



Determination Trace Levels of Vitamin C and Folic Acid in Urine Sample by Ultrasound-assisted Dispersive Liquid-Liquid Microextraction Method Coupled HPLC-UV

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ABSTRACT

A new, rapid and highly sensitive and rapid method was proposed for the simultaneous determination trace levels of vitamin C and folic acid in the human urine samples. The influence affecting factors on the UA-DLLME such as type and volume of the dispersive and extraction solvent, pH, ionic strength and temperature of sample solution, sonication condition investigated and optimized. Under the optimized extractions, relative standard deviations (RSD) of the analyses less than 6% (n= 3) and detection limit of 1.65-2.05 ng mL⁻¹ respectively. USAEME - HPLC-UV was successfully applied for the simultaneous determination of trace levels of vitamin C and folic acid in human urine Samples.

Keywords: Dispersive liquid-liquid microextraction, Folic acid, HPLC, Vitamin C, Ultrasound-assisted.

INTRODUCTION

Vitamin C and folic acid are water-soluble vitamins, necessary for a healthy diet. They are involved in tissue growth and normal cell function. They also serve as cofactors for at least eight enzymatic reactions, perform antioxidant activity as well as have protective roles for the cell membranes, and are crucial for maintaining optimal health¹⁻³. Many analytical methods including spectrophotometry⁴, spectrofluorimetry⁵, chemiluminescence^{6,7}, capillary electrophoresis^{8,9}, electrochemical methods¹⁰ and HPLC method^{11,12} have been reported for the

determination of vitamin C and folic acid. Most of these methods are expensive instruments and have a high limit of detection (LOD).

In 2006, a new microextraction technique called dispersive liquid-liquid microextraction (DLLME) was introduced^{13,14}. It is as an effective preconcentration and separation of technique among these microextraction method for inorganic and organic species determination^{15,16}. This method has a higher enrichment factor, is safe for use with hazardous samples, has a minimal cost because it consumes little reagent, is environmentfriendly,

low cost and easier to incorporate into automated analytical methods^{17,18}. The goal of this research was to develop a new and rapid method for the simultaneous determination of vitamin C and folic acid in human urine samples by high performance liquid chromatography-ultraviolet detection (HPLC-UV) after preconcentration by UA-DLLME. Experimental factors affecting the extraction recoveries, including type and volume of extraction and disperser solvents, pH, ionic strength and temperature of sample solution, and sonication time were considered and optimized. This method was aimed to investigate and validate a method for the determination of C vitamin concentrations in human urine and synthesis samples by HPLC after optimization by UA-DLLME method.

EXPERIMENTAL

Chemicals and reagents

The analytical-reagent grade of vitamins (>99%) were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC-grade methanol was purchased from Fisher Scientific (Waltham, MA, USA). Standard stock solutions were prepared by dissolving vitamin in deionized water. Working standard solutions at different concentrations were prepared freshly by mixing the appropriate volumes of the stock solutions and diluting with deionized water Millipore, Bedford, MA, USA).

Instrumentation and software

The chromatography runs for the separation and determination of vitamin C and folic acid were performed using a Shimadzu Corporation HPLC (Japan) system equipped with UV-VIS detector (SPD-20Avp), LPG system (LC-20ADvp), micro vacuum degasser and a C₁₈ - ODS column (250 × 4.0mm, 5 μm) from Shim pack VP (Japan). UV detection was performed at 200 to 300 nm. The mobile phase was a mixture of 60 % methanol and 40 % 0.03 M phosphate buffer pH 2.5 at a flow of 1 mL min⁻¹ at 25 °C. The pH was measured using a pH meter (Metrohm 827, Switzerland) combined with a glass electrode.

Preparation of sample

Actual urine samples were supplied from sick volunteers in Taleghani Medical Center (Abadan, Iran) and were kept frozen at -10°C before analysis.

The frozen urine samples were thawed at room temperature and centrifuged for 10 min at 4000 rpm in order to white lipid solid was sedimented in the bottom of the conical test tube. For protein precipitation a solution containing 50 μL of 1% trichloroacetic acid (TCA) was added to 1.0 mL of human urine. This mixture was shaken for 10 s and then filtered through 0.45 μm nylon ultra membrane. Then 0.5 mL filtered sample was transferred to a new tube and was alkalized with 200 μL (NaOH 1 mol L⁻¹) for the hydrolysis of acyl glucuronic acid conjugates; thereafter it was neutralized with 200 μL (HCl 1 mol L⁻¹). The matrix effects were lowered by two times dilution of urine sample and was then directly subjected to UA-DLLME procedure.

