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Modulation of Nonspecific Binding in Ultrafiltration Protein Binding Studies

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Purpose. The aim of this study was to reduce or prevent nonspecific binding (NSB) of compounds to ultrafiltration (UF) protein binding (PB) testing units.

Methods. UF units (regenerated cellulose, MWCO 10K) were used for PB and NSB measurements with or without pretreatment with 5% tween 80 (TW 80) or 5% benzalkonium chloride (BAK) on the filter membrane. Dosing solutions (10 μM) in human serum and pH 7.4 phosphate-buffered saline were centrifuged at 3,000 *g* and room temperature after 1-h incubation in UF testing units. In parallel, a 96-well equilibrium dialyzer was used for PB and NSB measurements in equilibrium dialysis (ED) at 37°C for 4 h. Samples of UF and ED were analyzed by LC/MS or LSC.

Results. Severe NSB was observed for etoposide, hydrocortisone, propranolol, and vinblastine in UF. In contrast, TW 80 or BAK pretreatment on the filter membrane decreased the NSB from 87–95% to 13–64% without causing a significant change in membrane integrity. When NSB was below 50% as a result of pretreating agents, PB data of marker compounds were comparable to those of ED.

Conclusions. The pretreated membrane with TW 80 or BAK showed significantly less NSB for compounds that had a tendency toward high membrane binding. A modified UF method with pretreatment improved the performance of UF and was able to produce comparable PB results to ED.

KEY WORDS: Protein binding; nonspecific binding; ultrafiltration; equilibrium dialysis.

INTRODUCTION

Protein binding (PB) plays an important role in the pharmacokinetics and pharmacodynamics of a drug. The extent of PB in the plasma or tissue controls the volume of distribution and affects both hepatic and renal clearance (1,2). In many cases, the free drug concentration, rather than the total concentration in plasma, is correlated to the effect (3). Drug displacement from drug–protein complex can occur by direct competition of two drugs for the same binding site and is important with drugs that are highly bound (>95%), for which a small displacement of bound drug can greatly increase the free drug concentration in the plasma. Recently, Benet and Hoener showed that changes in plasma protein binding by drug–drug interactions or disease–drug interactions would usually not influence the clinical exposure such as AUC of a patient to a drug (4). However, precise information on the free drug fraction is still essential for drug discovery and development and for the safety assessment of drugs.

In order to measure free fraction or PB of a drug, ultratfiltration (UF), ultracentrifugation, equilibrium dialysis (ED), chromatography, spectrophotometry, electrophoresis, etc. have been used (1,2). Essential methodologic aspects of PB study include the selection of assay procedures, devices, and materials. The most commonly used method for PB measurement is ED, which is believed to be less susceptible to experimental artifacts. However, it is time consuming and is not suitable for unstable compounds because it requires substantial equilibration time (3 to 24 h) depending on drugs, membrane materials, and devices.

Many researchers have used UF centrifugal devices for PB measurement. UF is a simple and rapid method in which centrifugation forces the buffer containing free drugs through the size exclusion membrane and achieves a fast separation of free from protein-bound drug. However, the major disadvantage of this method is nonspecific binding (NSB) of drugs on filter membranes and plastic devices. When the drug binds extensively to the filtration membrane, the ultrafiltrate concentration may deviate from the true free concentration. In spite of its advantages such as convenience and short processing time, several authors raised a question on the adequacy of UF for PB measurement because of the high NSB (5–8).

In recent years, more lipophilic compounds have been observed in pharmaceutical industries, so adsorption problems are expected to increase. Therefore, a modification of the UF method was needed to overcome NSB. Presaturation of filter membranes has been attempted in order to reduce or prevent NSB (8,9). Saturation of the adsorbing sites with unlabeled compound is, however, questionable because of the possibility of uncontrolled desorption or displacement of adsorbed compounds that would cause the overestimation of the free concentration (8,9).

We investigated the modulation of NSB to reduce or prevent the NSB of compounds in UF units. This report describes the utility of pretreating the filter membrane for the accurate measurement of PB values in UF method.

