Human Serum Albumin Binding in a Vial: A Novel UV-pH Titration Method To Assist Drug Design

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ABSTRACT: The knowledge on human serum albumin (HSA) binding is of utmost importance as it affects pharmacokinetic behavior and bioavailability of drugs. In this article, we report a novel method to screen for ionizable molecules with high HSA binding affinity based on pKₐ shifts using UV-pH titration. We investigated the HSA binding of 27 drugs and compared the results to experimental data from conventional methods. In most cases, significant shifts (ΔpKₐ > 0.1) were observed for drugs with high HSA binding, while no change could be detected for low-affinity binders. We showed the pivotal role of ionization centers in the formation of strong interactions between drug and HSA using molecular docking studies. We also verified our findings by testing five modified analogues designed by structural considerations. Significant decreases in their HSA binding proved that the UV-pH titration method combined with an in silico support can be used as a medicinal chemistry tool to assist rational molecular design.

INTRODUCTION

Active pharmaceutical ingredients (API) entering the systemic circulation may interact with various components of the blood, which directly affects their pharmacokinetic and pharmacodynamic behaviors. 1−5 Blood plasma consists of approximately 7−9% of plasma proteins (albumins, glycoproteins, and fibrinogen), which contribute a major part to API-specific interactions. Among them, human serum albumin (HSA) is the most abundant (about 54% of plasma proteins (albumins, glycoproteins, and fibrinogen), which contribute a major part to API-specific interactions. Among them, human serum albumin (HSA) is the most abundant (about 54%−60% of blood proteins), 6−7 which is present in the blood at particularly high concentrations (35−50 g/L). 8−11 It plays an important role in maintaining the osmotic pressure of the blood, serves as a reservoir for plasma components (e.g., fatty acids and steroid hormones), and it is also the main contributor to the binding of drug molecules. 8,7,9,10 The protein’s heart-shaped structure is composed of three main domains (I−III), each containing two subdomains (A and B). 8−10 Besides the several recently identified low- to high-affinity binding sites of HSA, 12,13 the two specific drug binding sites (site I: warfarin site on the IIA subdomain and site II: indole-benzodiazepine site on the IIIA subdomain) are considered to be the most significant, regarding plasma protein binding. 2,11,14−18 Drug molecules usually form reversible complexes with HSA by electrostatic and hydrophobic interactions. These HSA-API complexes cannot cross biological membranes; therefore, they become therapeutically inactive. 5,19 Moreover, strong binding may also have a significant effect on the pharmacokinetic (PK) behavior of APIs (e.g., altered clearance, 19−22 distribution, 19−21 drug−drug interactions, 19,23 and toxicity 24). The HSA binding affinity shows wide diversity for drug-like compounds; thus, it is essential to predict the unbound fraction of APIs from the early stages of drug discovery for the estimation of PK behavior.

For the measurement of HSA-binding properties of APIs, several in vitro methods exist,18,19,25−27 including equilibrium dialysis (ED), 28,29 ultrafiltration (UF), 30 ultracentrifugation, 31 chromatographic methods, 32−34 NMR spectroscopy, 35,37 X-ray crystallography, 36 capillary electrophoresis (CE), 38 etc. These methods differ in throughput and time of the measurement have different sample requirements and limitations, and each has its advantages and disadvantages (Table 1). Also, most of the methods are able to provide information solely about the APIs’ affinity to HSA, while structural information regarding the interactions between HSA and APIs can be acquired by only a few timely and expensive techniques (e.g., NMR and X-ray diffraction). Besides the experimental methods, in silico models can be used to predict HSA binding, while molecular modeling studies can also be used to explore possible binding modes between HSA and APIs, providing additional structural information on complex formation. 40,41 To give a thoroughly detailed description of the complexes, data from orthogonal
methods are needed to be evaluated and compared simultaneously. This study aims to present a UV-pH titration method as a novel approach to screen for high-affinity HSA-binding ionizable APIs. By means of the method, additional structural information might also be gathered, and the role of ionization centers in the interaction between HSA and APIs can be further elucidated to help medicinal chemists in their efforts toward rational molecular design.

