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Measurements of drug–protein binding by using immobilized human serum albumin liquid chromatography–mass spectrometry

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Abstract

An HPLC/MS based method was used for fast and convenient determination of drug plasma–protein interactions in early drug discovery screening by employing a human serum albumin affinity column. Results from this methodology were compared with data from ultrafiltration or dialysis methods, and good agreement was observed. A compound not suitable for ultrafiltration due to the very high non-specific binding to artificial membrane of ultrafiltration device was also successfully analyzed by this method, and the protein binding determined by this chromatography method was very similar to data obtained by dialysis technique employing biological membranes. The immobilized HSA column LC/MS method also proved to be more reproducible and precise compared to ultrafiltration method in drug protein binding measurements.
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Keywords: Drug–protein binding; Human serum albumin

1. Introduction

The degree of plasma–protein binding has a significant effect on pharmacokinetic and pharmacodynamic outcomes in vivo. Many critical pharmacokinetic parameters such as hepatic metabolism rate, renal excretion rate, biomembrane partition rate, and steady-state distribution volume are a function of unbound drug fraction (unbound/bound concentration ratio) [1,2]. Therefore, quantitative determination of drug–protein binding is important in clinical drug development.

There are several proteins in plasma, including human serum albumin (HSA), α_1-acid glycoprotein (AAG) and lipoproteins, that contribute to plasma protein binding of a drug. Among these proteins, drug–albumin binding has been most extensively studied because HSA is the most abundant protein found in human blood and plasma [3]. HSA is a 66 500 Da protein whose function in the body involves binding and transporting various small compounds such as hormones, fatty acids, and drugs. HSA is found to bind many neutral and acidic compounds, with its two major binding sites, known as the warfarin-azapropazone site and indole-benzodiazepine site [4]. However, other minor binding regions have also been proposed [5,6].

Many methodologies have been investigated for quantitative determination of drug–protein binding. Among those, equilibrium dialysis and ultrafiltration followed by HPLC analysis have been conventionally and most commonly used [7–10]. Equilibrium dialysis separates molecules across a semipermeable membrane according to molecular size (weight) by utilizing the driving force of concentration differential between solutions on each side of the membrane. Ultrafiltration is a method that rapidly (usually within 10 min) separates free small molecules from protein-bound ones using a membrane. These conventional methods suffer from relatively long analysis time (hours for equilibrium dialysis [11]), the need of an additional analytical step (such as GC, LC) to determine the actual final free drug concentration, non-specific binding of drugs onto the membrane [12], and leakage of bound drug through the membrane, which makes them not very applicable to highly protein-bound drugs [8,13].

Compared to conventional methods, chromatography based method is intrinsically simpler and faster in terms of sample preparation and analysis, and therefore has attracted more and more interest. High-performance frontal analysis (HPFA) is a newly developed chromatographic method for

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drug–protein binding studies [14–18]. Advantages of HPFA include accurate measurement of association constant between a drug and an immobilized protein, and simultaneous determination of total drug concentration and unbound drug concentration. The major disadvantage of frontal analysis is the relatively large quantity of drug compound required for each study [19,20].

Immobilized human serum albumin affinity chromatography by zonal elution has been developed for a wide variety of applications including chiral separation, binding equilibrium constant determination, drug protein binding measurements, etc. [21–26,32,34,5,18]. In this method, a small volume of solution containing the drug of interest is injected to immobilized HSA column, and the capacity factor (k') is used to evaluate the protein binding strength of the drug. Relatively fast drug–protein association/dissociation kinetics is assumed in this technique. The k' here is defined as the ratio of solute in the stationary phase to that in the mobile phase, as expressed by Eq. (1):

