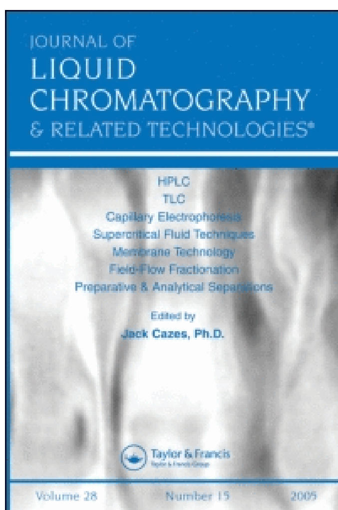
This article was downloaded by:
On: *23 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High Throughput Screening of Drug-Protein Binding in Drug Discovery

Hong Wan[a]; Fredrik Bergström[a]
[a] Lead Generation DMPK and Physical Chemistry, AstraZeneca R&D Mölndal, Mölndal, Sweden

To cite this Article Wan, Hong and Bergström, Fredrik(2007) 'High Throughput Screening of Drug-Protein Binding in Drug Discovery', Journal of Liquid Chromatography & Related Technologies, 30: 5, 681 — 700
To link to this Article: DOI: 10.1080/10826070701190989
URL: http://dx.doi.org/10.1080/10826070701190989

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Journal of Liquid Chromatography & Related Technologies®, 30: 681–700, 2007
Copyright © Taylor & Francis Group, LLC
ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701190989

High Throughput Screening of Drug-Protein Binding in Drug Discovery*

Hong Wan and Fredrik Bergström
Lead Generation DMPK and Physical Chemistry, AstraZeneca R&D
Mölndal, Mölndal, Sweden

Downloaded At: 17:47 23 January 2011

Abstract: Characterization of drug-protein binding is essential, since it has profound effects on pharmacokinetic and pharmacodynamic parameters of new chemical entities. Although the traditional equilibrium dialysis method is well established and widely used for drug-protein binding measurement, this assay suffers from its low throughput. Recent efforts have been made in developing and implementing high throughput assays for drug-protein screening in drug discovery. The present review highlights high-throughput assays, as well as potential new approaches for drug-protein binding screening in drug discovery. These primarily include automated 96-well plate, sample pooling based equilibrium dialysis combined with LCMS, immobilized HSA column HPLC, and electrodriven techniques such as frontal analysis combined with capillary electrophoresis (FACE), and affinity capillary electrophoresis (ACE). Fundamental drug-protein binding models are also discussed in order to (1) compare and discriminate HSA (human serum albumin) and AGP (alpha 1-acid glycoprotein) binding with regard to the whole plasma protein binding; and (2) elucidate the applicability of high throughput screening (HTS) from the viewpoint of a simplified binding model. Some strategies and recommendations are proposed in dealing with applications of high throughput assays in different situations.

Keywords: Review, High throughput screening, Drug-protein binding, Drug-protein binding model

*Invited review

Address correspondence to Hong Wan, Lead Generation DMPK and Physical Chemistry, AstraZeneca R&D Mölndal, Mölndal SE-431 83, Sweden. E-mail: hong.wan@astrazeneca.com

INTRODUCTION

In drug discovery, drug-protein binding data (fraction unbound drug, f_u) can be used (1) to better understand *in vivo* pharmacokinetic (PK) profile such as distribution volume, clearance and half-life; (2) to design optimal dose regimes and estimate safety margins; (3) to interpret pharmacodynamic (PD) data, as it is generally accepted that only unbound drug (free concentration) is pharmacologically active or responsible for a desired *in vivo* efficacy. It was estimated that about 40% development compounds fail to reach market due to poor pharmaceutical properties[1] (references, therein). This drives pharmaceutical companies to profile drug-like properties as early as possible in order to increase the success rate of compounds to the market. Therefore, the ability to screening of drug-protein binding becomes an important issue in drug discovery, even in early ADME in modern drug design. So far, equilibrium dialysis is the preferred and most widely used technique for protein binding measurement in most of pharmaceutical communities, because it offers accurate binding data due to the fact that the drug binding to plasma proteins is analyzed at equilibrium.[2] However, such a traditional equilibrium dialysis method, as depicted in Fig. 1, is labor-intensive and time-consuming with limited sample throughput capacity, as well as relatively large plasma sample consumption, which substantially limits screening a large number of compounds. Thus, development of alternative new methods and technologies

Downloaded At: 17:47 23 January 2011

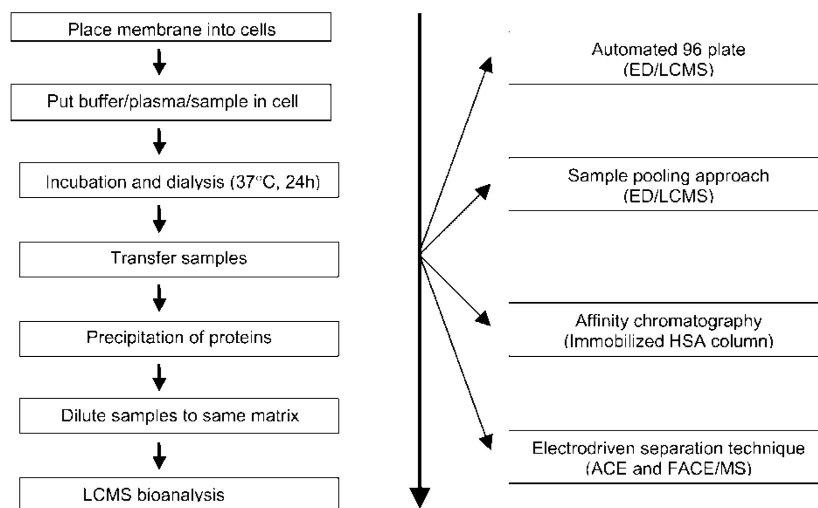


Figure 1. Traditional low throughput equilibrium dialysis versus high throughput screening technologies. ED: equilibrium dialysis; LCMS: liquid chromatography mass spectrometry; immobilized HSA column: immobilized human serum albumin; ACE: affinity capillary electrophoresis; FA: frontal analysis.

is highly desirable to increase throughput and reduce costs. Development of high throughput assays or novel pharmaceutical approaches while limiting the increasing costs enables the advance of improved *in vitro* models to accelerate drug designs. In this review, we will summarize recent developments with emphasis on high throughput technologies and new approaches applicable for drug-protein binding screening as outlined in Fig. 1.

