bound to biomacromolecules rather than have been further metabolized and eliminated as the end products of the activating pathway. Our preliminary work also indicates that ~5% of the ^{14}C-label is covalently bound to biomacromolecules at the end of the 3-h experiment[4]. Others have found carbon-14 bound to biomacromolecules after incubation of [2-^{14}C]nitrofurantoin with microsomal preparations from rat liver and lung (29). It is probable, therefore, that at least some portion of the dose of nitrofurantoin is covalently bound to the biomacromolecules in the perfused rat kidney with the remainder eliminated by as yet unidentified metabolic pathways. Since none of the nitrofurazone was excreted as the 5-amino or cyano metabolite, there is no direct evidence that any of the drug was reductively activated for binding to biomacromolecules. Other investigators have, however, found ^{14}C-label covalently bound to the proteins, DNA, and RNA, of the liver and kidney of rats fed [2-^{14}C]nitrofurazone (30), and our preliminary studies have found ~5% of the ^{14}C-label bound to biomacromolecules of the nitrofurazone perfused kidney[4]. The remaining 80% of the dose of nitrofurazone was accounted for by as yet unidentified metabolites in the perfusate and urine. These studies indicate that the perfused kidney does metabolize both nitrofurantoin and nitrofurazone. Furthermore, the more toxic nitrofurazone appears to be more extensively metabolized.

(1) P. L. Olive, in "Nitrofurans," G. T. Bryan, Ed., Raven, New York, N.Y., 1978, p. 131.

(2) M. R. Boyd, G. L. Catignani, H. A. Sasame, J. R. Mitchell, and A. W. Stiko, *Am. Rev. Respir. Dis.*, **120**, 93 (1979).

(3) K. G. Tolman, *Ann. Int. Med.*, **92**, 119 (1980).

(4) C. Yiannikas, J. D. Pollard, and J. G. McLeod, *Aust. N.Z. J. Med.*, **11**, 400 (1981).

(5) S. M. Cohen, in "Nitrofurans," G. T. Bryan, Ed., Raven, New York, N.Y., 1978, p. 171.

(6) J. J. Gavin, F. F. Ebetino, R. Freedman, and W. E. Waterbury, *Arch. Biochem. Biophys.*, **113**, 399 (1966).

(7) J. E. Biaglow, B. E. Jacobsen, and O. F. Nygaard, *Cancer Res.*, **37**, 3306 (1977).

(8) F. J. Peterson, R. P. Mason, J. Hovespian, and J. L. Holtzman, *J. Biol. Chem.*, **254**, 4009 (1979).

(9) M. B. Aufrere, B. Hoener, and M. Vore, *Drug Metab. Dispos.*, **6**, 403 (1978).

(10) M. B. Aufrere, "Analytical and Metabolic Studies with Nitrofurantoin," Ph.D. Thesis, University of California, San Francisco, Calif., 1978.

(11) B. Hoener and S. E. Patterson, *Clin. Pharmacol. Ther.*, **29**, 808 (1981).

(12) J. Olivard, G. M. Rose, J. M. Klein, and J. P. Hoetis, *J. Med. Chem.*, **19**, 729 (1976).

(13) H. G. Jonen, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **315**, 167 (1980).

(14) H. G. Jonen and I. Kaufman, *Biochem. Pharmacol.*, **29**, 263 (1980).

(15) H. G. Jonen, F. Oesch, and K. L. Platt, *Drug Metab. Dispos.*, **8**, 446 (1980).

(16) M. F. Paul, H. E. Paul, R. C. Bender, F. Kopko, C. M. Harrington, V. R. Ellis, and J. A. Buzard, *Antibiot. Chemother.*, **10**, 287 (1960).

(17) H. E. Paul and M. F. Paul, in "Experimental Chemotherapy," Vol. 2, R. J. Schnitzer and F. Hawking, Eds., Academic, New York, N.Y., 1964, p. 307.

(18) H. E. Paul, K. J. Hayes, M. F. Paul, and A. R. Borgmann, *J. Pharm. Sci.*, **56**, 882 (1967).

