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Journal of Chromatography B, 795 (2003) 187–194

JOURNAL OF CHROMATOGRAPHY B

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Semi-automatic high-throughput determination of plasma protein binding using a 96-well plate filtrate assembly and fast liquid chromatography–tandem mass spectrometry

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Received 26 May 2003; received in revised form 7 July 2003; accepted 14 July 2003

Abstract

A semi-automatic, high-throughput method has been developed to rapidly assess plasma protein binding of new chemical entities in drug discovery phase. New chemical entities are mixed with plasma and the unbound fractions are separated from the bound fraction by ultrafiltration in a 96-well filtrate assembly. The unbound fractions are then analyzed by fast liquid chromatography–tandem mass spectrometry (LC–MS/MS). Sample handling is automated by a robotic system. Employing a cocktail approach where multiple new chemical entities are allowed to bind to plasma proteins in the same well has further increased the throughput. We have validated the method with 12 commercially available compounds. The plasma protein binding data obtained by this method are comparable with the literature values. This method enables the determination of protein binding for 32 compounds in one single experiment instead of 1–2 compounds using the conventional methods.
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Keywords: Plasma protein binding; High-throughput; Proteins

1. Introduction

Plasma protein binding has profound effect on the pharmacokinetic and pharmacodynamic parameters of new chemical entities [1]. It also affects the calculation of in vivo hepatic clearance based on in vitro intrinsic clearance [2]. Therefore, protein binding is an important factor to consider in lead optimization. Traditionally, protein binding has been determined by equilibrium dialysis [3,4], ultrafiltration [5,6] or ultracentrifugation [7,8]. Equilibrium dialysis and ultracentrifugation require multiple hours to establish equilibrium. Ultrafiltration using individual devices can be time-consuming and labor intensive. Besides these conventional methods, a chromatographic method using immobilized human serum albumin (HSA) columns has also been reported [9,10]. Although the plasma protein binding data obtained with these columns has good precision and reproducibility, their use limits the choice

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doi:10.1016/S1570-0232(03)00564-6

of mobile phases (incompatibility with solvents such as acetonitrile) and the column requires special care to prevent degradation of the HSA. In addition, none of these methods are suitable for automation and high-throughput screening format. Allen et al. [11] have briefly evaluated the possible use of a cocktail approach to increase throughput, however, the full validation was not reported. Therefore, there is a need to develop a semi-automated high-throughput assay in order to address the plasma protein binding issue early in drug discovery and to validate the cocktail approach.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been widely used in the pharmaceutical industry due to its excellent selectivity, sensitivity and ease of use [12–14]. Its application in in vitro studies has also been demonstrated by Lau et al. [15,16]. Sample throughput can be increased dramatically (on the order of >250 samples per day) by using a generic fast gradient, with a typical runtime of less than 3 min [17]. In addition, with the advances in robotic systems, sample handling and sample injection can be done automatically in 96-well format with minimal human intervention.

In this paper, we have developed an ultrafiltration method based on a 96-well filtrate assembly to increase the throughput. Sample handling is automated by using a robotic system. In addition, we have adopted and validated a cocktail approach, by dosing multiple compounds to the plasma to further increase the throughput.

2. Experimental

2.1. Chemicals

Human plasma was obtained from Bioreclamation (Hicksville, NY, USA). Desipramine, verapamil, imipramine, diazepam, lorazepam, midazolam, oxazepam, chloropromazine, propranolol, nifedipine and nicardipine were obtained from Sigma (St. Louis, MO, USA). Methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was obtained from Sigma (St. Louis, MO, USA). High-purity water was prepared using EasyPure UV water purification system (Barnstead, Dubuque, IA, USA).

2.2. Solutions for method development

Stock solutions of the compounds were prepared at 1–2 mM in methanol. The stock solutions were then diluted to 10 μM for method development. Method development was performed by manual infusion on Sciex API 3000 (PE Sciex, Toronto, Canada).