Multi-Site Binding and One-to-One Binding Models

Several drug-protein binding models have been applied to characterize the interaction between drug and protein. In most cases, the drug-protein interactions are analyzed by either multi-site binding model (Eqs. (1)–(5)) or a simplified one-to-one binding model (Eqs. (6, 7)).[3,4]

$$r = \frac{[PD]}{P} = \sum_{i=1}^{m} \frac{n_i k_i [D]}{1 + k_i [D]} \qquad (1)$$

m	classes of independent binding sites
n_i	the number of sites of class i
k_i	the corresponding association constant (binding constant)
r	fraction of bound ligand molecules per protein molecule
P	the total concentration of protein
$[D]$, $[PD]$	the concentration of free and bound drug, respectively.

If we assume only two classes of binding sites ($n = 2$); Eq. (1) can be expressed as:

$$r = \frac{n_1 K_1 [D]}{1 + K_1 [D]} + \frac{n_2 K_2 [D]}{1 + K_2 [D]} \qquad (2)$$

where K_1, K_2 are the primary and secondary binding constants for the corresponding first binding site (n_1) and second binding site (n_2).

Since total drug concentration, C, and total protein concentration, P, can be written as:

$$C = [D] + [PD] \Leftrightarrow [PD] = C - [D] \qquad (3)$$

$$P = [P] + [PD] \Leftrightarrow [P] = P - [PD] \qquad (4)$$

Then, r can be obtained by measuring free drug concentration $[D]$ using Eq. (5):

$$r = \frac{[PD]}{P} = \frac{C - [D]}{P} \qquad (5)$$

Downloaded At: 17:47 23 January 2011

By applying a wide range of known drug concentrations C, a series of free concentration $[D]$ can be determined. Accordingly, two independent binding constants (K_1 and K_2) can be obtained by Eq. (2) using a non-linear regression approach. This is a general approach to evaluate drug-protein binding associated with two binding sites where saturable (n_1 and K_1) and nonsaturable (n_2 and K_2) phenomena occur. Although this generalization does not include the fact that the drug most likely bind to the protein on several binding sites in a diverse order of affinity, it is often applicable to characterize drug-protein interaction. As the primary binding constant $K_1(\sim 10^5\ \mathrm{M}^{-1})$ is typically much stronger by a magnitude of one to two orders than the secondary binding constant K_2 $(\sim 10^3\ \mathrm{M}^{-1})$,[2] Eq. (2) can be simplified to Eq. (6), which concerns only first binding site (n_1) and its corresponding binding constant K_1.

$$r = \frac{n_1 K_1 [D]}{1 + K_1 [D]} \tag{6}$$

For the case where the number of primary binding sites are approximately 1 for both HAS[2–5] and AGP,[6] the rearrangements of Eq. (5) and (6) yield Eq. (7), which is eventually a simplified one-to-one model.

$$K_a = \frac{[PD]}{[D][P]} = \frac{C - [D]}{[D]^2 + [D](P - C)} \tag{7}$$

When binding constant K_a is obtained, fraction bound (bound %) and unbound (f_u %) can be calculated according to Eq. (8) and (9), respectively.[4]

$$\mathrm{Bound}\,(\%) = 100 * \frac{(K_\mathrm{a}C + K_\mathrm{a}P + 1) - \sqrt{(K_\mathrm{a}C + K_\mathrm{a}P + 1)^2 - 4K_\mathrm{a}^2 PC}}{2K_\mathrm{a}C} \tag{8}$$

$$f_u(\%) = 100 - \mathrm{drug\ bound}\,(\%) \tag{9}$$

It should be noted that the one-to-one model (Eq. (7)) can be rearranged and derived to another expression (Eq. (10)),[7,8] provided that drug concentration is much lower than protein concentration. Equation 10 has also been employed to estimate the tissue binding.

$$\mathrm{Undiluted}\, f_u = \frac{1/D_t}{(1/fu_{mea} - 1) + 1/D_t} \tag{10}$$

where $f_{u_{mea}}$ and D_t represent the measured free fraction in diluted matrices and dilution times, respectively.

The advantage of Eq. (10) over Eq. (8) is that unbound fractions in undiluted species like plasma or brain tissue homogenates can be re-calculated from measured fraction in diluted forms without the necessity of knowing the identity or the exact concentrations of binding components.

In summary, for the case of *in vitro* drug-protein binding screening where the total protein concentration (approximately 600 μM in plasma) is in excess of drug concentration (typically 10 μM), it is reasonable to apply a one-to-one

Downloaded At: 17:47 23 January 2011

binding model (nonsaturable) to characterize the drug-protein interaction by an explicit equation (Eq. (7)). Although the application of multi-binding model (Eq. (1)–(5)) can more sufficiently quantify drug-protein interaction where more than one-binding site exists in a saturable condition, a number of experiments are required, thus constraining high throughput screening. It should be addressed that, in principle, the determination of binding constant (K_a) is more suitable for high throughput screening since K_a is independent of drug concentrations. As a result, a number of compounds can be pooled and simultaneously screened. On the other hand, the concentration-dependent assay, though to some extent, would limit the sample pooling; it is still applicable for high throughput screening provided that a low total drug concentration is applied as to minimize possible binding site saturation. An example of this will be further elucidated in a later section.

Downloaded At: 17:47 23 January 2011

Comparative Analysis of HSA and AGP Binding

HSA and AGP are abundant proteins in plasma, which primarily governs the whole plasma protein binding. It has been generally believed and demonstrated that acidic drugs bind strongly to HSA, while basic and neutral drugs bind more to AGP.[6,9,10] As shown in Table 1, the binding affinity constants of basic compounds to HSA are greater than AGP. According to Schuhmacher et al.[11] the fraction of unbound drug in a mixture of proteins can be calculated by single protein binding data using the following equation (derived by a one-to-one binding model):

$$f_{u(HSA+AGP)} = 1\Big/\left(1 - n + \sum_{i}^{n} \frac{1}{f_{ui}}\right) = 1\Big/\left(\frac{1}{f_{uHSA}} + \frac{1}{f_{uAGP}} - 1\right) \qquad (11)$$

Table 1. Comparative binding affinity and fraction unbound of HSA and AGP

Compound	Lidocaine	Imipramine	Propranolol	Chlorpromazine
pK_a	7.79	9.52	9.55	9.12
$LogKa_{HSA}$ (M^{-1})	2.85	3.26	3.34	4.05
$f_{u(HSA)}$%	71	49	44.3	13.5
$LogKa_{AGP}$ (M^{-1})	4.5	4.74	5.67	6.7
$f_{u(AGP)}$%	65.4	54.1	15.6	1.9
$f_{u(HSA+AGP)}$%	51.6	34.6	13.0	1.7
$f_{u(plasma)}$%	50	18	21	3[a]

pKa, $LogKa_{HSA}$ and $LogKa_{AGP}$ data adapted from ref 9; $f_{u(HSA)}$ and $f_{u(AGP)}$ calculated using Eq. (8) and (9) based on HSA concentration 580 μM and AGP 20 μM, respectively; $f_{u(HSA+AGP)}$ calculated according to Eq. (11) using individual protein binding data $f_{u(HSA)}$ and $f_{u(AGP)}$; a drug concentration of 10 μM is used in all cases.