$$k' = \frac{t_r - t_0}{t_0 - t_m} \quad (1)$$

In the above equation, t_r is the retention time of the drug, t_0 is the retention time of a non-binding compound, and t_m is the system void volume time. It has been claimed by many research groups [1,21,24] that the extent of drug protein binding correlates better with the expression of $k'/(k' + 1)$ than k'. Therefore, drug–protein binding percent (%binding) obtained by conventional method is then regressed against $k'/(k'+1)$, generating a quantitative working curve [21]. The validity of using immobilized albumin as a model for albumin in solution is supported by many studies showing that the binding properties of immobilized HSA are similar to those observed for HSA in solution [27–29]. This method is advantageous in that it requires much smaller sample volume compared to frontal analysis, and is generally feasible for medium to high throughput screening. By employing mass spectrometer as the detector for chromatography, a better sensitivity and specificity is acquired, which is essential in drug mixture analyses.

In this study, we used an immobilized human serum albumin column affinity chromatography, followed by electrospray ionization mass spectrometry equipped with a quadrupole mass spectrometer as the detector. Compared to UV detector, mass spectrometer offers much higher sensitivity and specificity, and has become widely used for both qualitative and quantitative detection. Zonal elution was performed on several commercial available drugs, and k' values for these drugs were obtained according to their retention time. Literature values of %binding of these drugs were then plotted against $k'/(k'+1)$ to generate the working curve. Proprietary compounds were analyzed in the same system, and their %binding was determined by fitting their $k'/(k' + 1)$ values in the quantitative working curve. Results were compared with the %binding obtained by ultrafiltration or dialysis method. A good correlation was found between the data obtained by the conventional methods and immobilized HSA chromatographic method, indicating the feasibility of using the chromatographic method as a means of fast and simple screening in early drug discovery stage.

2. Experimental

2.1. Samples and materials

Acetaminophen, glucose, phynetoin, dipyridamole, quinine, quinidine, tinidazole, salbutamol, salicylic acid, salicylamide, *p*-amino benzoic acid (PABA), and aniline (obtained from Sigma, St. Louis, MO, USA) and triamterene and warfarin (purchased from Aldrich, Milwaukee, WI, USA) were first dissolved as 2 mM stock solution in DMSO. Stock solution of each compound was further diluted as 20 μM working solution in HPLC mobile phase solvent for HPLC/MS/MS analysis. Proprietary compounds were first dissolved in DMSO as 2 mM stock solutions, and further diluted in HPLC mobile phase solvent to make 20 μM working solutions.

2.2. Chromatography

The HPLC system consisted of two Shimadzu LC-10AD-vp pumps with a SCL-10AVP Controller (Shimadzu Corporation, Kyoto, Japan), connected to a PE 200 autosampler (Perkin-Elmer Corporation, Norwalk, CT, USA). An immobilized HSA column (Advanced Separation Technologies Inc., Whippany, NJ, USA) 50 mm, 2.0 mm i.d., 5 μm was used. The mobile phase (pH = 7.4) was 50 mM ammonium acetate (Sigma) pH 7 buffer with two concentrations of isopropanol (4 and 20% by volume). The flow rate was 0.3 mL/min and the column and mobile phases were kept at 25 °C. The retention time (t_r) was the average of at least two consecutive measurements. The dead time t_0 was determined to be 0.57 min using a non-binding compound of glucose and the system void volume time was 0.17 min according to solvent front peak time.

2.3. Mass spectrometry

An API3000 turbo ion spray triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) was employed as the detector for HPLC. The temperature of turbo ion gas, N_2, was set at 400 °C. Selected ion monitoring (SIM) mode was used to monitor each compound.

2.4. Ultrafiltration

The Centrifree Micropartition device (Amicon, Millipore Corporation, Danvers, MA, USA) is designed specifically for rapidly separating unbound from bound drugs in small volume of serum, plasma and other biological fluids. An aliquot of 1 mL sample (10 μM spike in thawed human

plasma) was added to the sample reservoir and allowed to equilibrate at 37 °C for 10 min. Ultrafiltrate was obtained by centrifuging at 37 °C for 10 min (1000 × *g*) in a fixed angle rotor. The concentration of unbound drug in filtrate was determined by LC/MS analysis and the free fraction was calculated as the concentration of drug in the filtrate × 100% divided by the concentration of drug in the original spiked sample.