MATERIALS AND METHODS

Materials

Our UF unit (Ultrafree-MC regenerated cellulose membrane, MWCO 10K, UFC3LGC00) was obtained from Millipore (Bedford, MA), and the 96-well equilibrium dialyzer (Amika dialyzer, regenerated cellulose membrane, MWCO 10K, MB 74-2301) was obtained from Harvard Bioscience (Holliston, MA). Tween 80 (TW 80, product P-1754) and benzalkonium chloride (BAK, product B-6275) were obtained from Sigma Chemical Co. (St. Louis, MO). Human serum (Biowhitaker 14-402E, Walkersville, MD) was used for the serum protein binding measurement. pH 7.4 phosphate-buffered saline (PBS, 0.05 M phosphate buffer/0.09M NaCl) was used for the NSB measurement. ^{3}H-Ketoprofen, ^{14}C-antipyrine, ^{14}C-caffeine, ^{14}C-ibuprofen, and ^{14}C-theophylline were obtained from ARC (St. Louis, MO). ^{3}H-Etoposide, ^{3}H-vinblastine, and ^{14}C-fluorocytosine were obtained from Moravek (Brea, CA). ^{3}H-Hydrocortisone and ^{3}H-propranolol were obtained from NEN (Atlanta, GA). A total

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of 18 compounds (antipyrine, caffeine, fluorocytosine, theophylline, ibuprofen, ketoprofen, propranolol, etoposide, hydrocortisone, vinblastine, diltiazem, chlorpheniramine, promethazine, clonidine, lorazepam, verapamil, imipramine, and diclofenac) were purchased from Sigma Chemical Co. (St. Louis, MO). All other materials and reagents were obtained from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical Co. (St. Louis, MO) and were used as received.

Ultrafiltration Methods

PB measurements were conducted with or without pretreatment of filter membranes. In case of pretreatments, UF units were wet with 25 μL of 0.5 to 5% TW 80 or 0.5 to 5% BAK for 5 min, and the units were centrifuged for 10 min at 3,000 *g*. The remaining TW 80 or BAK in the filter cup was washed out with 200 μL of phosphate-buffered saline.

Drug solutions (10 μM) were prepared in human serum and PBS. The final drug solutions contained 0.1% DMSO, which originated from the chemical library (10 mM). Drug solutions (400 μL) were added to pretreated and untreated filter cups of UF units and were equilibrated for 1 h at room temperature. Fifty microliters of PBS donor sample was taken from the filter cup for analysis before centrifugation. The serum and PBS samples were centrifuged at 3,000 *g* and room temperature until the filtrate samples were collected about 60 μL. Fifty-microliter filtrate samples from the bottom reservoir were transferred for analysis. In addition, a calibration curve from 0.01 to 10 μM was prepared in PBS. The samples and calibrators were combined with the equivalent volume of methanol before analysis.

Equilibrium Dialysis Methods

Drug solutions (10 μM) were prepared in human serum and PBS with 0.1% DMSO. The donor samples (200 μL) were placed into the top wells of a 96-well equilibrium dialyzer and were sealed with caps. The bottom wells were added with equivalent volume of PBS and were sealed with caps. The assembled 96-well equilibrium dialyzer was attached into the Plate Rotator (Harvard Bioscience, 74-2320) and was allowed to rotate at 25 rpm for 4 h in a 37°C-humidified incubator. Samples (50 μL) were taken from both donor and receiver wells. Equivalent volumes of serum or PBS were combined with the receiver or donor samples to match the sample matrix before addition of extraction solution [100 μL of acetonitrile: water (1:1) with 1% $ZnSO_4$]. In addition, a calibration curve from 0.01 μM to 10 μM was prepared in serum. Initial donor samples (50 μL) of serum and PBS drug solutions, and calibrators followed the same extraction procedure. The extracted samples were kept at –20°C for 1 h and were centrifuged at 2,000 *g* and 4°C for 1 h. The supernatant was transferred for analysis.

Sample Analysis

Aliquots of samples (10–20 μL) were analyzed by high-performance liquid chromatography/mass spectrometer techniques (HPLC/MS) to assess the concentration of samples. The HPLC unit consisted of a HP1100 binary pump (Agilent Technologies, Foster City, CA), HP1100 vacuum degasser (Agilent Technologies, Foster City, CA), HTS PAL autosampler (Leap Technologies, Inc., Carrboro, NC), and column-switching valve (Valco Instruments, Inc., Houston, TX). Composition of the mobile phase, precolumn and main column selection, and flow rates varied from compound to compound. The mobile phases consisted of 0.1% formic acid in water and 0.1% formic acid in methanol. Flow rate varied over a range of 0.2 to 1 mL/min with isocratic or gradient elution and Betacil C-18 reverse phase (5 μm particle size, 50 mm × 2 mm i.d.) or YMC C-8 reverse phase (5 μm particle size, 50 mm × 4 mm i.d.) columns were used.