2.3. Ultrafiltration

Plasma protein binding was assessed in human plasma individually and/or by a cocktail approach (“four-in-one”). The human plasma was spiked with compound(s) individually or “four-in-one” at a concentration of 10 μM in glass tubes and incubated in a shaking water bath at 37 °C for 30 min. Plasma samples were then transferred in quadruple to ultrafiltration devices by a robotic system (Tecan Genesis 150 Workstation, Tecan, Durham, NC, USA). The ultrafiltration devices used were either a Microcon-96 filtrate assembly YM-30 or individual Centrifree filtrate assembly with a molecular weight cutoff of 30,000 Da (Millipore, Bedford, MA, USA). The unbound fraction of the compound was isolated from the Microcon-96 filtrate assembly by centrifuging at $3000 \times g$ for 45 min. The Centrifree filtrate assemblies were centrifuged at 16 g for 5 min. The ultrafiltrates were then added to an equal volume of acetonitrile containing the internal standard that is a proprietary compound, to determine the unbound fraction in the matrix. Samples from the initial spiked plasma, 30 min after incubation, and the retentate after ultrafiltration were also analyzed following the same treatment as the ultrafiltrates.

2.4. HPLC instrumentation

The HPLC consisted of a Shimadzu binary pump (Shimadzu, Columbia, MD, USA) and a CTC PAL autosampler (Leap Technologies, Carboro, NC, USA). The column used was Synergi MAX-RP, 4 μm, 2.0 mm × 30 mm (Phenomenex, Torrance, CA, USA). The HPLC pump and autosampler were controlled by the Mac software (PE Sciex). A divert valve (Electronic Valve Actuator, Jones Chromatography) was incorporated to divert the initial 0.5 min of the eluent to waste. The mobile phases were (A) 95:5 water:methanol with 0.01 M ammonium acetate, 60 μl/l

of acetic acid and (B) 100% methanol with 0.01 M ammonium acetate, 60 μl/l of acetic acid. A gradient of 40% B at 0.4 ml/min for 0.4 min, increase to 100% B at 0.6 ml/min in 0.2 min, followed by 100% at 0.6 ml/min for 0.7 min and then back to 40% B at 0.4 ml/min in 0.2 min was used. The total run time was 1.5 min. For method development, direct infusion was used instead of a HPLC column. Compounds were optimized in positive mode of ionization manually.

2.5. LC–MS/MS analysis

The samples were analyzed in positive mode using the electrospray interface of PE/Sciex API 3000 triple quadrupole mass spectrometer. Generic MS/MS conditions were used for all analysis with an interface temperature of 400 °C. The dwell time was 300 ms. The MRM transitions and optimized MS conditions developed during method development were then used to generate MS method files for subsequent data acquisition and data analysis.

2.6. Data analysis

Quantification of analysis was performed by MacQuant. Plasma protein binding was calculated using the following equation.

$$\text{percent unbound} = \frac{C_u}{C_m} \times 100$$

$$\text{percent bound} = 100 - \text{percent unbound}$$

where C_u is the concentration of analyte in the ultrafiltrate (μM) and C_m is the concentration of analyte in the plasma following 30 min incubation (μM).

3. Results

The performance of the Microcon-96 ultrafiltration assembly was evaluated by randomly choosing 12 in-house compounds that have protein binding data obtained using individual Centrifree ultrafiltration devices. The Microcon-96 assembly and individual Centrifree ultrafiltration devices were used to measure the percent unbound of in-house compounds in human plasma, Table 1. The percent unbound determined by the two methods were comparable with an $R^2 = 0.60$. Twelve commercial compounds were also tested in the Microcon-96 filtrate assembly, Table 2. The percentage unbound for the 12 commercial compounds obtained individually using the Microcon-96 filtrate assembly versus the literature values are plotted in Fig. 1. A good correlation of these data was observed with $R^2 = 0.90$. By using the 96-well format, we are able to increase the throughput by processing 96 samples at the same time. In addition, the 96-well format is amendable to automation with robotic system.

