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IMMOBILIZATION OF ALPHA 1-ACID GLYCOPROTEIN FOR CHROMATOGRAPHIC STUDIES OF DRUG-PROTEIN BINDING II. CORRECTION FOR ERRORS IN ASSOCIATION CONSTANT MEASUREMENTS

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Abstract

A new method for the immobilization of α_1 -acid glycoprotein (AGP) in HPLC columns was recently described for applications such as drug binding studies. Part of this earlier work used self-competition zonal elution studies to measure association equilibrium constants between immobilized AGP and *R*- or *S*-propranolol. It was later found that analysis of these data by a common equation derived for linear elution conditions gave erroneous values for experiments actually conducted under nonlinear conditions. This report discusses the nature of this error and uses frontal analysis to estimate the true binding strength between *R*- and *S*-propranolol and HPLC columns containing immobilized AGP.

A recent paper examined the binding of *R*- and *S*-propranolol to a new type of α_1 -acid glycoprotein (AGP) column (1). Part of this earlier work used self-competition zonal elution studies for association equilibrium constant measurements, an approach which has since been noted to contain some errors that are not uncommon in the literature but that do need to be corrected to better estimate the true binding strength of *R*- and *S*-propranolol with AGP.

The column used in Ref. (1) was prepared through the controlled and mild oxidation of AGP, followed by the immobilization of this protein to hydrazide-activated silica. Part of this paper evaluated binding of this AGP column to *R*- and *S*-propranolol by using a self-competition zonal elution experiment (i.e., the "perturbation method", "step and pulse method" or "system peak" method) (2–4). In this approach, a small sample of *R*- or *S*-propranolol was injected as a pulse onto the AGP column while a known, fixed concentration of the same compound was applied in the mobile phase as a competing agent. The resulting shift in the retention factor (k') for the observed peak was measured as a function of the mobile phase concentration of the analyte [A]. Plots of $1/k'$ versus [A] appeared to give a linear response for mobile phases containing 0 to 5 μ M propranolol.

In these experiments (1), association equilibrium constants were obtained by fitting the data to an equation from the literature that pertains to an analyte at infinite dilution, or "linear elution conditions" (see Eq. 1 in Ref. 1 and equivalent equations in Refs. 5 and 6). However, for an analyte at a finite concentration, this same equation can introduce errors when obtaining

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association equilibrium constants. The error arises because Eq. 1 in Ref. 1 is strictly valid only for the bolus of molecules that are injected into the column but not for the peak that is actually observed in the chromatogram. This latter peak is called the “perturbation peak” or “displacement peak” and is caused by perturbation of the liquid/solid equilibrium in the column due to sample injection. Although the injection of sample causes this perturbation, the sample and this perturbation do not elute at the same time from the column. This phenomenon is predicted under nonlinear elution conditions by chromatographic theory and has been observed in recent experiments (7–10). Molecules contained in the injected pulse exit as a “mass” peak which cannot be observed directly unless these molecules are distinguishable from the competing agent by an appropriate detector (e.g., using an isotopically-labeled analyte and a radioactivity detector or mass spectrometer; see chromatograms in Ref. 8 for examples). Failure to consider the difference in the mass and perturbation peaks leads to a systematic error in the measurement of an equilibrium constant by zonal elution when nonlinear elution conditions are present unless more complex expressions than Eq. 1 in Ref. (1) are used for data analysis (see Supplementary materials). The size of this error will depend on the concentration of the competing agent and the equilibrium constant for the system (7–9).

In this current report, improved estimates of the association equilibrium constants for *R*- and *S*-propranolol on the AGP column were obtained by using frontal analysis, or “breakthrough curve” analysis (3,10–12). Frontal analysis is a well-established technique that can be performed under nonlinear conditions by determining the amount of analyte required to reach the mean saturation point of a column at various applied concentrations of the analyte. Both zonal elution and frontal analysis have been shown to give comparable binding parameters when their data are fit to the appropriate equations [13]. However, frontal analysis gives raw adsorption isotherm data while zonal elution gives the best estimates of the isotherm parameters [9]. As a result, frontal analysis is not subject to errors due to differences in mass peaks versus perturbation peaks.