**Theoretical Basis of UV-pH Titration.** Spectrophotometry can be applied for the measurement of proton dissociation constants ($pK_a$) provided that the compound has a chromophore in proximity to the ionization center and the absorbance changes sufficiently as a function of pH. In aqueous solutions, the dissociation equilibrium of a monoprotic acid and the acid dissociation constant ($K_a$) can be written as

$$HA \rightleftharpoons A^- + H^+$$

$$K_a = \frac{[A^-][H^+]}{[HA]}$$

where $HA$ and $A^-$ are the neutral and dissociated forms of the acid, respectively. After rearrangement of eq 2 and taking logarithms, we acquire the Henderson–Hasselbalch equation

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

If there is a pH-dependent change in absorbance, the ratio of $HA$ and $A^-$ can be determined spectrophotometrically. At a pH where the molecule exists entirely in its neutral form, its absorbance at a given wavelength is given by

$$HA \rightleftharpoons A^- + H^+$$

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where $HA$ and $A^-$ are the neutral and dissociated forms of the acid, respectively. After rearrangement of eq 2 and taking logarithms, we acquire the Henderson–Hasselbalch equation

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

If there is a pH-dependent change in absorbance, the ratio of $HA$ and $A^-$ can be determined spectrophotometrically. At a pH where the molecule exists entirely in its neutral form, its absorbance at a given wavelength is given by
A_{HA} = \varepsilon_{HA} \times l \times [HA] \quad (4)

where \varepsilon_{HA} and [HA] are the molar absorption coefficient and concentration of HA, respectively, and l is the optical path length. At a pH where the molecule exists entirely in its dissociated form, the absorbance of A\(^-\) is given by

A_{A^-} = \varepsilon_{A^-} \times l \times [A^-] \quad (5)

where \varepsilon_{A^-} and [A\(^-\)] are the molar absorption coefficient and concentration of A\(^-\), respectively. At an intermediate pH where both species are present, the absorbance is given as

A_{HA} = \varepsilon_{HA} \times l \times [HA] + \varepsilon_{A^-} \times l \times [A^-] \quad (6)

Combining eqs 4–6, the ratio of HA and A\(^-\) can be written as

\frac{[A^-]}{[HA]} = \frac{A_i - A_{HA}}{A_X - A_i} \quad (7)

Combining eqs 3 and 7 provides a modified Henderson–Hasselbalch equation, which can be used to calculate pK\(_a\) values based on absorbance changes\(^3\)

\text{pH} = \text{pK}_a + \log \left( \frac{A_i - A_{HA}}{A_X - A_i} \right) \quad (8)

If the conditions for UV-pH measurements coincide, then this method is the fastest way for the determination of pK\(_a\) values (approximately 20–25 min/3 parallel measurements). Furthermore, the sample requirement of built-in UV-pH methods of the SiriusT3 instrument is also minuscule, and only 5 \(\mu\)L of a 10 mM stock solution (DMSO, MeCN, etc.) of the API is needed in a titration volume of 1 mL. Also, measurements in the presence of co-solvents, macromolecules, or additives may be possible, making it an ideal choice for complexation studies as well.

Upon complexation with macromolecules (e.g., cyclodextrins, crown ethers, proteins, etc.), the dissociation equilibrium and the acid dissociation constant can be written as

HA-G \rightleftharpoons A^-G^- + H^+ \quad (9)

K\(_a^G\) = \left( \frac{[A^-G^-][H^+]}{[HA-G]} \right) \quad (10)

where G is the guest molecule, and K\(_a^G\) is the apparent proton dissociation constant of the complexed API. Due to different absorption properties of the complexed species, a significant spectral change may occur.\(^4\) If absorption of the guest molecule is negligible and addition of absorbances is assumed, then the absorbances of the molecular species can be given as

A_{HA} = \varepsilon_{HA} \times l \times [HA] + \varepsilon_{HAG} \times l \times [HA-G] \quad (11)

A_{A^-} = \varepsilon_{A^-} \times l \times [A^-] + \varepsilon_{AG^-} \times l \times [A^-G^-] \quad (12)

A_i = \varepsilon_{HA} \times l \times [HA] + \varepsilon_{HAG} \times l \times [HA-G]
+ \varepsilon_{A^-} \times l \times [A^-] + \varepsilon_{AG^-} \times l \times [A^-G^-] \quad (13)

Substituting eqs 11–13 into eq 8, the apparent pK\(_a\) values (pK\(_{a,app}\)) can be calculated with shifts from pK\(_a\) values that are proportional to the ratio of bound and unbound fractions.

