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Use of a linear gradient flow program for liquid chromatography–mass spectrometry protein-binding studies

Hanlan Liu*, Chantel Sabus, Guy T. Carter, Mark Tischler

Discovery Analytical Chemistry, Wyeth-Ayerst Research, 401 N. Middletown Road, Pearl River, NY 10965, USA

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Abstract

A rapid screening method to measure drug–protein binding using an immobilized human serum albumin (HSA) column was developed. This method utilizes a linear gradient flow-rate to accelerate the elution of strong binders to the HSA column. Post-column addition of a pressure relief valve enables mass spectrometric detection at relatively high mobile phase flow-rates (i.e., 2 ml/min).

Keywords: Gradient elution; Immobilized proteins; Proteins; Albumin; Drugs

1. Introduction

Human serum albumin (HSA), a carrier protein in the blood, reversibly binds a variety of endogenous as well as exogenous compounds (e.g., drugs). In addition to HSA, other proteins in the serum such as α_1-acid glycoprotein, lipoproteins, trascortin, and thyroid-binding globulin also have specific affinities for a small number of drugs. Combining in vitro drug–protein binding parameters with in vivo pharmacokinetic data can give valuable insight into the disposition of a drug molecule [1–3]. Currently, a variety of methods are available for measuring drug–protein binding. These include: equilibrium dialysis; ultrafiltration; ultracentrifugation [4]; capillary electrophoresis [5,6]; spectroscopic methods (e.g., circular dichroism, optical rotatory dispersion, fluorescence, nuclear magnetic resonance) [7]; high-performance liquid chromatography [8,9]; and the recently reported biosensor affinity analysis using microchip immobilized HSA [10].

Chromatographic methods used to obtain drug–protein binding data usually offer high precision and reproducibility. These methods also allow for simplified automation and less sample consumption. Binding analyses using chromatographic methods are usually performed under isocratic mobile phase conditions so that the compounds can be compared and ranked on the same scale. Isocratic conditions, however, often require long running times for the elution of strong binders. One chromatographic method previously reported by Tiller et al., coupled immobilized HSA liquid chromatography to mass spectrometry [11]. In their work, the protein binding of 10 compounds was measured simultaneously under isocratic mobile phase conditions with detection by mass spectrometry. The work described in this paper improves upon the method developed by Tiller et al. by using a linear gradient flow-rate to measure drug–protein binding with an immobilized

*Corresponding author. Tel.: +1-845-602-2659; fax: +1-845-602-2969.

E-mail address: liuh2@war.wyeth.com (H. Liu).

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HSA column and an ion trap mass spectrometer. High mobile phase flow-rates at the end of the chromatographic run speeds up the elution of strong HSA binders and thus shortens the total analysis time. The combination of the linear gradient flow-rate with the multiple sample analyses afforded by mass spectrometry greatly reduces the time necessary to perform drug–protein binding studies.

2. Experimental

2.1. Materials

The analyte compounds glucose, salbutamol, cyclophosphamide, phenacetin, 4-acetamide phenol (acetominophen), triamterene, 4,4′-diaminodiphenyl sulfone (DDS or Dapsone), quinidine, and *R*/*S*-warfarin were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and *n*-propanol were obtained from EM Science (Gibbstown, NJ, USA), and ammonium acetate was purchased from J.T. Baker (Philipsburg, NJ, USA).

2.2. Equipment

The LC–MS analysis was performed using an Agilent 1100 series MSD Trap (Agilent Technologies, Palo Alto, CA, USA) equipped with an electrospray interface that can be operated in either the positive or the negative mode. The MSD ion trap was coupled to an Agilent Model 1100 series HPLC system equipped with a binary pump, an autosampler and a diode array UV detector. HP Chemstation software version 8.03 was used to control the HPLC system and Bruker Daltonics Trap Control software (version 6.03) was used to control the MSD ion trap mass spectrometer. Data analysis was performed using Bruker Daltonics Data Analysis (2.0) software.

2.3. Sample preparation

Compounds were dissolved in DMSO or water to make 1 mg/ml solutions with the exception of *R*,*S*-warfarin which was dissolved in DMSO to make a 2 mg/ml (1 mg/ml each isomer) solution. An analyte sample was prepared by diluting the test compounds with *n*-propanol–50 m*M* aqueous ammonium acetate (4:96). The resulting mixture contained each of the 10 analytes (glucose, salbutamol, cyclophosphamide, phenacetin, acetominophen, triamterene, dapsone, quinidine, *R*-warfarin, and *S*-warfarin) at 10 μg/ml.