(19) M. Veronese, D. Salvaterra, D. Barzaghi, and I. Setnikar, *Arzneim.-Forsch.*, **24**, 39 (1974).

(20) K. Tatsumi, S. Kitamura, and H. Yoshimura, *Arch. Biochem. Biophys.*, **175**, 131 (1976).

(21) M. C. Dodd, *J. Pharmacol. Exp. Ther.*, **86**, 311 (1946).

(22) K. Tatsumi, T. Ou, H. Yoshimura, and H. Tsukamoto, *Chem. Pharm. Bull.*, **19**, 330 (1971).

(23) I. Berkersky, W. A. Colburn, L. Fishman, and S. A. Kaplan, *Drug Metab. Dispos.*, **8**, 319 (1980).

(24) A. Heyrovsky, *Clin. Chim. Acta.*, **1**, 470 (1956).

(25) M. B. Aufrere, B. Hoener, and M. Vore, *Clin. Chem.*, **23**, 2207 (1977).

(26) B. Hoener and J. L. Wolff, *J. Chromatogr.*, **182**, 246 (1980).

(27) F. H. Epstein, J. T. Brosnan, J. D. Tange, and B. D. Ross, *Am. J. Physiol.*, **243**, F284 (1982).

(28) M. F. Paul, R. C. Bender, and E. G. Nohle, *Am. J. Physiol.*, **197**, 580 (1959).

(29) M. R. Boyd, A. W. Stiko, and H. A. Sasame, *Biochem. Pharmacol.*, **28**, 601 (1979).

(30) K. Tatsumi, S. Kitamura, and H. Yoshimura, *Chem. Pharm. Bull.*, **25**, 2948 (1977).

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Pharmacokinetic Implications of Stereoselective Changes in Plasma-Protein Binding: Warfarin/Sulfinpyrazone

Keyphrases □ Warfarin—plasma-protein binding, stereoselective displacement, drug-drug interaction with sulfinpyrazone □ Sulfinpyrazone—plasma-protein binding, stereoselective displacement, drug-drug interaction with warfarin

To the Editor:

The intensity of the observed pharmacological response produced by any drug is invariably related, directly or indirectly, to its plasma concentration. In the systemic circulation most drugs are bound to plasma proteins, and thus an equilibrium is established between bound and free drug. Since free drug alone is the pharmacologically active agent, the drug-plasma protein interaction may not only influence overall drug disposition but also affect the magnitude and time-course of the pharmacological response. In theory, numerous models could describe the relationship between hepatic clearance and plasma protein binding. However, a widely accepted model which has been shown to explain the vast majority of empirical observations, and one which is consistent with the results to follow, is the so-called well-stirred model (1-4). For drugs exhibiting a low extraction ratio (ER <0.3), it may be shown that their observed total body clearance (CL) is directly related to their unbound fraction within plasma (f_u) (5, 6). Consequently, for low-extractration ratio drugs which are highly protein bound (>98%), a small change in the fraction of the drug bound to plasma protein will not only result in a relatively large change in f_u but also in a proportional change in CL and may thus have potentially important consequences in the in-

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terpretation of the relationship between pharmacokinetic parameters and the observed pharmacological response.

Since albumin (the principal plasma binding protein for weakly acidic drugs) is chiral, its interaction with the enantiomers of racemic drugs might be expected to be different. Although stereoselective differences in protein binding have been previously noted (7–10), the implications of the phenomenon have not been generally recognized. The potential importance of stereoselective changes in plasma protein binding was demonstrated with the oral anticoagulant warfarin in its interaction with the uricosuric agent sulfinpyrazone[1]. Warfarin is administered as a racemic mixture despite the fact that the (*S*)-enantiomer is approximately five times as potent as the (*R*)-enantiomer. Moreover, the drug is highly bound (>98%) to plasma proteins.