## RESULTS AND DISCUSSION

**HSA-Binding Measurements by UV-pH Titration.** We investigated the possibility of measuring HSA binding of APIs based on changes of proton dissociation constants of their free and complexed molecular forms using UV-pH titration. First, we collected UV-vis absorbance data of HSA during the UV-pH titration assay to determine if the presence of HSA would interfere with the signal of APIs. As can be seen in Figure 1 (left), HSA has two pK\(_a\) values (pK\(_{a1}\) = 3.87 and pK\(_{a2}\) = 11.56), which are measurable by UV-pH titration. If we add HSA to the blank UV-pH titration assay (Figure 1, right), it annuls the spectral changes belonging to the first pK\(_a\) value; however, the

![Figure 1. pH-dependent UV-vis absorbance changes and detectable pK\(_a\) values of HSA in the absence (left) and in the presence (right) of blank correction.](https://dx.doi.org/10.1021/acs.jmedchem.0c00046)
second pK_a value remains detectable even after blank correction. Therefore, even though the intensity of the signals of the second pK_a value is decreased, we must consider the presence of overlapping absorption bands during titrations of APIs.

Next, we investigated HSA binding of APIs by measuring their pK_a values in aqueous medium in the absence of HSA and in the presence of HSA (pK_a,HSA). Figure 2 shows some examples of the acquired spectra and experimental pK_a values in the presence and absence of HSA. We observed significant pK_a shifts (ΔpK_a) for several APIs, while others showed no change of pK_a values upon addition of HSA (Table 2 and Table S1, Supporting Information). In the case of molecules with multiple protonation centers, their pK_a values changed to varying extent; we proposed that this might indicate which part of the molecule contributes more to the binding to HSA. This was later confirmed by molecular docking studies (see the next section for further details). In some cases, absorption bands of HSA suppressed the absorbance of the API; therefore, pK_a,HSA could not be determined. This usually occurred in the case of APIs with only minuscule pH-dependent absorbance changes in their spectrum in aqueous medium (e.g., diltiazem, imipramine, indomethacin, and propranolol) or in the case of pK_a values higher than ~10 (e.g., furosemide pK_a2 and amodiaquine pK_a3) where the signals of pK_a of HSA interfered. We could avoid this latter problem if the API had significant absorption at wavelengths higher than 320 nm (e.g., chloroquine pK_a2, nitrazepam pK_a2, and oxazepam pK_a2).

Figure 2. Absorbance spectra and detectable pK_a values of diclofenac (DIC), phenylbutazone (PHB), piroxicam (PIR), and procaine (PRC) in the presence and absence of HSA.
where no interference with HSA absorption bands was observed and thus evaluation of data was possible.

Comparison of the Results with Reference Values. To compare our results with data from orthogonal methods, we also measured HSA binding of the APIs using chromatographic measurements on an immobilized HSA column using rapid equilibrium dialysis (RED) and carried out molecular docking calculations of the APIs with HSA (as described in the Methods section). The results of the RED measurements and the molecular docking calculations were compared with literature data and experimental data from UV-pH titration measurements.

Table 2. Literature Data and Experimental Values of HSA Binding by Chromatographic, Rapid Equilibrium Dialysis (RED), and UV-pH Titration Measurements