2.4. Chromatographic and mass spectrometric conditions

An isocratic mobile phase of *n*-propanol–50 m*M* aqueous ammonium acetate (4:96) was used throughout the experiment as described in [11]. Compounds were eluted from the Hypersil Human HSA column, 7 μm, 50×4.6 mm, (Keystone Scientific, Bellefonte, PA, USA) using a linear gradient flow-rate program with the column temperature set at 37 °C. The linear gradient flow-rate program was set to increase from 0.5 to 2.0 ml/min. in 20 min, hold at 2 ml/min. for 5 min, then return to 0.5 ml/min in 2 min giving a total run time of 27 min. To relieve the problems of ineffective vaporization and ionization in the mass spectrometer caused by elevated flow-rates (>1.0 ml/min), a 100-p.s.i. (1 p.s.i.≡ 6894.76 Pa) pressure relief valve (Upchurch, Oak Harbor, WA, USA) was installed in between the column and nebulizer. The injection volume for the 10-μg/ml standard mixture was 10 μl with a total of 0.1 μg of each compound actually injected.

The mass spectrometer was operated using an electrospray ionization (ESI) source. The nebulizing gas was obtained from a high-purity nitrogen source available in the laboratory. The drying gas temperature was set at 300 °C with a gas flow of 12 l/min. The ion charge control parameter was turned on and targeted at 50 000 with a maximum accumulation time of 15 ms. Other values for the MS operating parameters were 60 p.s.i. for the N_2 nebulizer pressure, 50% for the compound stability, 100% for the trap drive level, and 4 for the rolling average. The mass spectrometer was set to scan from m/z 100 to m/z 700 with a scan resolution of 13 000 m/z per scan.

3. Results and discussion

Fig. 1 is a schematic diagram of the set-up used in this study. The coupling of the ion trap mass spectrometer with HPLC allowed for simultaneous

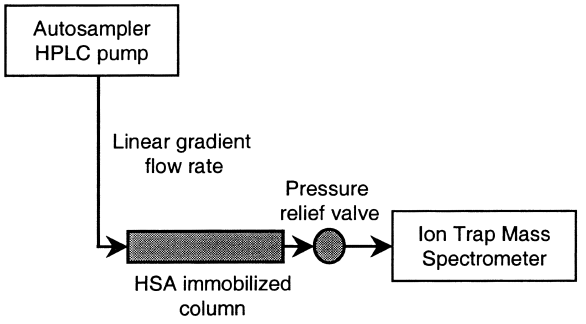

Fig. 1. Schematic of the set-up used for HSA binding measurements.

multiple-sample analyses due to the specificity of mass spectrometry. This configuration also enabled the analysis of compounds containing minor impurities. Furthermore, high sensitivity within the mass scan function of the ion trap mass spectrometer allowed for sample analyses at low concentrations (i.e. 10–50 μg/ml). Low sample concentrations are critical for equilibrium protein binding studies to avoid saturation of the protein surface. Simultaneous multiple-sample analyses also require low individual sample concentrations to avoid column overloading.

Fig. 2 shows the gradient flow-rate program used in this HSA-binding experiment, as described in the Experimental section. A constant high flow-rate is always desirable for rapid elution of strong binders from an immobilized HSA column. However, at a high constant flow-rate, the peak width of early-eluting peaks is too narrow for the mass spectrometer to collect a sufficient number of data points across the entire ion chromatographic peak. With an insufficient number of data points, an accurate retention time (the time point at the highest ion

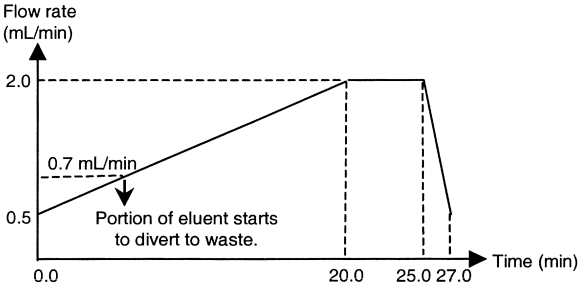

Fig. 2. Linear gradient flow-rate program used for HSA-binding measurements.

intensity) cannot be measured. The use of a linear gradient flow-rate overcame this problem. The low flow-rate in the beginning allowed for accurate retention time measurements of the early eluting peaks while the high flow-rate during the later stage of the analysis accelerated the elution of strong protein binders from the immobilized HSA column. The low flow-rate in the beginning also enabled the separation of early eluting compounds and reduced the chances of peak overlap and ion suppression.

