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Research Article

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Investigating the physicochemical properties of phenazopyridine hydrochloride using high-performance liquid chromatography and UV-visible spectrophotometry

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Received: 27 February 2018 / Revised: 26 April 2018 / Accepted: 27 April 2018

ABSTRACT: The purpose of this study is to develop high-performance liquid chromatography (HPLC) and ultraviolet-visible (UV-Vis) spectrophotometry methods for the determination of pKa, log P, and log D values of phenazopyridine hydrochloride (PHCl). For the HPLC analysis, the pH of the mobile phase was plotted against the capacity factor, and the pKa value was obtained from a sigmoidal relationship. Meanwhile, for the UV-Vis spectrophotometric study, the absorbance of PHCl was plotted against pH at 332 nm to obtain the pKa value. The log P and log D (at pH 2.0) values were then calculated based on the concentration of PHCl in the octanol/water phase. Using the HPLC method, the pKa value was established as 5.05 (± 0.15). Meanwhile, the spectroscopic technique afforded pKa values of 5.20 (± 0.22) and 5.17 (± 0.17) via a classical sigmoidal curve and the Albert and Serjeant method. The log D (at pH 2.0) and log P values of PHCl were 1.61 (± 0.16) and 2.62 (± 0.11), respectively. The obtained results are in good agreement with each other. The pKa, log P, and log D values of PHCl can be determined by HPLC and UV-Vis spectrophotometry with reproducible results.

KEYWORDS: Phenazopyridine hydrochloride; physicochemical properties; pKa; log P.

1. INTRODUCTION

A drug has to cross several semipermeable cell membranes, which act as biological barriers that selectively inhibit the passage of drug molecules, before they reach systemic circulation. Passive and facilitated passive diffusions, active transport, or pinocytosis are different ways for drugs to pass through the bimolecular lipid matrix of cell membranes, and the characteristics of cell membranes determine membrane permeability. Key physicochemical properties like hydrophobicity and lipophilicity are highly associated with the ability or inability of drug molecules to cross the membranes. Since the cell membrane is lipoid in nature, lipid-soluble and small molecules diffuse more rapidly across it compared to the passive diffusion of hydrophilic and large molecules [1,2].

Defining the physicochemical properties of any drug is a prerequisite in pharmaceutical research. As such, a number of experimental studies have been performed to establish the required information [3-8], and some in silico models have also been proposed [9,10]. The aim of the present study is to develop a set of analytical methods based on high-performance liquid chromatography (HPLC) and ultraviolet-visible (UV-Vis) spectrophotometry for the determination of pKa, log P, and log D values of drugs, or any synthesized pharmaceutical candidates. For this purpose, phenazopyridine hydrochloride (PHCl), a monoprotic drug with low solubility and high permeability (a Class II drug according to the Biopharmaceutics Classification System (BCS)), was selected as a model molecule. The dissolution of non-ionized phenazopyridine in aqueous medium is difficult owing to its physicochemical properties. The methodology proposed in this study could easily be adapted to any other monoprotic drugs or synthesized pharmaceutical candidates. In this study, the pKa results obtained from the HPLC and UV-Vis spectrophotometric methods were also compared.

How to cite this article: Celebier M, Kocak E, Dogan A, Altınoz S, Bascı NE. Investigating the physicochemical properties of phenazopyridine hydrochloride using high-performance liquid chromatography and UV-visible spectrophotometry. Marmara Pharm J. 2018; 22 (4): 528-535.

https://doi.org/10.12991/jrp.2018.94
ISSN: 1309-0801

2. RESULTS

The partition coefficient is the ratio of the concentrations of a substance that exists in an equilibrated system containing two immiscible liquids. Solubility differences of a compound in two different phases can be obtained using the partition coefficient. The partition coefficient of any ionizable solute can be measured in the aqueous and octanol phases, in which the pH value is adjusted for its non-ionized form. The concentration ratio of non-ionized solute in the solvents is calculated using the log P values. The log P value is a measure of lipophilicity (Eq. 1). Equation 1 is a general formula for the log P formulation of a water/octanol system (where, $C_{oc.}$ is the concentration in octanol, and $C_{union.water}$ is the concentration of the unionized form in water)

$$\log P_{oct/wat} = \log\left(\frac{C_{oc.}}{C_{union.water}}\right) \quad \text{(Eq. 1)}$$