| API name              | literature data (PPB%<sup>4.13−4.17</sup>, HSA% (HPLC)<sup>8.49</sup>) | experimental data (HSA% (HPLC), HSA% (RED)) | UV-pH titration (ΔpK<sub>a1</sub>, ΔpK<sub>a2</sub>) |
|-----------------------|---------------------------------------------------------------|---------------------------------------------|--------------------------------------------------------|
| amodiaquine           | 33 ± 3; 31.5                                                  | 78.8                                        | 0.12 ± 0.04, 0.64 ± 0.03                               |
| cefuroxime            | (S): 66.6 ± 3.3; (R): 42.7 ± 2.1                              | 40.1                                        | n.d.<sup>a</sup>, n.d.<sup>a</sup>                    |
| chloroquine           | >99.5; 99.5                                                  | 50.5<sup>b</sup>                           | 0.21 ± 0.03, 0.75 ± 0.05                               |
| diazepam              | 98.7 ± 0.2; 99                                               | 89.2                                        | 0.09 ± 0.03                                           |
| diclofenac            | 99.0                                                      | 98.2                                        | n.d.<sup>a</sup>                                      |
| diclofenac ethyl ether| >99.5; 99.5                                                  | 98.1                                        | 0.47 ± 0.01                                           |
| difusilas             | 99                                                         | 98.7                                        | 0.38 ± 0.04, n.d.<sup>a</sup>                         |
| difusilas ethyl ether | ~0                                                          | 97.5                                        | n.d.<sup>a</sup>                                      |
| diltiazem             | 78                                                         | 53.9                                        | n.d.<sup>a</sup>                                      |
| famotidine            | 20                                                         | 14.5                                        | 0.02 ± 0.03                                           |
| furosemide            | 98.4                                                       | 63.8                                        | 0.10 ± 0.01, n.d.<sup>a</sup>                         |
| imipramine            | 90.1 ± 1; 92.6                                              | 83.1                                        | n.d.<sup>a</sup>                                      |
| indomethacin          | 90; 92−99                                                   | 99.5                                        | 0.05 ± 0.03, n.d.<sup>a</sup>                         |
| isoniazid             | ~0                                                          | 6.8                                         | 0.55 ± 0.05                                           |
| isoxicam              | 97.3                                                       | 80.4 ± 0.8                                  | 0.22 ± 0.05                                           |
| lornoxicam            | 100.0                                                      | 98.0 ± 0.2                                  | 0.87 ± 0.23<sup>d</sup>, 1.00 ± 0.07                  |
| meloxicam             | 99                                                         | 99.9                                        | 0.16 ± 0.23<sup>d</sup>, 0.55 ± 0.05                  |
| O-methyl meloxicam    | 98.4                                                       | 96.7 ± 0.1                                  | 0.00 ± 0.01                                           |
| metronidazole         | 10; 11 ± 1                                                  | 5.4                                         | 0.06 ± 0.03, 0.98 ± 0.06                               |
| nitrazepam            | 82.3                                                       | 76.7                                        | 0.05 ± 0.03, 0.98 ± 0.06                               |
| oxazepam              | 98.4                                                       | 94.2                                        | 0.06 ± 0.03, 0.27 ± 0.03                               |
| phenylbutazone        | 97.8; 98−99                                                 | 98.4                                        | 0.53 ± 0.05                                           |
| C-methyl phenylbutazone| 85.0                                                     | 64.1 ± 1.8                                  | 0.27 ± 0.03                                           |
| O-methyl phenylbutazone| 92.9                                                     | 92.9 ± 1.9                                  | 0.16 ± 0.06                                           |
| physostigmine         | 2.0                                                        | 9.7 ± 6.6                                   | 0.00 ± 0.04, 0.55 ± 0.09                               |
| piroxicam             | 99; 99                                                      | 96.8                                        | 0.00 ± 0.04, 0.55 ± 0.09                               |
| procaine              | 6                                                          | 36.0                                        | 0.02 ± 0.06, 0.00 ± 0.06                               |
| propranolol           | 87 ± 6; 87                                                 | 62.5                                        | n.d.<sup>a</sup>                                      |
| sulindac              | 94; 93.5                                                   | 92.0                                        | 0.13 ± 0.11<sup>d</sup>, 0.72 ± 0.09                  |
| tenoxicam             | 100.0                                                      | 96.8 ± 0.0                                  | 0.01 ± 0.02                                           |
| trimetaphor           | 37.5; 41.5                                                 | 37.6                                        | 0.41 ± 0.05                                           |
| warfarin              | 99 ± 1; 99                                                 | 97.9                                        | 0.41 ± 0.05                                           |

<sup>a</sup>Experimental data has been measured using a racemic compound. <sup>b</sup>HSA binding could not be determined due to chemical decomposition during incubation. <sup>c</sup>HSA suppressed the absorbance of the API, and pK<sub>a</sub> was not determined. <sup>d</sup>High standard deviations originate from extrapolated pK<sub>a</sub> values, out of the measurement range of UV-pH titration.

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scores between the ligands and the mentioned charged residues confirm this implication (Figure 4).