A Sciex API 2000 using Turbo IonSpray or APCI sources (Applied BioSystems, Foster City, CA) was used for diltiazem, chlorpheniramine, clonidine, imipramine, promethazine, verapamil, and diclofenac, and a Finnigan LCQ IonTrap using APCI sources (ThermoFinnigan, San Jose, CA) was used for lorazepam. Diclofenac was analyzed in negative ion mode, and the rest of compounds were analyzed in positive ion mode, with source temperatures of 300 to 400°C. Single or multiple reaction monitoring was performed using nitrogen as the collision gas (LCQ MS used helium as the collision gas) with a dwell time of 400 ms. Total analysis time was 1 to 4 min per sample.

A trace amount of radioactive compounds (0.2–1 μCi/mL) was added to drug solutions of antipyrine, caffeine, fluorocytosine, theophylline, ibuprofen, ketoprofen, propranolol, etoposide, hydrocortisone, and vinblastine, and drug concentrations were determined using 1900 TR Liquid Scintillation Analyzer (Packard, Downers Grove, IL).

All experimental procedures were performed with more than three replicates, and NSB and PB were reported as mean with c.v. unless otherwise noted. Statistical analysis was performed using unpaired Student's *t* test between two mean values, and a probability of less than 0.05 ($p < 0.05$) was considered to be statistically significant.

Protein Binding (%) Calculation

UF Methods

NSB of UF units (NSB_{UF}) was determined from the measured concentrations of filtrate and PBS donor samples using Eq. 1:

$$NSB_{UF} = (C_{BD} - C_{BF})/C_{BD} \quad (1)$$

where C_{BD} is the donor (total) drug concentration in PBS before centrifugation and C_{BF} is the drug concentration in the PBS filtrate after centrifugation. When $C_{BF} = C_{BD}$, NSB is 0, and there is no need of NSB correction for the PB calculation. When $C_{BF} < C_{BD}$, it can be assumed that a fraction of drug disappeared in UF. The NSB correction of PB was made using Eqs. 2 and 3:

$$f_U = C_{SF}/[(1 - NSB_{UF}) \cdot C_{SD}] \quad (2)$$

$$\% \text{ PB} = 100 \cdot (1 - f_U) \quad (3)$$

where f_U is the free fraction, C_{SF} is the drug concentration in the serum filtrate, and C_{SD} is the nominal serum donor concentration, 10 μM. It was found that the donor concentration in serum did not change before or after filtration; therefore, the nominal serum donor concentration was used in C_{SD} for f_U calculation.

ED Methods

Drug concentrations in receiver (buffer) wells and donor (serum) wells were measured. At equilibrium both free drugs (C_{EF}) and protein-bound drugs (C_{EB}) are present in serum ($C_{ET} = C_{EB} + C_{EF}$), whereas only free drugs (C_{EF}) are present in buffer. Thus, the free fraction (f_U) and % PB were calculated as follows:

$$\% \text{ PB} = 100 \cdot (1 - f_U) = 100 \cdot (C_{ET} - C_{EF})/C_{ET} \quad (4)$$

NSB of equilibrium dialyzer (NSB_{ED}) was estimated from mass balance of PBS wells at 4 h for reference using Eq.5:

$$NSB_{ED} = (C_I - C_D - C_R)/C_I \quad (5)$$

where C_I is the measured initial concentration, C_D is the concentration of donor wells at 4 h, and C_R is the concentration of receiver wells at 4 h.

RESULTS

NSB Measurement

Table I lists NSB of 10 compounds tested in UF units without pretreatment. NSB values varied from 2 to 95% and were high for hydrocortisone, etoposide, propranolol, and vinblastine. PB was calculated with and without NSB correction using Eq. 2. As listed in Table I, PB was quite similar for low-NSB compounds with and without NSB correction. However, high-NSB compounds such as hydrocortisone, etoposide, propranolol, and vinblastine showed a significant difference between PB values with and without NSB correction ($p < 0.05$).

Pretreatment with TW 80

Four compounds that had high NSB were hydrophobic and basic ones (Table I). We attempted to modulate interactions between drug molecules and filter membranes utilizing a surface-active agent, TW 80. The nonionic surfactant was applied to the filter membrane to reduce potential hydrophobic interaction. Propranolol and hydrocortisone were selected for the feasibility test of this pretreatment.