The distribution coefficient is a ratio describing the sum of concentrations for all forms of a compound (ionized as well as non-ionized) in each phase, and it is also pH dependent. Thus, the pH of the aqueous phase should be buffered to a specific value. The distribution coefficient is also defined as a function of the total concentration ratio of the solute species in each phase (where, $\log D_{oct/wat}$ is the distribution coefficient between water and octanol, $C_{oc.}$ is the total concentration in octanol, and C_{water} is the total concentration of solute in water) (Eq. 2).

$$\log D_{oct/wat} = \log\left(\frac{C_{oc.}}{C_{water}}\right) \quad \text{(Eq. 2)}$$

For a non-ionized drug, log P is equal to log D at any pH value; however, the effective lipophilicity for any ionized drugs at any specific pH value is directly related to their pKa values (Eq. 3 for acidic drugs and Eq. 4 for basic drugs).

$$\log D = \log P - \log(1 + 10^{pH-pK_a}) \quad \text{(Eq. 3)}$$

$$\log D = \log P - \log(1 + 10^{pKa-pH}) \quad \text{(Eq. 4)}$$

Most drugs are weak organic acids or bases and thus, their non-ionized and ionized forms dominate according to their respective pKa values and the pH of the media. This relationship is described by the Henderson-Hasselbalch equation (Eq. 5) [11].

$$pH = pK_a + \log\left(\frac{[A^-]}{[HA]}\right) \quad \text{(Eq. 5)}$$

In Equation 5, [HA] is the molar concentration of the undissociated weak acid, $[A^-]$ is the molar concentration of this acid's conjugate base. The pKa is -log (Ka), where Ka is the dissociation constant for acids. The non-ionized form is usually lipid soluble (lipophilic) and diffuses readily across cell membranes. Meanwhile, the ionized form has low lipid but high water solubility (i.e., hydrophilic), along with high electrical resistance, and thus cannot penetrate cell membranes easily [12]. The biopharmaceutics classification system (BSC) separates drugs on the basis of their solubility and permeability, and is used as a guide by the U.S. Food and Drug Administration to predict intestinal drug absorption [13, 14]. Therefore, identifying the pKa and log $P_{oct/wat}$ values of a drug or any proposed potential active pharmaceutical ingredients is important in order to understand and classify their permeability behavior in the body.

The capacity factor of a drug in a reversed-phase HPLC system is related to its lipophilicity and pKa values, which then allows HPLC to be used for their measurements [15, 16]. In reversed-phase HPLC, an equilibrium exists between the analyte concentration in the lipophilic stationary phase and that in the hydrophilic mobile phase. The equilibrium constant, K, is the partition coefficient; it is defined as a ratio of the molar concentrations of an analyte in the stationary and mobile phases. Lipophilic molecules tend to be distributed in the lipophilic stationary phase instead of the hydrophilic mobile phase. The capacity factor (k') describes the migration rate of an analyte through the column. In reversed-phase HPLC, molecules with an increasing degree of hydrophobicity will typically spend more time on the lipophilic stationary phase, and a higher concentration of organic solvent will be required for their elution. If the organic solvent ratio is constant, the elution of the analyte will be associated with the proportion of its non-ionized form at the pH value of the mobile phase.

In this study, the initial HPLC experiments were performed to optimize the organic solvent ratio in the mobile phase. At basic pH values, PHCl exists as its non-ionized form – phenazopyridine, and would be retained longer on the lipophilic C18 stationary phase. PHCl was eluted at 24 min using a phosphate buffer:ACN (70:30 v/v) mixture at pH 5.8 as the mobile phase (Figure 1).