For the remaining five APIs, Glide XP was used to predict their binding modes in the two drug sites of HSA by docking to the X-ray structures 2BXC and 2BXE. The poses were inspected with regard to the vicinity of the protonatable groups (specifically, the ones with the significant pK_a shifts) to potential ionic interaction partners. For four APIs, such a binding pose was identified in drug site I (as the most favorable binding pose in each case), and the differences of the Coulomb interaction scores confirm the preference toward the deprotonated forms (Figure 5). For CHQ (the only API with a formal positive charge in its protonated form), no such binding mode was identified in either drug site. We propose that in the case of CHQ, the deprotonated form is favored because ligand entry to both drug sites is hindered for the protonated form due to the repulsive interactions with the positively charged residue clusters at the entry points of the sites (K195-K199-R218-R222 in drug site I and R410-K414 in drug site II, see also Figure 4).51

Due to the contradictory UV-pH titration results of DZP, to find possible explanations, we compared X-Ray structures (where available) and predicted binding modes of DZP and its structural analogue NZP (Figure 6). In the X-ray structure 2BXD, DZP occupies drug site II (A), with its only protonatable nitrogen being too far from the R485 residue to make direct contact. Although its predicted binding mode in site I (B) would justify a pK_a shift (the same nitrogen being only 3.0 Å away from R257), binding to this site is not observed in the crystal structure, suggesting that site II is clearly preferred. Thus, the protonatable nitrogen will not be able to directly interact with HSA, which can explain the smaller than expected pK_a shift. On the contrary, in the predicted binding mode of NZP to drug site II (C), the deprotonatable amide nitrogen is too far from R410 to make direct contact, but the predicted binding mode in drug site I (D) nicely supports the observed pK_a shift of 0.56 units (by...
placing the amide nitrogen 3.4 Å away from R257), suggesting the preference of NZP toward drug site I.

**Use of UV-pH Titration To Assist Medicinal Chemistry.** As the previous examples show, UV-pH titration combined with molecular modeling can be used to identify the role of ionization centers in the formation of strong API–HSA interactions. The information gathered this way might be used to help chemists to design molecules with decreased HSA binding, which might improve the API pharmacokinetics and increase their bioavailability. For validation purposes, some of the APIs with considerable \( pK_a \) shifts (DIC, DIF, MEL, and PHB) were modified by ester formation or alkylation to neutralize their protonatable groups; these analogues were evaluated with the same protocols as described above.

The chromatographic measurements showed a significant decrease in retention time for each modified API, indicating a lower HSA binding affinity (Table 2 and Figure 7). We observed moderate decreases in binding for DIC ethyl ester

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**Figure 5.** Refined predicted binding modes for the deprotonated (green) and protonated (magenta) forms of (A) meloxicam, (B) nitrazepam, (C) piroxicam, and (D) tenoxicam. Coulomb interaction scores with the most important interacting residues are shown in matching colors, the smaller the better.

**Figure 6.** Comparison of crystallographic and predicted binding modes of (A, B) diazepam and (C, D) nitrazepam, with distances of the relevant protonatable groups to the closest charged residues.
(100% → 98.1%) and DIF ethyl ester (100% → 97.5%) and extensive decreases in the case of O-methyl MEL (99.9% → 84.2%), O-methyl PHB (99.9% → 92.9%), and C-methyl PHB (99.9% → 85.0%). RED measurements also showed significant decreases in the binding of modified MEL (99.9% → 65.9%) and PHB (99.9% → 92.9% (O-methyl PHB) and 99.9% → 85.0% (C-methyl PHB)), while HSA binding could not be determined this way for the esters of DIC and DIF due to their instability under the measurement conditions of RED (Table 2).

To further support the experimental results, molecular docking studies have also been carried out to compare binding modes of the analogues. For the ethyl ester of DIF, the O- and C-methylated PHB, and the ethyl ester of DIC, the top binding modes predicted by Glide XP docking were closely similar to their respective unmodified counterparts. Nonetheless, the Coulomb interaction scores against the charged residues were small, as expected (Figure 8). For O-methyl MEL, unrestrained Glide XP docking could not identify a binding mode similar to that of the unmodified API. When restricted to the reference binding mode of meloxicam, the modified analogue exhibits repulsive Coulomb interaction scores, while in its unrestricted binding mode, the interaction scores confer slightly and in one case (K199), moderately attractive contributions, similar to the other analogues.

As the results of experimental measurements and molecular modeling show, we succeeded in using UV-pH titration as a screening tool for identifying structural moieties that make major contribution in formation of complexes with strong binding. By the modification of the APIs, the ionization centers responsible for strong interactions with HSA could be neutralized, resulting in a lower HSA binding. Since even a few percent decreases have a considerable effect on the distribution of APIs, the observed decreases in HSA binding (from 2–3 to 10–35%) support the technique’s applicability to help the design of novel molecules with favorable pharmacokinetic behavior.

