

# Quantitative HPLC Analysis of Active Pharmaceutical Ingredients in Syrup Vehicle Using Centrifugal Filter Devices and Determination of Xanthan Gum in Syrup Vehicle Using Rheometry

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## Abstract

Using rapid centrifugal filtration ( $\leq 30$  min) of diluted samples, the filter membrane prevented compounds with molecular weight higher than the nominal molecular weight limit (NMWL) from transporting through the membrane, thus separating them from compounds with molecular weight smaller than NMWL, which would pass through the membrane. The purpose of this study aims to remove high molecular weight matrix (such as xanthan gum) interferences while achieving a quantitative analysis of the active pharmaceutical ingredients of interest. Two model active pharmaceutical ingredients, L-arginine and amphotericin B, were quantitatively recovered from the diluted syrup vehicle after centrifugation with the filter devices. The reproducibility [% relative standard deviation (RSD), peak area] of the filtered samples was less than 0.5%. For amphotericin B samples. The linear range was 0.28  $\mu\text{g/mL}$  to 28.2  $\mu\text{g/mL}$ . The limit of detection was 0.06  $\mu\text{g/mL}$ . The limit of quantification was 0.28  $\mu\text{g/mL}$ . The viscosity of a syrup vehicle changed linearly with the concentration of xanthan gum. A method was thus developed to determine xanthan gum in the syrup vehicle. The accuracy was within 95.0% to 105.0% at different concentration levels.

## Introduction

Quantification of active pharmaceutical ingredients (APIs) in complex pharmaceutical matrices, such as syrup vehicles, is very challenging due to the incompatibility of the matrices with modern instruments [e.g., high-performance liquid chromatography (HPLC)]. Direct injection of such samples into analytical systems often contaminates and even damages the systems. Moreover, the concentrations of target analytes are often too low to allow direct detection of analytes even with advanced detection systems. A sample preparation step, including clean up and possible enrichment, is usually required prior to introduction of

samples into analytical instruments (1).

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are two popular sample preparation methods used in most analytical laboratories. LLE utilizes a solvent that is immiscible with sample matrices to extract target analytes while SPE utilizes an extraction phase adsorbed on fine particles packed in a cartridge to retain target analytes, which are eluted separately from interfering components. However, both of these methods are time-consuming (2).

Ultra-filtration is a pressure-driven membrane separation and purification process. It is being widely used in pharmaceutical and food industries and in water and waste water treatment processes (3–11). Ultra-filtration membranes may be considered porous membranes that are characterized by their nominal molecular weight limit (NMWL) (12). Molecules with molecular weights larger than NMWL are retained while those with molecular weight smaller than NMWL pass through the membrane. Separations of molecules with different molecular weights are thus achievable. Cross-flow filtration is widely used in the industry to provide continuous separation and purification of various products (13). However, centrifugal ultra-filtration is easier to operate in analytical laboratories for small sample sizes.

Syrup vehicles are complex mixtures, consisting of a variety of excipients such as diluters, solubilizers, stabilizers, and preservatives. Direct injection of diluted syrup vehicle into an HPLC system resulted in rapid reduction of column efficiency, split peaks, and severely reduced column lifetimes. It was suspected that high molecular weight polymers caused these problems by adsorbing onto the stationary phase of the HPLC column. This work investigated the use of centrifugal filter devices to remove high molecular weight interfering ingredients from the matrix to allow quantification of APIs in the syrup by HPLC. Unlike the traditional use of ultra-filtration to retain and concentrate high molecular compounds like proteins for subsequent analysis, this work was focused on the analysis of small molecular compounds in the filtrate after removing high molecular weight interferences with the filter devices. This work was extended to develop a method for the determination of xanthan gum based on measurement of viscosity.

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## Experimental

### Materials and chemicals

L-arginine, amphotericin B, HPLC-grade methanol, HPLC-grade triethylamine, and 1-hexanesulfonic acid sodium salt were purchased from Aldrich (Oakville, Ontario, Canada). Sodium phosphate monobasic, monohydrate, was purchased from EMD Chemicals (Gibbstown, NJ). Syrup vehicle (Ora Sweet SF) was purchased from Paddock Laboratories (Minneapolis, MN). Centrifugal filter devices (Amicon Ultra-15, 10K NMWL) were purchased from Millipore (Billerica, MA).

Sucrose, sorbitol, sodium saccharin, strawberry flavor, citric acid, sodium citrate, methylparaben, propylparaben, and xanthan gum were provided by The Pharmaceutical Development Service Department of Patheon (Toronto, Canada).

