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# Application of RP-HPLC for Determination of the Dissociation Constants of Rosuvastatin Calcium

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## *Introduction*

Rosuvastatin calcium (RC), is a member of a drug group called statins which are used to lower hypercholesterolemia and to prevent cardiovascular diseases, a synthetic lipid-lowering agent, inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) which is a catalyst in the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis in liver[1, 2]. The empirical formula of RC is $(C_{22}H_{27}FN_3O_6S)_2Ca$ and its chemical structure is given in Figure 1.

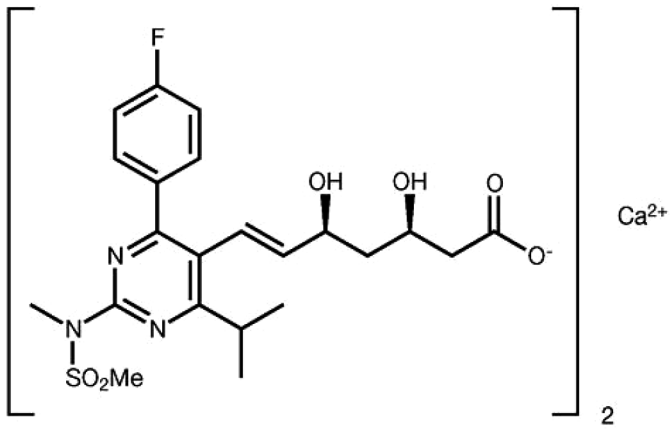


**Figure 1**
Chemical structure of RC

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It is well known for a long time that the dissociation constant is one of the most important physicochemical properties of a pharmaceutical compound to understand the extent of dissociation in the system of body[3-5]. The pH, basically the hydrogen ion concentration, influences the physical and chemical properties associated with absorption, such as solubility of the drug, lipid/water partition coefficient, electrical membrane potential, permeability of the membrane, and chemical reactivity[6-8]. In addition, development of dosage forms are directly related with the dissociation constant of a drug due to the fact that adjustment of the pH of a dosage form provides the optimum bioavailability[9-11]. The drugs are generally either weak acids or weak bases and most of the drugs are absorbed by passive diffusion of the nonionized moiety. Therefore, it is a great importance to know if a drug is in its ionized or nonionized form at a certain pH. Briefly, the properties of a drug molecule inside the body depends on the dissociation constant of the drug[12]. Hence, it is important to calculate properly the drug dissociation constant value of a pharmaceutical compound. The Henderson–Hasselbalch equation (Eq 1) describes the derivation of pH as a measure of acidity (using pKa, the negative log of the acid dissociation constant) in biological and chemical systems.

$$\mathrm{pH} = \mathrm{pK}_a + \log\left(\frac{[\mathrm{A}^-]}{[\mathrm{HA}]}\right) \qquad \text{(Eq 1)}$$

Here, [HA] is the molar concentration of the undissociated weak acid, [A–] is the molar concentration of this acid's conjugate base and pKa is -log (Ka) where Ka is the acid dissociation constant.

Several analytical methods was proposed for determination of the pKa values of drugs. These methods include spectrophotometry[13-16], chromatography[17-22], capillary electrophoresis[23-26] and potentiometric titrations[27-29]. Since the whole biochemical processes occur in aqueous solutions in biological fluids, the determination of acid dissociation constant in aqueous solutions through HPLC is a fitting alternative to the other techniques. Small amount of sample is enough to study with HPLC and somewhat impurity in sample and low solubility in water are not problem to determine dissociation constants in HPLC analysis.

The aim of this study is to calculate the dissociation constants of RC through using HPLC and by that way to show the capability of HPLC to determine the dissociations constants of polyprotic compounds.

## *2. Materials and Methods*

### 2.1. Chemicals and Apparatus

Rosuvastatin calcium used in this work was obtained from Reddy's Lab. Sodium dihydrogen phosphate (Merck) was used in mobile phase to prepare the buffer solutions. Acetonitrile (Sigma Aldrich) was used in mobile phase as organic phase. Sodium hydroxide (Rieldel-de Haen) was used to adjust the pH of mobile phases. Uracil (Sigma Aldrich) was used to determine the dead volumes. Water obtained from the Milli-Q water system (Barnstead, USA) was used to prepare the buffer solutions.