The conversion of retention time ($t_{r/conversion}$) measured by a linear gradient flow-rate to retention time measured by a constant flow-rate is shown in Eq. (1):

$$t_{r/conversion} = \frac{t_{rg} \cdot (V_o + V_t)}{2V_c} \quad (1)$$

where t_{rg} is the retention time of a compound using a linear gradient, V_o is the starting flow-rate of the linear gradient, V_t is the flow-rate at t_{rg}, and V_c is the constant flow-rate (i.e., 0.8 ml/min for this experiment). V_t can be calculated as:

$$V_t = \frac{V_e - V_o}{T} \cdot t_{rg} + V_o \quad (2)$$

where T is the gradient duration (i.e., 20 min for this experiment, see Fig. 2), and V_e is the flow-rate at the end of the linear gradient (i.e., 2 ml/min for this experiment). Combining Eqs. (1) and (2) gives:

$$t_{r/conversion} = \frac{t_{rg}\left(2V_o + \frac{V_e - V_o}{T} \cdot t_{rg}\right)}{2V_c} \quad (3)$$

Eq. (3) applies only to those compounds whose retention times fall in the up-slope region of the linear gradient diagram (Fig. 2). If the retention time of a compound lies beyond the gradient in the constant flow-rate portion of the program (i.e., 20–25 min), $t_{r/conversion}$ is then calculated using the following equation:

$$t_{r/conversion} = \frac{T \cdot (V_o + V_e)}{2V_c} + \frac{(t_{rg} - T) \cdot V_e}{V_c} \quad (4)$$

Eq. (4) is derived from the addition of Eq. (3) by setting $t_{rg} = T$ in Eq. (3) and the equation for calculating the retention time at a constant flow-rate (i.e., V_e). Thus, Eq. (4) takes into account the linear

gradient flow-rate as well as the constant flow-rate conditions of the gradient flow-rate diagram shown in Fig. 2. This equation still holds true even if the constant flow-rate period is extended beyond 25 min, which may be necessary for the elution of tightly bound compounds (data not shown).

In order to achieve sufficient and effective vaporization and ionization of samples, mass spectrometers have an upper flow-rate limit of approximately 1.0 ml/min when used in the electrospray ionization mode. The linear gradient flow-rate program designed for this experiment exceeds this upper limit. Addition of a post-column pressure relief valve helped resolve this problem. A regular ''T'' connector can also perform a similar function to that of a pressure relief valve however it only transfers a fixed ratio of the flow volume to the mass spectrometer instead of a fixed flow-rate, which is the case when utilizing a pressure relief valve. Since the flow-rate constantly changes during the gradient program, the use of a regular ''T'' connector introduces variable flow-rates to the mass spectrometer. A time adjustment for the nebulizer parameters is then necessary in order to achieve optimal ionization and not all mass spectrometry application software programs possess this function. The variable flow-rates introduced by a regular ''T'' connector also introduce a baseline drift, which could affect peak retention time measurements. Since a pressure relief valve delivers the eluent to the mass spectrometer at a constant flow-rate, no time adjustment is required for the nebulizer parameters. Also, the pressure relief valve used has a void volume of less than 5 μl, so there is little post-column dilution effects observed on the eluted peaks.

The function of the pressure relief valve is simple. During the linear gradient flow period, pressure gradually builds up in the tubing linking the pressure relief valve and the mass spectrometer, as well as in the nebulizer. Once the back pressure to the relief valve reaches a threshold of 100 p.s.i., the valve opens. A portion of the eluent then diverts from the column to a waste reservoir by flowing through the regulator cartridge aligned perpendicular to the pressure relief valve tee (Fig. 3). Thus, the mass spectrometer receives the eluent only at or below a specific flow-rate, regardless of the flow-rate change during the gradient program. This specific flow-rate