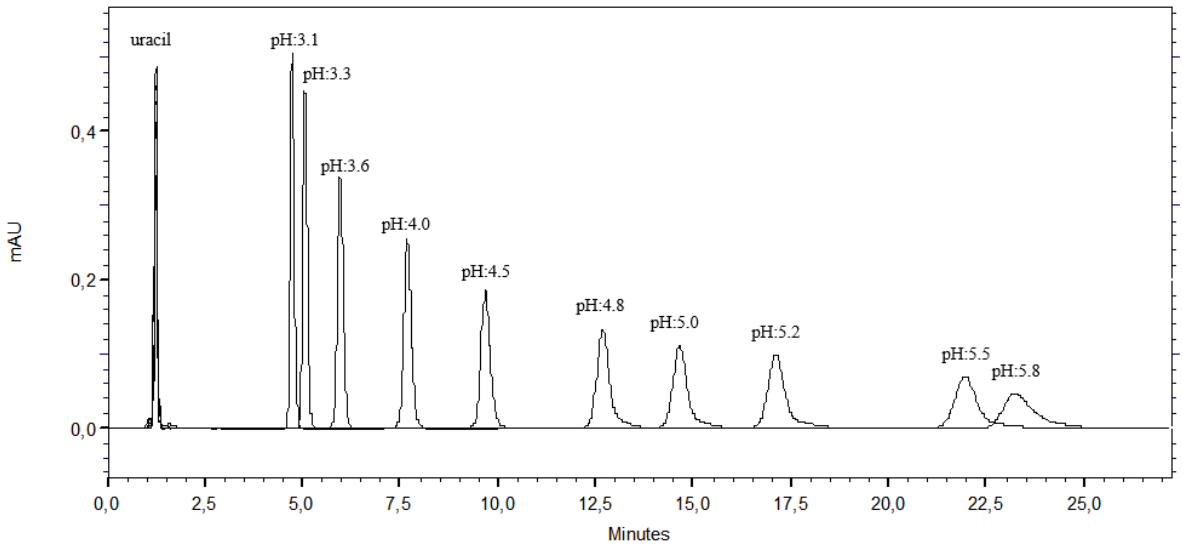

Figure 1. Representative overlaid chromatograms of PHCl obtained under optimum conditions at various pH values (pH 3.1 – 5.8). Experimental conditions: ACE C_{18} (125 × 4.6 mm ID, 5 µm) column, phosphate buffer: ACN (70:30 v/v) mixture at pH 3.1, 3.3, 3.6, 4.0, 4.5, 4.8, 5.0, 5.2, 5.5, and 5.8 as the mobile phase, flow rate: 1 ml min^{-1}, injection volume: 20 µL, and detection wavelength (λ): 332 nm.

When the pH of the mobile phase was plotted against k', a sigmoidal relationship between pH and the capacity factor was obtained (Figure 2), and the pKa value of PHCl was found to be 5.05.

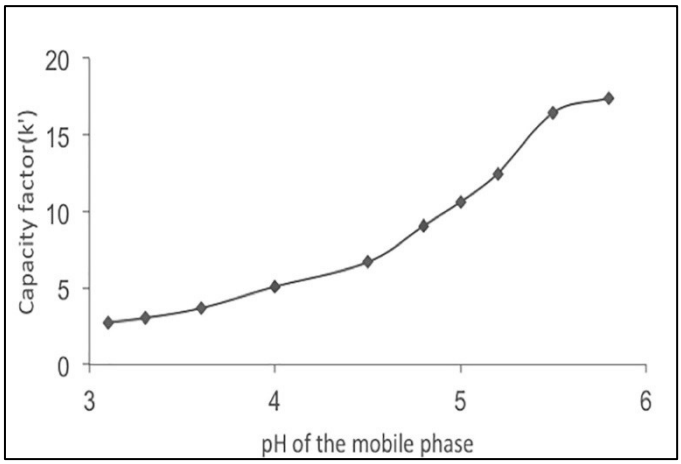

Figure 2. Plot of capacity factor (*k'*) values as a function of pH.

UV-Vis spectrophotometry is another technique used to determine the pKa value of PHCl in the present study. The absorption of organic compounds (including most drugs) is based on the transitions of n or π electrons to the π* excited state, and the absorption peaks for these transitions fall in the 200 – 700 nm range, which is an experimentally convenient spectral region. The referred transitions require an unsaturated group in the molecule to provide the π electrons. The solvent in which the absorbing species is dissolved may have serious effects on the spectrum [20]. In this study, the spectra of PHCl in phosphate buffers that have the same ionic strength but different pH values (i.e., 3.5 – 8.3) were recorded. The absorption spectrum for PHCl responded to the changing pH values of the buffered aqueous media (Figure 3). The changes in the absorbance values are usually monitored using overlaid plots of the recorded spectra, and the largest change occurs when the acidity of the aqueous solution is equal to the pKa of the studied compound [17].