The RPLC method was performed on a Shimadzu CBH-20 A HPLC system (Shimadzu Technologies, Kyoto, Japan) equipped with an UV visible detector (Shimadzu SPD-M20A) and a column (ACE C18 column 125x4,6mm, 5 μm). pH adjustments of the mobile phases were carried out with a Mettler Toledo MA 235 pH/ion analyzer (Schwerzenbach, Switzerland) using M-T combination pH electrode.

### 2.2. Standard Stock Solutions and Buffer Solutions

Standard stock solution of RC was prepared by dissolving 10 mg of RC with methanol in a 10 mL of volumetric flask. The mobile phase was acetonitrile : buffer (40 mM phosphate buffer in various pH values) (40:60 v/v) solutions and they were prepared by dissolving 1.38 gram of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) in 100 mL of water and 100 mL of acetonitrile mixture and then adjusting the pH from 3.04 to 6.18 by adding 0.1 M sodium hydroxide solution. The final volume of the buffer solutions was filled up to 250 mL by adding water.

### 2.3. Chromatographic procedure

Three analyte injections were performed at each pH value of the mobile phases. The injection concentration of RC was 20 μg $mL^{-1}$ and the volume of the samples injected into the column was 20 μL for each run. The dead volume ($t_0$) was measured by injecting uracil solution before each injection. The flow rate was 1 mL $min^{-1}$ while the temperature was 25 $^{\circ}$C. Rosuvastatin shows maximum absorbance at 205 nm where the UV detection was performed.

## 3. Result and Discussion

Acidic and basic molecules could be either in ionized or in nonizonized forms according to the pH value of the matrix solution. The capacity factor of an analyte peak in a RP-HPLC experiment is directly related with the polarity of the analyte. Since, the polarity of an acidic or basic analyte is related with the ratio of ionized and nonionized forms in a matrix, the capacity factor of an analyte could be changed by modifying the pH values. Thus, it could be observed a sigmoidal relationship between the capacity factor and the pH of the mobile phase. Another parameter affecting the capacity factor of an analyte is the organic solvent ratio in the mobile phase for a RP-HPLC experiment. In this study, increasing the acetonitrile ratio of the mobile phase decreased the capacity factor of RC dramatically. Therefore, the organic solvent ratio kept constant while the pH values were changing. Optimization of the acetonitrile ratio was performed with initial studies and acetonitrile : buffer 40:60 (v/v) mixture was considered as the optimum ratio due to the fact that even in basic pH values where the RC is more ionized, the analysis time was more than 4 minutes and the retention time of RC covered a wide range by modifying pH of the mobile phase. Figure 2 presents the variation on the elution time of RC by changing the pH value and Figure 3 shows the relationship between the capacity factor and pH of the mobile phase.