In order to further increase the sample throughput, we have investigated the possibility of dosing multiple compounds. With the highly selective LC–MS/MS method, multiple compounds can be monitored in one sample, as long as the compounds have distinctive

Table 1
Comparison of plasma percentage unbound determined using Microcon 96-well format and individual Centrifree devices

Compound	96-Well format		Individual Centrifree
	Individual percent unbound (%CV) ($n = 4$)	Cocktail percent unbound (%CV) ($n = 4$)	Individual percent unbound (%CV) ($n = 4$)
SCH 1	0 (0)	0 (0)	0 (0)
SCH 2	17 (21)	22 (9.3)	30 (3.2)
SCH 3	23 (10)	30 (9.9)	15 (4.3)
SCH 4	6 (12)	6.1 (12)	5.6 (3.6)
SCH 5	5.8 (19)	6.2 (20)	5.3 (27)
SCH 6	0.25 (22)	0.21 (25)	0 (0)
SCH 7	13 (18.5)	12 (27)	6.2 (17)
SCH 8	0.66 (45)	0.66 (48)	1.5 (27)
SCH 9	7.6 (48)	16 (34)	12 (3.2)
SCH 10	5.9 (14)	6.5 (12)	6.6 (6.1)
SCH 11	1.2 (82)	3.3 (29)	5.5 (4.5)
SCH 12	3.2 (42)	2.8 (12)	3.5 (4.9)

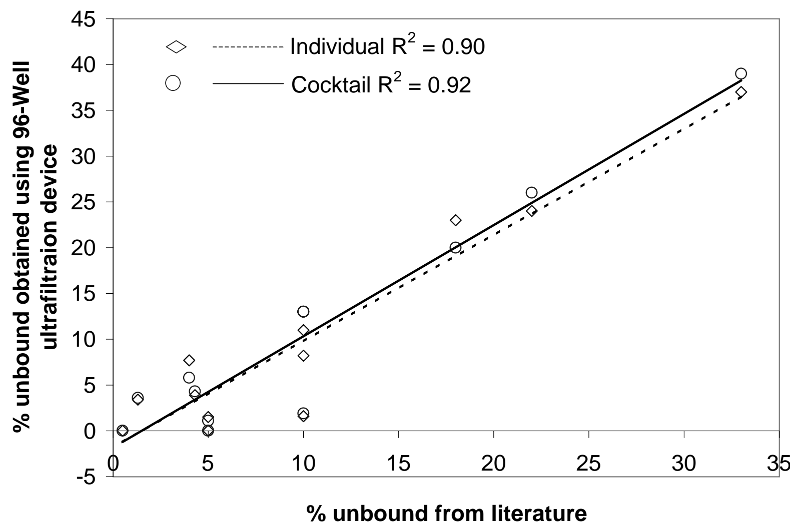

Fig. 1. Correlation of percentage unbound obtained individually and cocktail approach using 96-well ultrafiltration devices and the literature value.

parent and fragment ion masses as shown in Fig. 2. A "four-in-one" cocktail approach was investigated in this study. The percent unbound of new chemical entities and the commercial compounds are comparable for individual dosing and the cocktail approach (Tables 1 and 2). The percent unbound values obtained individually and by the cocktail approach using the Microcon-96 are plotted in Fig. 3. A very good correlation of $R^2 = 0.94$ was observed. A very good correlation was also observed between values obtained using the cocktail approach and the literature values for the 12 commercial compounds with an R^2 of 0.92 (Fig. 1).

The stability of the compounds was determined by comparing the concentration of the plasma following 30 min incubation at 37 °C (C_m) and the initial spiked plasma concentration. We have also determined the recovery of the compounds following ultrafiltration by comparing the retentate concentration with C_m. The results suggested that all compounds tested here are stable within the experimental time frame and the recovery were more than 90%.

Table 2
Comparison of plasma fraction unbound determined using Microcon 96-well format and literature values

Compound	96-Well format		Literature Percent unbound
	Individual percent unbound (%CV) ($n = 4$)	Cocktail percent unbound (%CV) ($n = 4$)	
Desipramine	23 (25)	20 (15)	18 [23]
Verapamil	1.6 (19)	1.9 (26)	10 [23]
Imipramine	8.2 (19)	13 (12)	10 [23]
Diazepam	3.4 (20)	3.6 (13)	1.3 [23]
Lorazepam	11 (15)	13 (14)	10 [24]
Midazolam	1.5 (24)	1.1 (18)	5 [23]
Oxazepam	3.9 (15)	4.3 (13)	4.3 [23]
Sildenafil	7.7 (23)	5.8 (17)	4 [25]
Chlorpromazine	0.03 (56)	0.01 (130)	5 [23]
Propranolol	37 (19)	39 (14)	33 [21]
Nifedipine	24 (5)	26 (7.4)	22 [23]
Nicardipine	0.02 (28)	0.02 (30)	0.5 [23]

