Use of entrapment to prepare columns containing alpha$_1$-acid glycoprotein for rapid studies of drug-protein binding by high-performance affinity chromatography

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Introduction

In recent decades, many studies have focused on the interactions between drugs and serum proteins because these processes are important in determining the transport, excretion and metabolism of many drugs in the body [1]. One serum protein involved in these studies is alpha$_1$-acid glycoprotein (AGP). This protein is a major constituent in plasma, and has the ability to bind and to transport numerous basic and neutral drugs in the blood stream [2].

High-performance affinity chromatography (HPAC) uses a biologically-related ligand as a stationary phase in a high-performance liquid chromatographic system. This method is a powerful means for studying the interactions between an applied analyte and the ligand [1,3]. One of the challenges in the immobilization of proteins and other biomolecules to the solid support of an HPAC column is to attach these ligands to supports and produce immobilized agents that closely mimic the behavior of the same biomolecules in their native form [3].

This work examined the use of a slurry-based entrapment method to immobilize AGP in HPAC microcolumns for rapid studies of drug-protein binding. The conditions needed for this entrapment process were studied and optimized. The behavior of the immobilized AGP prepared under optimum immobilization conditions was evaluated by frontal analysis and zonal elution experiments to examine its binding to carbamazepine, *S*-propranolol and other drugs. The columns that were prepared were found to give entrapped AGP that had good agreement with the binding behavior that is seen for soluble AGP.

Methods

I. AGP entrapment and column preparation

1. Entrapment method

Protein entrapment consists of three general steps: preparation of hydrazide-activated silica, preparation of oxidized glycogen, and entrapment of the protein (see **Fig. 1**). The AGP and control supports were packed into 10 mm × 2.1 mm i.d. columns.

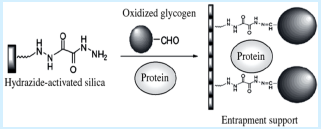

Figure 1. General scheme for entrapment of a protein using a glycogen-capped and hydrazide-activated support [2]

2. Optimization of conditions for AGP entrapment

HPLC-grade Nucleosil Si 100-7 and 300-7 silica (pore sizes in Å = 100 or 300, particle size in µm = 7) were evaluated for the preparation of the entrapped AGP supports.

The amount of oxalic dihydrazide needed for the preparation of hydrazide-activated silica supports was also investigated. The ratio of moles of oxalic dihydrazide vs. initial diol groups on the supports was selected to be 5:1, 3:1, 1:1, or 0.5:1.

Glycogen was initially oxidized by using periodic acid. After oxidation, both desalting columns and centrifugal filters (30 kDa MWCO) were evaluated for use in purifying the oxidized glycogen to remove any remaining periodic acid or soluble oxidation products.

II. Chromatographic studies

1. Frontal analysis

A known concentration of carbamazepine was continuously applied to an AGP or control column, while the amount of analyte passing through the column was monitored. As the column became saturated with the analyte this produced a breakthrough curve (**Fig. 2**). The moles of analyte need to reach the mean point of the breakthrough curve (m_{Lapp}) was determined, and the data were then fit to a single- or two-site binding model to obtain the total amount of active ligand in the column and the association equilibrium constant(s) for the analyte with the ligand or support [4].

Acknowledgement

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One-site binding model $$\frac{1}{m_{Lapp}} = \frac{1}{K_A m_{Ltot}[A]} + \frac{1}{m_{Ltot}}$$

Two-site binding model $$m_{Lapp} = \frac{m_{L1,tot}K_{A1}[A]}{1+K_{A1}[A]} + \frac{m_{L2,tot}K_{A2}[A]}{1+K_{A2}[A]}$$

[A]: molar concentration of anlayte, A
K_A: association equilibrium constant for the analyte with the column
m_{Lapp}: apparent moles of analyte required to reach the mean point of the breakthrough curve at a given analyte concentration
m_{Ltot}: total moles of active binding sites in the column
$m_{L1,tot}$ & $m_{L2,tot}$: total moles of sites L_1 and L_2 in the column
K_{A1} & K_{A2}: association equilibrium constants for A with sites L_1 & L_2

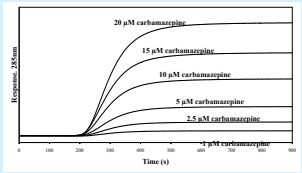

Figure 2. Frontal analysis chromatograms obtained for carbamazepine with entrapped AGP

2. Zonal elution

Solutions of 5 µM carbamazepine, disopyramide, imipramine, lidocaine, *S*-propranolol, or sodium nitrate was injected onto the AGP and control columns while the elution time of the solute was monitored. The k′ value of a drug with AGP and V_M were easily measured by zonal elution experiments, and m_L was determined by frontal analysis. Thus, the values of K_A for the various drugs with AGP could be estimated by using the equation shown below [4].

Correlation between k′ and K_A $$k' = K_A \frac{m_L}{V_M}$$

k′: retention factor for A after correcting for non-specific binding
m_L: moles of active binding sites in the column
V_M: void volume of the column

Results

I. Effect of support pore size on AGP entrapment

Table 1. Amount of entrapped AGP for various pore sizes of HPLC-grade silica, as determined by a protein assay

Pore size	Entrapped AGP (µg/mg silica)
300 Å	49.1 (± 5.1)
100 Å	63.6 (± 7.4)

Based on both the amount of entrapped AGP and the retention of *S*-propranolol on AGP columns, silica with a pore size of 100 Å was selected for all later studies.