3. Results and discussion

A total of 12 commercially available compounds, with protein binding ranging from less than 10% to above 90%, were injected individually onto the HSA affinity column using 4% isopropanol and 96% 50 mM aqueous ammonium acetate as the mobile phase. For each compound, only a very small amount was injected (2 μL of 20 μM solution) to assure that linear elution conditions were preserved. For an analyte having a single type of binding site on an immobilized ligand and no other interactions involved, the retention of the analyte on the immobilized ligand is described as [33]:

$$k' = \frac{K_a m_L}{V_m} \tag{2}$$

In the above equation, k' is the capacity factor for analyte, as in Eq. (1), K_a stands for the equilibrium constant for association between the analyte and the immobilized ligand, m_L is the total moles of active binding sites on immobilized column for the analyte, and V_m represents the void volume of the column. It is assumed that the amount of injected analyte is smaller than the total moles of column active binding sites, so that the linear elution condition is maintained. Eq. (2) indicates that k' is proportional to both K_a and m_L. Therefore, a strong binding interaction and a high ligand load will both lead to larger k' value, thus longer retention time.

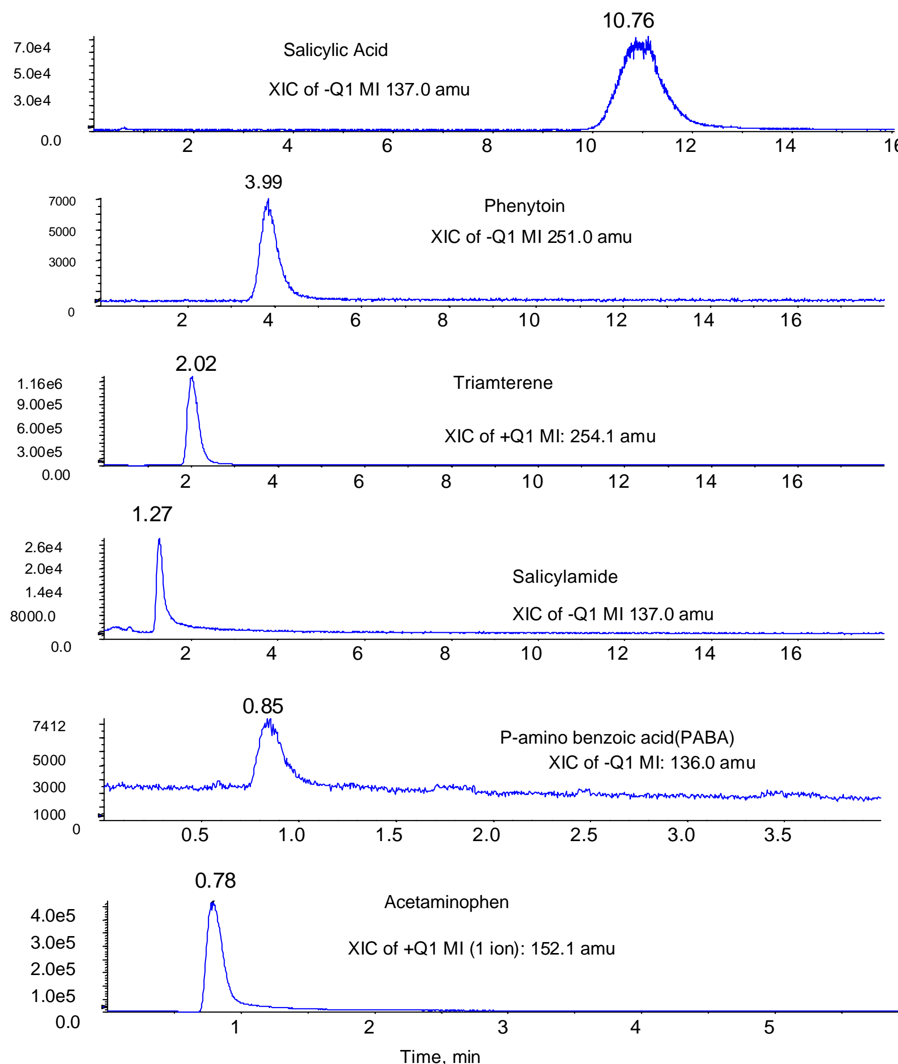

Fig. 1. Extracted ion mass chromatogram of salicylic acid, phenytoin, triamterene, salicylamide, PABA, and acetaminophen. Mobile phase: 96% aqueous 50 mM ammonium acetate, 4% isopropanol. Column: HSA column, 50 × 2.0 mm i.d., Advanced Separation Technologies Inc., Whippany, NJ.

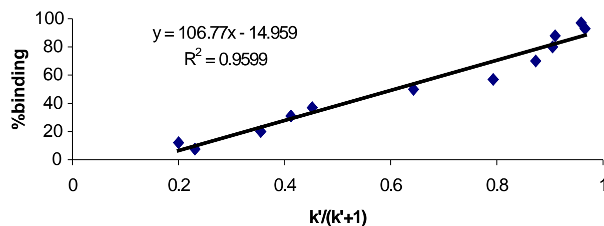