UA-DLLME procedure

A 5 mL aqueous sample solution (pH 3.5) with the ionic strength of 0.05 % (w/v) NaCl, containing 25 ng mL⁻¹ of vitamin C and folic acid were placed in a 10.0 mL screw cap glass test tube with conical bottom. A mixture of 50 μL of methanol containing 100 μL carbon tetrachloride were rapidly injected into the sample solution with a 1.0 mL glass syringe and then the glass test tube was immersed in an ultrasonic water bath for 4 min to enhance the extraction of vitamin C and folic acid from aqueous solution into the microdroplets of carbon tetrachloride. Then the mixture was centrifuged at 5500 rpm for 4 min in order to obtain the extraction solvent at the bottom of the centrifuge tube. The sedimented phase was completely transferred to another test tube using 100 μL microsyringe (Hamilton) and blown to dryness with a mild nitrogen stream. After slowly discarding the aqueous solution, the resulting droplet and lipidic solid were dissolved at 20 μL with HPLC grade methanol and then filtrated through a 0.45 μm membrane to throw out the white floccule from the extract of urine.

RESULTS AND DISCUSSION

It is well known that the extraction recoveries of UA-DLLME can be affected by different factors, such as types and volume of extraction solvent, pH and ionic strength of sample solution, extraction time and temperature as well as centrifugation time. The optimization was performed for high extraction recovery. Samples of 5 mL of aqueous solutions containing 25 ng mL⁻¹ of vitamin C and folic acid were

used to investigate the best extraction conditions for the proposed UA-DLLME method.

Selection of extraction and disperser solvents

In UA-DLLME, the selection of the most suitable extraction and dispersive solvents is the prerequisite condition which needs to be considered¹⁴. In the selection of extraction solvent, several factors should be considered: (1) low solubility in water, (2) higher density than water, (3) high extraction recoveries of interested compounds and (4) good chromatographic behavior. Taking into account these characteristics, four high density solvents such as dichloromethane (CH_2Cl_2 : 1.32 g mL^{-1}), chloroform (CHCl_3 : 1.47 g mL^{-1}), carbon tetrachloride (CCl_4 : 1.59 g mL^{-1}), and chlorobenzene ($\text{C}_6\text{H}_5\text{Cl}$: 1.11 g mL^{-1}) were investigated for this purpose. The disperser solvent would influence the formation of the cloudy solution and the produced infinite interface increases the mass transfer. The dispersive solvent must be miscible with both aqueous and organic phases. Acetonitrile, methanol, ethanol and acetone were evaluated to select the best dispersive solvent in this study. It was found that the maximal peak area was attained when carbon tetrachloride and methanol

solvents were used as extraction and dispersive solvents, respectively. In order to study the effect of extraction solvent volume on the extraction efficiency, different volumes of CCl_4 (20 - 120 μL at 10 μL interval) and a 50 μL of methanol were tested. According to the obtained results, with the increase of CCl_4 volume (90 -100 μL), the peak areas of vitamin C and folic acid increased; however, more increasing the volume of CCl_4 than 100 μL , the peak areas was decreased. As a result, 100 μL of CCl_4 was used as extraction solvent for further experiments. The influence of the volume of the methanol was investigated by changing its volume from 10 – 60 μL (at 10 μL interval). It was found that the extraction efficiency is raised with increasing methanol to 50 μL and then decreased by increasing the volume of methanol for the vitamin C and folic acid. The reason for this could be that at a low volume of methanol, a cloudy state could not be formed well, therefore, resulting in a low recovery. At a higher volume of methanol, the solubility of the vitamin C and folic acid in water was increased, leading to the decreased extraction efficiency. Based on the experimental results, 50 μL of methanol was chosen in subsequent experiment.