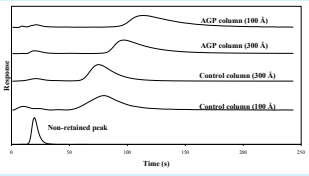

Figure 3. Zonal elution for *S*-propranolol and sodium nitrate (which gave a non-retained peak) on AGP and control columns packed using silica supports with various pore sizes

II. Optimization of preparation for hydrazide-activated silica

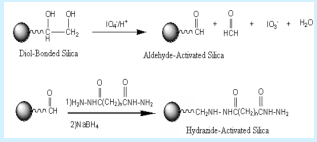

Figure 4. Synthesis of hydrazide-activated silica [5]

Table 2. Retention for *S*-propranolol on AGP columns prepared using various mole ratios of oxalic dihydrazide vs. diol groups (t_r: retention time)

Ratio of oxalic dihydrazide vs. diol groups	t_r on AGP column (s)	t_r on control column (s)	Specific retention (s)
5:1	131.5 (± 0.5)	97.37 (± 1.6)	34.2 (± 17)
3:1	371.8 (± 1.6)	114.6 (± 0.2)	257.2 (± 1.6)
1:1	414.3 (± 4.6)	104.2 (± 0.3)	310.3 (± 4.6)
0.5:1	117.8 (± 3.8)	102.6 (± 0.1)	15.2 (± 3.8)

The overall retention and specific retention of *S*-propranolol on the AGP columns increased as the mole ratio for dihydrazide vs. diol groups was decreased during the entrapment process when the ratio was greater than one and showed little specific binding when the mole ratio was less than one (**Table 2**). Thus, the final mole ratio chosen for use in entrapment was 1:1.

References

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III. Optimization of preparation for oxidized glycogen

As shown in **Fig. 5**, the oxidized glycogen that was purified by using desalting columns precipitated after one day of storage at 4ºC while oxidized glycogen that was purified by centrifugation was stable under the same conditions.

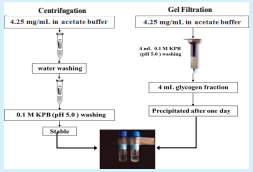

Figure 5. Purification of oxidized glycogen

IV. Chromatographic studies

1. Frontal analysis for determination of K_A and m_L for carbamazepine with AGP

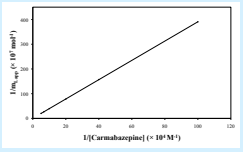

Figure 6. Linear regression of double-reciprocal plots for carbamazepine on a control column using a one-site binding model

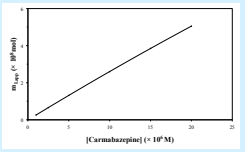

Figure 7. Non-linear regression for carbamazepine on an AGP column using a two-site binding model

The equation for the best fit-line in **Fig. 6** using a one-site binding model was y = 3.903x + 0.5101, with a correlation coefficient of 0.999 (n = 6). The values of K_A and $m_{L,tot}$ that were determined from the control column represented non-specific binding by carbamazepine both in this column and in the AGP column; these results were then used to also help fit the data obtained from the AGP column when using a two-site binding model (**Fig. 7**). The correlation coefficient for this second fit was 0.999 (n = 6). The estimated K_A for carbamazepine with AGP, as determined for this fit using K_{A2}, was close to the previously reported value of 1.0 (± 0.1) × 10^5 M^{-1} at pH 7.4 and 37°C [6].

Table 3. Estimates of K_A and $m_{L,tot}$ for carbamazepine on the control and AGP columns

Column	K_{A1} (M^{-1})	$m_{L1,tot}$ (mol)	K_{A2} (M^{-1})	$m_{L2,tot}$ (mol)
Control	1.3 (± 0.5) ×10^3	2.0 (± 0.1) ×10^{-6}	—	—
AGP	1.3 (± 0.5) ×10^3	2.0 (± 0.1) ×10^{-6}	1.2 (± 0.6) × 10^5	1.4 (± 0.3) ×10^{-9}

2. Zonal elution for determination of K_A

Table 4. Measured retention factors, calculated association equilibrium constants and reported association equilibrium constants on the entrapped AGP column for various drugs

Drug	k′	$K_{A,calc}$ (M^{-1})	$K_{A,ref}$ (M^{-1})	Reference
Carbamazepine	2.0 (± 0.4)	6.1 (± 1.8) × 10^4	1.0 × 10^5	[6]
Disopyramide	36 (± 2)	1.1 (± 0.2) × 10^6	1.0 × 10^6	[2]
Imipramine	24.6 (± 0.2)	7.6 (± 1.6) × 10^5	9.4 × 10^5	[7]
Lidocaine	6.0 (± 2.5)	1.8 (± 0.7) × 10^5	(1.1-1.7) × 10^5	[8]
S-Propranolol	69 (± 4)	2.1 (± 0.5) × 10^6	4.2 × 10^6	[9]

The consistence between the calculated K_A values and previously reported K_A values indicated that the behavior of the entrapped AGP gave good agreement to the behavior of soluble AGP.

Conclusion

A slurry-based method for AGP entrapment in HPAC microcolumns was investigated for use in the rapid studies of drug interactions with AGP. The optimum conditions needed for this entrapment process were determined by evaluating the effect of the silica pore size, optimizing the purification of the oxidized glycogen, and investigating the amount of oxalic dihydrazide needed for the preparation of hydrazide-activated silica. The chromatographic behavior of the entrapped AGP was found, by means of frontal analysis and zonal elution experiments, to be consistent with the results expected for solution-phase AGP.