Fig. 2. Quantitation working curve (with 4% isopropanol + 96% NH4OAc water as the mobile phase) by linear regression of literature %binding against $k'/(k'+1)$. Mobile phase: 96% aqueous 50 mM ammonium acetate, 4% isopropanol. Column: HSA column, 50 × 2.0 mm i.d., Advanced Separation Technologies Inc., Whippany, NJ.

The LC/MS chromatogram of some of the tested drugs is shown in Fig. 1. It was found that drugs of lower plasma protein binding tend to elute earlier from HSA column than drugs of higher protein binding. Another observation is that the strong binding drugs tend to elute as broad chromatographic peaks [21], and their run-to-run retention time reproducibility is inferior to the weak binding drugs.

The $k'/(k'+1)$ of each drug was determined according to its retention time. Literature values of %binding (by either ultrafiltration or dialysis method) were then regressed against $k'/(k'+1)$. The working curve, shown in Fig. 2, has good linearity with a correlation coefficient of 0.96 which is quite acceptable considering the diversity of drugs tested and the variety of %binding data resources used. The retention time t_r, $k'/(k'+1)$, literature values of %binding [21,31] for the above tested compounds are found in Table 1.

Seven proprietary compounds (**A**, **B**, **C**, **D**, **E**, **F**, and **G**) were analyzed under the identical chromatographic conditions, and the chromatogram of selected compounds is shown in Fig. 3. Compounds **F** and **G** could not be eluted with the 4% isopropanol and 96% aqueous ammonium acetate mobile phase in 30 min. The $k'/(k'+1)$ of compounds

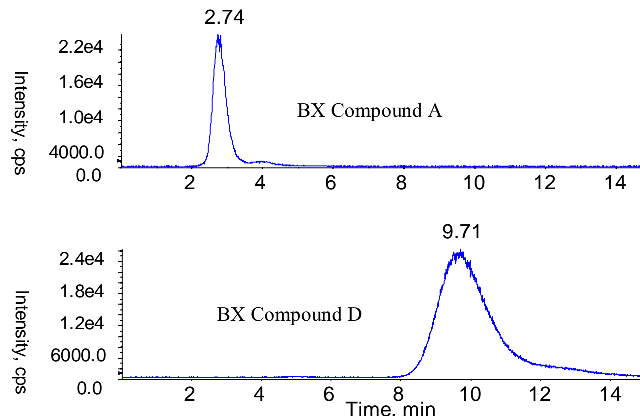

Fig. 3. Extracted ion mass chromatogram of selected proprietary compounds. Mobile phase: 96% aqueous 50 mM ammonium acetate, 4% isopropanol. Column: HSA column, 50 × 2.0 mm i.d., Advanced Separation Technologies Inc., Whippany, NJ.

