

## DETERMINATION OF PROTEIN BINDING WITH ISRP COLUMNS

### Analytes:

#I: 10 uM Warfarin + 550 uM BSA, (Reference 5)  
 #II: Imirestat (20 ug/ml) in Serum, (Reference 2 and 3)

<b>Flow Rate:</b>	#I: 2.0 ml/min	<b>Detection:</b>	#I: 310 nm	<b>Sample Size:</b>	#I: 200 µl
	#II: 1.0 ml/min		#II: 254 nm		#II: 200 µl

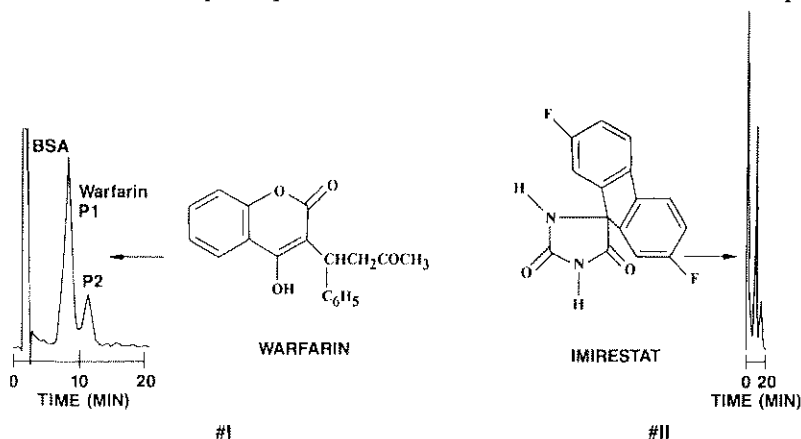
**Column #I:** 5 micron GFF ISRP, 25 cm x 4.6 mm ID  
**Regis Product Number:** 731452

**Column #II:** 5 micron GFF ISRP, 5 cm x 4.6 mm ID  
**Regis Product Number:** 731450

### Mobile Phase:

#I: 95% 0.03M KH<sub>2</sub>PO<sub>4</sub> (pH 6.8), 5% THF  
 #II: 0.01M KH<sub>2</sub>PO<sub>4</sub> (pH 6.8)

**Discussion:** Due to the unique nature of the ISRP packing material, drugs which are strongly bound to proteins may elute as two peaks when a serum sample is directly injected onto an ISRP column, under very select conditions. It has been determined that the drug molecules bound to a strong primary binding site experience a delayed release, while drugs bound to weaker secondary binding sites are released immediately on introduction to the mobile phase. As a result of the exclusion of proteins from the packing, drug which had been bound to the primary site elutes in a major peak ahead of a second smaller peak, which contains both the drug bound initially to secondary sites and the original free drug. The peak splitting has been found to be very dependent on column length, sample size, and the mobile phase conditions. The peak positions can be controlled with mobile phase ionic strength, pH, and organic



**Discussion (continued):** modifier concentration. The peaks can be easily merged into one peak if desired. The phenomenon has been observed for phenytoin and its derivatives in human serum (1-3), and warfarin combined with bovine serum albumin (BSA) (4,5). If the drug is combined with pure protein and the free drug concentration is

measured by an independent method (e.g. ultrafiltration), then the concentration of the drug bound to the secondary site can be determined from the second peak by difference. With this and a knowledge of number of moles bound per site, the binding constants for each binding site can be directly calculated (4).

## Results:

**Table: Concentration of P-1(D2), P-2(D2) and unbound warfarin(Df) and calculated binding constants (log K1 and log K2) (Reference 4)**

sample	injection vol.	D1(uM)	D2(uM)	Df(uM)	log K1	log K2
500 uM W - 600 uM BSA	200 ul	433	70.4	10.8	4.68	3.27
500 uM W- 400 uM BSA	300 ul	433	66.3	27.7	4.58	2.85
200 uM W - 600 uM BSA	200 ul	171	29.5	4.4	4.55	3.28

**References:** (1) T.C. Pinkerton, Plenary lecture presented to the 30th Annual Symposium on Chromatography, Kyoto, Japan, Jan. 27, 1987.

(2) T.D. Miller, PhD Thesis, Department of Chemistry, Purdue University, West Lafayette, Indiana, August 1987.

(3) T.C. Pinkerton, T.D. Miller, and L.J. Janis "The Effect of Protein Binding on the High Performance Liquid Chromatography of Phenytoin Imirestat in Human Serum by Direct Injection onto Internal Surface Reversed-Phase Columns", Anal. Chem. 61, 1171-1174 (1989).

(4) A. Shibukawa, T. Nakagawa, H. Tanaka, and J. Haginaka, "Effect of Protein Binding on the Drug Assays of Plasma Sample using Internal Surface Reversed-Phase Silica Columns", 8th Conference on Liquid Chromatography, Tokyo, Japan, Oct. 27-29, 1987 (abstract).

(5) A. Shibukawa, T. Nakagawa, M. Miyake, and H. Tanaka, "Analysis of Warfarin-Albumin Binding by HPLC with Internal Surface Reversed-Phase Silica Column", Chem. Pharm. Bull. 36(5) 1930-1933 (1988).

Thomas C. Pinkerton, The Upjohn Company,  
Control Division, Bldg 259-12 Kalamazoo, MI 49001