[a]Data from pharmacokinetic database Goodman & Gilman 1996.

$f_{u(HSA+AGP)}$	fraction of free (unbound) drug in a mixture of proteins
n	number of proteins involved in plasma protein binding (mainly HSA and AGP)
f_{ui}	fraction of free (unbound) drug in an individual protein solution.

Based on the fact that HSA and AGP are primary proteins contributing to the whole plasma protein binding, fraction unbound drug in a mixture of proteins of HSA (580 μM) and AGP (20 μM) can be calculated by individual HSA and AGP binding data using Equation (11). Consequently, the contribution of drug binding to HSA and AGP can be compared with the whole plasma proteins. Table 1 gives the calculated f_u data, which exemplifies the stronger binding of the basic compounds (pK_a from 8 to 9.5) to AGP than that to HSA. As indicated in Table 1, if the binding affinity of the basic compounds to AGP is more than two orders of magnitudes higher than that to HSA, the contribution of AGP binding to the whole plasma protein tends to be significant. More specifically, AGP binding, rather than HSA, influences the whole plasma protein binding more, as exemplified by propranolol and chlorpromazine whose binding affinity to AGP affinity is two orders of magnitude stronger than that to HSA. As a result, the fraction unbound $f_u(AGP)$ is close to that of $f_u(HSA + AGP)$. Furthermore, in Table 1, the calculated $f_u(HSA + AGP)$ is in line with the measured f_u in plasma, although the variability of reported plasma protein data was noticed due to different techniques and conditions.[12] On the other hand, as demonstrated in Table 2, if the binding affinity constant of HSA is not less than one order of magnitude weaker than that of AGP, the HSA, rather than AGP, binding predominates the whole plasma protein binding (as marked in gray area in Table 2). In other words, the AGP contribution to plasma protein binding can be ignored in such circumstance. This is attributed to the fact that the HSA concentration is much higher than the AGP concentration in the plasma, thus dominating the whole plasma protein binding. Because of binding specificity and concentration differences of AGP and HSA in the plasma, a caution should be taken when employing HSA binding data instead of plasma protein binding, in particular in interpretations of binding data from various species. Although the plasma concentration of AGP is much lower than that of HSA, AGP can become the major drug binding macromolecule in plasma with significant clinical implications.[10] Current comparative analysis of HSA and AGP binding will help us better understand the binding differences of two abundant plasma proteins, thus facilitating the appropriate applications of available techniques for high throughput screening of plasma protein binding.

Downloaded At: 17:47 23 January 2011

Downloaded At: 17:47 23 January 2011

Table 2. Simulated fraction unbound $f_u(HSA+AGP)$ at various binding affinity

$f_{u(HSA+AGP)}\%$	LogKa = 2 $f_{u(HSA)}\% = 95$	LogKa = 3 $f_{u(HSA)}\% = 63$	LogKa = 4 $f_{u(HSA)}\% = 15$	LogKa = 5 $f_{u(HSA)}\% = 1.7$	LogKa = 6 $f_{u(HSA)}\% = 0.18$	LogKa = 7 $f_{u(HSA)}\% = 0.018$
LogKa = 2 $f_{u(AGP)}\% = 100$	94	63	15	1.7	0.18	0.018
LogKa 3 $f_{u(AGP)}\% = 98$	93	63	15	1.7	0.18	0.018
LogKa = 4 $f_{u(AGP)}\% = 84$	80	57	14	1.7	0.18	0.018
LogKa = 5 $f_{u(AGP)}\% = 41$	40	33	12	1.7	0.17	0.018
LogKa = 6 $f_{u(AGP)}\% = 8.4$	8.4	8.1	5.7	1.5	0.17	0.018
LogKa = 7 $f_{u(AGP)}\% = 0.98$	0.98	0.98	0.93	0.63	0.15	0.017

$f_{u(HSA)}$ and $f_{u(AGP)}\%$ calculated according to Eq. (8), a drug concentration of 10 μM is used in all cases; $f_{u(HSA+AGP)}$ calculated according to Eq. (11) on the basis of individual binding data $f_{u(HSA)}$ using HSA as 580 μM and $f_{u(AGP)}$ using AGP as 20 μM.

HIGH THROUGHPUT ASSAYS FOR DRUG-PROTEIN BINDING SCREENING

Equilibrium Dialysis with Automatic 96-Well Plate and LC/MS

Recently, throughput of the traditional equilibrium dialysis method has been improved by implementation of a 96-well format. Kariv et al. presented a 96-well equilibrium dialysis device for plasma protein binding measurement and validated three drugs, propranolol, paroxetine, and losartan, with low, intermediate, and high binding properties, respectively.[13,14] They concluded that high-throughput 96-well equilibrium dialysis device showed good correlation to the traditional method and it is compatible with the HTS format for automated liquid handling and bioanalytical mass spectrometry. Banker et al. also presented a novel 96-well format dialysis apparatus for measuring plasma protein binding[15] which is a vertical design having advantages over current 96-well dialysis on the market in terms of surface-to-volume ratio and reduction of non-specific binding (NSB). The device, made of Teflon material, is not only accessible by a robotic system for easy automation, but is also reusable. In addition, a semi-automatic, high-throughput, 96-well plate ultrafiltration has also been employed to rapidly assess plasma protein binding of new chemical entities.[16] It is well known that ultrafiltration is not suitable for measuring highly bound compounds due to the NSB effect. However, this drawback seems to be overcome by a recently modified ultrafiltration methodology, i.e., mixing of control plasma retentate with the filtrate, thus eradicating the NSB effect.[17] Currently, several 96-well equilibrium dialyzers are also commercially available from Linden Bioscience (rapid equilibrium dialysis device, Ricerca). Obviously, the application of 96-well format dialyzer improves throughput as compared to the traditional single chamber based devices. However, the assay is usually based on single compound measurements; hence they are not amenable for screening large sets of compounds. It should be addressed that, for each compound, a number of samples from both buffer and plasma sides has to be analyzed by LCMS, which essentially limits throughput of the whole assay.