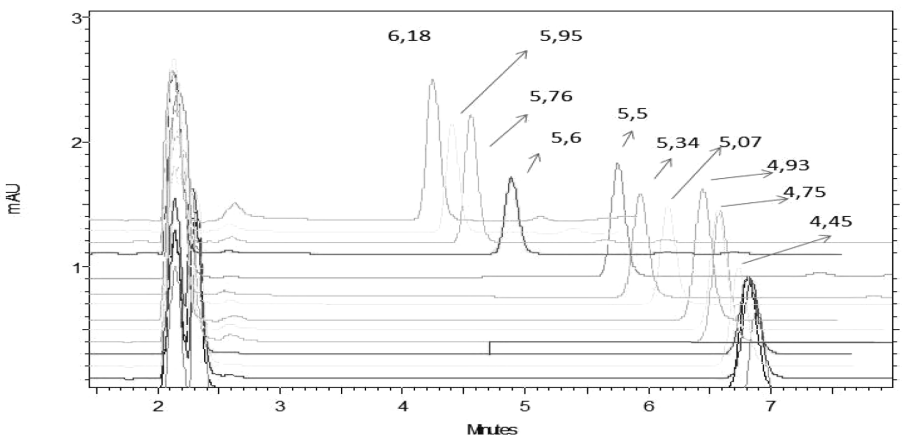


**Figure 2**
Representative chromatograms of RC taken under different pH values of the mobile phase.

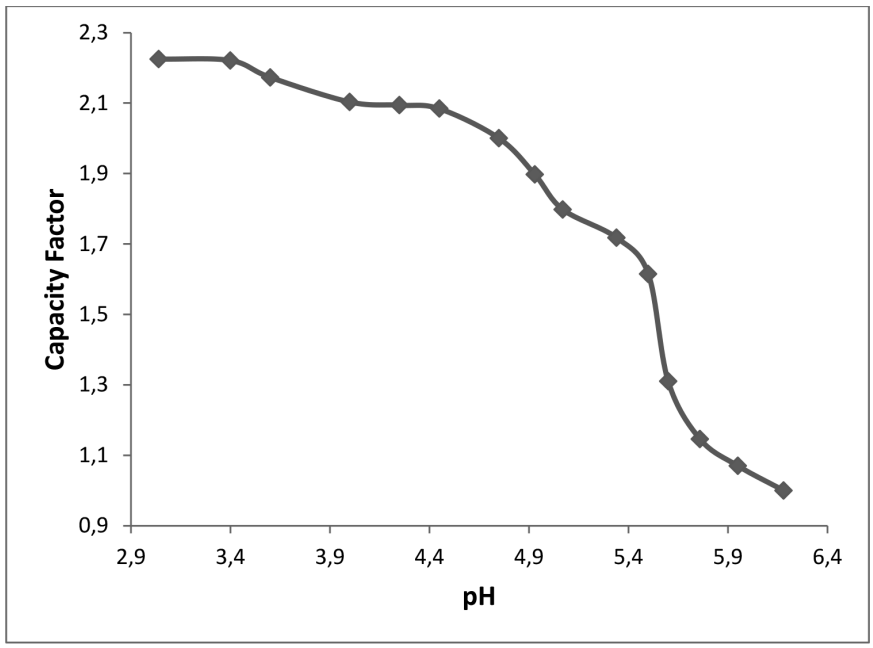


**Figure 3**
Figure 3. Variation of the capacity factor with the pH of the mobile phase.

RC has three ionization constants. In literature, it is indicated that the first ionization is about carboxylic acid group of RC and it is at pH 3.8. The second ionization is at pH 4.6 and it is related with the amine group. The last ionization is at pH 5.5 and it depends on amine groups as well as the second ionization step. In order to observe the ionization steps of RC, the pH values of mobile phase were adjusted between 3.04 - 6.18 in which limits the HPLC column is stable. The RC standard solutions (20 μg $mL^{-1}$) were injected as three replicates for each pH values of the mobile phase. The capacity factor of RC peak for each pH values were calculated with the average of three replicate injections by using uracil as the dead volume ($t_0$) indicator. In the Figure 3, It could be observed three sigmoidal curves which are about the relationship between the capacity factors and the pH values of the mobile phases. These sigmoidal curves were focused in Figure 4, Figure 5 and Figure 6 for pH 3.04 - 4.45, pH 4.00 - 5.34, and pH 5.05 - 5.60, respectively. By using these graphs, the cross points were drawn in order to find three acid dissociation constants of RC. Thus measured acid dissociation constants of RC were found to

be 3.7, 4.8 and 5.6, respectively. The results show that RP-HPLC method could be used for determination of the dissociation constants of acidic or basic active pharmaceutical ingredients even they are triprotic acids or bases. It has been proposed for a long time that potentiometric titration is one of the unique method to determine the dissociation constants of pharmaceuticals. The disadvantage of potentiometric titrations is that the concentration of the pharmaceutical solution must be high enough to indicate the equation point of analysis but it is not always possible to work with high concentrations if the compounds are slightly soluble in aqueous solutions, on the other hand, sometimes it is really hard to supply such high amounts of pharmaceutical standards. Since most of the pharmaceuticals show that ultraviolet absorbance, and the maximum absorbance values show bathochromic and hypochromic shifts depend on the pH value of the matrix, spectrophotometric methods are capable

