

## EFFECTS OF SERUM LIPID CONTENT ON THE BINDING OF MINOCYCLINE

MARTIN J. RAFF, JAMES T. SUMMERSGILL, FRANK J. FONTANA, PATRICIA A. BARNWELL,  
NORTON G. WATERMAN and LOUIS SCHARFENBERGER

The Section of Infectious Diseases, Department of Medicine, and  
Department of Surgery, Price Institute, University of Louisville  
School of Medicine, Louisville, Kentucky 40201 U.S.A.

(Received for publication March 14, 1977)

Minocycline was added to normal and hyperlipemic serum samples in concentrations of 1~10 mcg/ml. These specimens had similar protein contents. Chemically extractable minocycline was quantitated fluorometrically. Hyperlipemic serum (cholesterol 480 mg/100 ml; triglycerides 321 mg/100 ml) yielded an average of 50% less minocycline than did normal serum (cholesterol 170 mg/100 ml; triglycerides 114 mg/100 ml). When ultrafiltrates of serum containing 6, 12 and 20 mcg/ml minocycline were assayed microbiologically, it was evident that variations in serum triglyceride and cholesterol levels did not alter the ratio of bound to free drug. Minocycline appears to be reversibly associated with, and/or soluble in, triglyceride-cholesterol components of serum.

Minocycline appears to have greater lipophilic properties than other tetracyclines<sup>1,2)</sup>, which may account for high ratios of tissue to serum concentrations<sup>2)</sup>. SCHACH VON WITTENAU and YEARY<sup>3)</sup> have suggested that increased lipophilicity of a tetracycline antibiotic should result in greater tissue concentrations, provided that protein binding is not increased proportionately. Values of 56% and 76% binding to human serum proteins have been reported for tetracycline and minocycline, respectively<sup>2)</sup>. Tissue penetration may be enhanced by the increased lipophilic nature of minocycline, even in the presence of higher protein binding<sup>2)</sup>. In view of minocycline's lipophilic properties, an investigation was initiated to determine the possible role of hyperlipidemia in the binding and antimicrobial activity of minocycline.

### Materials and Methods

#### Lipoprotein Serum Assay

All human serum specimens employed in this study were subjected to lipoprotein electrophoresis to determine levels of cholesterol and triglycerides. Total protein content was also determined. Normal serum specimens (NS) averaged cholesterol levels of 170 mg/100 ml and triglyceride levels of 114 mg/100 ml. Hyperlipemic serum specimens (HS) averaged cholesterol levels of 480 mg/100 ml and triglyceride levels of 321 mg/100 ml. Total protein content of both types of serum was similar (7,200 mg/100 ml). All serum specimens were adjusted to pH 7.2 with 0.15 M phosphate buffer prior to use in experimentation.

#### Ultrafiltration

Ultrafiltration of aliquots of NS and HS containing minocycline was accomplished using a teflon ultrafiltration cell designed by Dr. C. E. TRAITOR of Lederle Laboratories, Pearl River, New York. Teflon was employed as the construction material as minocycline appears to have a high affinity for, and adheres to, glass surfaces but not to teflon. The membrane used was a semipermeable dialyzer tubing (Cat. No. 3787-D40, A. Thomas Co., Philadelphia, Pa.) with an average pore diameter of 48Å.

Supported by grants from the Louisville Medical Research Foundation, Louisville, Kentucky, and Lederle Laboratories, Pearl River, New York.

The membrane, previously soaked in sterile saline, was opened along one crease and mounted tightly between the two chambers of the ultrafiltration cell. A 10-ml aliquot of the filtration sample, previously prepared at the appropriate minocycline concentration, was introduced into the upper chamber. Two hundred millimeters Hg pressure was applied for approximately 18 hours to collect 1~2 ml of filtrate. The binding capacity of serum components is expressed as a percentage of the total drug concentration from the following formula;

$$\frac{C_s - C_f}{C_s} \times 100 = \text{Percent antibiotic bound}$$

where  $C_s$  = initial concentration in the serum at the time of preparation and  $C_f$  = final concentration in the filtrate. Binding values for minocycline in NS and HS were determined at therapeutically attainable levels of 6, 12 and 20 mcg/ml. Experimental controls were conducted using normal-serum ultrafiltrate that proved void of protein and lipid, but which retained normal levels of glucose and electrolytes. All ultrafiltrations were conducted at 25°C.

#### Biological Assay

Minocycline levels in serum prior to ultrafiltration ( $C_s$ ) and in final ultrafiltrate volumes ( $C_f$ ) were measured using a disc-agar diffusion method with *Bacillus subtilis* ATCC 6633 as the assay organism. Standard curves, prepared in normal serum, were calculated using the method of least squares described by HOCKENHULL<sup>13</sup>. No appreciable differences were observed when NS or HS were used to calculate standard curves.