A–**E** was determined based on their retention time according to Eq. (1), and the %binding was calculated by fitting the $k'/(k'+1)$ data into the working curve in Fig. 2. Table 2 lists $k'/(k'+1)$ values, the calculated %binding, and the %binding obtained in-house by ultrafiltration method for compounds **A**–**E**. The results show that for those compounds, the protein binding obtained by chromatographic and ultrafiltration methods are in good agreement, while chromatography offers protein binding data with much lower standard deviation, indicating it as a more reproducible and precise method [20,23]. Minor discrepancy between the two methods could stem from: (1) in chromatographic method, only albumin is involved in the protein binding model, while ultrafiltration or equilibrium dialysis method is carried out with all plasma proteins. Hence, correlating the percent binding data from ultrafiltration or equilibrium dialysis with $k'/(k'+1)$ in chromatographic method could either overestimate or underes-

Table 1
Commercial drugs tested, their retention time (with standard deviation), $k'/(k'+1)$ values, literature %binding, and %binding calculated based on $k'/(k'+1)$ according to the equation from quantitation working curve in Fig. 2

Compound	t_r (min)	k'	$k'/(k'+1)$	%Binding by chromatography	%Binding from literature [16,31]
System void	0.17 (0.005)				
Glucose	0.57 (0.01)	0	0		
Triamterene	2.10 (0.029)	3.83	0.79	70	57
Salicylamide	1.29 (0.01)	1.80	0.64	54	50
PABA	0.85 (0.01)	0.70	0.41	29	31
Aniline	0.89 (0.00)	0.83	0.45	33	37
Acetaminophen	0.79 (0.00)	0.55	0.36	23	20
Tinidazole	0.67 (0.005)	0.25	0.20	6	12
Salbutamol	0.69 (0.006)	0.30	0.23	10	7.5
Quinidine	3.32 (0.012)	6.88	0.87	78	70
Quinine	4.38 (0.015)	9.53	0.91	82	80
Phenytoin	4.63 (0.02)	10.15	0.91	82	88
Salicylic acid	9.95 (0.17)	23.45	0.96	87	97
Dipyridamole	12.03 (0.17)	28.65	0.97	88	93

Mobile phase: 96% aqueous 50 mM ammonium acetate, 4% isopropanol. Column: HSA column, 50 × 2.0 mm i.d., Advanced Separation Technologies Inc., Whippany, NJ.

Table 2
Proprietary compounds **A**–**E**, their retention time (with standard deviation), $k'/(k'+1)$ values, %binding by ultrafiltration (with standard deviation), and %binding (with standard deviation) calculated based on $k'/(k'+1)$ according to the equation from quantitation working curve in Fig. 2

Compound	t_r (min)	$k'/(k'+1)$	%Binding by ultrafiltration	%Binding by chromatography
BX compound **A**	2.74 (0.03)	0.844	61 (2.58)	75 (0.194)
BX compound **B**	16.17 (0.31)	0.975	78 (3.11)	89 (0.052)
BX compound **C**	14.98 (0.09)	0.973	86 (0.96)	89 (0.018)
BX compound **D**	9.71 (0.15)	0.958	85 (0.96)	87 (0.071)
BX compound **E**	9.35 (0.04)	0.956	87 (1.15)	87 (0.021)

Mobile phase: 96% aqueous 50 mM ammonium acetate, 4% isopropanol. Column: HAS column, 50 × 2.0 mm i.d., Advanced Separation Technologies Inc., Whippany, NJ.

