± 2.75
3 h	98.68 ± 1.60	89.31 ± 4.41	13.31 ± 1.43	–	–	8.31 ± 0.89
6 h	98.02 ± 3.08	73.22 ± 5.60	–†	–	–	–

*Measured concentration compared to theoretical concentration.
†Concentration of VC cannot be detected.

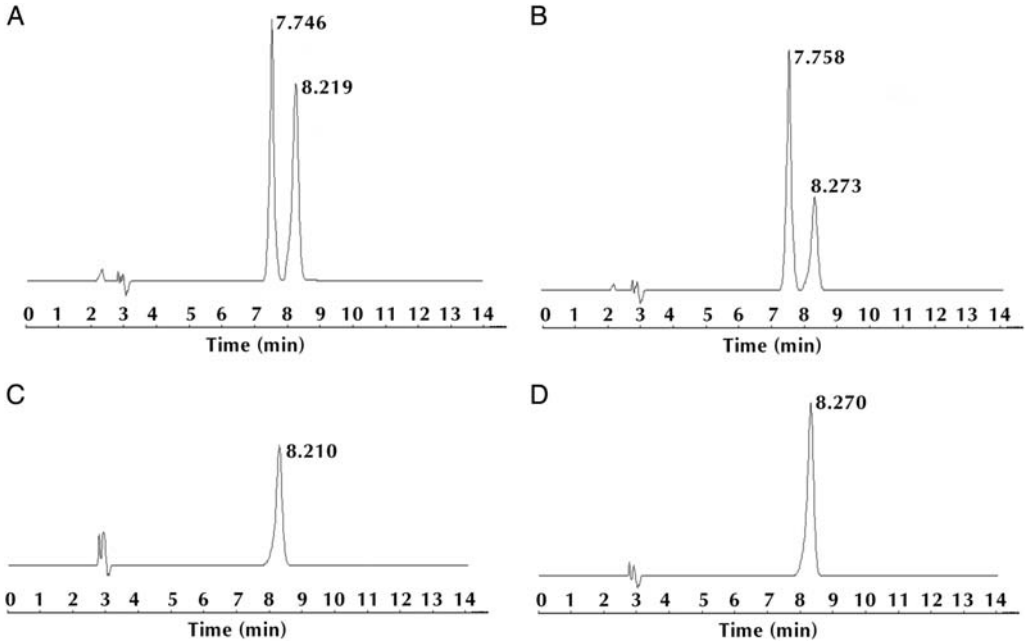

Figure 2. Representative chromatograms of vitamin C samples after treatment with 1 mol/L hydrochloride (A), 1 mol/L sodium hydroxide (B), 10% H_2O_2 (C); and light (D).

the peak profile was clearly improved. There is a reversal peak of water at the time of 3.3 min; this was because calcium gluconate was instable when in contact with the mobile phase, so the sample was diluted with water in the determination.

This method was convenient and easy for the quality control of low concentration of vitamin C than other reports. Adriana and Neli also used an HPLC method to quantify vitamin C, but there was a combination of pre-column derivatization used, and these approaches require a derivatization step which makes the method time consuming (15). Zhou et al. used the HPLC method with gradient elution to determine vitamin C in human plasma (19), but this method was complicated and inconvenient.

Table IV
Determination of VC in Compound Sample Solutions ($n = 3$)

Batch number	Found ± SD (%)	RSD%
B1	99.54 ± 1.65	1.66
B2	100.22 ± 2.18	2.18
B3	98.97 ± 1.72	1.74

Method validation

Linearity

The linearity of the method was determined by linear regression. The calibration curve for vitamin C was linear in the range of 10.0–100.0 μg/mL. The linear regression equation was $y = 46938x + 15589$ with a correlation coefficient $r = 0.9997$. The values of the correlation coefficient were close to unity, indicating good linearity.

Limit of detection and quantitation

The LOD and LOQ were determined. The LOD was 0.03 μg/mL, and the LOQ was 0.1 μg/mL for vitamin C. The results indicated that the method is sensitive for the determination of very small concentrations of vitamin C.

