REPORTS

Correction for Volume Shift during Equilibrium Dialysis by Measurement of Protein Concentration

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Abstract: Volume shift during equilibrium dialysis produces errors in estimating the fraction of drug unbound. This study describes a method in which protein concentration in the plasma is used to correct binding data for volume shifts. Data are presented for phenytoin, a drug that does not bind to the dialysis system, and for verapamil, a drug that does. The conventional method of not correcting for volume shift, the method described previously by one of us (TNT) for a drug that does not bind to the dialysis system, and the proposed method of determining fraction unbound are compared and discussed. It is concluded that the second method is simple and can be used to determine the unbound fraction for a drug, such as phenytoin, which does not bind to the dialysis system. If a drug binds to the dialysis system, as does verapamil, the proposed method of measuring protein concentration before and after dialysis can be reliably used to correct for volume shift.

Equilibrium dialysis is a commonly used method to determine the unbound fraction (fu) of a drug in plasma. Because volume shifts occur during dialysis, errors in the determination of fraction unbound result. These errors have been the subject of recent studies (1–5) in which a method was presented to correct the fraction unbound for the volume shift that occurs during dialysis (1). Briefly, the method involves the determination of the drug concentration in the buffer after dialysis (CC'_B) and in the plasma before dialysis (C_p) and knowledge of the original ratio of volumes of buffer to plasma. If radiotracer is added to the buffer, as in these studies, then the radiotracer concentration in the buffer before and after dialysis is used. To use this method correctly, as pointed out by Tozer et al. (1), mass balance of drug in the solution during the dialysis procedure is assumed. This assumption implies that the drug neither binds to the dialysis membrane or system nor decomposes. For many drugs, binding does occur. This paper describes a method for correction of volume shift which can be used in this situation. Theoretically, this method, as well as that of Tozer et al. (1), is based on the principle that the transfer of fluid during dialysis does not change the unbound concentration; rather, the bound concentration is decreased. The method involves the measurement of protein concentration in the plasma before and after dialysis. To test this method, we studied the plasma protein binding of verapamil, a drug that binds to the dialysis system, and phenytoin, a drug which does not. The fraction unbound that for both compounds was estimated by this method, the method of Tozer et al., and by making no correction for volume shift (conventional method). The results demonstrate that for both verapamil and phenytoin, protein concentration measurements carried out in the plasma before and after dialysis can be reliably used to measure the fraction unbound in the presence of a volume shift.

Materials and Methods

Blank plasma was obtained from four healthy volunteers and stored at −20°C pending dialysis. Equilibrium dialysis was carried out in 2 ml dialysis cells (Dianorm System, Spectrum, Inc., Los Angeles, CA). One milliliter blank plasma from each subject was dialyzed in duplicate against 1 ml of Sorensen's phosphate buffer (0.13 M, pH 7.4) containing 200 ng unlabeled verapamil and 122 pg of ^{3}H-verapamil (75 Ci/mmol; New England Nuclear, Boston, MA) or 7.6 μg of phenytoin and 48.4 ng of ^{14}C-phenytoin (47 mCi/mmol; New England Nuclear). Plasma was separated from buffer by a cellophane membrane (Spectra/Por2, Spectrum, Inc.) with a molecular weight cut-off of 12000–14000. Dialysis was carried out at 37°C for 6 h during which time the cells were gently rotated. The 6 h time period was established in preliminary experiments to be sufficiently long (greater than five times the time required to reach 50% of equilibrium value) to ensure that equilibrium had been achieved for both compounds. One-half milliliter buffer before and after dialysis, and 0.5 ml plasma after dialysis were analyzed by liquid scintillation counting.

Radiolabeled compounds were checked for radiochemical purity (> 97%) by thin-layer chromatography and liquid scintillation counting. We found ^{3}H-verapamil had to be purified by thin layer chromatography before use. The tritiated compound, however, remained stable during the dialysis procedure.

Concentration of protein in the plasma before and after dialysis was determined by the method of Gornall et al. (6).

Recovery of both phenytoin and verapamil from the dialysis system was assessed by dialyzing only the radiolabeled compound in buffer against blank buffer under the conditions of the experiment. Phenytoin was almost completely recovered (> 97%) from the system, whereas the recovery of verapamil varied from 50 to 80% in replicate determinations.