Table 3
Proprietary compounds **F** and **G**, their retention time (with standard deviation), $k'/(k'+1)$ values, and %binding (with standard deviation) calculated based on $k'/(k'+1)$ according to the equation from quantitation working curve (20% isopropanol)

Compound	t_r (min)	$k'/(k'+1)$	%Binding by other methods	%Binding by chromatography
BX compound **F**	4.05 (0.09)	0.89	96	98 (0.066)
BX compound **G**	4.93 (0.16)	0.92	98 (2.38)	99 (0.078)

Mobile phase: 50 mM ammonium acetate, 20% isopropanol. Column: HSA column, 50 × 2.0 mm i.d., Advanced Separation Technologies Inc., Whippany, NJ.

timate the actual protein binding of those compounds (e.g. quinidine and dipyridamole are known to have significant binding to α_1-acid glycoprotein [31]). Affinity columns immobilized with other plasma proteins could be a potential supplement, such as the immobilized α_1-acid glycoprotein column. Although immobilized AAG column has been commercially available and widely used for separation of enantiomers of different compounds, its binding properties were found to be quite different from AAG in solution [19,20]. Therefore, using immobilized AAG column for quantitation of drug protein binding still remains questionable. (2) Compound structure diversity, which leads to binding mechanism complexity [24,26]. De Jong and coworkers [24] have shown that a better correlation was obtained with a series of structurally analogous piperazines compared to the structurally heterogeneous compounds.

In order to elute compounds **F** and **G**, the mobile phase was modified with higher organic composition [30]. Organic modifier such as methanol, 1-propanol, isopropanol, etc. has been reported to decrease the retention time on protein columns while still maintaining stereoselectivity and offering improved peak shape [17,21,24]. Ashton et al. [30] used mobile phases containing 30–40% of isopropanol to elute indolocarbazole derivatives which showed very strong binding to HSA column. They claimed that the column retained its binding property although the mobile phases in their study had much higher organic concentration than is recommended by the column manufacturers. In the present study, by employing the mobile phase of 20% isopropanol and 80% 50 mM aqueous ammonium acetate, Compounds **F** and **G** eluted within 5 min. In Ashton et al. study, the estimation of %binding was realized by extrapolation to 0% isopropanol for every single compound, which was very time consuming and required multiple chromatographic analysis under different isopropanol concentrations. However, we used several strong binding reference compounds to create a quantitative working curve for 20% isopropanol mobile phase, which proved to be a fast and convenient method for analysis of relatively large number of samples. Fig. 4 shows the chromatogram of **F** and **G**, and also warfarin, quinine,

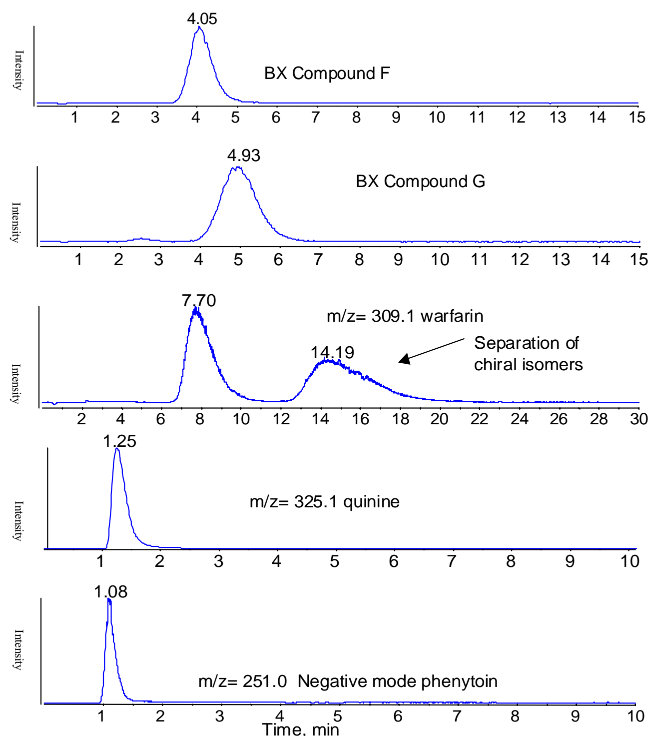

Fig. 4. Extracted ion mass chromatograms of proprietary compounds **F** and **G**, and warfarin, quinine, and phenytoin. Mobile phase: 50 mM ammonium acetate, 20% isopropanol. Column: HSA column, 50 × 2.0 mm i.d., Advanced Separation Technologies Inc., Whippany, NJ.