Table 1: Analytical performance of UA-DLLME method for determination of vitamin C and folic acid in blank urine

| Analyte | r^2 | LOD ^a (ng mL^{-1}) | LOQ ^b (ng mL^{-1}) | LR ^c (ng mL^{-1}) | RSD (n=6) |
|------------|-------|---|---|--|--------------|
| Vitamin C | 0.997 | 1.69 | 5.63 | 7-500 | 3.7 |
| Folic acid | 0.998 | 2.05 | 6.83 | 8-500 | 5.3 |

^a LOD: limit of detection; ^b LOQ: limit of quantification; ^c LR: linear range

Table 2: Comparison of the UA-DLLME with other methods for determination of Vitamin C and folic acid

| Method | Analyte | Matrix | LDR (ng mL^{-1}) | LOD (mg L^{-1}) | RSD% | Ref |
|-----------------------|------------|---------|--------------------------------|-------------------------------|------|------|
| HPLC-MS/MS | Folic acid | Plasma | 0.249–19.9 | - | 15 | 19 |
| HPLC-DAD | Vitamin C | Mineral | 410.2–1230.7 | 137 | - | 20 |
| | Folic acid | Tablets | 2952–8856 | 10 | - | |
| Ion-exclusion HPLC-UV | Vitamin C | Plasma | 5–200 | 0.306 | - | 21 |
| UA-DLLME-HPLC-UV | Vitamin C | Urine | 7-550 | 1.69 | 3.7 | This |
| | Folic acid | | 8-500 | 2.05 | 5.3 | work |

Effect of pH on extraction efficiency

The pH of sample solution is an important factor and can be affected on the extraction recoveries. Therefore, its effect was evaluated in the range of pH 2-8. According to the obtained results, extraction recovery is maximum at pH = 3.5. This could be explained by the pK_a values of vitamin C and (4.5) and folic acid (3.5). Thus, when the pH is around the pK_a value of the analyte, it is mainly in its neutral form and can easily extract into the organic phase.

Ionic strength

The influence of ionic strength on the performance of DLLME method was investigated by adding different amounts of NaCl (0-2%w/v).

The results indicated that the extraction efficiency decreases, with addition of salt up to 0.05 % (w/v) salt content. Therefore, 0.05 % (w/v) NaCl was chosen in further experiment.

Effect of extraction time and temperature

In this method, for improve the homogeneity, ultrasound- and shaking-assisted methods were investigated with a series of experiments. The effect of ultrasonic time on the extraction efficiency was investigated in the range of 0 - 5 min. The maximum recovery was obtained for ultrasonication of 4.0 min and no improvement was achieved by further sonication time. This was probably due to the fact that the ultrasonic water bath could generate the emulsion quickly and rapidly make a very large contact surface

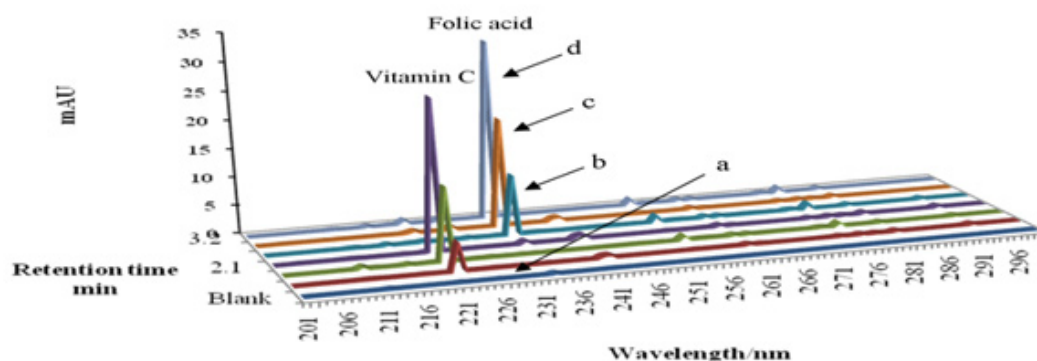


Fig. 3: Chromatograms of vitamin C and folic acid by HPLC at optimum extraction condition (a) blank urine; (b), (c) and (d) blank urine samples spiked with 15, 30 and 45 ng mL⁻¹ of vitamin C and folic acid, respectively