### Preparation of standard and sample solutions

#### Preparation of standard stock solutions

51.50 mg of L-arginine was accurately weighed into a 50-mL volumetric flask, diluted to volume with the mobile phase, and mixed well.

88.15 mg of amphotericin B was accurately weighed into a 250-mL volumetric flask, and about 50 mL of methanol was added. The mixture was sonicated until the amphotericin B was dissolved, diluted to volume with methanol, and mixed well.

#### Preparation of standard working solutions

5.0 mL of standard stock solutions were pipetted into a 50-mL volumetric flask and diluted to volume with the mobile phase for L-arginine or with methanol for amphotericin B.

#### Preparation of sample solutions

5.0 mL of standard stock solutions and 3.0 mL of the syrup vehicle (Ora Sweet SF) were pipetted into a 50-mL volumetric flask and diluted to volume with the mobile phase for L-arginine or with methanol for amphotericin B.

### Centrifugal ultra-filtration

Centrifugal filter devices were cleaned by centrifugation with 10 mL of deionized water prior to immediate use. 10 mL of standard and sample solutions were transferred into centrifugal filter devices, and centrifugation was performed at 5000 rpm for 30 min.

### Determination of viscosity

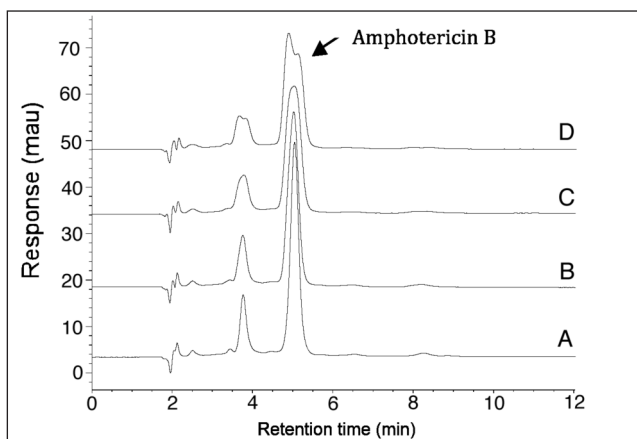
The viscosities of xanthan gum solutions were determined with Brookfield Digital Rheometer model DV-III Ultra (Brookfield Engineering Laboratories, Middleboro, MA). 8.0 mL of solutions were transferred into the sample chamber (13 RP) which was maintained at 25°C. A SC4-18 spindle was used, and its speed was set to 50 rpm.

### HPLC analysis

Agilent (Santa Clara, CA) 1100 HPLC system with data acquisition was utilized for the analysis. For the analysis of L-arginine, the separation was carried out on a YMC Pack C<sub>8</sub> column (S-5  $\mu$ m, 150 mm  $\times$  4.6 mm), purchased from YMC (Milford, MA) with a total analysis time of 10 min for standards and 30 min for samples. The column was maintained at 20°C. A mixture of buffer solution (50 mM 1-hexanesulfonic acid sodium salt, 25 mM sodium phosphate and 0.2% triethylamine, pH = 2.3), and methanol with the volume ratio of 75:25 was used as the mobile phase. The flow rate of the mobile phase was 1.0 mL/min, and the sample injection volume was 20  $\mu$ L. The effluent was monitored by UV detection at 210 nm. For the analysis of amphotericin B, the separation was carried out on a YMC Pack C<sub>8</sub> column (S-5  $\mu$ m, 12 nm, 150 mm  $\times$  4.6 mm) with a total analysis time of 20 min. The column was maintained at 25°C. A mixture of buffer solution (0.1 M sodium phosphate, pH = 3.0) and methanol with the volume ratio of 30:70 was used as the mobile phase. The flow rate of the mobile phase was 1.0 mL/min, and the sample injection volume was 20  $\mu$ L. The effluent was monitored at 405 nm.

## Results and Discussion

Attempts at quantification of a drug substance in a commercialized syrup focused on diluting the syrup to 10 to 20 times and subsequently injecting the diluted syrup solution into an HPLC system. The first injection resulted in good separation and quantitation. However, peak shape (tailing factor, *T*, and peak width,  $W_{1/2}$ ) and column efficiency (theoretical plate number, *N*) deteriorated rapidly from the second injection and afterward. Figure 1 illustrates the deterioration of the peak shape and column efficiency. With the injection of less diluted syrup solution (four times dilution), a brand new column was destroyed after the first injection. Because the injected solution had been filtered through a 0.45- $\mu$ m filter and the column pressure did not increase significantly, it was unlikely that particulates had blocked the frits. After examining the composition of the syrup, attention was drawn to the presence of xanthan gum, a water-soluble polymer widely used as stabilizer and rheology modifier in the pharmaceutical industry. The typical molecular weight range of xanthan gum is  $2 \times 10^6$  to  $5 \times 10^7$  (v). Adsorption of xanthan