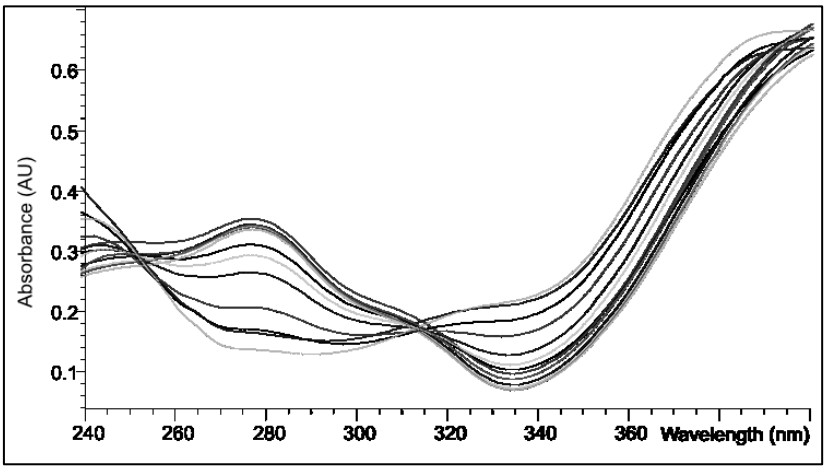

Figure 3. Representative overlaid spectra of PHCl under optimum conditions at various pH values (pH 3.5 – 8.3).

A clear hypochromic shift was observed at 332 nm whereas a hyperchromic shift was observed at 277 nm. When the absorbance of PHCl was plotted against pH at 332 nm, a sigmoidal relationship was obtained (Figure 4). When a sigmoidal curve fitting was applied, the pKa value of PHCl was found to be 5.20.

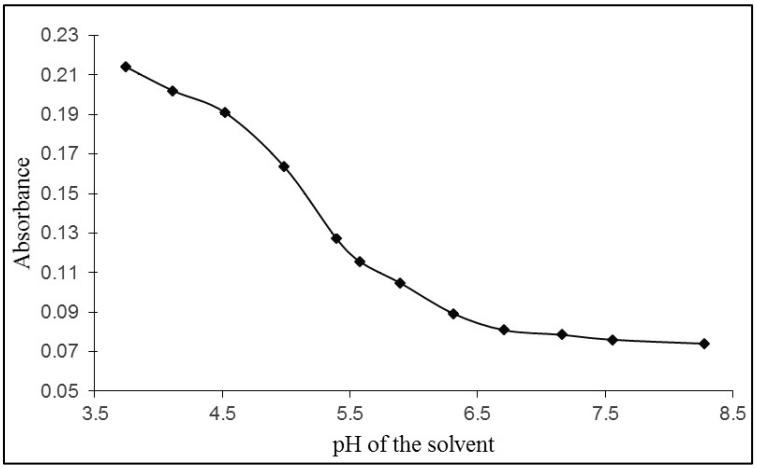

Figure 4. Plot of absorbance values at 332 nm as a function of pH.

When the Albert-Serjeant procedure was applied to the data collected from the UV/Vis spectrophotometry study (in the pH range of 4.5 – 7.2), a linear regression was obtained, where y = -0.7288x + 3.8215, R^2=0.9989. The intersection point of the linear regression line with the y-axis at zero gives the pKa of PHCl, which was found to be 5.17.

To determine the log P of PHCl in this study, its log D value at pH 2.0 (at which it is fully ionized) was used. The log D value of PHCl was found using the percentage amount of PHCl in the organic and aqueous phases. The log D value of PHCl was 1.61, resulting in a log P value of 2.62 (see Eq. 4).

PHCl is chemically designated as 2,6-pyridinediamine-3-(phenylazo) monohydrochloride. The phenazopyridine molecule has two hydrogen accepting amine groups, and it exists in its ionized form at acidic pH values. In this study, the HPLC column worked optimally in the stable pH range of 3.0 – 6.0, and the extreme change in the lipophilicity of PHCl could be followed by the retention time shifts that occurred at various pH values of the mobile phase. Thus, it is easy to see the relationship between the log D of PHCl and the pH of a medium (Table 1).

Table 1. Physicochemical properties of PHCl (n=3).

Physicochemical Property	pK_a			Log D at pH 2.0	Log P
Method	HPLC	UV-Vis Spect.	Albert and Serjeant Method	UV-Vis Spect.	UV-Vis Spect.
Value	5.05 (±0.15)	5.20 (±0.22)	5.17 (±0.17)	1.61 (±0.16)	2.62 (±0.11)

The difference observed between pK_a values with spectrophotometric, potentiometric and chromatographic methods was statistically insignificant (t-Test, $p > 0.05$).