and phenytoin, used as standards for creating quantitation working curve under the condition of 20% isopropanol mobile phase. Thus, generated working curve has an equation of $y = 27.4725x+73.0687$, and correlation coefficient $R^2 = 0.9974$. The retention time, $k'/(k' + 1)$, %binding by other methods, and %binding by chromatography for **F** and **G** are listed in Table 3.

While performing ultrafiltration on compound **F**, the non-specific binding of this compound to the Centrifree Micropartition device membrane was found to be greater than 90%. Therefore, ultrafiltration method was not suitable for determination of protein binding of this compound. An alternative method using human red blood cell as a biological equilibrium dialysis (BED) vehicle was used. The red cells from 30 ml of freshly heparinized human blood were washed and suspended in either plasma or buffer at pH 7.4. After incubation of the suspension with the compound allowing equilibration between the unbound concentration, the analyte levels in the red cell and in plasma or buffer were determined. The plasma protein binding can then be calculated from the concentration ratios obtained in the red cell suspensions. Compared with BED method, which requires complicated sample preparation and long analysis time, HSA affinity chromatography LC/MS is much faster and more convenient, while offering quite precise results at the same time. This observation also indicates that for compounds with high non-specific binding to artificial membrane, which makes them not suitable for normal ultrafiltration and equilibrium dialysis devices, chromatography seems to be a very promising choice.

4. Conclusion

A fast and convenient method, HSA immobilized column chromatography was employed to determine the plasma protein binding of compounds in early drug discovery process. Compounds of protein binding <95% are eluted out with 4% isopropanol 96% 5 mM ammonium acetate mobile phase in practical analysis time (within 15 min). Compounds of high protein binding (>95%) are eluted out with mobile phase of much higher organic concentration (20% isopropanol). Fig. 5 shows the strategy flowchart of analyzing compounds of a great variety of protein-binding properties. The integrity of the column was ascertained periodically by injecting previously studied reference compounds using 4% isopropanol mobile phase. No discernable differences in the binding properties were observed after the usage of 20% isopropanol in mobile phase.

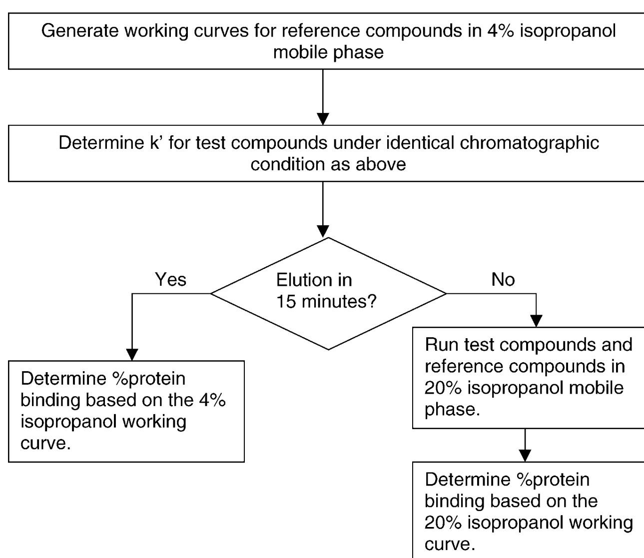

Fig. 5. Strategy flowchart of analyzing large number of samples covering wide range of protein binding.

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