**Figure 1.** Chromatograms of consecutive injections of a diluted syrup sample spiked with amphotericin B (retention time: 5.1 min): (A) First injection ( $T = 1.0$ ,  $N = 2613$ ,  $W_{1/2} = 0.228$  for the major peak amphotericin B unless otherwise specified), (B) second injection ( $T = 0.95$ ,  $N = 1537$ ,  $W_{1/2} = 0.302$ ), (C) third injection ( $T = 0.96$ ,  $N = 946$ ,  $W_{1/2} = 0.413$ ), and (D) fourth injection (peak split).

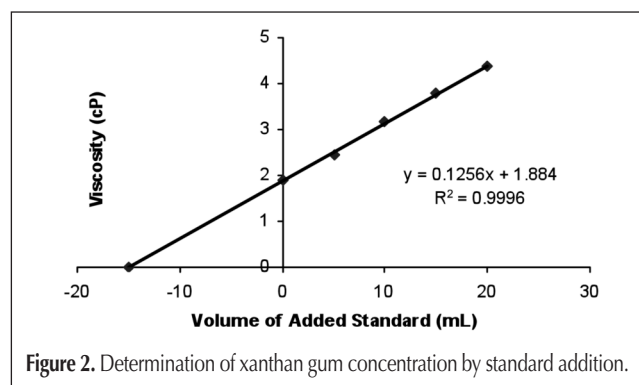
gum on the packed particles of the column would be extremely difficult to eliminate under normal HPLC conditions, thus deteriorating the column efficiency or even damaging the column. It was therefore critical to separate xanthan gum from sample solutions prior to injection.

### Determination of xanthan gum in the syrup

Xanthan gum is a long chain polysaccharide composed of the sugars glucose, mannose, and glucuronic acid. Xanthan gum is widely used as a thickener, stabilizer, emulsifier, and foaming agent in food and pharmaceutical industry. As demonstrated previously, adsorption of xanthan gum onto the stationary phase destroys columns. In order to perform quantitative analyses of active pharmaceutical ingredients of interest in a syrup sample, it is important to remove xanthan gum from the syrup and to analyze the concentration of xanthan gum in the syrup.

A series of xanthan gum aqueous standard solutions were prepared by serial dilution of a stock standard solution and the viscosities of each standard solution were determined. It was found that the viscosity changed linearly with the xanthan gum concentration from 12.6  $\mu\text{g/mL}$  to 1.26  $\text{mg/mL}$  (linear correlation coefficient: 0.995,  $a = 0.0232$ ,  $b = 1.2999$ ). To estimate the concentration of xanthan gum in the syrup, two calibration methods were used. The first one was standard addition. 5 mL of the syrup was diluted to 100 mL, and the solution was used as the sample. The sample was spiked with a standard solution with 5 mL increments. The viscosity of the sample and the spiked samples was determined. Figure 2 presents the results from which the concentration of xanthan gum was estimated as ca. 3.8  $\text{mg/mL}$  (equivalent concentration, assuming the xanthan gum used in the syrup is the same as the xanthan gum used as the standard). The second calibration method was external calibration. An analytically prepared syrup vehicle, consisting of sucrose, sorbital, sodium saccharin, strawberry flavor, citric acid, sodium citrate, methylparaben, propylparaben, and deionized water, was used as the matrix. The viscosities of the standard xanthan gum solutions prepared in the syrup vehicle were determined. The repeatability from the six standard solutions (0.08%, w/w) was 1%. The accuracy was within 95.0% to 105.0% at two concentration levels (0.04% and 0.2%, w/w). The linear correlation coefficient was 0.994 from concentrations of 0.02% to 0.4% (w/w). It was, therefore, demonstrated that both methods are suitable for quantitative analysis of xanthan gum in the syrup vehicle.

