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Technical Note

Serum Protein Binding of AL01576, a New Aldose Reductase Inhibitor

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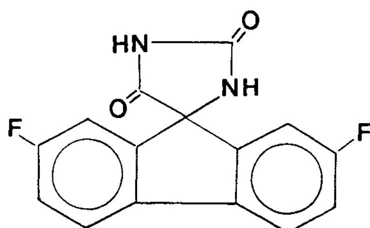
INTRODUCTION

AL01576 [spiro-(2,7-difluro-9*H*-fluorene-9,4′-imidazolidine-2′,5′-dione] (Scheme I) is an aldose reductase inhibitor which is currently undergoing evaluation for the treatment of some of the medical complications of diabetes (e.g., prevention of cataract formation and peripheral neuropathy). AL01576 is structurally similar to phenytoin. Serum protein binding plays an important role in determining the time course and therapeutic activity of phenytoin (1,2). In a similar fashion, the binding of AL01576 to serum proteins may impact on its pharmacokinetic/pharmacodynamic profile. Therefore, the following study was undertaken to characterize the protein binding of AL01576.



Scheme I

MATERIALS AND METHODS

Radiolabeled (^{14}C) AL01576 was supplied by Alcon Laboratories (Fort Worth, Tex.) and was radiochemically pure (>98%; 42.3 mCi/mmol) using an established high-performance liquid chromatographic (HPLC) method (3). Radiolabeled AL01576 was determined to be stable (>98% intact drug) in both serum and buffer at 37°C for 5 hr. Human serum albumin (Fraction V; lot No. 1051-9328) and human α-1-acid glycoprotein (purified from Cohn Fraction VI; lot No. 36F-9335) were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Human blood samples were collected as part of a companion study (4). Following an overnight fast, blood was obtained from 10 healthy adult male volunteers and insulin-dependent patients who were otherwise healthy. Serum was harvested from these blood samples, aliquots of these samples were pooled, and both the individual and the pooled serum samples were stored at −20°C until used.

Equilibrium Dialysis. The serum protein binding of AL01576 was determined by equilibrium dialysis (5). Serum or protein samples were dialyzed against an equal volume (0.3 ml) of pH 7.35, 0.133 *M* sodium–potassium phosphate buffer, except when examining the effect of pH on AL01576 binding. In that case, buffer components were manipulated and the initial pH ranged between 6.2 and 7.8; the ionic strength was maintained (μ = 0.75) with the addition of sodium chloride (0 to 10.6 g/liter). Unless otherwise stated the initial buffer (or protein) concentration of AL01576 was 1 μg/ml. Equilibration time was 5 hr (established in preliminary studies). Postdialysis samples of both the buffer and the serum side were obtained. No significant volume shifts (<5%) were detected.

Ultrafiltration. Ultrafiltration studies of AL01576 binding were performed using the Centrifree system (Amicon, Danvers, Mass.) in a temperture-controlled centrifuge (AccuSpin Fr; Beckman, Palo Alto, Calif.). Aliquots of serum were incubated at 37°C with drug for 1 hr prior to filtration. Serum samples (1 ml) were subject to ultrafiltration at 37°C and 1500*g* for 15 min, producing an ultrafiltrate which was less than 20% of the original volume.

Blood to Plasma. Blood-to-plasma ratios were determined by adding a known amount of radiolabeled AL01576 to blood (containing EDTA); following a 1-hr incubation (37°C) the sample was centrifuged and the plasma concentration of AL01576 was determined. The resulting plasma samples were also subjected to ultrafiltration.

AL01576 Analysis. Postdialysis serum and buffer as well as ultrafiltration samples were analyzed for AL01576 by liquid scintillation counting. Quench correction was performed using the external standards method. The binding results are expressed as the fraction unbound (f_p), the ratio of buffer to serum (or protein) drug concentration.

RESULTS AND DISCUSSION

As depicted in Fig. 1, the serum protein binding of

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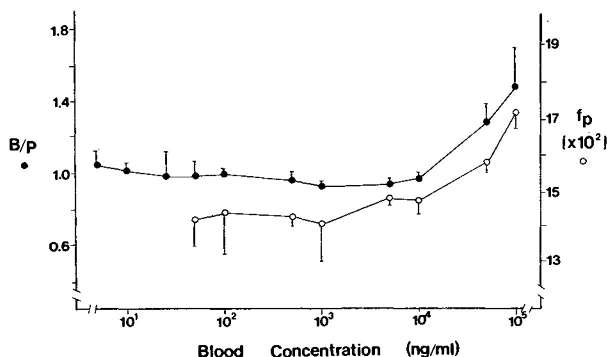

Fig. 1. The blood-to-plasma ratio (B/P; ●) and the fraction unbound (f_p; ○) for AL01576 as a function of the total AL01576 concentration in blood.