Frontal analysis was performed by utilizing the same type of AGP support as employed for the zonal elution studies (see Ref. (1) for details on the preparation of this material). These experiments were conducted using 50×4.1 mm I.D. stainless steel columns that contained immobilized AGP or a control support prepared under identical conditions but with no AGP being added during the immobilization step. All columns were enclosed in water jackets and attached to a circulating water bath for temperature control (1). Breakthrough curves were generated on these columns at 1.00 mL/min using solutions that contained 0.10 μ M to 1.50 mM *R*- or *S*-propranolol in pH 7.4, 0.067 M phosphate buffer. After sample application, the retained analyte was eluted by passing pH 7.4, 0.067 M phosphate buffer alone through the column. All results were corrected for the column void time and any nonspecific binding of propranolol to the control column (note: this latter value made up less than 22% of the total binding capacity measured on the AGP column).

Figure 1 shows a plot of m_{Lapp} versus [R-Propranolol] for frontal analysis data obtained at 37.0°C, where m_{Lapp} represents the moles of applied analyte required at a given analyte concentration to reach the mean point of the breakthrough curve. This plot has a sharp increase in m_{Lapp} versus [R-Propranolol] at low-to-moderate concentrations (i.e., 0.1–100 μ M), followed by a more gradual increase in m_{Lapp} at higher propranolol concentrations. Similar results were obtained for *S*-propranolol. This behavior has been noted previously with immobilized or soluble AGP at 37.0°C over a comparable concentration range of beta-blockers (12,14) and has been attributed to the presence of two populations of binding sites: 1) a selective high affinity site present in approximately a stoichiometric amount versus AGP, and 2) a group of lower affinity, non-selective sites. When the results in Figure 1 were fit to such a “two-site” model, this gave a best-fit response with a correlation coefficient of 0.9964 ($n = 15$) for *R*-propranolol and 0.9901 ($n = 17$) for *S*-propranolol.

As shown in Table 1, the selective site/non-selective two-site model gave an association equilibrium constant for the selective site (K_{a1}) equal to $1.1 \times 10^6 \text{ M}^{-1}$ for *R*-propranolol and $1.4 \times 10^6 \text{ M}^{-1}$ for *S*-propranolol at 37.0°C. The stoichiometric ratio for this selective site (n_1) was 0.53–0.57 when compared to a total content of $73 (\pm 2) \text{ nmol AGP}$ in the column. This result is similar to a previous one obtained for alprenolol on AGP (12). The second group of non-selective sites gave a best-fit value for the product $n_2 K_{a2}$ that was $2,160 (\pm 70) \text{ M}^{-1}$ or $1,340 (\pm 70) \text{ M}^{-1}$ for *R*- and *S*-propranolol. The resulting fit was practically equivalent to that obtained for a bi-Langmuir model in which the denominator in the term $(m_{L2} K_{a2} [\text{Propranolol}] / (1 + K_{a2} [\text{Propranolol}]))$ is approximately equal to one, where m_{L2} represents the total moles of "non-selective" sites in the column. This behavior occurs when the product $K_{a2} [\text{Propranolol}]$ is small compared to one and may explain why non-saturable behavior has been observed for the second class of sites on AGP. The values of K_{a1} obtained with the bi-Langmuir model were statistically equivalent to those in Table 1 for the selective site/non-selective site model.