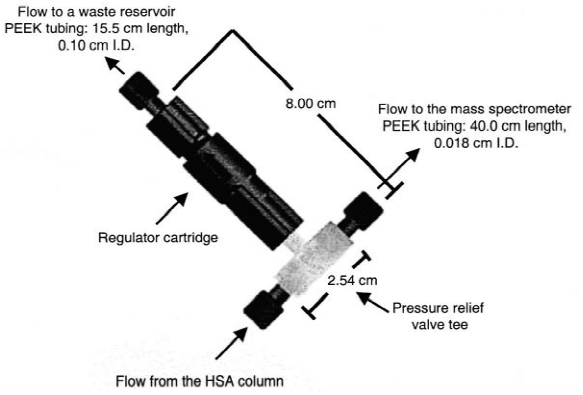

Fig. 3. Schematic of the 100-p.s.i. pressure relief valve.

is determined at the point where the pressure relief valve reaches its threshold of 100 p.s.i. The size and length of tubing linking the two ends of the pressure relief valve are critical in order to deliver the proper amount of eluent to the mass spectrometer during the linear gradient program. As shown in Fig. 3, the PEEK (polyether ether ketone) tubing connecting the pressure relief valve to the waste line has a length of 15.5 cm with a 0.10-cm I.D. whereas the tubing connecting the pressure relief valve to the mass spectrometer has a length of 40.0 cm with a 0.018-cm I.D. Under these conditions, the 100-p.s.i. threshold is reached and a portion of the eluent begins to divert to the waste reservoir at flow-rates greater than or equal to 0.7 ml/min. Thus, the mass spectrometer never sees a flow-rate greater than 0.7 ml/min and the LC–MS system is able to sustain the high flow-rates (i.e., 2.0 ml/min) in the method without significant loss of ionization efficiency due to incomplete vaporization. A quick check to test the viability of the pressure relief valve before starting an experiment can be done by running a flow-rate greater than 0.7 ml/min (or other specific flow-rate as determined by experimental set-up) through the system for several min and observing the appearance of eluent from the waste line. The same should then be done using a flow-rate less than 0.7 ml/min (or other specific flow-rate as determined by experimental set-up) to check that no eluent comes from the waste line. This quick check ensures that the pressure relief valve is working properly.

While developing this system, a question arose as to whether the linear gradient flow-rate in conjunc-

tion with the pressure relief valve would cause a shift in the retention times of analyte peaks when compared to analyte peaks observed under constant flow-rate conditions without a pressure relief valve. Two experiments were performed to address this concern. First, samples were analyzed using the linear gradient flow program described in Fig. 2 in conjunction with the pressure relief valve shown in Fig. 3. The same group of samples was then analyzed under constant flow-rate conditions (i.e., 0.8 ml/min) without the pressure relief valve. Fig. 4 shows the extracted ion chromatograms of the eight analytes run using the immobilized HSA column chromatography–ion trap ESI-MS with a linear gradient flow and pressure relief valve. When converted using Eq. (3), the retention times measured in Fig. 4 are the same as those measured using a constant flow-rate of 0.8 ml/min with no pressure relief valve. This is verified by the linear regression analysis shown in Fig. 5. The resultant regression line has a slope close to 1 (0.916) and a *y*-intercept close to 0 (0.104) with a correlation coefficient of 1.000.

The comparison of the warfarin racemate peaks analyzed under both linear gradient and constant flow-rate conditions is shown in Fig. 6. Warfarin is a known strong HSA binder and generally elutes at longer retention times resulting in broad peaks.

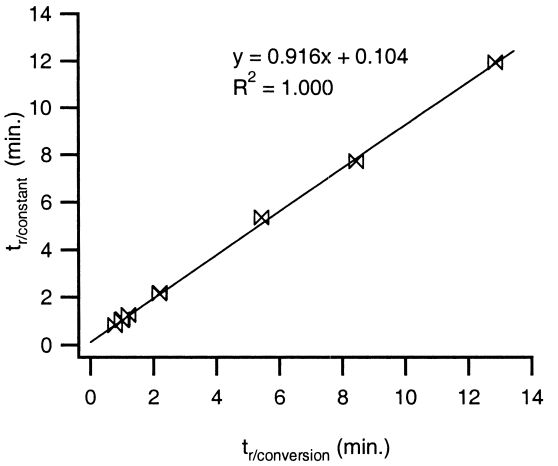

Fig. 5. Plot of retention times measured using a constant flow-rate ($t_{r/constant}$) of 0.8 ml/min vs. retention times measured using a gradient flow-rate and converted to a constant flow-rate value ($t_{r/conversion}$) with Eq. (3). Data points are from the experiment shown in Fig. 4.