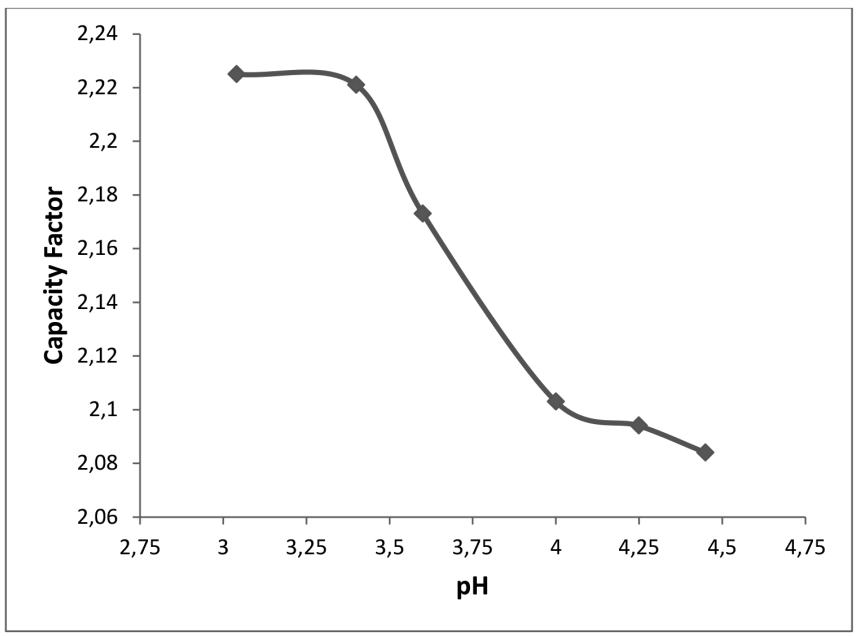


**Figure 4**
The first ionization step of RC (pH 3.04 - 4.45)

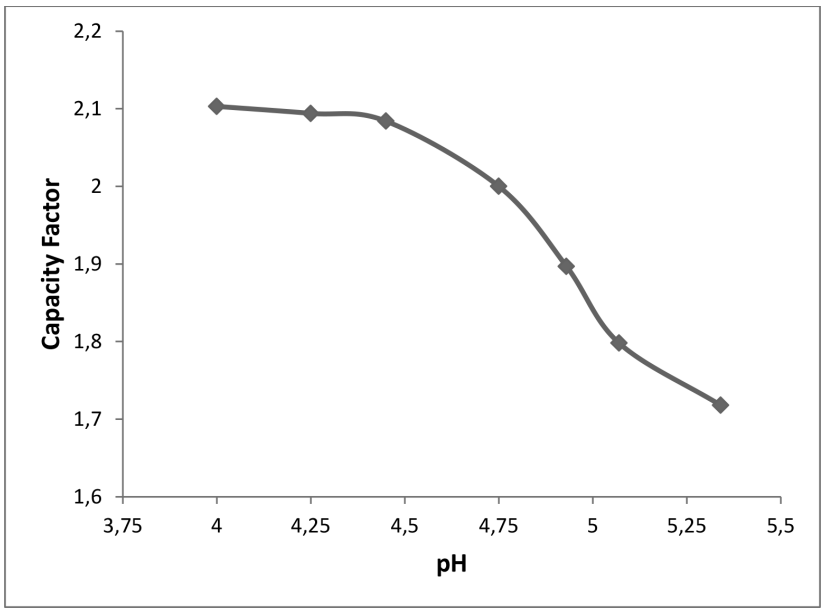


**Figure 5**
The second ionization step of RC (pH 4.00 - 5.34)

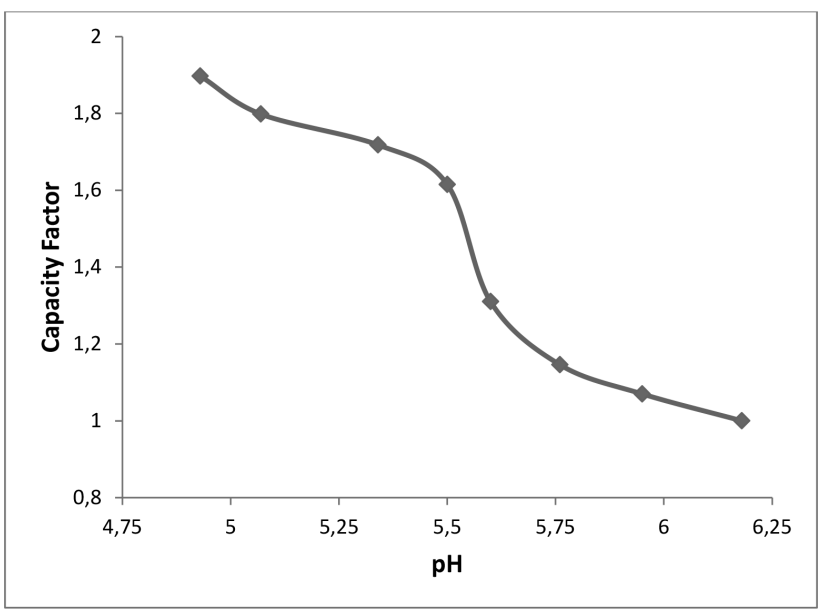


**Figure 6**
The third ionization step of RC (pH 5.05 -5.60)

of determining dissociation constants easily even for diluted solutions of most pharmaceuticals. The important point is that the shifts on maximum absorbance value could not be useful for triprotic acids or bases due to the fact that the precision for such a study is not enough to determine the three dissociation constants properly. Capillary electrophoresis (CE) methods make it easy to determine dissociation constants by working in various pH values and also there is no stability problem for a capillary in high or low pH values. However, it is known that the repeatability between the injections are poor in CE studies, and it causes an unknown random error on each analysis. The mechanical strength of newly developed HPLC columns cover a wide range of pH values such as from 1.0 to 11.0, and these columns provide working in a wide range of pH in comparison to the previous silica based C18 columns. It is believed that HPLC would be one of the unique technique for the determination of the dissociation constants of active pharmaceutical ingredients in the near future for its precision, accuracy and fast analysis time.

## *4. Conclusion*

It is always difficult to determine multiple dissociation constant values of a compound. This work shows that HPLC methods are reliable and simple for this purpose. Three dissociation constants of the basic drug RC were found by using HPLC. The sigmoidal relationships were found between the pH of the mobile phase and the capacity factor of the RC peaks. Experimentally found results are in harmony with the literature. Extracted data of this work can be used in other pharmalogical studies and this study is important to present the usage of HPLC for determination of the dissociation constants of a polyprotic compound.