4. Discussion

The extent of protein binding is critical in predicting the interaction between the drug candidate and its intended in vivo target; and the clearance mechanisms. In order to characterize the pharmacokinetic profile and pharmacodynamic behavior of potential drug candidates and also to rationalize in vitro/in vivo extrapolations, it is important to understand the extent of plasma protein binding and any species differences that may exist. Protein binding is also important

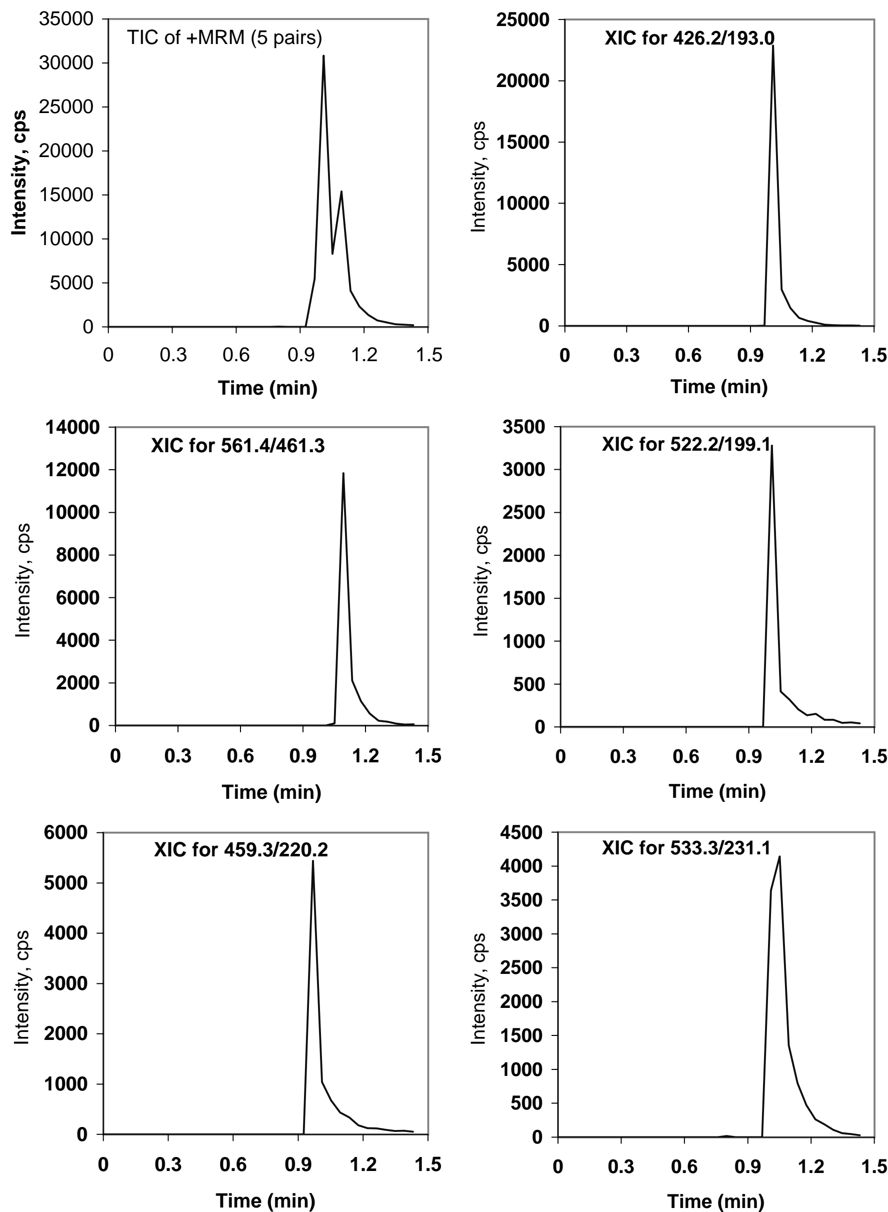

Fig. 2. Representative mass chromatograms.

for highly protein-bound (>90%), low clearance drugs with a narrow therapeutic index and a small distribution volume following intravenous administration due to the possibility of binding displacement interactions in vivo [18–20] (warfarin, phenytoin and tolbutamide). The method validated here provides a relatively simple and effective screening method for the evaluation of protein binding. This protein binding data can also be used to estimate the impact of protein binding on a drug's clinical activity.