Sample Pooling Based Equilibrium Dialysis and LC/MS

Recently, Wan and Rehngren have presented a new approach for screening plasma protein binding, based on equilibrium dialysis combined with rapid generic LC/MS bioanalysis.[12] Using a simple sample pooling, they have been able to simultaneously screen ten compounds and demonstrated good correlations between single compound and pooled compounds. An example of this is illustrated in Fig. 2 for a test set of structurally diverse compounds including commercial drugs and proprietary compounds. Compared with the

Downloaded At: 17:47 23 January 2011

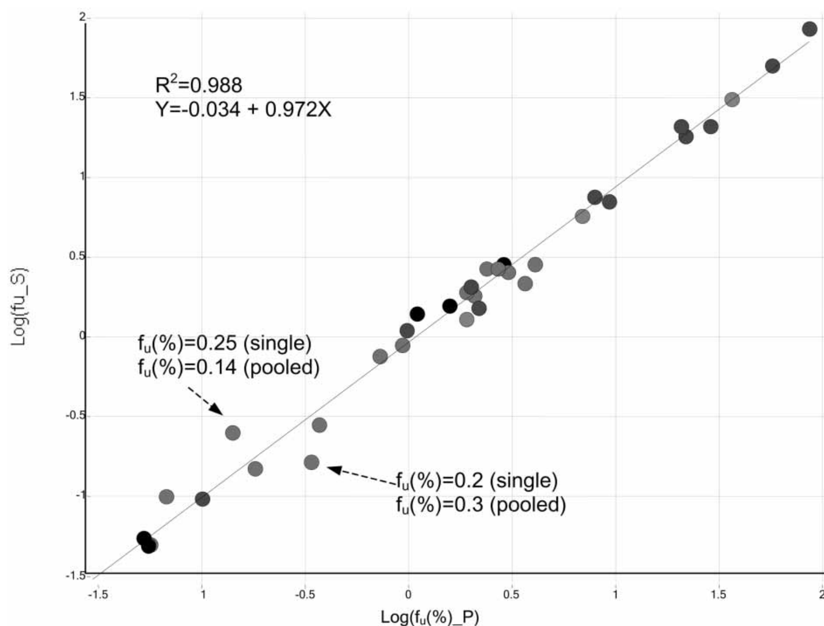


Figure 2. Correlation of $f_u(\%)$ between single and pooled compounds.[12] $f_u(\%)$ measured in different plasma species: black: mouse plasma; blue: human plasma for reference standards; green: human plasma for AZ compounds; red: guinea pig plasma for AZ compounds; sample pooling from 3 to 11 compounds; total number of tested compounds n = 36; Log($f_u(\%)$_S) and Log($f_u(\%)$_P) are the Log units of $f_u(\%)$ from single and pooled compounds, respectively. Reproduced from [12], Wan, H.; Rehngren, M. High-throughput screening of protein binding by equilibrium dialysis combined with liquid chromatography and mass spectrometry. Journal of Chromatography A **2006**, *1102*, 125, with permission from Elsevier B.V.

Downloaded At: 17:47 23 January 2011

variations of f_u by conventional single compound measurements, the sample pooling affords acceptable accuracy for high throughput screening of protein binding. Another distinct advantage of sample pooling is that this approach allows simultaneous determination of new compounds of interest and reference control standards under the same conditions, thereby ensuring data quality. Their results also support an earlier study by Fung et al. who showed reasonably good correlations between single and pooled compounds by an ultrafiltration technique.[16] Tight correlations between single and pooled compounds by both equilibrium dialysis and ultrafiltration techniques can be attributed mainly to the fact that (1) HSA binding predominates the whole plasma binding; (2) drug-protein is equilibrated in a rapid and reversible way without primary the binding site being saturated. Based on the theoretical calculation by Equations (7) and (8), the applicability of sample pooling could be elucidated by a one-to-one binding model, concluding that f_u is

constant or independent of a drug's concentration if the protein concentration is more than five-fold higher than the drug concentration[12] (Fig. 3). Bearing in mind that this calculation was based on the assumption that the drug predominantly binds to HSA in an unsaturable condition and only the first primary binding constant is taken into account. In addition to increased throughput capacity, the sample-pooling approach for protein binding screening is particularly beneficial for measuring *in vitro* protein binding for small volume plasma, such as mouse plasma where the plasma is available only in limited amounts. A further potential application of sample pooling is its suitability to screening the fraction of unbound brain homogenates for rapid CNS penetration classification (unpublished data). The sample pooling approach is expected to more efficiently and rapidly assess plasma protein binding of new chemical entities in the drug discovery phase.

Downloaded At: 17:47 23 January 2011

Immobilized HSA HPLC Column

As human serum albumin (HSA) is a major determinant of whole plasma proteins, immobilized HSA has been utilized as a viable alternative to rapidly screen plasma protein binding. The fundamental theory and practice of using immobilized HSA has been reviewed by Hage[18] and Valko.[19] Advantages of this approach include its speed, precision, and good correlation versus solution based methods, ease of automation, and the ability to reuse the same column for more than hundreds of samples.[19] More repeatable binding data are obtainable from the immobilized column than by free solution based dialysis. In brief, the method is based on the assumption that the chemically bonded HSA retains the binding specificity and conformational mobility of the native serum albumin. Accordingly, the percent of drug bound to HSA can be rapidly obtained by simply measuring the retention time/factor (k') to correlate HSA bound percent by Eqs. (12)–(13).[19]

$$k = \frac{(t - t_0)}{t_0} = \frac{[PD]}{[D]} = K_a[P] \tag{12}$$

$$\%HSA = 100\frac{[PD]}{[D] + [PD]} = 100\left(\frac{k}{1 + k}\right) \tag{13}$$

Experimentally, a group of reference compounds with a wide range of plasma protein binding can be used for calibration of %HSA and k', providing condition independent binding data. It should be pointed out that the immobilized HSA column is very important for measuring kinetic binding, while dialysis with free solution is an equilibrium based measurement. In general, good agreement is obtained between the two different methodologies, which proves the validity of using chromatography as a tool to study or screen drug-protein interaction. Beaudry et al. demonstrated a good correlation coefficient ($R^2 = 0.799$) for a wide variety of 40 structurally