Table I. NSB (%) and PB (%) of Test Compounds in UF without Pretreatment

	No pretreatment		
Compounds	NSB[a] (%)	PB[b] (%) without NSB correction	PB[c] (%) with NSB correction
Fluorocytosine	1.9	0	0
Theophylline	8.1	42.6	37.5
Antipyrine	10.4	12.7	2.61
Caffeine	16.5	29.5	15.5
Ibuprofen	29.3	99.4	99.2
Ketoprofen	33.6	99.4	99.1
Hydrocortisone	86.6	86.8	1.7
Etoposide	91.3	95.4	47.4
Propranolol	90.6	95.7	54.2
Vinblastine	95.4	97.9	53.3

Note: PB values were calculated with and without NSB correction.
[a] Mean ($n = 3$) with c.v. less than 10% except ibuprofen (13%).
[b] Mean ($n = 3$) with c.v. less than 10% except antipyrine (40%).
[c] Mean ($n = 3$) with c.v. less than 10% except antipyrine (220%) and hydrocortisone (163%).

Figure 1 shows the effect of TW 80 treatment on the NSB modulation of propranolol and hydrocortisone. With 0.5% TW 80 pretreatment, NSB of propranolol decreased from 91% to 41%, and NSB of hydrocortisone decreased from 87% to 40% at 50 μM. As shown in Fig. 1, the extent of NSB modulation was dependent on the concentration of TW 80 and drug concentrations. The higher the concentration of the TW 80 pretreatment, the smaller were the NSB values achieved, and the lower drug concentrations, the higher was the observed NSB. The protein leakage in UF was assessed with and without 5% TW 80 pretreatment, which showed the least NSB of propranolol and hydrocortisone. UF units had 0.75 and 1.2% of serum protein leakage without and with pretreatment, respectively, which were quite comparable. The results of protein leakage indicated that the filter membrane retained its integrity with pretreatment.

Table II presents NSB of 10 test compounds with 5% TW 80 pretreatment on the filter membrane. NSB was not observed for compounds such as fluorocytosine, theophylline, antipyrine, and caffeine, and NSB of ibuprofen, ketoprofen,

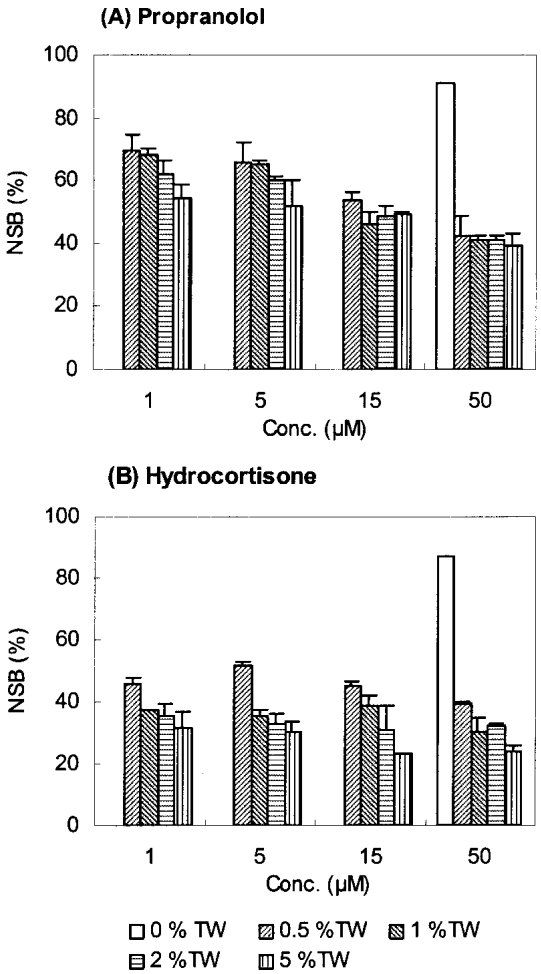

Fig. 1. The effect of TW 80 pretreatment (at five different concentrations from 0 to 5%) on the mean NSB of propranolol and hydrocortisone (at four different concentrations from 1 to 50 μM). The bar represents the standard deviation of replicates ($n \geq 3$).