3. DISCUSSION

To the best of our knowledge, there is currently no analytical method that describes the determination of the pKa, log P, and log D values of PHCl together and all experimentally in the literature with simple chromatographic, spectrophotometric and potentiometric instruments. In terms of potentiometric determination usage of Albert-Serjant equation is also alternative to the papers. The papers published stating the pKa before mostly use software supported pKa determinations which are considered to be predicted values. Our study puts forward the benefits of experimental findings which should be considered to be real values. Also our work is conducted with simple experimental instruments which would be find in any laboratory without the need of expensive predictor softwares other than the log D experiments performed with hyphenated techniques [18-20]. In this study, we developed a set of analytical methods to determine the aforementioned parameters using PHCl as a model drug.

4. CONCLUSION

This study was carried out to gain a better understanding of the physicochemical properties of PHCl. It is the first instance that demonstrates the development of spectroscopic and chromatographic methods to determine the pKa, log P, and log D values of PHCl. With these known physicochemical parameters, phenazopyridine can now be used more reliably in many pharmaceutical applications. Moving forward, our group will use the same analytical techniques to analyze other drugs and drug candidate molecules.

5. MATERIALS AND METHODS

5.1. Chemicals

$NaH_2PO_4.2H_2O$ and NaOH were purchased from Merck (Darmstadt, Germany). $Na_2HPO_4.H_2O$, uracil, acetonitrile (ACN), methanol (MeOH), and 1-octanol were obtained from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were of HPLC grade. HPLC grade water obtained from the Milli-Q water system (Barnstead, USA) was used for the preparation of the mobile phase, standard solutions, and buffers.

5.2. Instrumentation

The HPLC system consisted of a Spectra-SYSTEM P2000 gradient pump, a SpectraSYSTEM SCM 1000 degasser, a Rheodyne manual injector with a 20 µL loop, and a SpectraSYSTEM UV2000 detector (Thermo Separation Products, USA), which was controlled using a ChromQuest software. The wavelength detector was set at 332 nm. Compound elution was performed on a reversed-phase ACE C18 (125 × 4.6 mm ID, 5 µm) column (Aberdeen, Scotland).

Spectrophotometric measurements were carried out using an Agilent model 8453 UV-Vis spectrophotometer, equipped with a diode array detector (DAD) (190 – 1100 nm). UV spectra of the reference and sample solutions were recorded in the wavelength range of 200 to 600 nm using 1 cm quartz cells.

5.3. Solutions

5.3.1. *Standard stock solution of PHCl (500 µg ml^{-1} in water and in MeOH)*

The PHCl standard stock solution was prepared by dissolving 50 mg of PHCl in a 100 ml of volumetric flask using 70 ml of water. The solution was vortexed, ultrasonicated, and the volume was then filled up to 100 ml using water. The same procedure was used to prepare PHCl standard stock solutions in methanol.

5.3.2. Standard stock solution of uracil (500 µg ml^{-1})

The uracil standard stock solution was prepared by dissolving 50 mg of uracil in a volumetric flask containing 100 ml of water. The mixture was vortexed and sonicated to afford a clear uracil solution.

5.3.3. Phosphate buffer:ACN (70:30 v/v) solutions for HPLC studies (40 mM, pH 3.1 –5.8)

A total of 3.12 g of $NaH_2PO_4.2H_2O$ was first dissolved in 350 ml of water, before adding 150 ml of ACN. The pH of this mixture was adjusted to the desired value within the range of 3.1, 3.3, 3.6, 4.0, 4.5, 4.8, 5.0, 5.2, 5.5, and 5.8) using 0.5 M NaOH.

5.3.4. Phosphate buffer solutions for UV-Vis spectrophotometric studies (pH 3.5 – 8.3)

$NaH_2PO_4.2H_2O$ and $Na_2HPO_4.H_2O$ buffer solutions (each with a concentration of 0.1 M) were prepared by dissolving 15.6 and 13.8 g of $NaH_2PO_4.2H_2O$ and $Na_2HPO_4.H_2O$, respectively, in 1,000 ml of water. Mixing appropriate volumes of the stock buffer solutions with water (in a volumetric ratio range between 99:1 (v/v) and 1:99 (v/v)) to give a final solution volume of 100 ml provided a wide range of final pH values. The ionic strength of the respective solutions was calculated according to Eq. 6, where Ci is the molar concentration of ion i (M, mol L^{-1}), zi is the charge number of that ion, and the sum is taken over all ions in the solution.

$$I = \frac{1}{2} \sum_{i=0}^{n} Ci\ Zi^2 \qquad \text{(Eq. 6)}$$

Since $H_2PO_4^-$, HPO_4^{2-}, and Na^+ ions exist in the solutions, the ionic strengths of all prepared mixtures were calculated using the molar concentrations of these ions in 100 ml solutions. The ionic strengths of these solutions at different pH values were between 0.150 and 0.250. Appropriate amounts of these solutions were taken and diluted with water to a final volume of 25 ml for the preparation of solutions that had an ionic strength of 0.020. The pH values of the final solutions were in the range of 3.5 – 8.3 (i.e., 3.5, 3.7, 4.1, 4.5, 5.0, 5.4, 5.6, 5.9, 6.3, 6.7, 7.2, 7.6, and 8.3).