LC–MS/MS is a very important technique in pharmaceutical industry. The excellent sensitivity and selectivity with short analysis time permit high-throughput screening of a large number of compounds

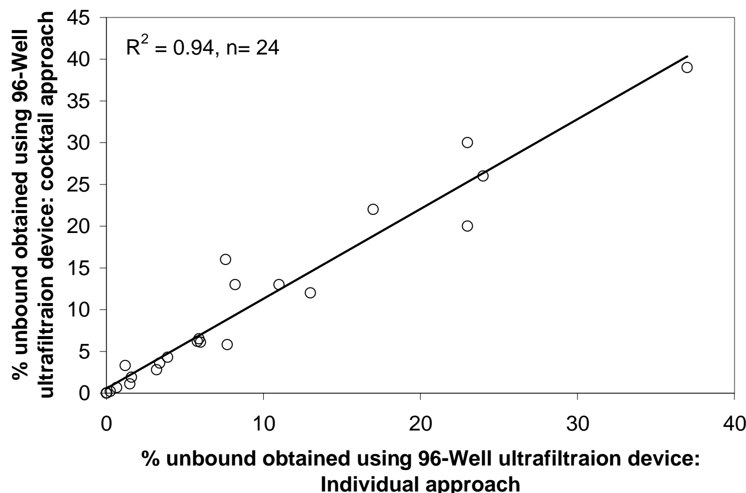

Fig. 3. Correlation of percentage unbound obtained individually and cocktail approach using 96-well ultrafiltration devices.

in support of drug discovery. We have developed a generic HPLC method which uses a Synergi MAX-RP column (Phenomenex). No additional chromatographic method development was necessary for individual compounds. A runtime of 1.5 min was achieved by ramping up the flow rate from 0.4 ml/min to 0.6 ml/min at 0.4 min to speed up the elution of analytes. The flow rate was then returned to 0.4 ml/min, allowing the column to equilibrate. With the excellent selectivity of tandem mass spectrometer, the compounds can be separated by their characteristic parent and fragment ion masses and quantitated with little or no chromatographic separation.

Besides fast sample analysis, sample preparation is another important area in high-throughput screening. Equilibrium dialysis has been the "gold" standard for the determination of plasma protein binding. However, it can take more than 20 h to reach equilibrium and plasma stability will be an issue with less stable compounds. Higher throughput equilibrium dialysis method were also reported by Kariv et al. [21] and Banker et al. [22]. However, these methods still suffered from long equilibrium time of 6–20 h, plasma stability, volume shifts, extensive preparation of dialysis membranes before use, and high leakage rate. Alternatively, ultrafiltration with individual devices can shorten the time drastically to 5 min for each sample with minimal leakage. For higher throughput, the Microcon-96 filtrate assembly ultrafiltration assembly can be used. The ultrafiltration time for Microcon-96 is only 45 min and plasma stability is less likely to be an issue. Another advantage of the Microcon-96 is the small amount of sample required; 250 μl compared with 1 ml for either equilibrium dialysis or individual ultrafiltration devices. This is important in drug discovery since only a small amount of compound is available. With the advances in robotics, sample preparation can be automated in 96-well format.

It is interesting to note that for compounds SCH 1–4, the data correlated very well between the two (individual and cocktail) approaches, even when the compounds exhibited large differences in the extent of protein binding. This suggests there was no interference between different compounds when dosed together for protein binding determination.

The unbound fraction determined by using individual devices and 96-well filtrate assembly are comparable except for the compounds SCH 2 and 9. A low correlation with an $R^2 = 0.6$ was observed between the two methods. The two sets of data were obtained in two different laboratories using different lots of human plasma. As suggested by Kariv [21], the concentration of the plasma protein will affect the percent bound fraction. Human plasma contains more than 60 different proteins with a wide range of concentrations and binding affinities and capacities. The significant variation in the protein content in human plasma due

to genetic variation and diseases may result in the low correlation between the two methods.