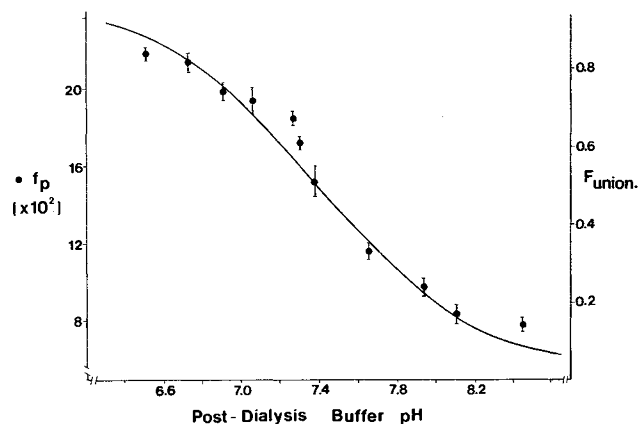

Fig. 2. AL01576 binding (unbound fraction; f_p; ●) as a function of postdialysis buffer pH. The line represents a plot of the fraction unionized for a weak acid with a pK_a of 7.4.

AL01576 was established to be similar in extent to that of phenytoin (f_p = 0.15) and was concentration independent over the concentration range observed to date in clinical trials (50–150 ng/ml) (3). The blood-to-plasma concentration ratio for AL01576 (approx. 1) was also established to be concentration independent, varying only in response to increases in f_p at AL01576 concentrations well above 10 μg/ml. AL01576 was bound to serum albumin (mean AL01576 f_p in a 580 μ*M* albumin solution was 0.260) and not to α-1-acid glycoprotein (mean AL01576 f_p in 20 μ*M* α-1-acid glycoprotein was 0.964). This information should prove useful in evaluating the pharmacokinetic disposition of AL01576, since AL01576 appears to be a low-extraction ratio drug (3) in which the systemic clearance would be expected to be dependent upon the fraction unbound.

The pH dependency of AL01576 was remarkable. The data in Fig. 2 strongly suggest that the observed binding of AL01576 can be described very simply in terms of differences in the degree of binding between the ionized and the unionized species. This relationship can be described mathematically by the following equation:

$$f_p = F_u f_{p,u} + F_i f_{p,i}$$

where F_u and F_i are the fraction unionized and ionized, respectively, and $f_{p,u}$ and $f_{p,i}$ are the fraction unbound of the unionized and ionized, respectively. Rearranging this relationship and substituting the term $(1 - F_u)$ for F_i yields

$$f_p = (f_{p,u} - f_{p,i})F_u + f_{p,i}$$

The excellent correlation ($r = 0.991$) observed between f_p and F_u strongly supports this model. Based on this analysis, the binding of the ionized (f_p = 0.060) and unionized (f_p = 0.248) species can also be estimated. This analysis would suggest that at least part of the binding of AL01576 to albumin is electrostatic in nature.

Although the changes in binding could be explained in terms of the ionization of AL01576, the present data do not rule out the possible role of pH on a conformational change in albumin structure. This conformational change, called the N > B transition, has been shown to alter the binding of drugs at both the warfarin and the benzodiazepine binding site on serum albumin (6,7). The present information does suggest that minor alterations in blood pH could add to the variability in AL01576 pharmacokinetics by contributing to the inter- and intrasubject variability in protein binding. This pH dependency of f_p may be clinically relevant since the target population (diabetic patients) can exhibit an altered blood pH (7.0 to 7.3) due to ketoacidosis. Moreover, it should be recognized that the correct estimation of *in vivo* binding must take into account the strong pH dependency of AL01576 binding and the pH drift observed during the time course of equilibrium dialysis experiments (8). Subsequent analysis of AL01576 binding in our laboratory (4) utilized an initial buffer pH which is 7.35 pH units; this allows for a final pH of 7.4.

The use of ultrafiltration devices to estimate serum protein binding has grown in use over the years. Preliminary studies revealed no appreciable loss of AL01576 to the filtration device from a buffer solution of AL01576 (>99% recovered from a 1 μg/ml buffer solution). Although the results from equilibrium dialysis and ultrafiltration experiments using serum obtained from a group of healthy subjects (N = 10) correlated with one another ($r^2 = 0.736$; $P < 0.01$), the ultrafiltration experiments produced consistently lower f_p values (mean f_p = 0.168 ± 0.020 vs 0.182 ± 0.017 for dialysis). This difference is probably the result of the pH-sensitive binding of AL01576; slight increases in pH have been noted for ultrafiltration and the nature of the samples that were filtered (i.e., serum where pH was not adjusted or buffered). The binding of AL01576 to a pool of control serum was temperature sensitive; mean ultrafiltration f_p values were lower at 24°C (mean = 0.016 ± 0.002) compared to 37°C (mean = 0.121 ± 0.003) ($P < 0.05$).

In summary, we have demonstrated the serum protein binding of AL01576 to be concentration independent, to involve a binding site on albumin, and to be strongly pH dependent. This information should help interpret the results of pharmacokinetic and pharmacodynamic studies involving AL01576. Moreover, AL01576 binding to serum albumin may contribute to the inter- and intrasubject variability of the AL01576 response.

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