#### Fluorometry

Extraction of minocycline from human serum for fluorometric assay was accomplished using a modification of the procedure described by KOHN<sup>15</sup>. Distilled water (4.5 ml) was added to 1 ml aliquots of NS and HS containing concentrations of minocycline ranging from 1 to 10 mcg/ml. This was followed by the addition of 1 ml 0.16 M  $\text{CaCl}_2$  in 1.8 N trichloroacetic acid. Following 10 minutes of vigorous agitation, the protein precipitate was sedimented by centrifugation for 10 minutes at  $480 \times g$ . A 3.0-ml aliquot of supernate from each tube was transferred to a corresponding second set of tubes containing 3.0 ml ethylacetate and 3.0 ml sodium barbital. Following a second 10-minute period of agitation the minocycline had been extracted into the ethylacetate phase. Mixing of 0.5 ml of this phase with 0.5 ml of 1.0 N acetic acid in absolute ethanol, prepared the samples for fluorometric assay. Samples were read in a G.K. Turner model 110 fluorometer equipped with a High Sensitivity Conversion Unit at an excitation wavelength of 390 nm and an emission wavelength of 490 nm. The primary filter was a Wratten #405 and the secondary filter was a #8. Control samples were determined in phosphate buffer (pH 7.2), and treated in the same manner as were the experimentals in terms of the extraction and subsequent assay.

### **Results**

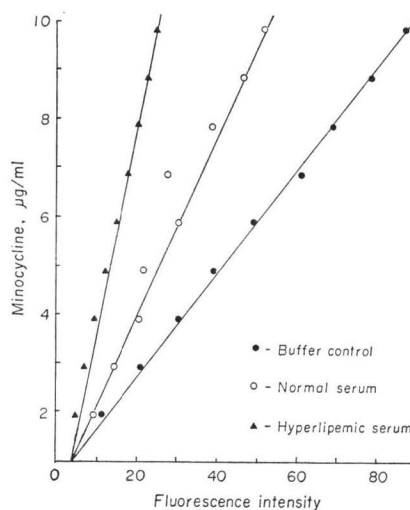
There is considerable difficulty in the quantitative ultrafiltration of samples containing minocycline due to the non-specific binding of the drug to membrane surfaces. Conventional "bag-type" ultrafiltration procedures resulted in non-specific binding values approaching 40% of the free drug (unpublished data). However, the use of the teflon ultrafiltration cell greatly reduced the extent of this problem. Controls in the ultrafiltration studies resulted in non-specific binding values averaging 4% of the free drug.

Ultrafiltration studies to determine the degree of binding of minocycline in NS and HS are summarized in Table 1. Microbiological assay of these filtrates revealed no significant differences between percentages of bound drug at therapeutically attainable serum levels (6, 12 and 20 mcg/ml). Average binding values of  $65.85 \pm 2.65\%$  and  $63.47 \pm 3.28\%$  for NS and HS respectively, were observed at a concentration of 6 mcg/ml. When serum concentrations were increased to 12 mcg/ml, there was a sig-

Table 1. Binding values for minocycline in normal and hyperlipemic serum as determined by ultrafiltration.

Initial conc. of minocycline in serum	Exp. No.	Percent antibiotic bound	
		Normal serum	Hyperlipemic serum
6 mcg/ml	1	69.10	67.00
	2	65.20	59.90
	3	62.70	65.40
	4	66.40	61.60
	Mean ±S.D.	65.85 ±2.65	63.47 ±3.28
12 mcg/ml	1	46.15	47.12
	2	44.50	45.82
	3	45.61	46.33
	4	44.20	46.14
	Mean ±S.D.	45.11 ±1.50	46.35 ±0.63
20 mcg/ml	1	38.10	39.60
	2	35.30	37.60
	3	31.50	36.70
	4	37.00	40.50
	Mean ±S.D.	35.47 ±2.89	38.60 ±1.75

Fig. 1. Fluorometric measurement of minocycline in normal and hyperlipemic serum following chemical extraction. Samples were incubated at 37°C for 24 hours prior to extraction, to allow for equilibrium of binding to serum components.



nificant ( $p < 0.01$ ) decrease in the percentages of bound minocycline in both NS and HS ( $45.11 \pm 1.50\%$  and  $46.35 \pm 0.63\%$  respectively). Further increasing the serum concentrations to 20 mcg/ml, resulted in a significant ( $p < 0.01$ ) decrease in binding percentages in NS and HS ( $35.47 \pm 2.89\%$  and  $38.60 \pm 1.75\%$ , respectively). Despite this, there were no significant differences between the two specimens at any concentration of minocycline.