With such a high concentration of xanthan gum in the syrup, injection of even diluted samples would destroy the column



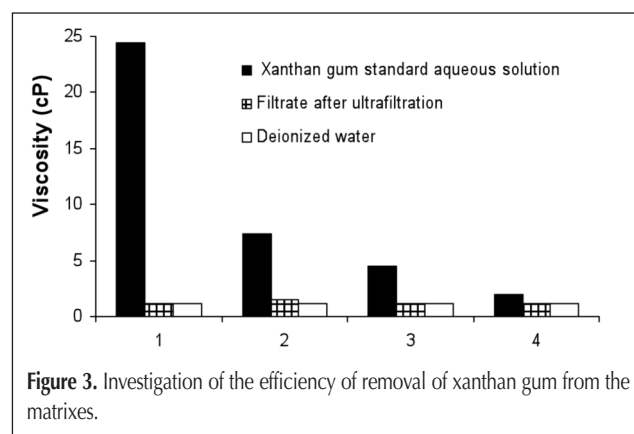
quickly. To investigate the efficiency of removing the interfering xanthan gum from the matrix by the use of ultra-filtration, a few experiments were carried out.

The first experiment was to determine the viscosity of xanthan gum standard aqueous solution before and after ultra-filtration. The experiment was performed at four concentration levels: 1261.5  $\mu\text{g/mL}$ , 267.5  $\mu\text{g/mL}$ , 126.15  $\mu\text{g/mL}$ , and 26.75  $\mu\text{g/mL}$  labeled as 1, 2, 3, and 4 in Figure 3. For each concentration level, the first column represents the viscosity of the xanthan gum standard aqueous solution; the second column represents the viscosity of the filtrate of the xanthan gum standard aqueous solution after filtration; the third column represents the viscosity of deionized water (see Figure 3 legend). The results demonstrate that the viscosity of the filtrates of the xanthan gum standard aqueous solutions after ultra-filtration decreased to the same value of deionized water, which means that most xanthan gum was efficiently retained in the filter after ultra-filtration. The second experiment was to determine the viscosity of diluted syrup (20 times dilution) and the same diluted syrup spiked with xanthan gum standard before and after ultra-filtration. The results from this experiment demonstrated that the viscosity of both solutions decreased significantly to the same value after ultra-filtration. This suggested that even with the presence of other matrix components, xanthan gum could be separated from the matrix with the filter device. Unfortunately, the concentrations of xanthan gum in the filtrates were much lower than the limit of quantitation of the previous two methods developed for the analysis of xanthan gum. Highly sensitive techniques, such as chromatography, are required to determine the concentrations of xanthan gum in the filtrate.

### Analysis of L-arginine and amphotericin B in the syrup

Dialysis has long been used for elimination of proteins from bio-samples. Dialysis membranes separate molecules according to their shape and size (14–16). However, hours and even days are required to reach an equilibrium. Ultra-filtration accelerates the separation in minutes by the use of high pressure. Special designed centrifugal ultra-filtration filter devices are widely used to concentrate high molecular weight compounds (e.g., proteins) after filtering low molecular compounds and solvents. In this study, the devices were investigated for the elimination of xanthan gum, while filtrates were collected for analysis.

L-arginine is an amino acid. It is a small molecule compound



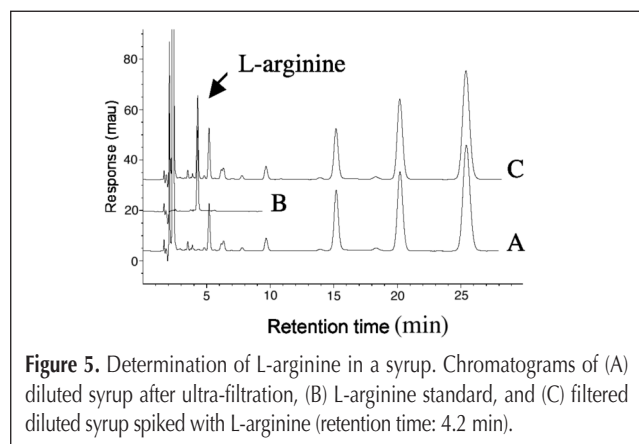
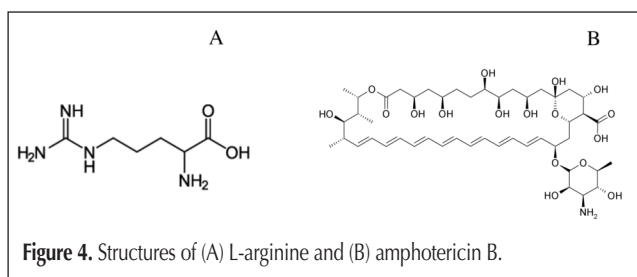
(molecular weight = 174) with high hydrophilicity (Figure 4). Its syrup samples were prepared by spiking L-arginine standard solutions into the syrup. The prepared samples were then diluted 10 times with the mobile phase prior to ultra-filtration. The filtrates were collected for HPLC analysis. Figure 5 shows the chromatograms of the syrup, L-arginine standard, and spiked samples. There were no significant interfering peaks from the syrup at the retention time of L-arginine, so quantitation for L-arginine was unambiguous. The mean recovery of three L-arginine samples was  $100.3 \pm 0.8\%$ , which implied that the filter devices neither retained nor adsorbed L-arginine. The reproducibility (%RSD, peak area) of L-arginine in the filtered samples was less than 0.5% for 20 injections, and the peak shape was maintained through out the whole analysis. The constant injection and peak shape could not be achieved without the removal of high molecular interfering compounds such as xanthan gum from the samples by ultra-filtration. The preliminary study suggested that the use of ultra-filtration as the sample preparation tool was feasible for quantifying L-arginine in the syrup.