Another issue that needs to be addressed is the possibility of saturation of binding sites on the proteins. By closely examining the data in Table 1, data obtained by dosing individually matches well with that obtained by the cocktail approach even in the presence of highly bound new chemical entities, SCH 6 and 8. This suggests that saturation of binding sites was not observed with the "four-in-one" cocktail approach at 10 μM. The concentration of human serum albumin (HSA) is 615 μM in plasma, therefore, the 40 μM (10 μM × 4) of total drugs used in this experiment will not saturate all the binding site, even if HSA has only one site for binding. As also suggested by Kariv et al. [21], the concentration of plasma protein is high and the amount of drug is far below the number of theoretical binding sites for total human serum protein content even at concentration as high as 1000 μg/ml. Therefore, saturation should not be an issue and our observation supports similar conclusion. Alpha-1 acid glycoprotein (AAG) is the second most abundant protein in plasma with much lower concentration. Drugs that bind to this protein might result in saturation. However, this phenomena was not observed in the compounds tested here. The results presented here also suggest that the cocktail dosing provided a fast, effective and accurate method in evaluating protein binding. This increased the sample throughput drastically and provided timely data to discovery scientists.

Higher variation between wells in the Microcon-96 filtrate assembly as compared with Centrifree devices was observed for both individual dosing and the cocktail approach as shown by the %CV in Table 1. In general, highly bound compounds showed higher variation due to the small amount of drug present in the filtrate and were more difficult to quantitate. This variation may be caused by the quality of the ultrafiltration devices. However, the average data from three to four replicates matches well with the individual devices and literature values.

Discrepancies between the data obtained using the 96-well filtrate assembly and the literature values were also observed for nicardipine and chlorpramazine. These data suggested that the ultrafiltration method employed here lacks precision and accuracy in determining protein binding for compounds with very low percentage unbound. Despite the observed lack of precision, the method discussed here would appear adequate for the rapid evaluation of compounds required in the drug discovery setting.

Some differences in the percent unbound were noticed between the present report and the published data for verapamil. However, the published percent unbound for verapamil varies from 4.3 to 23 in human plasma and serum [18] when different conditions and stereoisomers were used. In addition, McGowan et al. [26] showed that plasma verapamil binding varies with the AAG content in the plasma. Therefore, the difference observed in the present study may have resulted from (1) the difference in the methods (equilibrium dialysis, ultrafiltration, ultracentrifugation, etc.); (2) experimental conditions (pH, temperature, and ionic strength) used and (3) variation in protein content in human plasma (differences in AAG concentration).

With the use of 96-well filtrate assembly, 32 compounds can be tested in triplicate ($n = 3$) at the same time. Sample transfer can be done automatically by a Tecan Genesis 150 Workstation. When the cocktail approach with the 96-well filtrate assembly was adopted to assess the plasma protein binding, the throughput per plate was increased to 128 compounds per matrix ($n = 3$) or 32 compounds if four different matrices are used ($n = 3$).

5. Conclusion

The higher throughput method described here using a 96-well plate has several advantages over other methods: (1) high capacity (32 compounds, single matrix in triplicate per run); (2) short ultrafiltration time (45 min), less plasma stability issue than equilibrium dialysis; (3) compatible with automation; (4) low volume (250 μl plasma); and (5) multi-species capability within the same plate.

The present paper demonstrated the successful development of a 96-well format ultrafiltration procedure to assess protein binding. The data indicated that unbound fractions of new chemical entities in human plasma as determined by the cocktail approach using the 96-well format correlate with those determined individually. Although the protein binding data showed somewhat higher variability when determined using

the 96-well format, the data were comparable to those obtained using individual Centrifree devices and literature values. With the combination of fast LC–MS/MS method (1.5 min per sample), the use of Microcon-96 filtration device and a robotic system can result in a rapid and simple determination of plasma unbound fraction for a large number of compounds. A further increase in sample throughput can be achieved by adopting a cocktail approach.

Acknowledgements

Authors would like to thank Dr. Diana Montgomery and Ms. Ann Thomas, Schering-Plough Research Institute for her valuable suggestions and a critical review of the manuscript.

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