The measurement of the free fraction of a highly bound drug such as warfarin is technically difficult due to the very low concentration of unbound drug (nanograms per milliliter at therapeutic levels). The problem is further compounded if the stereochemical composition of the free fraction is to be determined. One solution to the problem of measuring differences in enantiomeric binding is to use pseudoracemic drugs (a 1:1 mixture of (*R*)-[^{12}C]warfarin and (*S*)-[2-^{13}C]warfarin[2]) and then analyze the enatiomer concentrations by GS–MS, as previously reported (12). The advantage of this approach is that it allows an assessment of the drug in essentially the exact same form (racemate) as that which is used clinically. In addition, since MS allows differentiation of the two enantiomers within the same experiment, error can be significantly reduced. For example, the ratio, and changes in the ratio, between corresponding ions representing the (*R*)- and (*S*)-enantiomers can be measured directly without reference to internal standards or the requirement of mathematical treatment of any kind. The calculation of the degree of binding does, however, require the presence of a suitable internal standard and further data treatment.

Blood was drawn from healthy normal male volunteers and collected into flint-glass tubes containing 15% w/v potassium EDTA solution (0.1 mL), one of the few anticoagulants found to have little or no effect on drug–protein binding (13). Care was taken to avoid contact with materials that might modify the protein binding interaction in any way (14). Plasma was separated from the cellular components by centrifugation and all plasma samples were pooled. To a 12-mL plasma sample was added a methanolic solution (10 μL) of pseudoracemic warfarin to yield a final plasma concentration of 10 $\mu g \cdot mL^{-1}$. The spiked plasma samples (10 mL) were transferred into polycarbonate ultracentrifuge tubes and centrifuged at 50,000 rpm (300,000×*g*) for 20 h at 30°C. The clear supernatant (5 mL), containing unbound drug, was removed and to it was added a known amount of [phenyl-$^{2}H_5$]warfarin, the internal standard. The ultracentrifugation technique was chosen for its ability to handle relatively large plasma volumes. This feature is important as the low unbound concentration of warfarin necessitates the extraction of large volumes of plasma water to obtain sufficient amounts of the enantiomers for analysis. There are, however, practical concerns with this method. Contamination of the supernatant with albumin may result in erroneous estimates of the unbound fraction. For highly bound drugs such as warfarin, even minor contamination with albumin yields unnaturally high f_u values. Clearly, this is not the case in our investigations. Artifactual f_u values would also be readily apparent if there were any dissociation of the drug–protein complex during the centrifugation process. Although studies were undertaken to investigate the possibility, no evidence was found to suggest a concentration gradient of unbound warfarin (a result of sedimentation/layering) existed within the supernatant. Of the remaining 2 mL of spiked plasma, containing total (bound plus unbound) drug, a 1-mL sample was similarly spiked with the internal standard. The plasma and supernatant were extracted and derivatized as previously outlined for plasma samples[1]. Relative and absolute quantitation of (*R*)-[^{12}C]- and (*S*)-[2-^{13}C]warfarin was obtained by measurement of ion ratios with reference to the internal standard *via* GC–MS analysis (12).

The results of the study are shown in Table I. Although differences in the degree of binding of the two enantiomers was not significant, these differences are greatly magnified when considered in terms of the pharmacologically active free fractions. These data indicate that the (*S*)-enantiomer is more highly bound than the (*R*)-enantiomer, as previously reported (9). It is interesting to note the good agreement between our results and those reported previously, particularly in light of the different methodologies that were employed.

We have previously shown that (*R*)-warfarin has a greater distribution volume than the (*S*)-enantiomer[1]. As only free drug is available to distribute throughout the body, consideration of the present data provides an explanation for this observation: (*S*)-warfarin, being more highly bound to plasma proteins than the (*R*)-enantiomer, would be expected to have a smaller distribution volume, since it is more confined to the circulatory system. Interestingly, the ratio of the apparent volumes of distribution of the (*R*)- and (*S*)-enantiomers (V_R:V_S = 1.6) is almost identical to the unbound fraction ratio (Table I).