5.3.5. Phosphate buffer solution for log $D_{oct/wat}$ (20 mM, pH 2.0)

$NaH_2PO_4.2H_2O$ (2.9 g) was dissolved in 350 ml of water. The pH of this mixture was adjusted to 2.0 by adding 0.1 M H_3PO_4 solution. The total volume was filled up to 500 ml by adding water.

5.4. Analytical procedures

5.4.1. HPLC determination of pKa

The LC system used a phosphate buffer:ACN (70:30 v/v) mixture with adjusted pH values of 3.1 – 5.8 as a mobile phase. The mobile phase was filtered through a 0.45-µm membrane filter, and the flow rate was set at 1.0 ml min^{-1} at room temperature (22 – 23 °C). PHCl samples were prepared via the dilution of standard stock solutions in the mobile phase and they were injected in triplicate for each pH value. The working sample and uracil solution (15 µg ml-1) were also filtered through a 0.45-µm membrane filter before injection.

Uracil solutions were injected into the HPLC system to detect the retention time of the dead volume. Capacity factors (k') of PHCl were obtained as per Eq. 7, where tr is the retention time of PHCl, and t0 is the retention time of uracil [21].

$$k' = \frac{(t_r - t_0)}{t_0} \qquad \text{(Eq. 7)}$$

5.4.2. UV-Vis spectrophotometric determination of pKa

The standard stock solution of PHCl was diluted using phosphate buffer solutions of different pH values but with the same ionic strength. The final concentration of the respective solutions was 10 µg ml^{-1}. The phosphate buffer solution was used as a blank while the spectra of PHCl solutions were recorded at 332 nm. The absorbance values were then plotted against the pH values.

To obtain pKa values using the UV-Vis spectrophotometric data, the Albert-Serjeant method [22], as defined in Eq. 8, was also utilized.

$$pKa = pH + \log (di - d / d - dm) \qquad \text{(Eq. 8)}$$

It describes the calculation of pKa, where di is the absorbance of the ionized species while d is the absorbance of the solution tested, and dm is the absorbance of the non-ionized species.

5.4.3. UV-Vis spectrophotometric determination of log $D_{oct/wat}$

The experimental design was structured based on the requirements stipulated in the OECD guidelines for the testing of chemicals [23]. The standard stock solution of PHCl was prepared in MeOH and diluted to 200 µg ml^{-1} with MeOH in a 5-ml volumetric flask. Subsequently, 100 µL of this solution was added into a volumetric flask containing 4.95 ml each of phosphate buffer (50 mM, pH 2.0) and octanol. The solution was first mixed on a vortex-mixer for at least 30 min, and then ultrasonicated for another 2 h. The two-phase heterogeneous solution was transferred into a centrifuge tube and centrifugated for 2 min at 2,000 rpm, before transferring the distinct phases using a pipette. Next, PHCl solutions at three concentration levels (1.0, 2.0, and 4.0 µg ml-1) were prepared from the PHCl stock solutions using phosphate buffer (20 mM, pH 2.0) as well as octanol. The spectra of these solutions were then recorded, using phosphate buffer (50 mM, pH 2.0) or octanol as a blank solution. The absorbance values at 405 nm were measured and a calibration curve was constructed, which was then used to calculate the PHCl concentrations in the aqueous and octanol phases.

Acknowledgements: All experimental procedures were performed in the laboratory of the Department of Analytical Chemistry at the Faculty of Pharmacy of Hacettepe University.

Author contributions: Concept – M.Ç., E.K., A.D.; Design – S.A., N.E.B.; Supervision – S.A., N.E.B.; Resource – S.A.; Materials – S.A., N.E.B.; Data Collection and/or Processing - M.Ç., E.K., A.D.; Analysis and/or Interpretation – M.Ç., E.K.; Literature Search – E.K., A.D.; Writing – M. Ç., N.E.B.; Critical Review – M.Ç., E.K., A.D., S.A., N.E.B.

Conflict of interest statement: The authors declared no conflict of interest.

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