Table II. NSB (%) and PB (%) of Test Compounds in UF with TW 80 and BAK Pretreatment

Compounds	5% TW 80 pretreatment			5% BAK pretreatment		
	NSB[a] (%)	PB[b] (%) without NSB correction	PB[b] (%) with NSB correction	NSB[a] (%)	PB[b] (%) without NSB correction	PB[b] (%) with NSB correction
Fluorocytosine	0	0	0	0	0	0
Theophylline	0	37.9	37.9	0	39.5	39.5
Antipyrine	0	6.0	6.0	0	12.3	12.3
Caffeine	0	21.8	21.8	0	25.8	25.8
Ibuprofen	2.3	99.4	99.4	91.7	99.6	95.1
Ketoprofen	4.1	99.2	99.2	79.1	99.5	97.5
Hydrocortisone	21.1	65.4	56.7	24.5	72.8	63.9
Etoposide	23.1	90.1	87.2	28.3	92.4	89.4
Propranolol	46.5	85.1	72.1	12.8	68.5	63.9
Vinblastine	95.2	95.4	4.2	63.7	94.8	85.7

Note: PB values were calculated with and without NSB correction.
[a] Mean ($n = 3$) with c.v. less than 10%.
[b] Mean ($n = 3$) with c.v. less than 10% except antipyrine (21%).

hydrocortisone, etoposide, and propranolol decreased significantly to below 50%. When NSB decreased, PB values of hydrocortisone, etoposide, and propranolol were similar with and without NSB correction. However, vinblastine, which had high NSB with TW 80 pretreatment, showed a big difference between PB values with and without NSB correction ($p < 0.05$).

Pretreatment with BAK

Because TW 80 pretreatment did not reduce NSB of vinblastine, benzalkonium chloride (BAK), a cationic surfactant, was selected as a possible way to prevent potential ionic interaction between basic compounds and filter membranes. NSB and PB results with BAK pretreatment are listed in Table II. With 5% BAK pretreatment, NSB of basic compounds such as propranolol and vinblastine decreased significantly, from 91% to 13% and from 95% to 64%, respectively. However, BAK pretreatment increased NSB of acidic compounds such as ibuprofen and ketoprofen, from 29% to 92% and from 34% to 79%, respectively. As expected, NSB of neutral compounds and hydrophilic compounds did not change with 5% BAK pretreatment. Similar to TW 80 pretreatment, low-NSB compounds with BAK pretreatment showed comparable PB results with and without NSB correction. However, PB of ketoprofen, ibuprofen, and vinblastine were significantly different with and without NSB correction because of their high NSB with BAK pretreatment ($p < 0.05$).

Protein Binding Measurement by 96-Well Format ED

The most important benefit of equilibrium dialysis (ED) is that PB can be accurately calculated regardless of NSB. It is also accepted that NSB in the ED method is much less than in the UF method. Kariv *et al.* (10) published PB data of propranolol, paroxetine, and lorsatan by a 96-well format equilibrium dialyzer. The report presented a good correlation between the traditional ED techniques and 96-well format equilibrium dialyzer for drugs with low, intermediate, and high PB properties.

Figure 2 compares PB (%) and free fraction of hydrocortisone, etoposide, propranolol, and vinblastine that were calculated by UF with no treatment/no NSB correction, no treatment/NSB correction, pretreatment/no NSB correction, and pretreatment/NSB correction, and ED methods. In no treatment group were the PB (or free fraction) data of the four marker compounds significantly different from those in ED regardless of NSB correction ($p < 0.05$). However, the pretreatment groups provided quite comparable PB (or free fraction) results for hydrocortisone, etoposide, and propranolol to those in ED. For vinblastine, NSB correction contributed significantly to PB calculation, and only PB with NSB correction was close to PB in ED. Overall results of Fig. 2 show that the modified UF method with a pretreatment step is capable of providing similar PB results to those in ED method.

A total of 18 compounds were run for PB in both UF and 96-well equilibrium dialyzer. Pretreated UF units were used, and NSB correction was made for the PB calculation. TW 80 pretreatment was made for neutral (fluorocytosine, antipyrine, theophylline, caffeine, hydrocortisone, and etoposide) and acidic (ketoprofen, ibuprofen, and diclofenac) compounds, and BAK pretreatment was made for basic (propranolol, vinblastine, diltiazem, chlorpheniramine, promethazine, clonidine, lorazepam, verapamil, and imipramine) compounds. The impact of NSB on PB calculation is significantly less in ED than UF, although NSB can occur to the dialysis membrane or to surfaces of the dialysis apparatus. PBS drug solutions were dialyzed against PBS to assess NSB and to examine equilibrium in ED units. No significant NSB (<15%) was observed in ED, and equilibrium was achieved with 4-h incubation for most of the compounds tested in this report (data not shown). Vinblastine and etoposide appeared to need more than 4 h of incubation time to reach equilibrium due to their high molecular weights, 811.0 and 588.6, respectively. Most of compounds showed similar PB results in both methods. Two PB data sets correlated well ($R^2 = 0.93$) with a slope of 1.01 and an intercept of 2.03, which were close to those of the unity line (Fig. 3). Free fraction data sets also showed a good correlation between the two methods (data not shown).