(*S*)-Warfarin has been shown to be about five times more potent an anticoagulant than its optical antipode. Determination of this difference, however, did not take into account the differential binding of the enantiomers (15) and thus does not reveal the true (intrinsic) difference in potency between the enantiomers. When protein binding is taken into account, the (*S*)-enantiomer has an inherent potency approximately eight times greater than that of the (*R*)-enantiomer. Clinically, it is sufficient to acknowledge the apparent differences in potency; however, the intrinsic difference in potency is important for the postulation of potential receptor sites and consideration of structure–activity relationships.

Changes in the plasma protein binding of a drug arising from multiple-drug therapy is well documented; probably, it is the most common explanation for any drug–drug interactions. Plasma is, however, an open compartment, and any drug that is displaced from plasma proteins will rapidly distribute into the tissue compartments. Consequently, the transient increase in f_u produced by the displacing drug is often of little pharmacological importance. Sulfinpyrazone is a widely used uricosuric agent, but more recently its effects on platelets has led to its use in the treatment of a variety of thromboembolic disorders. It is now well documented (16) that if warfarin and sulfinpyrazone are coadministered, the pharmacological response to warfarin is enhanced; the basis of the interaction appears to be metabolic in origin[1]. The important and poten-

[1] S. Toon, L. K. Low, W. F. Trager, R. A. O'Reilly, C. H. Motley, and A. Goulart, unpublished results.

[2] The preparation of the pseudoracemate and determination of stereochemical and isotopic purity was as previously described (11).

Table I—Enatiomeric Binding Data of Pseudoracemic Warfarin (10 μg·mL^{-1}) in the Absence and Presence of Sulfinpyrazone (15 μg·mL^{-1})

	Control	Sulfinpyrazone
(*R*):(*S*) Unbound fraction (f_u) ratio	1.58 (*SD* ± 0.15; *n* = 11)	2.25 (*SD* ± 0.26; *n* = 11)
t test		$p < 0.001$
Percentage bound[a]		
(*R*)	99.15 (*SD* ± 0.24) (f_u% = 0.85)	98.73 (*SD* ± 0.14) (f_u% = 1.27, increase of 49.41%)
(*S*)	99.47 (*SD* ± 0.16) (f_u% = 0.53)	99.42 (*SD* ± 0.10) (f_u% = 0.58, increase of 9.43%)
pesudoracemate	99.31 (f_u% = 0.69)	99.08 (f_u% = 0.92, increase of 34.06%)

[a] Calculated as the mean of the individual enantiomer binding data.

tially complicating role that stereoselective changes in protein binding play in the interaction is evident from a consideration of the equation for total body clearance which pertains to poorly extracted drugs (6):

$$CL = f_u \cdot CL'$$

Without acknowledging the stereoselective change in f_u between the two warfarin enantiomers brought about by concomitant sulfinpyrazone administration, one might incorrectly conclude that any inequivalent change in *CL* between (*R*)- and (*S*)-warfarin has its origins at the enzymatic level (as measured by the intrinsic clearance *CL'*), a result of metabolic induction or inhibition. In light of our present observations, it is apparent that stereoselective protein binding displacement of a poorly extracted drug may manifest itself as an inequivalent change in the *CL* of the two enantiomers in the absence of any dynamic change in metabolism. Sulfinpyrazone is metabolized in humans, and the plasma-protein binding of the parent drug and its metabolites have been investigated (17). Obviously, sulfapyrazone metabolites could complicate the protein binding interaction with warfarin *in vivo*; however, our evidence with volunteers who concomitantly receive warfarin and sulfinpyrazone (15) indicate this is not the case. Interestingly, sulfinpyrazone has been reported to induce the metabolism of (*R*)-warfarin while simultaneously inhibiting the metabolism of the (*S*)-enantiomer (18, 19). These conclusions were drawn from the sulfinpyrazone-induced changes in enantiomeric clearance.

(1) M. Rowland, L. Z. Benet, and G. G. Graham, *J. Pharmoacokinet. Biopharm.*, **1**, 123 (1973).

(2) K. S. Pang and M. Rowland, *J. Pharmacokinet. Biopharm.*, **5**, 625, (1977).