### CONCLUSIONS

In this study, we demonstrated the applicability of UV-pH titration as an orthogonal method for the identification of APIs with high-affinity binding. This fast and cost-effective method can be used as a screening assay in the case of molecules containing ionization centers, providing binding data faster than conventional methods. We showed that the observed pKₐ shifts (ΔpKₐ) are proportional to HSA binding of APIs. In the case of multiprotic molecules, by means of molecular docking, we demonstrated that the pKₐ shifts of different sizes provide structural information on the binding mode of the API. To elucidate the significance of ionization centers of molecules, we also investigated modified analogues with neutralized protonation centers. The results clearly showed that decreased protein binding can be achieved by this approach, resulting in molecules with improved pharmacokinetics. Therefore, the UV-pH titration method combined with an in silico support might be used as a novel medicinal chemistry tool to assist researchers in the rational drug design to decrease the high attrition rate in later stages of drug discovery. In the future, besides screening for APIs with high-affinity binding, this method might also be used for comparison of binding of a specific API with different kinds of modified HSA derivatives or in the case of different formulations of HSA.

### EXPERIMENTAL SECTION

**Materials.** Analytical grade solvents such as acetonitrile (MeCN), dimethyl sulfoxide (DMSO), ethanol (absolute), formic acid, trifluoroacetic acid (TFA), and potassium hydroxide (KOH) were purchased from Merck KGaA (Darmstadt, Germany). Analytical grade 2-propanol (IPA), 0.5 M hydrochloric acid, and 0.5 M potassium hydroxide were purchased from Honeywell International.
Inc. (New Jersey, USA). Albumin from human serum (lyophilized powder, ≥97% (agarose gel electrophoresis) cat. no.: A9511), ammonium acetate (NH₄OAc), dichloromethane (DCM), ethyl acetate (EtOAc), methyl iodide (MeI), phosphate-buffered saline (PBS) powder, potassium chloride, and the reference materials (acetaminophen, amodiaquine dihydrochloride dihydrate, amoxicillin, ampicillin, antipyrine, aspirin, atenolol, captopril, carbamazepine, cefotaxime sodium, cefuroxime sodium, cephalexin, chloroquine diphosphate, chlorothiazide, chlorpromazine hydrochloride, cimetidine, diclofenac sodium, diethylpropion, diltiazem hydrochloride, diphenhydramine hydrochloride, famotidine, furosemide, imipramine hydrochloride, indomethacin, isoniazid, isoxicam, ketoconazole, meloxicam, metronidazole, naproxen, nifedipine, nitrazepam, oxazepam, phenylbutazone, phenytoin, phystostigmine, piroxicam, procaine hydrochloride, pranopanol hydrochloride, ranitidine hydrochloride, rifampin, sulfamethoxazole, sulindac, terbutaline hemisulfate, trimethoprim, and warfarin) were purchased from Sigma-Aldrich Co., Ltd. (Budapest, Hungary). Diclofenac ethyl ester, ketorolac tromethamine, and O-methyl meloxicam were purchased from Toronto Research Chemicals Inc. (North York, Toronto, Canada). Lornoxicam and tenoxicam were purchased from Carbosynth Ltd. (Compton, Berkshire, United Kingdom). O-methyl phenylbutazone was purchased from Ambinter c/o Greenpharma S.A.S. (Orléans, France). Diethylpropion and C-methyl phenylbutazone were synthesized at Gedeon Richter Plc. Compounds possessed a purity of >95% by means of HPLC. (Diethylpropion ester: SHIMADZU prominence modular HPLC system equipped with a photodiode array detector (PDA) and a mass spectrometer equipped with an electrospray ionization source (ESI); column: CORTECS C18+, 90 Å, 2.7 μm (3.0 × 50 mm); retention time = 3.89 min; peak area, 98.76%; eluent A, 0.1% (v/v) formic acid in water; eluent B, MeCN/water 95:5 (v/v) with 0.1% (v/v) formic acid; gradient: 0–4.5 min/0–100% B and 4.5–5.6 min/100% B; flow rate of 1.25 mL/min; detection at 220 ± 4 nm; column temperature: 40 °C. C-methyl phenylbutazone: Agilent 1200 liquid chromatography system equipped with a diode array detector and coupled with an Agilent 6410 triple quadrupole mass spectrometer (QQQ-MS) equipped with an ESI source; column: Kinetex EVO C18, 100 Å, 2.6 μm (3.0 × 100 mm); retention time = 8.60 min; peak area, 99.77%; eluent A, 0.1% (v/v) TFA in water; eluent B, MeCN/water 95:5 (v/v) with 0.1% (v/v) TFA; gradient: 0–5 min/0–100% B and 5–15 min/100% B; flow rate of 1 mL/min; detection at 220 ± 4 nm; column temperature: 45 °C.) For detailed information on their syntheses and confirmation of structures (NMR and HRMS), see the Supporting Information (Sections 4–6). Neutral linear buffer (NLB) was purchased from Pion Inc. (U.K.) Ltd. (Forrest Row, United Kingdom). In all experiments, distilled water.
was purified by the Millipore Milli-Q 140 gradient water purification system.