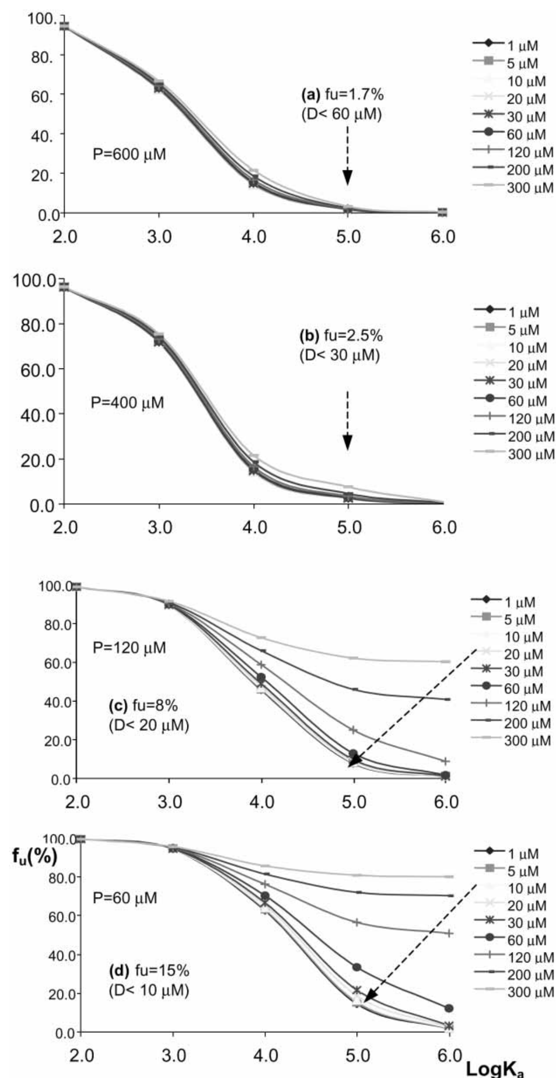


Figure 3. Simulated $f_u(\%)$ functions as different affinities (Log K_a) at different drug and protein concentrations.[12] (a) Albumin concentration 600 μM (representing 100% plasma); (b) protein concentration 400 μM; (c) protein concentration 120 μM (20% plasma); (d) protein concentration 60 μM (10% plasma). The calculation from the theoretical one-to-one binding model indicate $f_u(\%)$ value as marked in the figures is nearly independent of the drug concentrations when its concentration is maintained more than 5 times lower than total protein concentration, thereby implying the applicability of sample pooling. Reproduced from Ref. [12], Wan, H.; Rehngren, M. High-throughput screening of protein binding by equilibrium dialysis combined with liquid chromatography and mass spectrometry. Journal of Chromatography A **2006**, *1102*, 125, with permission from Elsevier B.V.

Downloaded At: 17:47 23 January 2011

unrelated compounds with different binding affinities (bound percent from 0 to 99%) and found a better correlation ($R^2 = 0.824$) using a quantitative structure-retention relationship approach.[20] Their experiments were performed with a mobile phase of 6% acetonitrile containing 0.2% (v/v) acetic acid. Another study by Chen et al.[21] demonstrated an excellent correlation ($R^2 = 0.9599$) for a group of 12 commercial compounds (bound percent from 7.5 to 97) as well as a good correlation for 7 proprietary compounds (bound percent 61 to 98) using 4% isopropranol-containing mobile phase. They suggested the use of 20% isopropranol instead of 4% isopropranol to screen highly bound compounds, e.g., 99% bound. Using similar conditions containing 5% isopropranol in the mobile phase, Buchholz et al. obtained a moderate correlation ($R^2 = 0.661$) for a variety of 69 chemical structures.[22] Impressively, employing a fast isopropranol gradient system, Valko et al. demonstrated a reasonably good correlation for a large number of diverse compounds and an example of correlation between HSA and plasma protein binding by conventional methods is shown in Fig. 4.[23] They also showed that the binding data obtained from a fast gradient condition was comparable to that of isocratic elution with much longer times for strongly bound drugs. Based on a simple and fast separation, Valko et al. have generated more than 4,000 HSA binding data sets for lead optimization in early drug discovery.[24] Interestingly, it was found that the HSA binding data resulted in better correlation with volume of distribution than plasma protein binding (HSA used for volume of distribution prediction). It was suggested that a combination of binding data from both HSA and AGP columns should further improve correlation with plasma protein binding.[24] In a more recent study with a mobile phase containing 7% isopropranol, a comparison of protein binding of a group of acidic, basic, and neutral compounds with literature values showed excellent correlation ($R^2 = 0.9857$, bound percent from 12 to 97), while a poor correlation was observed from the AGP column. They concluded that the current commercial HSA columns can be used for drug-protein binding studies, while AGP columns are not as appropriate for such work.[25]

In view of the published HSA data,[23] it appears that highly bound compounds exhibit poorer correlations than those with lower binding ranges, comparing plasma protein binding. A poor correlation between HSA binding and plasma protein binding for the compounds with higher plasma protein binding is noticed (plasma protein binding $>90\%$, $R^2 = 0.375$, cf. Fig. 4). This analysis agrees well with the observation by Buchholz et al. who obtained better correlations for compounds with a wide range of protein binding, in contrast to those with highly bound compounds.[22] This might be explained by the differences of HSA and AGP binding data, as presented in Tables 1 and 2. More specifically, if AGP binding affinity is greater than HSA, particularly at higher binding affinity, it will more significantly impact the whole plasma protein binding, and vice versa. However, this may not necessarily imply that the HSA immobilized column is less suitable