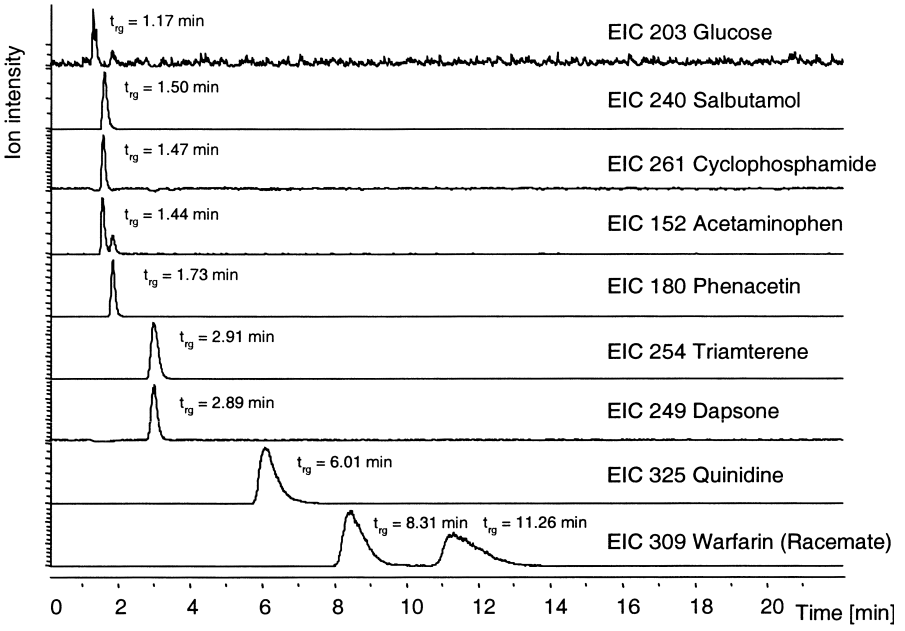

Fig. 4. Extracted ion chromatograms of 10 analytes (glucose, salbutamol, cyclophosphamide, acetaminophen, phenacetin, triamterene, dapsone, quinidine, and warfarin (racemate)) obtained using immobilized HSA column LC–ESI-MS in the positive mode with a linear gradient flow-rate and a pressure relief valve.

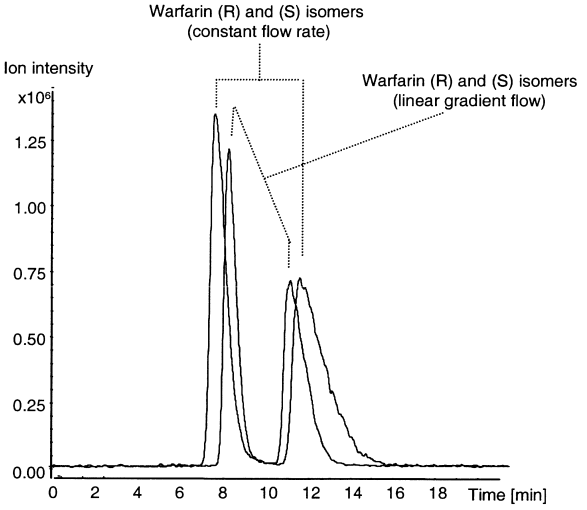

Fig. 6. Comparison of warfarin racemate peaks using both linear gradient and constant flow-rate conditions.

Under the gradient flow conditions, both racemate peaks eluted within a narrower retention time window than when run under constant flow-rate conditions. This evidence further supports the use of a linear gradient flow program.