## *Summary*

The acid-base dissociation constant (pKa) of a drug is a key physicochemical parameter influencing many biopharmaceutical characteristics. In this work, dissociation constant values of rosuvastatin calcium were determined by using RP-HPLC method. The elution of rosuvastatin

calcium was achieved by using C18 column (ACE 125x4,6 mm, 5 μm) while the mobile phase was acetonitrile: phosphate buffer (40 mM) (50:50 v/v) at various pH values. The flow rate was 1 mL $min^{-1}$ and the injection volume was 20 μL. The detector was set at 205 nm wavelength. Dissociation constant values were obtained through the relationship between the capacity factors and the pH values of the mobile phases. Thus, three ionization steps were properly observed from the sigmoidal curves and the dissociation constant values were found to be 3.7, 4.8 and 5.6, respectively.

*Keywords:* Rosuvastatin, HPLC, pKa, dissociation constant

## *Özet*

### RP-HPLC' nin Rosuvastatin Kalsiyumun İyonlaşma Sabitlerinin Bulunmasında Kullanımı

Bir ilaç molekülünün iyonlaşma sabiti (pKa), o ilacın biyofarmasötik davranışını etkileyen en önemli fizikokimyasal parametrelerden biridir. Bu çalışamda, rosuvastatin kalsiyumun iyonlaşma sabiti değerleri RP-HPLC kullanılarak tespit edilmiştir. Rosuvastatin kalsiyum elüsyonu C18 kolon (ACE 125x4,6 mm, 5 μm) kullanılırken farklı pH değerlerine ayarlanmış asetonitril:fosfat tamponu (40 mM) (50:50 h/h) hareketli faz olarak kullanılarak gerçekleştirilmiştir. Akış hızı 1 mL $dak^{-1}$ olarak ayarlanmıştır ve enjeksiyon hacmi 20 μL' dir. Dedektör 205 nm dalgaboyunda ölçüm yapmıştır. İyonlaşma sabiti değerleri, kapasite faktörü ile hareketli faz pH değerelri arasındaki ilişkiden yola çıkılarak bulunmuştur. Böylece elde edilen sigmoidal eğrilerden rosuvastatin kalsiyuma ait üç farklı iyonlaşma sabiti değeri sırasıyla 3,7; 4,8 ve 6,6 olarak bulunmuştur.

*Anahtar kelimeler:* Rosuvastatin, HPLC, pKa, iyonlaşma sabiti

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