**UV-pH Titration.** The UV-pH titrations were performed using a fully automated SiriusT3 instrument (Pion Inc., Forest Row, U.K.). Spectrophotometric determination of pKₐ values was performed using the built-in fast UV pKₐ method, and the absorbance changes during the titrations were monitored by the dip-probe absorption spectroscopy (D-PAS) method, and evaluation of calculation of pKₐ values were performed by the SiriusT3Refine software (version 1.1.3.0., Pion Inc., Forest Row, U.K.). For the determination of pKₐ values in aqueous media, 5 μL of 10 mM DMSO solution of the samples was titrated from pH 2.0 to pH 12.0 in 1.5 mL ionic strength adjusted water (ISA-water: 0.15 M KCl; the initial sample concentration is approximately 33.3 μM). In the case of determination of pKₐ values in the presence of HSA (pKₐiso), a modified fast UV assay was used: ISA-water solutions containing HSA (33.3 μM, with a 1:1 nominal molar ratio of HSA and APIs) were added manually in advance to the titration vials. All measurements were performed under a nitrogen atmosphere at T = 25.0 ± 0.1 °C. The pH region 2.0–10.0 and the spectral region of 250–450 nm were used in the analysis, and the results were calculated from a minimum of three replicates in each case.

**Molecular Docking.** Ligand and protein structures were prepared with the standard tools of the Schrödinger software package, based on the OPLS3 force field. For ligand docking and the generation of per-residue interaction scores, the extra precision mode (XP) of Glide was used. The publicly available PDB structures 2BXC, 2BXE, and 4Z69 were used for evaluating the experimental binding modes of PHB, DIF, and DIC, respectively. For the additional five APIs, the structures 2BXC and 2BXE were used to generate predicted binding modes with Glide XP, after validating them by redocking their cognate ligands into the respective binding pockets. (RMSD values between experimental and predicted poses were 1.40 Å for 2BXC and 2BXE, respectively, and 5.60 Å for 4Z69; therefore, this structure was omitted from further use.) The best identified binding pose was refined for both the protonated and deprotonated forms of the APIs. For detailed description of HSA-binding measurement by RED and biomimetic chromatography, see the Supporting Information.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00046.

Results of UV-pH titrations in the presence and absence of HSA, results of HSA-binding measurements by RED and biomimetic chromatography (CHIRALPAK HSA column), comparison of the HPLC systems used for HPLC-HSA measurements, and synthesis and structural confirmation (NMR and HRMS) of difunisal ethyl ester and C-methyl phenylbutazone (PDF)

Molecular formula strings of different APIs (CSV)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

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**ABBREVIATIONS**

CE, capillary electrophoresis; CHQ, chloroquine; DIC, diclofenac; DIF, difunisal; DZP, diazepam; DAD, diode array detector; ED, equilibrium dialysis; EIC, extract ion chromatogram; EtOAc, ethyl acetate; HT, high throughput; IPA, 2-propanol; ISA, ionic strength adjusted; KOH, potassium hydroxide; LT, low throughput; MeCN, acetonitrile; MeI, methyl iodide; MEL, meloxicam; MT, medium throughput; NIB, neutral linear buffer; NSB, nonspecific binding; NZP, nitrazepam; PDA, photodiode array (detector); PHB, phenylbutazone; PIR, piroxicam; RED, rapid equilibrium dialysis; SPME, solid-phase microextraction; TEN, tenoxicam; TFA, trifluoroacetic acid; UF, ultrafiltration

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