Utilizing the intensity of fluorescence as an indicator of the concentration of minocycline in the serum extract, there appears to be a significant decrease in the amounts of this compound that can be extracted from HS as opposed to NS (Fig. 1). This phenomenon is demonstrated by the differences in the slopes of the HS curve (3.75) and the NS curve (1.83). Control samples, conducted in pH 7.2 phosphate buffer, yielded a curve with a slope of 1.12.

### Discussion

Minocycline hydrochloride is less chemically extractable, by the techniques utilized here, from HS than from NS. This indicates that the drug appears to be associated with, or soluble in, the lipoprotein portion of the serum. It is suspected that minocycline, in view of its known lipophilicity, is somehow solubilized within the lipoprotein matrix but has not been bound to these substances. This hypothesis is supported by the results of the microbiological assay of ultrafiltrates, which indicate that there is no change in the activity of the compound in HS versus NS. This occurs despite the lipid "association" when measured by non-biological techniques. It is known that minocycline will chelate to divalent metal cations at an alkaline pH<sup>6</sup>. Thus, it might be speculated that a combined effect is taking place in the HS; (1) the chelation of minocycline to  $Ca^{++}$  (which is associated with serum albumin); and (2) an association with the increased lipoprotein matrix in the HS.

Ultrafiltration studies indicate that this association of minocycline with the lipoprotein portion does not, however, affect the free drug concentration, when measured utilizing biological activity as

a parameter. The level of drug recovered from serum ultrafiltrates is not effectively altered, even in the presence of abnormally elevated serum lipoproteins.

The decrease in protein binding seen with increases in serum concentrations of minocycline (6, 12, 20 mcg/ml) suggests that saturation of available protein binding sites may occur at relatively low serum levels. This may have biological and clinical significance when considering the question of protein binding of antimicrobial agents, as discussed by KUNIN<sup>7)</sup>. It is probable that high tissue levels of tetracycline and minocycline are attainable despite the high degree of protein binding. High free drug levels of other antimicrobial agents may be seen at therapeutically attainable serum concentrations once saturation of binding sites has occurred. Factors such as this may render simple determinations of the degree of protein binding of an antibiotic unreliable for evaluation of its clinical potential. These have been discussed previously<sup>8,9)</sup>.

#### Acknowledgements

Supported by grants from the Louisville Medical Research Foundation, Louisville, Kentucky, and Lederle Laboratories, Pearl River, New York.

The authors wish to thank Dr. CHARLES DUNCAN for his assistance.

#### References

- 1) COLAIZZI, J. D. & P. R. KLINK: pH-Partition behavior of tetracyclines. *J. Pharm. Sci.* 58: 1184~1189, 1969
- 2) MACDONALD, H.; R. G. KELLY, S. E. ALLAN, J. F. NOBLE & L. KANEGIS: Pharmacokinetic studies on minocycline in man. *Clin. Pharm. Ther.* 14: 852~861, 1973
- 3) SCHACH VON WITTENAU, M. & R. YEARY: The excretion and distribution in body fluids of tetracycline after intravenous administration to dogs. *J. Pharm. Exp. Ther.* 140: 258~267, 1963
- 4) HOCKENHULL, D. J. D.: *Progress in Industrial Microbiology.* p. 129, Heywood and Co., Ltd, London, 1959
- 5) KOHN, K. W.: Determination of tetracyclines by fluorescent complexes: Application to biological materials. *Anal. Chem.* 33: 862~866, 1961
- 6) BARRINGER, W. C.; W. SCHULTZ, G. M. SIEGER & R. A. NASH: Minocycline hydrochloride and its relationship with other tetracycline antibiotics. *Amer. J. Pharm.* 146: 179~191, 1974
- 7) KUNIN, C. M.: Enhancement of antimicrobial activity of penicillins and other antibiotics in human serum by competitive serum binding inhibition. *Proc. Soc. Exp. Biol. Med.* 117: 68~73, 1964
- 8) WATERMAN, N. G.; M. J. RAFF, L. SCHARFENBERGER & P. A. BARNWELL: Protein binding and concentrations of cephaloridine and cefazolin in serum and interstitial fluid of dogs. *J. Infect. Dis.* 133: 642~647, 1976
- 9) WATERMAN, H. G.; L. SCHARFENBERGER & M. J. RAFF: Rate of binding of antibiotics to canine serum proteins. *Antimicrob. Agents & Chemoth.* 5: 294~295, 1974