(3) K. S. Pang and M. Rowland, *J. Pharmacokinet. Biopharm.*, **5**, 655, (1977).

(4) K. S. Pang and M. Rowland, *J. Pharmacokinet. Biopharm.*, **5**, 681, (1977).

(5) G. R. Wilkinson and D. G. Shand, *Clin. Pharmacol. Ther.*, **18**, 377 (1975).

(6) G. Levy and A. Yacobi, *J. Pharm. Sci.*, **63**, 805 (1974).

(7) F. Karusch, *J. Am. Chem. Soc.*, **76**, 5536 (1954).

(8) W. E. Muller and U. Wollert, *Mol. Pharmacol.*, **11**, 52 (1975).

(9) A. Yacobi and G. Levy, *J. Pharmacokinet. Biopharm.*, **5**, 123 (1977).

(10) S. A. Bai, U. K. Walle, M. J. Wilson, and T. Walle, *Drug Metab. Dispos.*, **11**, 394 (1983).

(11) W. R. Porter, K. Kunze, E. J. Valente, and W. F. Trager, *J. Labelled Comp. Radiopharm.* **17**, 763 (1980).

(12) E. D. Bush, L. K. Low, and W. F. Trager, *Biomed. Mass Spectrom.*, **10**, 395 (1983).

(13) W. F. Van der Giesen and J. Wilting, *Methods Find. Exp. Clin. Pharmacol.*, **4**, 417 (1982).

(14) K. M. Giacomini, J. C. Giacomini, and R. T. Blaschke, *Biochem. Pharmacol.*, **29**, 3337 (1980).

(15) J. N. Eble, B. D. West, and K. P. Link, *Biochem. Pharmacol*, **15**, 1003 (1966).

(16) J. M. Davis and L. E. Johns, Jr., *N. Engl. J. Med.*, **299**, 955 (1978).

(17) P. Jakobson, A. K. Pedersen, and F. Andrease, *Acta Pharmacol. Toxicol.*, **51**, 243 (1982).

(18) J. O. Miners, T. Foenander, S. Wanwimolruk, A. S. Gallus, and D. J. Birkett, *Eur. J. Clin. Pharmacol.*, **22**, 327 (1982).

(19) R. A. O'Reilly, *Circulation*, **75**, 202 (1982).

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Thermodynamic Studies of Tolbutamide Polymorphs

Keyphrases □ Tolbutamide—crystalline polymorphs, solubility as a function of temperature, polymorphic conversion, transition temperature

To the Editor:

Aqueous suspensions of tolbutamide were found to thicken to an unpourable state after several weeks of occasional shaking (prior to daily dosing). Samples of the same suspensions that were not shaken showed excellent stability after years of storage at ambient and elevated temperature. Microscopic examination revealed that the thickening was due to partial crystalline conversion of the original plate-like tolbutamide crystals to very fine needle-shaped crystals, which tend to form a highly flocculated structure. The crystals were identified as a polymorphic form rather than a solvate or change in habit. IR spectra and X-ray diffraction confirmed that the acicular form is identical with Burger's form III (1). The polymorphic conversion was unexpected since published solubility data suggest that form I is the more stable polymorph at room temperature (1, 2). However, a transition temperature, above which form III converts to form I, has been reported by several investigators to be somewhere between 98.5°C and 118°C (1, 3, 4). An inconsistency here is clearly apparent: form I cannot be the more stable polymorph at temperatures both above and below a transition temperature. Furthermore, the reported solubility data (1) indicating that form I is less soluble than form III at 37°C conflict with suspension stability data generated in this laboratory, which indicate that form I converts to form III at room temperature. To resolve the discrepancy between the published literature and recent observations in this laboratory, the aqueous solubilities of polymorphs I and III were determined as a function of temperature.

The form I tolbutamide used in this study was prepared for commercial use by a final recrystallization from methanol–water[1]. Form III was made by stirring an aqueous suspension

[1] Lot No. 445HS; The Upjohn Co., Kalamazoo, Mich.

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