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Short communication

Vacuum ultrafiltration sampling for determination of plasma protein binding of drugs[1]

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1. Introduction

The extent of binding of drugs to plasma proteins is considered an important factor in determining their pharmacologic behavior. Studies of drug binding to proteins have typically been performed by either equilibrium dialysis or ultrafiltration [1–5]. Equilibrium dialysis suffers from the need for a long equilibration and nonspecific binding of the drug to the dialysis membrane [2,5]. Ultrafiltration also suffers from nonspecific binding to the ultrafiltration membrane and possible shifts in the protein concentration [4]. Microdialysis sampling has been demonstrated as an alternative technique to study drug binding both in vitro and in vivo [6–9]. However, accurate determinations of the free concentration of drug by microdialysis sampling require careful calibration of the microdialysis probe with respect to recovery.

Vacuum ultrafiltration makes use of fiber membrane probes similar to those used for microdialysis sampling [10,11]. However, the driving force for sampling is application of a slight negative pressure to pull bulk solution into the probe. Large solute molecules are excluded by the membrane but small molecules are transported with the bulk solution. This provides essentially a complete recovery for small molecules and removes the need for probe calibration. All of the advantages of microdialysis sampling relative to other techniques for the determination of protein binding are also provided by vacuum ultrafiltration.

The objective of this study was to evaluate the use of vacuum ultrafiltration for determining the free fraction of drugs in plasma. The binding of several drugs to human plasma was determined. The drugs were chosen to exhibit a wide range of binding to plasma proteins. Vacuum ultrafiltration was compared with normal ultrafiltration, i.e. ultrafiltration driven by centrifugal force.

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2. Materials and methods

2.1. Chemicals

Acebutolol, acetaminophen, cephalothin, chloramphenicol, isoniazid and salicylic acid were purchased from Sigma Chemical (St. Louis, MO). Recovered human plasma was obtained from the Kansas City Community Blood Center and the Topeka Blood Bank. All other chemicals were of reagent grade or better and used as received.

2.2. Ultrafiltration probes

Ultrafiltration probes were purchased from Bioanalytical Systems (West Lafayette, IN). UF-3-2 probes with polyacrylonitrile (PAN) dialysis fibers were used in these studies. The probe inlet was inserted into a 30 cm length of 0.19 mm i.d. Tygon peristaltic pump tubing (Cole-Parmer Instrument Co., Niles, IL), which was threaded through a cartridge of an Alitea-XV peristaltic pump (Alitea USA, Medina, WA) leading to a collection vial (Fig. 1).

2.3. Chromatographic analysis

The concentrations of the drugs in both vacuum and centrifugal ultrafiltrates were determined by liquid chromatography with either UV or electrochemical detection. The chromatographic system consisted of a Shimadzu LC-6A pump, a Rheodyne Model 7125 injection valve with a 5 μl sample loop (Rainin Instrument Co., Woburn, MA), an SPD-6AV UV–Vis detector (Shimadzu, Columbia, MD) and an LC-4C amperometric detector (Bioanalytical Systems). Separation was performed with a Brownlee ODS-102 column (100 × 2.1 mm i.d.) with 5 μm spherical C18 packing material. The mobile phase was ammonium phosphate (pH 2.5; 0.05 M) for all compounds except for isoniazid, for which the mobile phase was sodium acetate (pH 5.2; 0.05 M). Acetonitrile was added to the mobile phase as an organic modifier as needed for each drug as listed in Table 1. A flow rate of 0.5 ml min^{-1} was used for all experiments.

Table 1
Analytical conditions

Drug	Detection conditions[a]	Mobile phase (% ACN)
Acebutolol	240 nm UV	15
Acetaminophen	750 mV EC	2.5
Cephalothin	272 nm UV	15
Chloramphenicol	280 nm UV	25
Isoniazid	800 mV EC	2.5
Salicylic acid	250 nm UV	15

[a] EC = electrochemical detection.

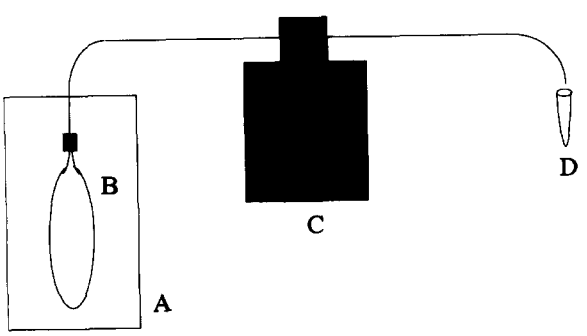

Fig. 1. Schematic diagram of the system for vacuum ultrafiltration determination of protein binding, A = plasma sample vial; B = ultrafiltration probe; C = peristaltic pump; D = collection vial.