The theoretical simulation of chromatogram peaks in the presence and absence of a pressure relief valve was also performed. All the simulations performed were based on Gaussian functions, which are typical for chromatographic peak shapes. Fig. 7 shows the chromatogram for simulated peaks using: (A) a constant flow-rate of 0.8 ml/min (–○–); (B) a linear gradient program (Fig. 2) without a pressure relief valve (–▽–); and (C) a linear gradient program (Fig. 2) with a pressure relief valve (–□–). A hypothetical peak with a retention time of 31.25 min under constant flow-rate conditions would have a theoretical bandwidth at half-height of 1.99 min when taking into account the column's efficiency ($N=$ 1360 for 50×4.6 mm I.D.) as stated in the column manufacturer's specifications. The simulated peak under a constant flow-rate was a straightforward manipulation of the Gaussian function. The derivation of peaks for the linear gradient flow-rate, however, involved several more calculation steps. First, each time point in chromatogram peak A (t_r) was converted to t_{rg} using Eqs. (3) or (4) by setting t_r equal to $t_{r/conversion}$. Next, the flow-rate at each t_{rg} (V_t) was calculated using Eq. (2). Finally, the ion intensity at t_{rg} (I_{trg}) was calculated by multiplying the ion intensity at each t_r (I_{tr}) by the ratio of V_t to V_c [Eq. (5)].

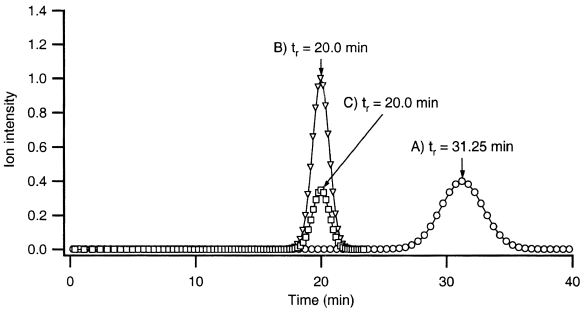

Fig. 7. Chromatographic peaks simulated using a gaussian curve function. (A) Peak (-○-) derived from a constant flow-rate of 0.8 ml/min; (B) peak (-▽-) derived from the linear gradient flow-rate program described in Fig. 2 without the pressure relief valve; and (C) peak (-□-) derived from the linear gradient flow-rate program described in Fig. 2 with the presence of the pressure relief valve.

$$I_{trg} = I_{tr}\left(\frac{V_t}{V_c}\right) \tag{5}$$

where $V_c = 0.8$ ml/min.

It is apparent from Fig. 7 that analyses performed with the linear gradient flow program can reduce the retention time of analytes by approximately 40% when compared to retention times of analytes run using a constant flow-rate of 0.8 ml/min. The bandwidth is also much narrower for the gradient flow peak than the constant flow peak. The simulated peak calculated for a gradient flow in the presence of the pressure relief valve has a signal intensity drop of approximately 60% when compared to the peak calculated for a linear gradient flow without a pressure relief valve. The signal reduction was due to the diversion of part of the eluent into the waste reservoir. However, the retention time of the chromatographic peak, which is the parameter of interest, remains the same. There also appears to be no observable changes in the chromatographic peak shape after the addition of the pressure relief valve.

In order to validate this procedure as a method for protein-binding measurement, the retention times ($t_{r/conversion}$) of the eight analytes were tested and compared (Table 1) with the values reported by Tiller et al. [11]. The corresponding percent protein

Table 1
Comparison of retention times measured in this work with the results reported by Tiller et al. [11]

Compound	t_{rg} (min) gradient flow-rate with pressure relief valve	$t_{r/conversion}$ (min) t_{rg} converted using Eq. (3)	t_r (min) isocratic flow rate [11]	% Binding [11,12]
Glucose	1.17	0.80	1.25	0
Salbutamol	1.50	1.04	1.40	7.5
Cyclophosphamide	1.47	1.02	Not available	13
Acetaminophen	1.44	1.00	1.55	24
Phenacetin	1.73	1.22	1.88	33
Triamterene	2.91	2.22	3.32	57
Dapsone	2.89	2.20	Not available	73
Quinidine	6.01	5.45	7.32	90
R-Warfarin	8.31	8.43	15.70	99
S-Warfarin	11.26	12.98	20.90	99

binding values were also included [11,12]. The elution order of analytes observed during this study was consistent with that reported by Tiller et al. [11]. This elution order directly correlates to the percent protein binding and allows a qualitative rank order to be established. The ability to establish a protein binding rank order can be useful for the discovery stage of pharmaceutical research where a series of compounds can be evaluated for desired protein binding characteristics.

The data summarized in this work further supports the use of HSA column LC–ion trap ESI-MS as a useful and simple screening method for drug–protein binding measurement. Both experimental and theoretical data also demonstrate that a linear gradient flow program combined with a pressure relief valve allows for a more rapid screening assay than when using isocratic flow-rates alone. This developed approach is also suitable for other binding studies where LC–MS is appropriate.

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