Downloaded At: 17:47 23 January 2011

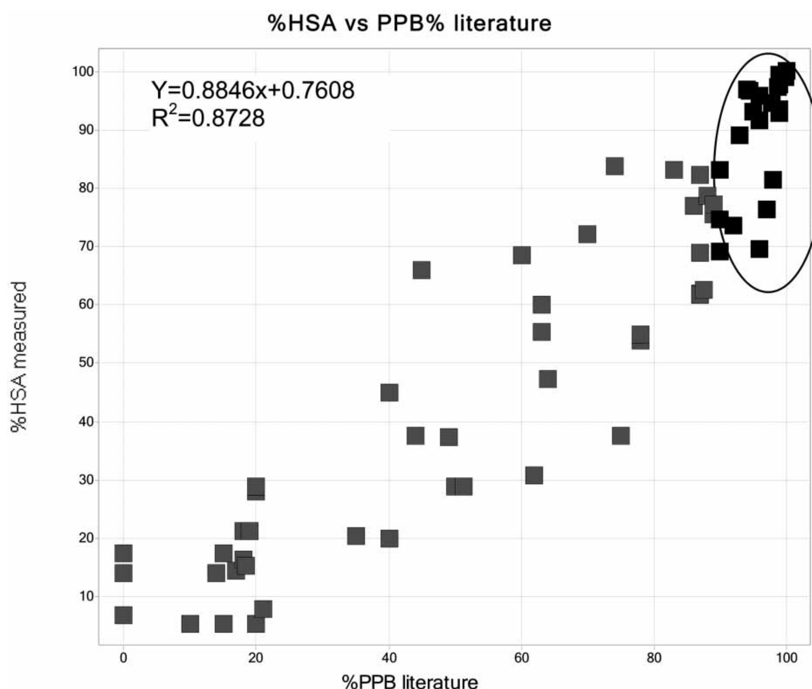


Figure 4. Plot of the literature plasma protein binding as a function of the measured HSA binding data.[23] Overall correlation for a wide range of bound percent (0–97%); data marked in circle: correlation for relatively strongly bound compounds with bound percent higher than 90 % (data with curtsey of Klara Valko). Reproduced from Ref. [23], Valko, K., Nunhuck, S., Bevan, C., Abraham, M. H., Reyncilds, D. P. Fast gradient HPLC method to determine compounds binding to human serum albumin. Relationships with octanol/water and immobilized artificial membrane lipophilicity. Journal of Pharmaceutical Sciences, ***2003,*** *92*, 2236, with permission from Wiley-Liss, Inc, a subsidiary of John Wiley & Sons, Inc.

Downloaded At: 17:47 23 January 2011

for screening highly bound compounds, rather than probably being useful for cut-off of highly bound compounds for the lead series with the goal of reducing the extent of binding. An important issue should be first to identify which protein (primarily HSA or AGP) binds to which drug. As pointed out,[23] it is not essential, at the earlier stages of the drug discovery process, to provide accurate measurements of binding. However, it is very important that the measured values provide a reproducible ranking order of the compounds. This makes possible the development of quantitative structure-property relationship (QSPR) in modification of the structure without decreasing the primary activity of the molecules on a particular target. For instance, Colmenarejo et al. were able to develop a general QSPR model to predict binding affinity to HSA by means of a readily accessible HSA column.[26,27]

It is speculated that high throughput screening from an immobilized column can considerably improve cost-benefit ratios without compromising data quality and providing relevant information for interpretation of PK/PD related parameters.

In addition to the immobilized HSA column, a solid supported membrane, such as a unilamellar liposomal membrane covalently bound to porous silica beads, has been developed for high throughput determination of the free fraction of drugs strongly bound to plasma proteins[28] (commercially available as Transil®), based on the distribution of drugs between plasma water, plasma proteins, and solid-supported lipid membrane. The method was validated with a wide range of lipophilicities in particular for highly bound compounds (log P = 1.9–5.6) and f_u values (35–0.018%), but few applications have been reported.

Downloaded At: 17:47 23 January 2011

Electromigration Technique

Frontal Analysis-Capillary Electrophoresis (FACE)

Frontal analysis, combined with capillary electrophoresis (FACE), is a new and alternative technique for drug-protein interaction studies by quantitatively measuring the frontal peak height of drug resolved from the drug-protein complex, compared to that of a standard drug in the absence of protein. Some typical applications were earlier reviewed,[29] and the potential of this technique has been recently reported.[30] The unique feature of this technique is that the measurements are typically done in a single step at near-physiological conditions without disturbing the binding conditions during equilibrium, thus offering the possibility of rapid protein binding screening. This approach turns out to be much less labor intensive than conventional equilibrium and ultrafiltration methods where separation steps by LC/MS are required. FACE is suitable for measurements of diverse structures, including basic, neutral, and acidic compounds with a wide range of binding constants from low to high binding affinities. Jia et al. reported a pressure-assisted FACE approach for rapid determination of binding constants of 17 drugs to HSA and of 4 drugs to AGP.[9] Martinez-Pia et al. used a short-end injection (back vacuum) for rapid evaluation of drug binding to HSA and human plasma.[5] With analysis times shorter than 3 min for each separation, the measured binding constants are consistent with those by much longer migration times, indicating that the FACE technique is well suited for high throughput screening of drug-protein binding. Reproducibility of measurements for this method is typically better than 10% coefficient of variation,[5,9] which is comparable to conventional equilibrium dialysis techniques. One major limitation of the technique is its low sensitivity in UV detection, which is unsuitable to screen *in vitro* drug-protein binding at a low drug concentration, e.g., 10 μM. In addition, if the

drug is not resolved from the protein, it is difficult to evaluate binding in some circumstances. However, these drawbacks might be overcome by the application of MS detection. Wan and co-workers have recently illustrated a single run measurement of protein binding by FACE coupled with MS detection.[31] This novel approach, as shown in Fig. 5, enables measurements of drug-protein binding by a single run separation without performing necessary off-line calibration curves, thereby leading to an increased throughput and more reproducible binding data as well. Meanwhile, recovery can also be evaluated. With current CEMS (Agilent MSD ion trap mass spectrometer), measurements of *in vitro* plasma protein binding with drug concentrations lower than 10 μM were achievable. Sensitivity is expected to be further improved by application of novel CEMS interfaces, particularly with

Downloaded At: 17:47 23 January 2011

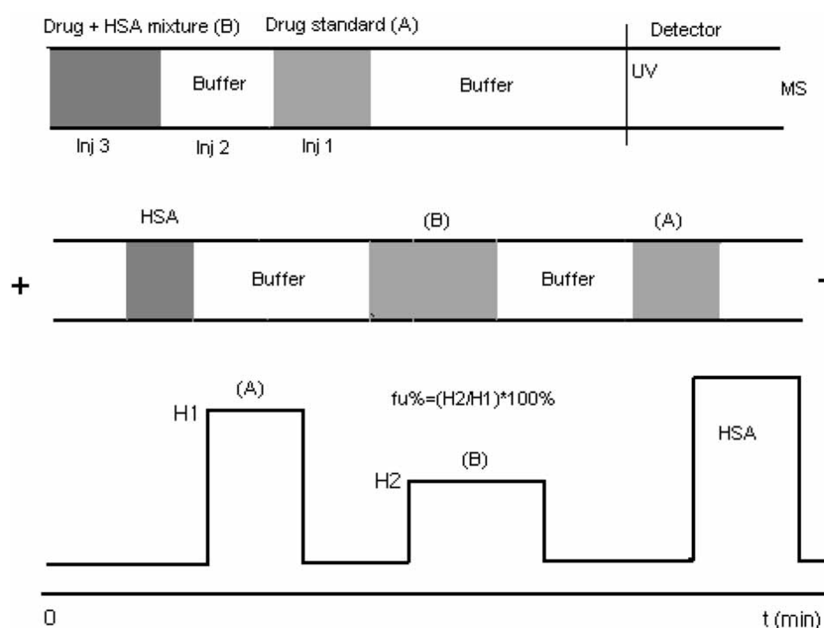


Figure 5. Illustration of single run measurement of protein binding by FACE/MS.[31] H_1 and H_2 are the peak plateau heights of drug standard and the drug resolved from drug-protein mixture, respectively. $f_u\%$ is the unbound free fraction of drug to specific protein ($f_u = H_2/H_1$). For plasma protein binding, diluted plasma concentrations such as 10 to 20 % can be utilized. The fraction unbound in 100% plasma can be re-calculated using Eq. 10. This novel approach enables unbound fraction measurement in a single run with potential of high throughput screening. Reproduced from Ref. [31], Wan, H., Östlund, Å., Jönsson, S., Lindberg, W. Single run measurements of drug-protein binding by high performance frontal analysis-capillary electrophoresis and mass spectrometry. Rapid Communications in Mass Spectrometry, ***2005***, *19*, 1603. with permission from John Wiley & Sons, Inc.