2.4. Protein binding experiments

Stock solutions of each drug were prepared in Ringer's solution at a concentration at least 400-fold that desired for the binding experiment. An aliquot of the stock solution was then pipetted into sufficient plasma to give the desired plasma concentration. The plasma concentrations of the various drugs used were chosen to be within their therapeutic range. the spiked plasma samples were thoroughly mixed by vortexing and allowed to equilibrate for 45 min at 37°C. After equilibration the spiked plasma samples were divided into two fractions, one for analysis by vacuum ultrafiltration and the other for analysis by centrifugal ultrafiltration.

Table 2
Percentage of drug bound to human plasma proteins as determined by vacuum and centrifugal ultrafiltration

Drug	Concentration (μg ml^{-1})	VUF[a]	CUF[b]	Recovery[c] (%)	Nonspecific adsorption[d] (%)
Acebutolol	10[e]	13.3 ± 2.7	14.3 ± 2.0	98.8 ± 1.1	1.9 ± 0.3
Acetaminophen	5	11.4 ± 1.3	12.3 ± 0.9	98.3 ± 2.1	0
	10	10.4 ± 0.7	10.1 ± 0.6	99.5 ± 0.2	0
	50	10.0 ± 0.2	10.9 ± 0.7	99.7 ± 0.2	0
Cephalothin	10	71.9 ± 1.7	72.9 ± 1.1	98.8 ± 4.1	0
Chloramphenicol	4	52.6 ± 4.3	50.6 ± 5.1	93.4 ± 14.0	8.3 ± 4.8
Isoniazid	5	20.0 ± 1.7	21.5 ± 1.2	98.9 ± 1.2	0
Salicylic acid	50	90.8 ± 0.5	89.6 ± 0.8	96.1 ± 1.1	2.7 ± 1.1
	100	87.8 ± 0.5	89.9 ± 0.8	94.2 ± 0.7	2.9 ± 0.5
	150[e]	86.4 ± 4.8	85.9 ± 1.2	86.1 ± 1.5	4.0 ± 0.6
	300	65.7 ± 0.6	70.1 ± 0.7	79.8 ± 0.7	2.5 ± 0.6
	500[e]	54.9 ± 2.3	56.2 ± 3.9	74.3 ± 0.8	3.4 ± 0.5

[a] VUF = vacuum ultrafiltration, $n = 3$.
[b] CUF = centrifugal ultrafiltration, $n = 3$.
[c] Recovery of the ultrafiltration probe at the concentration of the free drug found during the binding experiment reported as the concentration in the ultrafiltrate relative to the concentration in the sample.
[d] Nonspecific adsorption to the centrifugal ultrafiltration membrane determined at the concentration of the free drug found during the binding experiment reported as the amount adsorbed relative to the total drug initially in the sample.
[e] $n = 6$.

2.5. Centrifugal ultrafiltration

Centrifugal ultrafiltration was performed with an MPS-1 micropartition system with a YMT membrane filter (Amicon, Lexington, MA). The spiked plasma sample was brought to 37°C and three 1 ml aliquots were then ultrafiltered by centrifugation at 3000g for 45 min. The ultrafiltrate was analyzed for free drug by liquid chromatography as described above. Nonspecific adsorption of each drug to the ultrafiltration membrane was determined by ultrafiltering a drug standard in Ringer's solution. The extent of nonspecific adsorption to the membrane for each drug is listed in Table 2. The free drug concentrations were corrected for this nonspecific adsorption.

2.6. Vacuum ultrafiltration

Vacuum ultrafiltration was performed by first calibrating the probe at 37°C for recovery of the specific drug using standards in Ringer's solution. The standard used was of the same concentration and sampling volume as the plasma concentration to be studied. Prove recoveries of the drugs used in this study were determined as the ratio of the concentration of drug in the ultrafiltrate to the concentration in the standard solution (Table 2). An 8 ml aliquot of the spiked plasma sample was brought to 37°C in a dry heating block with constant stirring. Vacuum ultrafiltration was performed using a calibrated probe with the peristaltic pump set at 300 rpm. Samples of approximately 25 μl were collected (at about 8–10 min intervals) until at least three consecutive samples exhibited consistent free drug concentrations. Free drug was determined by direct injection of the vacuum ultrafiltrate into the chromatogaphic system using the conditions described above (Table 1). The free drug concentration in the plasma was then determined by dividing the concentration in the ultrafiltrate by the recovery.

3. Results and discussion

Vacuum ultrafiltration occurs by the pressure drop "pulling" sample into the probe. The molecular weight of the membrane was selected such

that only small molecules can be drawn through with the bulk solution while larger molecules are excluded. Convection in the sample continually renews the solution around the probe, keeping the concentration of all components adjacent to the probe at the bulk solution concentration. Since the amount of ultrafiltrate collected is negligible relative to the total sample volume, the overall drug and protein concentrations remain essentially constant during the experiment. Therefore, the drug binding equilibrium is not disturbed by this technique.