Table 3: Extraction recoveries of vitamin C and folic acid from actual urine samples with the UA-DLLME method (n =3)

| Analyte | Spiked (ng mL ⁻¹) | Found (ng mL ⁻¹) | ER ± RSD (%) |
|------------|-------------------------------|------------------------------|--------------|
| Vitamin C | 0.0 | 18.3 | - |
| | 15.0 | 32.7 | 96.2 ± 4.5 |
| | 30.0 | 46.8 | 95.1 ± 4.1 |
| | 45.0 | 62.5 | 98.2 ± 3.8 |
| Folic acid | 0.0 | 25.3 | - |
| | 15.0 | 39.4 | 94.3 ± 5.2 |
| | 30.0 | 54.5 | 97.3 ± 4.7 |
| | 45.0 | 68.9 | 96.8 ± 4.2 |

area between the extraction solvent and aqueous phase. Therefore, 4 min was found to be the optimum extraction time. Temperature affects the solubility of organic solvent in water, distribution coefficients and mass transfer of the target analytes. The effect of extraction temperature on extraction recoveries was evaluated over the range of 15 to 35°C. Results show (Fig. 2.) that increase in solution temperature decreases the amount of analytes extracted. Thus, 25°C was selected as optimum temperature.

Analytical features of proposed method

Under the optimized conditions, the analytical features of the developed method including linearity (correlation coefficient (r^2)) and linear range

(LR)), limit of quantification (LOQ), limit of detection (LOD), and precision (RSD%) were evaluated. LOD and LOQ were calculated as 3 and 10 times of the standard deviation of blank signal divided by the slope of the calibration curve, respectively. The calibration curves for the vitamin C and folic acid showed good linearity in the concentration range 7 – 500 ng L⁻¹, with correlation coefficients (r^2) greater than 0.997. The LODs and LOQs were found to be 1.69 and 5.63 ng L⁻¹ for vitamin C and 2.05 and 6.83 ng L⁻¹ for folic acid, respectively. The repeatability was studied for six replicate analyses of the spiked samples (at concentrations 25 ng L⁻¹ of vitamin C and folic acid) under the same operational parameters. The relative standard deviations (RSDs) were satisfactory, ranging from 3.7 % and 5.3 % for the vitamin C and folic acid, respectively, showing the good repeatability of the proposed method. The obtained results are summarized in Table 1. The repeatability (intra-day) and reproducibility (inter-day) precision of the UA-DLLME were assessed according to the results of six replicates at level of 25 ng mL⁻¹ of vitamin C and folic acid for six consecutive days. The values of intra-day relative standard deviation (RSD) and inter-day RSD were 6.4 and 6.8%, respectively. The chromatograms of the blank urine and spiked blank urine samples at three concentration levels are shown in Fig. 3. To demonstrate the superiority of the presented method, a comparison between the important analytical parameters of the developed UA-DLLME and those reported in the literature was made as summarized in Table 2¹⁹⁻²¹.

Analysis of actual samples

To evaluate the performance and applicability of UA-DLLME method for the extraction and subsequent determination of vitamin C and folic acid from actual urine samples was examined using

calibration curves. These samples were pretreated as described in section 2.4, extracted using UA-DLLME method and analyzed by HPLC-UV. These samples were spiked with the standards of vitamin C and folic acid at the concentration of 15.0, 30.0 and 45.0 ng mL⁻¹. For each concentration, three replicate experiments were undertaken and the results are given in Table 3. The extraction recovery for the spiked real samples was in the range of 94.3 % and 98.2% and the RSDs were between 3.8% - 5.2%. Obtained results (Table 3) indicate the applicability and efficiency of proposed UA-DLLME-HPLC-UV for a sensitive, fast, reproducible and simple extraction and determination of vitamin C and folic acid in actual urine samples.

CONCLUSION

A new method has been proposed for the simultaneous determination trace levels of vitamin C and folic acid in human urine samples using HPLC after optimization by UA-DLLME method. Optimum conditions were found at 100 μ L of CCl₄, 50 μ L of methanol, 0.05 % (w/v) of NaCl and 4 min for sonication time. The advantages of this method include high recovery, simplicity of operation, good repeatability and reproducibility, with good LODs. Finally, because of the low organic solvent consumption, the presented UA-DLLME proved an inexpensive and environmentally friendly method.

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