nano-spray based designs, or new ion traps and TOF instruments. Another benefit of using MS detection is that the drug molecules can be detected even without being resolved from the protein provided that ion suppression effect and protein absorption on the capillary wall are negligible. Currently, although the FACE technique has not been popularly recognized in drug discovery research, in our opinion, this technique has great potential as an alternative tool for screening drug-protein binding and would be especially beneficial for biological samples that are only available in minute quantities.

Affinity Capillary Electrophoresis (ACE)

In addition to FACE, affinity capillary electrophoresis (ACE) can also be applied for drug-protein interaction study by monitoring the electrophoretic mobility shifts of drugs as a function of protein concentrations. As a result, the binding constant is obtainable, which is used to estimate the fraction unbound by a simplified one-to-one binding model. In principle, the ACE enables a more rapid estimation of drug binding and compound ranking, since a number of compounds can be pooled and simultaneously screened on the basis of a 1:1 isotherm model, regardless of drug concentrations. An example of this was shown by Lewis et al. who applied ACE for the screening of novel antimicrobial targets from a small molecular library of 44,000 compounds that possessed drug-like properties and antimicrobial activity against drug-resistant clinical isolates. They concluded that ACE is a valuable tool for the fast, efficient detection of specific binding molecules that possess biological activity.[32] It can be anticipated that ACE and FACE, coupled with MS, should provide promising and alternative approaches for screening of drug-protein binding. Further applications of these techniques for real plasma proteins should be explored with regard to more sensitive MS detection and reduced adsorption of protein on the capillary inner wall.

Direct Screening Drug-Protein Interaction Without Separation

As discussed above, like many protein-binding assays, such as equilibrium dialysis and ultrafiltration, frontal analysis requires a follow-up separation step after the incubation to be able to quantify the free drug concentration [D]. In particular, dialysis assay results in a number of samples necessary for LC/MS analysis for each compound since both buffer and plasma samples have to be diluted to match the matrix and ensure a linear range, thus substantially limiting the throughput of the assay. In this regard, the performance of direct measurement of free concentration without separation would be beneficial. Based on rapid and reversible, non-covalent interaction between drug and protein, drug-protein can be evaluated by direct

Downloaded At: 17:47 23 January 2011

measurement of both free drug [D] and drug-protein complex [PD] concentrations in an equilibrium condition without involving a separation step, as presented by Jorgensen et al. using electrospray ionization MS.[33] Another similar study, performed with chip technology using automated nanoelectrospray MS detection demonstrated, directly, quantitative determination of a non-covalent binding constant with advantages of high throughput capacity, as well as low sample consumption.[34] Currently, the major limitation of direct measurement is that the complex [PD] and the matrix (mainly salts) present in the plasma would affect the accurate detection of both the free drug and the bound complex form. Nevertheless, these results confirm that the speed of an on-line separation step should not be a limiting factor in developing a high throughput method for protein binding screening, as the drug-protein interaction is a kinetically rapid on/off complexation and reversible equilibrium.[3,4] In other words, a rapid separation process, such as use of an immobilized HSA column or FACE and ACE, should offer relevant binding data as compared to that with slow separation conditions. The further development of a matrix independent MS ion source would be a key issue for direct screening drug-protein binding.

Downloaded At: 17:47 23 January 2011

CONCLUSIONS

Currently, conventional equilibrium dialysis is still the most widely used method for plasma protein binding measurements, as it is well established, accurate, and less susceptible to experimental artifacts. Implementations of automated 96-well plate and/or sample pooling approaches will further strengthen the applications of this technique for drug-protein binding screening in drug discovery. In particular, sample pooling using the same apparatus as conventional assays, but with reduced plasma volume, should be a more cost-effective approach. One unique feature of equilibrium dialysis is that the method is readily adaptable for screening brain tissue homogenates, while other techniques are hitherto rather restricted. Moreover, the immobilized column has been shown to be a powerful tool to rapidly screen and rank protein binding, due to its unique high throughput capacity and reproducibility. Although this method has shown reasonably good correlations between HSA and plasma protein binding for large sets of diverse compounds, large discrepancies have also been observed, particularly for the compounds strongly bound to AGP. Comparative analysis of HSA and AGP binding clearly indicates that the HSA binding will dominate plasma protein binding if the compound binds to HSA equally, or stronger than AGP. Otherwise, the contribution of AGP to the whole plasma protein binding can be significant if the binding affinity to AGP is more than two orders of magnitude higher than that to HSA. Therefore, to identify which drug binds to which protein becomes an important issue before one can use

immobilized HSA column binding data in place of plasma protein binding, in particular, for the interpretation of *in vivo* data. Nevertheless, at the earlier stage of discovery, the high throughput screening HSA column can be used to identify strongly bound compounds and to build structure-binding relationships that help to design compounds with reduced binding, i.e., below 90–95%.[23] In addition, novel minimized electrodriven techniques such as FACE and ACE show potential for high throughput screening of drug-protein binding. From the methodological point of view, ACE and FACE have significant advantages over equilibrium dialysis, in terms of very small quantities of biomacromolecules, simplicity, and speed. Further applications of these new techniques are demanded, although, currently, time and efforts necessary to validate rapid and reliable techniques for each class of compounds would limit its potential widespread use in drug discovery. It is evident that separation methods such as the immobilized HSA column HPLC, ACE, and FACE generate similiar binding data as compared with membrane based equilibrium dialysis, in general. It should be noted that the correlation score might also be restricted by the accuracy of the plasma protein binding data at variable conditions. For instance, it has been demonstrated that a shift of pH in plasma after dialysis could result in variable binding data under certain conditions.[12] The preference of assays will depend on the familiarity and availability of respective technique, as well as where/how binding data are to be used. In the earlier drug discovery phase, sample pooling and the HSA immobilized column are expected to provide relevant information to optimize drug-like lead compounds in a more cost-effective way. However, considering the protein concentration differences among various species, a conventional dialysis method may be required to provide more accurate binding data for *in vivo* scaling for a small number of compounds. It should be addressed that a discrimination of 99% and 99.9% bound is of crucial importance, giving a 10-fold free concentration difference. On the other hand, in order to build QSPR models for reliable predictions, high quality data are essential for necessary model validation. In this respect, the limited numbers of accurate data should be more beneficial than much that is not accurate enough or inconsistent. Besides acceptable accuracy, high throughput assays should not be just the increasing numbers of compounds measured, but rather an issue of time-savings and cost reduction, and data quality (where to use) and throughput (cost) may have to be compromised when applying high throughput screening assays.