Because solute molecules are drawn through the membrane as a result of the bulk flow of solution, if their molecular weights are much less than the molecular weight cut-off of the membrane, their concentration in the filtrate will be the same as their concentration in the bulk solution. By comparison with microdialysis sampling, which uses similar membrane probes, the recovery of vacuum ultrafiltration is 100%. Therefore, in contrast to microdialysis sampling, it is not necessary to calibrate the ultrafiltration probe if the membrane cut-off is chosen properly. The recoveries of the tested compounds are listed in Table 2. As can be seen, they are all essentially 100% except for salicylic acid. The reason for the apparent concentration dependence of the recovery of salicylic acid using vacuum ultrafiltration is not known. None of the other compounds studied showed a concentration dependence of recovery.

The results from vacuum ultrafiltration and centrifugal ultrafiltration determination of drug binding to human plasma proteins are compared in Table 2. The precision of the two methods was equivalent. Comparison of the vacuum and centrifugal ultrafiltration data exhibits no significant differences. As can be seen from Fig. 2, there is no bias between the two methods. Vacuum ultrafiltration can be used to determine protein binding over a wide range of free drug concentration and extent of protein binding.

4. Conclusions

These data demonstrate that vacuum ultrafiltration can be used to determine the binding characteristics of small drug molecules to proteins. Vacuum ultrafiltration offers the advantage of not significantly changing the volume of the sample or disturbing the binding equilibrium. Therefore, relative to centrifugal ultrafiltration, much smaller sample volumes can potentially be used. If the molecular weight cut-off of the ultrafiltration probe membrane is chosen properly, no calibration is needed, in contrast to using microdialysis sampling. Finally, the advantages of the microprobe technique demonstrated previously using microdialysis sampling also apply to vacuum ultrafiltration [6–9]. Because this is an in situ sampling technique, the sample temperature can be maintained at any desired value during the entire experiment. Because the surface area of the membrane is much smaller than that for vacuum ultrafiltration and multiple samples can be collected, problems of nonspecific absorption can be overcome.

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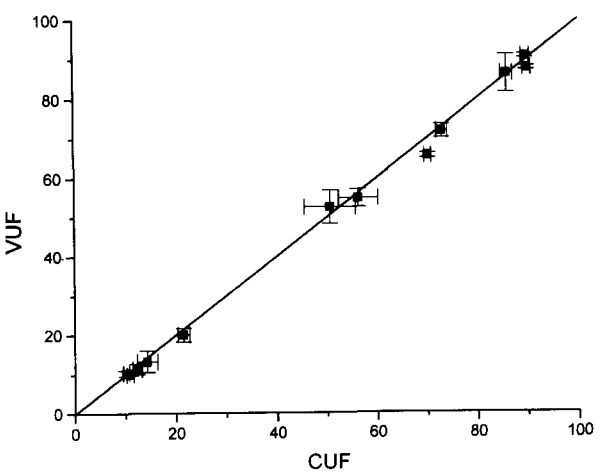

Fig. 2. Comparison of plasma protein binding determination by vacuum ultrafiltration and centrifugal ultrafiltration.

References

[1] V.P. Shah, S.M. Wallace and S. Riegelman, J. Pharm. Sci., 63 (1974) 1364–1367.
[2] W.F. Bowers, S. Fulton and J. Thompson, Clin. Pharmacokinet., 9 (Suppl 1) (1984) 49–60.
[3] J.B. Whitlam and K.F. Brown, J. Pharm. Sci., 70 (1981) 146–150.
[4] Y.A. Zhirkov and V.K. Piotrovskii, J. Pharm. Pharmacol., 36 (1984) 844–845.
[5] J. Barre, J.M. Chamouard, G. Houin and J.P. Tillement, Clin. Chem., (1985) 60–64.
[6] A.M. Herrera, D.O. Scott and C.E. Lunte, Pharm. Res., 7 (1990) 1077–1081.
[7] M. Ekblom, M. Hammarlund-Udenaes, T. Lundqvist and P. Sjöberg, Pharm. Res., 9 (1992) 155–158.
[8] S. Sarre, K. Van Belle, I. Smolders, G. Krieken and Y. Michotte, J. Pharm. Biomed. Anal., 10 (1992) 735–739.
[9] A.L. Quelle, S. Dupin, A.E. Tufenkji, P. Genissel and G. Houin, Pharm. Res., 11 (1994) 835–838.
[10] M.C. Linhares and P.T. Kissinger, Anal. Chem., 64 (1992) 2831–2835.
[11] M.C. Linhares and P.T. Kissinger, J. Pharm. Biomed. Anal., 11 (1993) 1121–1127.