Data Analysis

The three methods used to calculate the fraction unbound (fu) of verapamil and phenytoin were:

Method 1 (conventional):

$$fu_1 = \frac{C'_B}{C'_p}$$

Method 2 (Tozer et al. (1):

$$fu_2 = \frac{C'_B}{C_B - C'_B}$$

Method 3 (present):

$$fu_3 = \frac{C'_B}{C^0_{bnd} + C'_B}$$

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where C'_B and C'_P are the concentrations of radioactivity after dialysis in the buffer and plasma, C_B is the concentration of radioactivity in the buffer before dialysis and C^0_{bnd} is the concentration of bound drug had no volume shift occurred (1). The value of C^0_{bnd} is obtained as follows:

$$C^0_{bnd} = C'_{bnd}(1+\delta)$$

where C'_{bnd} represents the bound drug concentration obtained after dialysis. C'_{bnd} can be obtained as follows:

$$C'_{bnd} = C'_P - C'_B$$

δ is the fractional increase in the volume of the plasma due to the osmotic water shift and can be approximated as follows:

$$\delta = \frac{P}{P'} - 1$$

where P and P' are the protein concentrations in the plasma before and after dialysis.

Statistical analysis of the three methods was carried out by one-way analysis of variance. The F-statistic was evaluated to test if there were differences among the three methods. Specific differences between pairs of methods were evaluated using the Newman-Keuls multiple range test.

Results

The fractions unbound of phenytoin calculated by the three methods for each subject are shown in Table I. Both fu_2 and fu_3 differed significantly from fu_1, the conventional method. However, fu_2 and fu_3 which were corrected for volume shifts were not significantly different from each other. Values of δ varied between 0.066 and 0.353.

The respective fractions unbound of verapamil in the plasma of each subject are shown in Table II. In this case, fu_1, fu_2, and fu_3 differed significantly from each other. Values of δ here varied from 0.075 to 0.418.

Discussion

Correction for volume shift is important when using equilibrium dialysis as a method to obtain the fraction unbound of a drug *in vivo*. As discussed by Tozer et al., this correction is particularly important for drugs that are highly bound to plasma proteins and under conditions in which substantial volume shift occurs (1). In the present study, we found considerable variability in volume shift (δ) between dialysis cells (ranging from 0.066 to 0.418). The factors which produce volume shifts are related to the osmotic pressure of the nondiffusible substances in the plasma and the dialysis time and were not further investigated.

In this study, the volume shift could be accounted for in the determination of fraction unbound for phenytoin by either measuring protein concentration before and after dialysis (method 3) or using the method of Tozer et al. (method 2). The mean values of fraction unbound calculated from methods 2 and 3 were 0.136 and 0.139, respectively, and did not differ significantly from each other. This is in agreement with theory and demonstrates that for a drug which is not lost (bound to system components or degraded) during dialysis, the method of Tozer et al. is reliable and has the advantage of requring only one post-dialysis measurement (C'_B).

In contrast to phenytoin, the recovery of verapamil from the dialysis system was incomplete and erratic. In this case, as pointed out by Tozer et al., their method cannot be used to correct the fraction unbound for volume shift. As shown in Table II, and in accordance with theory, fu_2 calculated by their method underestimates the fraction unbound calculated by method 3 (fu_3). For drugs such as verapamil that are lost during the dialysis procedure, errors may also occur in the calculation of post-dialysis drug concentration. This problem has been discussed elsewhere (7).

The proposed method has the disadvantage of requiring additional measurements (protein concentration before and after dialysis) to determine the fraction unbound. These measurements introduce error. This may be the cause of the larger standard deviation (S.D.) observed with method 3 (see Tables I and II) and suggests that special care be given to measuring the protein concentration when applying this method.

In summary, volume shift during equilibrium dialysis produces errors in the estimation of the fraction unbound. If a drug is not lost during dialysis, as was the case of phenytoin in this study, the method of Tozer et al. can be used to correct for volume shift and to accurately obtain the fraction unbound. For a drug such as verapamil, whose loss during the dialysis procedure is erratic, measurement of protein concentration before and after dialysis can be used reliably to correct for volume shift.

Acknowledgments

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Table I. The fraction unbound of phenytoin calculated by the three different methods[a]

Subject (#)	fu_1	fu_2	fu_3	δ
1	0.152[b]	0.131	0.140	0.066
2	0.179	0.151	0.157	0.172
3	0.172	0.129	0.141	0.259
4	0.153	0.132	0.118	0.353
Mean	0.164	0.136	0.139	0.213
S.D.	0.014	0.010	0.016	0.120

[a]Methods are described in text.
[b]Values represent means of duplicates.

Table II. The fraction unbound of verapamil calculated by the three different methods[a]

Subject (#)	fu_1	fu_2	fu_3	δ
1	0.122[b]	0.095	0.115	0.075
2	0.123	0.095	0.090	0.418
3	0.129	0.088	0.105	0.235
4	0.133	0.092	0.117	0.163
Mean	0.127	0.092	0.107	0.223
S.D.	0.005	0.003	0.012	0.146

[a]Methods are described in text.
[b]Values represent means of duplicates.