The best-fit results for the selective site/non-selective site model indicate that there are two types of binding sites for *R*- and *S*-propranolol on AGP. These results also suggest that, at propranolol concentrations below $50 \mu\text{M}$, most of the binding by AGP involves the high affinity selective sites. It is estimated that binding to the selective sites accounts for a large fraction of the binding by AGP when propranolol concentrations approach infinite dilution (i.e., below $1 \mu\text{M}$), with this contribution decreasing to approximately 80% of the total binding measured for $50 \mu\text{M}$ propranolol (on a column with an effective AGP concentration of $140 \mu\text{M}$). This observation explains why many former binding studies using similar concentrations of AGP and propranolol have not observed the non-selective sites. These results also suggest that the non-selective sites play a negligible role in the binding of AGP at the typical therapeutic levels of propranolol ($0.2\text{--}0.4 \mu\text{M}$ versus $13\text{--}34 \mu\text{M}$ AGP) (14); however, such nonselective binding must be considered on a case-by-case basis and may play a more important role in the binding of other compounds to AGP.

Table 1 compares the frontal analysis results for K_{a1} with the apparent values measured in Ref. (1) by self-competition zonal elution experiments. The frontal analysis results at 37.0°C were 2.5 to 3-fold lower than those reported in Ref. (1); a similar comparison made at 15°C gave 4 to 5-fold lower values (data not shown). The lower values measured by frontal analysis can be explained by the expected differences in the retention factors of the displacement and the mass peaks in the self-competition zonal elution studies. Another important difference in the results shown in Table 1 is that the zonal elution values were interpreted using a one-site interaction model while the frontal analysis values were acquired over a broader range of propranolol concentrations and were analyzed with a two-site model, making it possible to consider and correct for binding at the non-selective sites for propranolol on AGP.

The K_{a1} values determined by frontal analysis were closer than the zonal elution results to solution-phase values reported for the propranolol/AGP system (see literature cited in Table 1 of Ref. (1)). This includes better agreement with association equilibrium constants of $0.38\text{--}0.63 \times 10^6 \text{ M}^{-1}$ measured at 37.0°C for propranolol enantiomers or $0.4\text{--}0.54 \times 10^6 \text{ M}^{-1}$ for racemic propranolol, and values of $0.84\text{--}1.13 \times 10^6 \text{ M}^{-1}$ reported at room temperature for racemic propranolol. These results support the conclusion reached in Ref. (1) that the new AGP columns can be used as a model for soluble AGP in drug-protein binding studies. This study also demonstrates the need for caution in the use of self-competition zonal elution studies for evaluating the binding properties of immobilized proteins in chromatographic columns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AGP, α_1 -acid glycoprotein.