ACKNOWLEDGMENTS

The authors thank Dr. Walter Lindberg, Johan Gabrielsson, and Anders Holmen for invaluable comments on the manuscript and for helpful discussions; Mikael Rehngren for the previous contribution (Ref. 12) as well as

Downloaded At: 17:47 23 January 2011

continuous involvment in development of a high throughput assay for screening brain tissue binding.

REFERENCES

1. Di, L.; Kerns, E.H. Curr. Opin. Chem. Biol. **2003**, *7*, 402–408.
2. Sebille, B.J. Chromatogr. **1990**, *531*, 51–77.
3. Lindup, W.E. In *Progress in Drug Metabolism*; Bridges, J.W., Chasseaud, L.F., Gibson, G.G., Eds.; Taylor & Francis Ltd, 1987; Vol. 10, Chapter 4, 141–185.
4. McDonnell, P.A.; Caldwell, G.W. In *New Advances in Analytical Chemistry*; Atta-ur-Rahman, Eds.; Harwood Academic Publisher: Amsterdam, 2000; 487–525.
5. Martinez-Pla, J.J.; Martinez-Gomez, M.A.; Martin-Biosca, Y.; Sagrado, S.; Villanueva-Camanas, R.M.; Medina-Hernandez, M.J. Electrophoresis **2004**, *25*, 3176–3185.
6. Herve, F.; Urien, S.; Albengres, E.; Duche, J.C.; Tillement, J.P. Clin. Pharmacokin. **1994**, *26*, 44–58.
7. Romer, J.; Bickel, M.H. J. Pharm. Pharmacol. **1979**, *31*, 7–11.
8. Kalvass, J.C.; Maurer, T.S. Biopharm. Drug Dispos. **2002**, *23*, 327–338.
9. Jia, Z.J.; Ramstad, T.; Zhong, M. J. Pharm. Biomed. Anal. **2002**, *30*, 405–413.
10. Israili, Z.H.; Dayton, P.G. Drug Metab. Rev. **2001**, *33*, 161–235.
11. Schuhmacher, J.; Buhner, K.; Witt-Laido, A. J. Pharm. Sci. **2000**, *89*, 1008–1021.
12. Wan, H.; Rehngren, M. J. Chromatogr. A **2006**, *1102*, 125–134.
13. Kariv, I.; Cao, H.; Oldenburg, K.R. J. Pharm. Sci. **2001**, *90*, 580–587.
14. Kariv, I.; Rourick, R.A.; Kassel, D.B.; Chung, T.D.Y. Combinat. Chem. High Throughput Screen. **2002**, *5*, 459–472.
15. Banker, M.J.; Clark, T.H.; Williams, J.A. J. Pharm. Sci. **2003**, *92*, 967–974.
16. Fung, E.N.; Chen, Y.H.; Lau, Y.Y. J. Chromatogr. Anal. Technol. Biomed. Life Sci. **2003**, *795*, 187–194.
17. Taylor, S.; Harker, A. J. Pharm. Biomed. Anal. **2006**, *41*, 299–303.
18. Hage, D.S. J. Chromatogr. B **2002**, *768*, 3–30.
19. Valko, K.J. Chromatogr. A **2004**, *1037*, 299–310.
20. Beaudry, F.; Coutu, M.; Brown, N.K. Biomed. Chromatogr. **1999**, *13*, 401–406.
21. Cheng, Y.; Ho, E.; Subramanyam, B.; Tseng, H.L. J. Chromatogr. Anal. Technol. Biomed. Life Sci. **2004**, *809*, 67–73.
22. Buchholz, L.; Cai, C.H.; Andress, L.; Cleton, A.; Brodfuehrer, J.; Cohen, L. Eur. J. Pharm. Sci. **2002**, *15*, 209–215.
23. Valko, K.; Nunhuck, S.; Bevan, C.; Abraham, M.H.; Reyncilds, D.P. J. Pharm. Sci. **2003**, *92*, 2236–2248.
24. Valko, K. *The Chromatographic Society: Chiral Separation Technologies Symposium*; Swallow Churchate Hotel: Old Harlow, Essex, 2005.
25. Singh, S.S.; Mehta, J. J. Chromatogr. Anal. Technol. Biomed. Life Sci. **2006**, *834*, 108–116.
26. Colmenarejo, G.; Alvarez-Pedraglio, A.; Lavandera, J.L. J. Med Chem. **2001**, *44*, 4370–4378.
27. Colmenarejo, G. Med. Res. Rev. **2003**, *23*, 275–301.
28. Schuhmacher, J.; Kohlsdorfer, C.; Buhner, K.; Brandenburger, T.; Kruk, R. J. Pharm. Sci. **2004**, *93*, 816–830.
29. Wan, H.; Thompson, R.A. Drug Disc. Today: Technol. **2005**, *2*, 171–178.

Downloaded At: 17:47 23 January 2011

30. Heegaard, N.H.H. Electrophoresis **2003**, *24*, 3879–3891.
31. Wan, H.; Östlund, Å.; Jönsson, S.; Lindberg, W. Rapid Comm. Mass Spectrom. **2005**, *19*, 1603–1610.
32. Lewis, L.M.; Engle, L.J.; Pierceall, W.E.; Hughes, D.E.; Shaw, K.J. J. Biomolec. Screen. **2004**, *9*, 303–308.
33. Jorgensen, T.J.D.; Roepstorff, P.; Heck, A.J.R. Anal. Chem. **1998**, *70*, 4427–4432.
34. Zhang, S.; Van Pelt, C.K.; Wilson, D.B. Anal. Chem. **2003**, *75*, 3010–3018.

Received July 9, 2006
Accepted November 20, 2006
Manuscript 6980C

Downloaded At: 17:47 23 January 2011