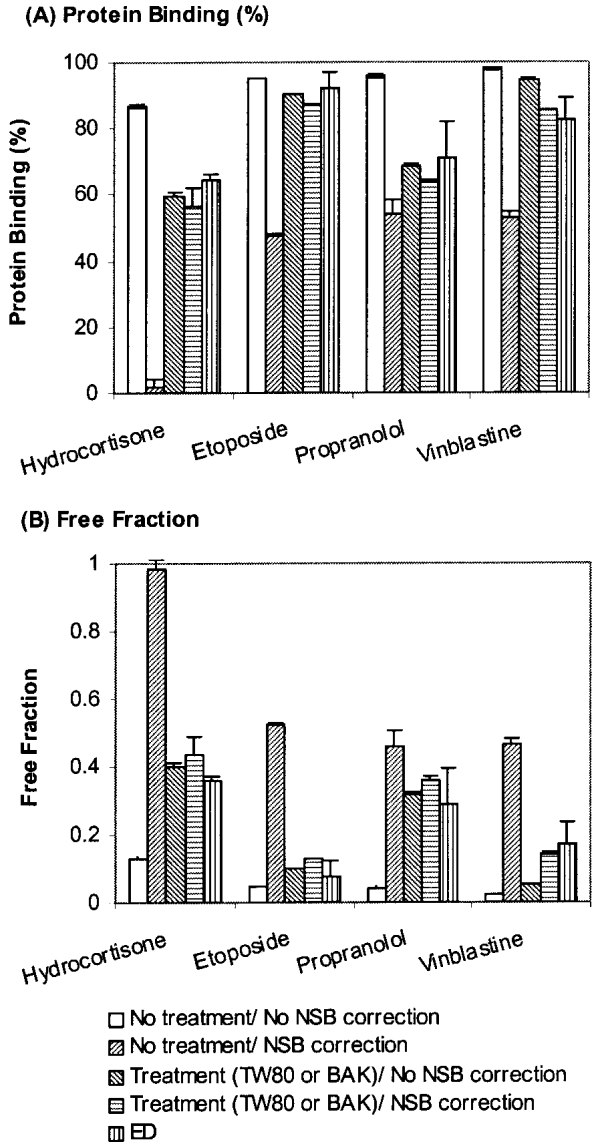

Fig. 2. Comparison of PB (%) and free fraction of hydrocortisone, etoposide, propranolol, and vinblastine between UF and ED methods. The values were calculated by four different methods (no treatment/no NSB correction, no treatment/NSB correction, pretreatment/no NSB correction, and pretreatment/NSB correction) in UF, and they were compared to those of the ED method. The bar represents the standard deviation of replicates ($n \geq 3$).

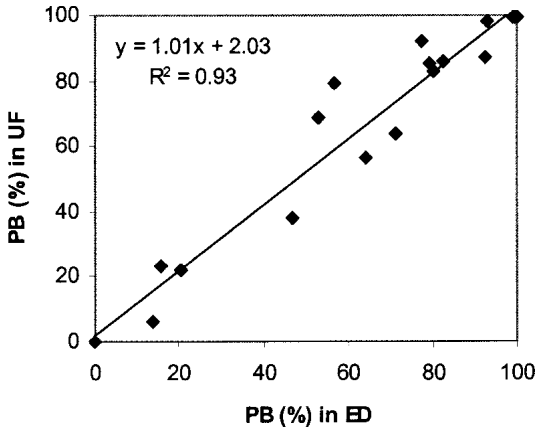

Fig. 3. Correlation of mean PB (%) of 18 compounds between UF and ED. The PB data in UF were calculated with pretreatment (TW 80 for neutral and acidic compounds and BAK for basic compounds) and NSB correction.