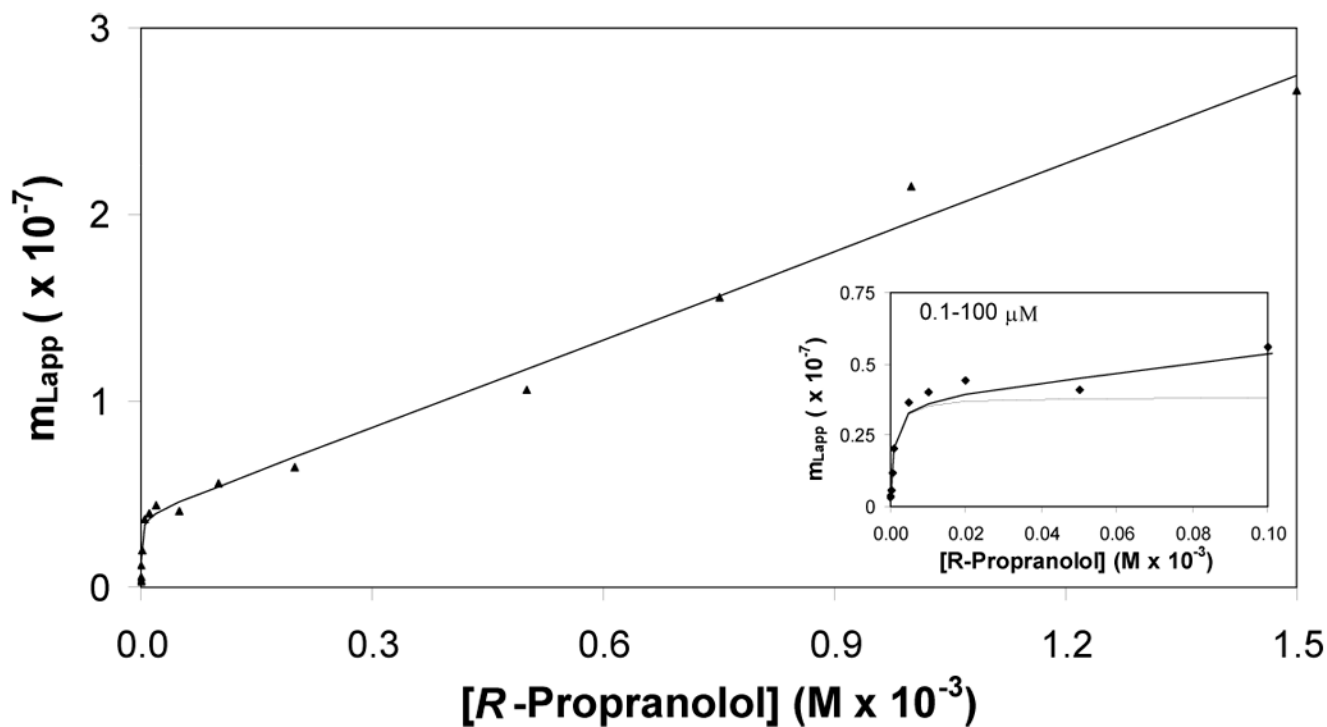


Figure 1.

Frontal analysis results for *R*-propranolol at pH 7.4 and 37.0°C on a hydrazide-linked AGP column. The measured values for m_{Lapp} had precisions of ± 0.4 to 13% over the range of propranolol concentrations that were examined. The best-fit line shown in this graph is for the equation $m_{Lapp} = (m_{L1}K_{a1} [\text{Propranolol}]) / (1 + K_{a1}[\text{Propranolol}] + (m_{L2}K_{a2}) [\text{Propranolol}])$, where K_{a1} and K_{a2} are the association equilibrium constants for a set of selective and non-selective sites for propranolol on AGP, with m_{L1} and m_{L2} being the moles of these binding sites that are present in the AGP column. Using the total estimated amount of AGP in the column ($m_{L,AGP}$), the following relationships were used with the best-fit results to obtain the values for n_1 and $n_2 K_{a2}$ that are given in the text: $m_{L1}/m_{L,AGP} = n_1$ and $(m_{L2} K_{a2})/m_{L,AGP} = n_2 K_{a2}$. The dashed line in the inset shows the response predicted for the high affinity selective site in the two-site model.

Table 1

Association equilibrium constants measured at 37.0°C for binding by *R*- and *S*-propranolol to the high affinity selective site on AGP

Enantiomer	Zonal elution result	Association equilibrium constant, K_{a1} (M^{-1})	
		Frontal analysis result ^b	
<i>R</i> -Propranolol	$2.7 (\pm 0.2) \times 10^6$	$1.1 (\pm 0.6) \times 10^6$	
<i>S</i> -Propranolol	$4.2 (\pm 0.3) \times 10^6$	$1.4 (\pm 0.7) \times 10^6$	

^aThese results were determined in pH 7.4, 0.067 M phosphate buffer. The numbers in parentheses represent a range of ± 1 S.D.

^bThese values are for the selective/non-selective site model. The values obtained for K_{a1} when using a bi-Langmuir model were $1.1 (\pm 0.6) \times 10^6 M^{-1}$ and $2.0 (\pm 0.8) \times 10^6 M^{-1}$ for *R*- and *S*-propranolol, respectively (see discussion in the text).