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Experimental Combination Chemotherapy with Thymidylate Synthetase and Ribonucleotide Reductase Inhibitors

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Abstract: The synergistic cytotoxic effects on exponentially growing 9L rat brain tumor cells of several inhibitors of thymidylate synthetase (TS) and ribonucleotide reductase (RNR) used in combination were investigated using a colony forming efficiency assay as the experimental endpoint. A 24 h treatment with nontoxic (0.1 μg/ml) or low (1.0 μg/ml) doses of 5-fluorouracil (FUra), 5-fluorodeoxyuridine, 5,8-dideazaisofolic acid, or 2'-deoxy-2'-fluoro-ara-uracil markedly enhanced cell kill caused by subsequent administration of 100 μg/ml hydroxyurea (HU) for 6 h. When a similar dose of HU or 1-formylisoquinoline thiosemicarbazone was administered for 6 h immediately after a 24 h treatment with either a 0.1 μg/ml or 1.0 μg/ml of FUra, a cell kill of approximately 1 log in addition to that caused by each drug alone was obtained. Thus a synergistic cell kill was consistently obtained when a low dose of TS inhibitors was administered 24 h before a 6 h treatment with another low dose of agents that act as RNR inhibitors. This synergism was not observed when FUra-treated cells were treated with methotrexate, 6-mercaptopurine, vincristine, or 1,3-bis(2-chloroethyl)-1-nitrosourea. Similarly, a 6 h treatment with 1 μg/ml of FUra of cells that had been treated for various periods with 100 μg/ml of HU did not increase cell kill more than that obtained with HU alone (30 % cell kill).

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Patients harboring malignant brain tumors are often treated on chemotherapeutic regimens that use various combinations of drugs that have different modes of action. Combinations are selected to obtain an enhanced cell kill by metabolic interaction of two agents, to take advantage of cytokinetic perturbations induced in tumor cells treated with different agents, or to decrease drug-induced side effects. Because of the limited number of available, efficacious chemotherapeutic agents, use of these agents in combination provides a reasonable approach to the treatment of cancer patients. Even though biological and/or biochemical mechanisms are not well-understood, there are many combinations of drugs that enhance cytotoxicity in experimental settings.

5-Fluorouracil (FUra) has been used extensively for the treatment of various neoplasms and, because it crosses the blood-brain barrier, has been used in combination with other drugs for treatment of malignant brain tumors (1). We have shown (2) that treatment of exponentially growing 9L rat brain tumor cells with low nontoxic doses of FUra resulted in the accumulation of cells in S-phase. Treatment of such cells with hydroxyurea (HU) resulted in a greatly enhanced cell kill (3). Because cells blocked at the G_1/S border by moderately toxic doses of FUra also showed enhanced sensitivity towards HU, this synergism is not simply a result of cytokinetic perturbations induced by FUra nor of the phase specificity of HU.

In this report, we describe experiments in which inhibitors of thymidylate synthetase (TS), which are more specific than FUra, and more potent inhibitors of ribonuclease reductase (RNR) than HU were used in combination against 9L rat brain tumor cells *in vitro*. Results obtained support the hypothesis that synergism is the result of a blockade of TS followed by inhibition of RNR.

Materials and Methods

9L Cell Culture

9L rat brain tumor cells (1 to 2 x 10^6 cells) were seeded into 75 cm^2 tissue culture flasks and grown in 16 ml of Eagle's minimum essential medium (MEM) supplemented with 10 % newborn calf serum, nonessential amino acids, and gentamicin (50 μg/ml) (CMEM). Before treatment, cells were incubated for approximately 24 h at 37 °C in a humidified 5 % CO_2 : 95 % air atmosphere to establish early log phase growth. Cell survival was determined with a colony forming efficiency (CFE) assay (2, 4). Surviving fractions (SF's) were calculated as the ratio of the CFE's of treated cells to the CFE's of control cells.

Drugs and Treatment

FUra (fluorouracil injectable, Roche Laboratories, Nutley, NJ), 5-fluorodeoxyuridine (FdUrd, Sigma, St. Louis, MO), 5,8-dideazaisofolic acid (H-338, kindly supplied by Dr. John B. Hynes, Department of Pharmaceutical Chemistry, Medical University of South Carolina), 2'-deoxy-2'-fluoro-5-fluoro-ara-uracil (FFdAU, kindly supplied by Dr. K. A. Watanabe, Walker Laboratory of the Memorial Sloan-Kettering Cancer Center, New York), HU (Calbiochem-Behring, La Jolla, CA), 1-formylisoquinoline thiosemicarbazone (IQ-1, a gift of Dr. A. Sartorelli), methotrexate (MTX, Lederle Laboratories, Pearl River, NY), 6-mercaptopurine (6-MP,