DISCUSSION

ED has been the preferred method to determine PB compared to UF because ED is less susceptible to experimental artifacts, and UF suffers from NSB of drugs to the filter membrane. As shown in this report, the serious NSB in UF yielded questionable PB data, especially for hydrocortisone, etoposide, propranolol, and vinblastine (Table I). NSB of drugs in UF units has also been reported by several authors: serious NSB of disopyramide and its metabolite to Centriflo conical membranes (Amicon, USA) (5); NSB of diazepam, ibuprofen, and quinidine sulfate for PTLC membranes (Millipore, USA) (6); NSB of theophylline, acetaminophen, and warfarin to PM 10 (Amicon, USA) (7); and NSB of verapamil, nifedipine, and prazosin to CF 50A (Amicon, USA), YMT (Amicon, USA), and YMB (Amicon, USA) (8).

Because of the limitations of conventional UF and ED methods, different PB techniques were explored such as charcoal adsorption (13) and high-performance frontal analysis for a drug–protein binding study (14). Yet, the relatively new PB techniques have not been widely used in the pharmaceutical industries and academia. A different type of filter membrane was also tested to improve performance of UF. It was reported that polysulfone filter membrane (Millipore, UFC3TTK00) showed less NSB for theophylline than Millipore MC filter units and Amicon Centrifree system (15). Authors of this report also tested the same material in UF units. However, NSB to polysulfone membranes was not significantly different from that to regenerated cellulose acetate membranes for more hydrophobic compounds (hydrocortisone, etoposide, propranolol, and vinblastine) than theophylline (data not shown). Zhirkov and Piotrovskii pretreated the filter membrane of CF 50A, YMT, and YMB with serum ultrafiltrate to reduce NSB (8). However, the serum ultrafiltrate pretreatment did not improve/lower NSB of verapamil, nifedipine, and prazosin to CF 50A, YMT, and YMB at all.

UF units that were used in this report consisted of a polypropylene plastic upper filter cup and a bottom reservoir. Regenerated cellulose acetate membrane was placed in the upper filter cup. The regenerated cellulose acetate was produced by the reaction of cellulose with anhydrides of acetic acids in the presence of sulfuric acid catalyst (11). The cellulose esterification reaction represented a hydrophobic modification of the hydrophilic cellulose polymer backbone, and the reported average acetyl content was around 30 to 40% (11). Kwong reported that positive or negative charges on a membrane could influence the ultrafiltration of ionized drug (12). It was claimed by the manufacturer that the regenerated cellulose acetate membranes did not carry any charge. However, it was assumed that acetyl groups (CH_3CO^-) could be charged negatively in part and the charged acetyl groups would interact with positively charged basic compounds at neutral pHs. Thus, it was hypothesized that TW 80 could prevent potential hydrophobic interaction for neutral and acidic compounds such as hydrocortisone, etoposide, ibuprofen, and ketoprofen, and BAK could reduce potential ionic

interaction for basic compounds such as vinblastine and propranolol. No treatment showed severe NSB of etoposide, hydrocortisone, propranolol, and vinblastine in UF (Table I), whereas pretreatment of the filter membrane with TW 80 or BAK decreased the NSB from 87–95% to 13–64% without causing a significant change in membrane integrity (Table II). In contrast to other efforts to reduce NSB in UF (8,15), the results of Table I and Table II showed that the TW 80 or BAK pretreatment could reduce NSB in UF units. Further, the results can provide a decision tree regarding the selection of pretreatment agents such that TW 80 pretreatment reduces NSB of neutral or acidic compounds and BAK pretreatment reduces NSB of basic compounds (Fig. 4).

Authors have attempted to demonstrate the utility of pretreating the filter membrane by comparing PB data between the UF and ED methods. As shown in Fig. 3, there was a good correlation ($R^2 = 0.93$) between two methods with a slope of unity, although UF was conducted at room temperature, whereas ED was conducted at 37°C in this study. Ha *et al.* (17) reported that a correlation between PB of quinidine in UF (25°C) and ED (37°C) was high ($R^2 = 0.972$), and the slope was 0.884. The authors suggested that the slight difference in PB values resulted from a temperature difference between the two methods. PB of demethylchlorimipramine was measured in both UF and ED by Bertilsson *et al.* (18). It was claimed that UF at room temperature provided a closer PB result (96%) to the *in vivo* results than ED (92%) at 37°C because of loss of plasma binding capacity during ED incubation. It was also observed that PB values of disopyramide and thiopental were similar regardless of temperature (22°C vs. 37°C) and methods (UF vs. ED) (19–21). As seen in Fig. 3, PB in UF was slightly higher than that in ED with an intercept of 2.03%. The slight discrepancy might be attributed to the NSB correction of PB in UF and a temperature difference (25°C vs. 37°C) between the two methods.