The analysis of L-arginine appeared to be a simple case because the interaction of L-arginine with the matrix and the filter devices was negligible. A more complicated situation is the analysis of amphotericin B in the syrup. Amphotericin B is a larger molecular compound (molecular weight = 924) with high hydrophobicity. The interaction of amphotericin B with the matrix and the filter devices would be expected to be higher than that of L-arginine with the matrix and the filter devices. It was observed that amphotericin B was not detected when a mixture of methanol and water (20:80, v/v) was used as the diluent. The recovery of amphotericin B increased to only 26% when the volume ratio of methanol to water in the diluent was increased to 70:30. The low recovery of amphotericin B was easily ascribed to the adsorption of amphotericin B on the membranes of the filter devices and

maybe the retaining of amphotericin B with high molecular weight polymers. Because amphotericin B is a yellow compound, the change of the white color of the membrane of the filter devices to yellow clearly demonstrated the adsorption of amphotericin B on the membrane. To completely recover amphotericin B, the strength of the diluent was further increased. Only when 100% methanol was used as the diluent did the recovery of amphotericin B reach from 98.5% to 100.6% at different concentration levels. Further investigation demonstrated that the reproducibility (%RSD, peak area) for the filtered samples was 0.3% for 30 injections. The linear range was 0.28–28.2  $\mu\text{g/mL}$ . The limit of detection (LOD) was estimated to be 0.06  $\mu\text{g/mL}$  [signal-to-noise ratio (S/N) = 4.1]. The limit of quantification (LOQ) was estimated to be 0.28  $\mu\text{g/mL}$  (S/N = 22.7). The %RSD of the recovery of three samples at the concentration of LOQ was 1.7%. These results suggested that the developed method was suitable for quantifying amphotericin B in the syrup.

Finally, it was interesting to notice that precipitates were observed when the samples were diluted with methanol. It was assumed that the precipitates were salts, which are not soluble in methanol. But we learned later that xanthan gum is not soluble in methanol. This phenomenon suggested an alternative approach to quantifying amphotericin B in the syrup: That is, dilute the samples with methanol, centrifuge the diluted samples in centrifuge tubes, dilute the supernatant with water to decrease the strength before injection, and inject into HPLC.

It was found that amphotericin B was completely recovered (recovery = 98.8%,  $n = 3$ ) using this method while the column efficiency and the peak shape were not jeopardized. This seemed to be simpler than the ultra-filtration method. However, it has to be realized that this is a case-by-case situation. For example, for the case of L-arginine, ultra-filtration might be simpler because dilution prior to injection was not required. In addition, some other polymers in other syrups may not be precipitated by methanol, etc. Nevertheless, this is still an interesting method for sample preparation.



## Conclusion

The preliminary results demonstrated that the developed sample preparation method was suitable to remove interfering high molecular weight components from syrup vehicle by the use of the filter devices, making possible quantitative analysis of APIs in the complex matrix by HPLC. The sample preparation procedure was simple and efficient. The recoveries of target analytes are mostly within 98–102% under optimized extraction and centrifugation conditions. The drawback of the method is that analytes in the filtrate are not concentrated, which might prevent its use for the analysis of analytes with extremely low concentrations in the samples. In these cases, enrichment of analytes are generally required to meet instrumental detection limits.

A viscosity method was developed and validated for the determination of xanthan gum. The validation challenged the reproducibility, accuracy, and linearity, and it demonstrated that the method is suitable to determine the xanthan gum in a syrup with the concentration of xanthan gum from 0.02% to 0.4% (w/w).

Future works will include further characterizing the filter devices, investigating robustness of the method, and applying the method for the analysis of analytes in other complex matrixes containing interfering ingredients of high molecular weight molecules.

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