Table V
Stability Data for the Compound Oral Solution in an Accelerated Test and Long-Term Test

Month	Accelerated test			Long-term test		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
0	99.73 ± 0.98	100.45 ± 1.79	98.79 ± 1.32	99.73 ± 0.98	100.45 ± 1.79	98.79 ± 1.32
0.5	97.61 ± 1.18	98.73 ± 2.17	97.02 ± 2.03	98.91 ± 1.74	99.18 ± 1.48	98.37 ± 2.33
1	93.26 ± 2.83	95.02 ± 2.26	93.41 ± 1.67	97.12 ± 2.49	98.26 ± 1.86	97.27 ± 1.90
2	90.21 ± 1.91	92.16 ± 1.09	90.67 ± 0.97	96.26 ± 1.28	97.73 ± 2.88	96.28 ± 2.31
3	88.95 ± 2.19	89.48 ± 1.57	87.84 ± 2.26	95.02 ± 2.73	95.79 ± 2.04	94.65 ± 2.22
6	81.74 ± 1.52	83.05 ± 2.26	80.84 ± 1.69	93.21 ± 1.89	93.71 ± 1.75	93.07 ± 2.08

Accuracy

A recovery study was performed by the analysis of simulated preparations at three concentrations (25, 50, and 75 μg/mL), and the results are summarized in Table I. The mean recovery was 99.72%, and the RSD% values were less than 5%. The results indicated that the proposed HPLC method is accurate for the determination of the investigated drugs.

Precision

The precision of the method was determined for both intra-day and inter-day repeatability in oral solutions. The intra- and inter-day variability of the assayed method was determined at low, medium, and high concentrations. The results were shown in Table II. These data indicate that the assayed method is reproducible within the same day and three different days.

Stability test

Stability is a key problem of vitamin C analysis, because it is known to be very unstable in an aqueous solution. There are lots of factors that negatively influence their stability (i.e., light, increased temperatures, pH, and the presence of oxide). It is therefore necessary to keep the influences of these variables to a minimum (17).

Vitamin C was not stable for 6 h when stored at room temperature, but vitamin C was relatively stable when stored at 4°C for 6 h. When in contact with acid, basic, oxide, or light, vitamin C can be destroyed acutely. The results are shown in Table III. Figure 2 shows the chromatograms of a vitamin C destruction test after treatment with acid, base, light, and oxidation by H_2O_2. The peak of vitamin C can be detected and separated effectively. There is another peak, appearing next to the peak of vitamin C, after acid and base were added. It is known that dehydroascorbic acid, 2, 3-diketogulonic acid, 2-diketogulonic acid, and xylose are the main oxidative product of vitamin C, but those compounds have no strong UV absorption expect dehydroascorbic acid (20). In this study, the same retention time was found between the dehydroascorbic acid standard solution and the vitamin C sample; this peak was the oxidative product of vitamin C, dehydroascorbic acid.

The results indicate that vitamin C samples should be stored at a low temperature (4°C) in the brown flask, and it should avoid contact with acid, base, oxide, and light.

Application of the method

Determination of vitamin C in the compound solution

The proposed validated HPLC method was applied to the determination of vitamin C in three different batches of the compound solution. Satisfactory results were obtained as shown in Table IV; the mean percentage found and the RSD values indicate that the proposed method could be adopted for the determination of vitamin C in a compound solution.

Determination of vitamin C in the accelerated test and long-term test

The developed method was applied to the determination of the content of vitamin C in a compound oral solution in the accelerated test and long-term test. The stability data of the three batches are summarized in Table V. The content of vitamin C was reduced by about 20% under accelerated conditions, and by 7% under 25°C after a storage period of 6 months. Therefore, the vitamin C compound oral solution can be considered to be a stable preparation.

Conclusion

A new reliable and sensitive ion-pair HPLC method had been successfully developed and applied to the assay of vitamin C in the compound oral solution. No research about the preparation of calcium gluconate and vitamin C compound oral solution has been reported, and this is the first time for the determination of vitamin C in this compound oral solution. Validation experiments showed a very good precision and accuracy of the method, with coefficients of variation and relative errors less than 5%. This method is very practical for the determination of vitamin C. The stability of vitamin C was also determined, and the results show that vitamin C should be stored at a low temperature (4°C) in the brown flask. The vitamin C compound oral solution was stable in an accelerated test and a long-term test. The compound oral solution can be considered to be a stable preparation.

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