It has been known that the Donnan effect could affect the determination of protein–drug binding in ED (23), particularly for those drugs that are highly ionized and weakly bound. It was reported that addition of small electrolytes in the system could abolish the unequal distribution of diffusible ions (24). Suter *et al.* showed that addition of 0.1 M KCl to 0.04 M phosphate buffer achieved a Donnan ratio close to unity for cesium ions irrespective of protein concentrations up to 30 mg/mL (25). The pH 7.4 isotonic phosphate-buffered saline (PBS) of this report consisted of 0.05 M phosphate buffer and 0.09 M NaCl; therefore, it was believed that Donnan effect on PB in ED was insignificant.

The accurate NSB estimation and correction of PB in the UF method are still challenging. It is difficult to predetermine what drug concentration should be used for NSB measurement. In this report, NSB was measured at 10 μM, which was the same as the serum drug concentration. Based on the fact that NSB is inversely dependent on drug concentrations, and free drug concentrations in serum samples are lower than 10 μM when PB occurs, NSB at 10 μM in PBS may be an underestimation of a real NSB in serum samples. Despite the potential NSB underestimation, results of Figs. 2 and 3 suggested that the modified UF method with pretreatment could measure PB to a similar extent of accuracy as in ED when NSB correction was made.

There is a high chance of NSB underestimation as the PBS filtrate volume increases. In this report, the volume of drug solutions in UF units was fixed at 400 μL, and the volume of ultrafiltrate was less than 60 μL (approximately 15% of the initial volume) in order to lower the risk of NSB underestimation.

NSB of high-molecular-weight compounds (MW > 500) could be intrinsically high because of potential molecular sieving effects. Kurz *et al.* reported molecular sieving effects for drug molecules with high molecular weights in UF (16). The error in a protein-free solution was around 2% for a drug of molecular weight of 300, but it increased rapidly to 13% for streptomycin, which has a molecular weight of 581 (16). The concentration of high-molecular-weight drugs was found to be lower in the ultrafiltrate than in the nonfiltered solution. Thus, it is possible that NSB could be confounded with molecular sieving effect for vinblastine (MW 811.0) in this report, and this can be one of the reasons for measurable apparent NSB of vinblastine even with BAK pretreatment. Authors of this report observed high NSB for compounds of high molecular weights regardless of pretreatment (data not shown).

As NSB increases, the extent of NSB correction of PB increases, and the precision of this method decreases. If NSB is greater than 50%, the extent of NSB correction of PB becomes more than 100% (Eq. 2). When NSB is significantly high, such as >80%, PB with and without NSB correction can be significantly different. Therefore, it is recommended to use a NSB correction method when NSB is less than 50% to minimize the risk of inaccurate PB estimation.

Currently LION Bioscience is developing an *in silico* ADME predictive model (iDEA™), that can predict absorption, metabolism, distribution, and excretion properties of compounds in the human body using *in vitro* measurement of permeability, solubility, protein binding, and metabolic stability. The iDEA™ metabolism model (v2.1) was successfully developed by using PB data measured by the method described here.

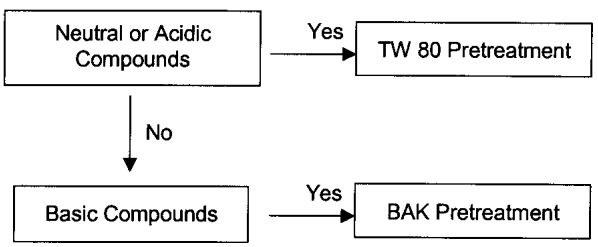

Fig. 4. A decision tree for the selection of pretreatment method depending on the physicochemical characteristics of compounds in the UF method.

CONCLUSIONS

Decreasing or preventing NSB is essential for the accurate measurement of PB in the UF method. This work presented the utility of methods of filter membrane pretreatment for the reduction of NSB. The membrane pretreated with TW 80 or BAK showed significantly less NSB for compounds that had a tendency for high membrane binding. The results of two different pretreatment methods demonstrated that NSB in UF units could be reduced significantly, and when NSB was reduced, PB in the UF method was similar to those in ED.

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