# Scatchard Plot Analysis of Ligand–Erythrocyte Interactions

**Keyphrases** □ Scatchard plots—constructed from ligand–erythrocyte interaction data, use of binding capacities evaluated □ Ligand–erythrocyte interactions—Scatchard plots constructed from data, use of binding capacities evaluated □ Erythrocyte–ligand interactions—Scatchard plots constructed from data, use of binding capacities evaluated □ Binding capacities—use in Scatchard plots constructed from ligand interaction data evaluated

*To the Editor:*

Over the past few years, several different methods have been used to construct Scatchard plots from ligand–erythrocyte interaction data. For example, a 1% concentration of protein, determined by the biuret reaction, with an assumed molecular weight of 69,000 was used (1) to construct Scatchard plots of a drug–erythrocyte interaction[1]. More common is the use of the binding capacity of the erythrocytes (2, 3) based on the Rosenthal plot (4). The binding capacity is defined as the product of the number of binding sites and the macromolecule concentration. Such a plot may be necessary to describe binding by systems such as plasma that contain more than one species of binding macromolecules with different concentrations. It does not appear necessary, however, to use binding capacities to describe ligand–erythrocyte interactions.

Data from ligand–erythrocyte interactions have been reported as molecules of ligand bound per erythrocyte (5). This use of erythrocyte concentration is more accurate than the commonly used volume per volume concentration of packed red blood cells. The amount of extracellular fluid trapped between packed erythrocytes varies from 1 to 9%, depending on the speed and length of time of centrifugation (6). Thus, the number of erythrocytes per milliliter of packed erythrocytes also varies with the method of centrifugation. Reliable red cell counts are obtainable with an automated counter[2] (7). Data reported in this way can be analyzed by use of the Scatchard plot (8).

Reversible, noninteracting binding of a ligand by a macromolecular binding site is described by:

$$A + S \overset{k}{\leftrightarrow} D_b$$

*Scheme I*

where $A$ is the concentration of the free ligand, $S$ is the concentration of the free site, $D_b$ is the concentration of the ligand–site complex, and $k$ is the intrinsic association constant. Thus:

$$k = \frac{D_b}{AS} \qquad \text{(Eq. 1)}$$

and:

$$D_b = kAS \qquad \text{(Eq. 2)}$$

Let $S_t$ be the total concentration of the binding site in the system. Then:

$$S_t = D_b + S \qquad \text{(Eq. 3)}$$

When the system consists of a ligand and erythrocytes, $S_t$, in molecules per milliliter, equals $C_t$, the concentration of erythrocytes in cells per milliliter, with the assumption that all erythrocytes are identical in composition. One molecule of any particular site on an erythrocyte is equivalent to one erythrocyte cell. This situation is analogous to the molar concentration of any particular site on a protein molecule being equivalent to the molar concentration of the protein. Therefore:

$$S = C_t - D_b \qquad \text{(Eq. 4)}$$

Substitution of Eq. 4 into Eq. 2 yields:

$$\frac{D_b}{C_t} = \frac{kA}{1 + kA} \qquad \text{(Eq. 5)}$$

When the erythrocytes contain more than one site per cell, the total concentration of ligand bound, $(D_b)_t$, is the summation of the concentrations bound to each site. Thus, by grouping identical sites:

$$\frac{(D_b)_t}{C_t} = r = \sum_{i=1}^{m} \frac{n_i k_i A}{1 + k_i A} \qquad \text{(Eq. 6)}$$

where $n_i$ is the number of identical sites of class $i$ per erythrocyte.

Equation 6 is of the form normally used to analyze Scatchard plots. However, in a Scatchard plot of ligand–erythrocyte interaction data, $r$ is in units of molecules of drug bound per milliliter divided by the number of cells per milliliter while $A$ is in its normal molar concentration unit. From such a plot, values of $k_i$ are obtained in the usual units of $M^{-1}$ and the number of binding sites per cell of each class is obtainable. Such a plot has the additional value of allowing one to postulate which intracellular component(s) of the erythrocyte is involved in the interaction. For example, if a linear Scatchard plot is obtained with an $x$ intercept of approximately $2.8 \times 10^8$, it is reasonable to postulate a ligand interaction with a single site on hemoglobulin since there are approximately 280 million hemoglobulin molecules per erythrocyte (9).

In conclusion, the use of binding capacities apparently is not necessary in the study of ligand–erythrocyte interactions. With the assumption that the erythrocytes are identical, the concentration of sites per cell is analogous to the concentration of sites per molecule of protein. If the erythrocytes are sufficiently different so that this method does not hold true, then any results obtained by any method of analysis will have little meaning.

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[2] Coulter.



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# Site Dependence for Topical Absorption of Nitroglycerin in Rats

**Keyphrases** □ Nitroglycerin—percutaneous absorption in rats, effect of application site □ Absorption, percutaneous—nitroglycerin in rats, effect of application site □ Vasodilators, coronary—nitroglycerin, percutaneous absorption in rats, effect of application site

*To the Editor:*

Topical nitroglycerin ointment produces clinically significant reductions in the frequency and severity of exercise-induced angina attacks (1, 2) and reductions in heart workload and determinants of myocardial oxygen consumption (3). This dosage form enjoys a growing popularity because it provides sustained hemodynamic effects (4), its administration is noninvasive, and unabsorbed drug can be removed conveniently.

Little is known about the physical and physiological factors that influence percutaneous nitroglycerin absorption. Such information is important with nitroglycerin since systemic availability is the primary goal of topical application. Furthermore, because of the relatively short nitroglycerin elimination half-life (5), the percutaneous absorption rate becomes the limiting kinetic factor and essentially determines the plasma concentration–time profile after topical drug application ["flip-flop pharmacokinetics" (6)]. The application mode and the topical preparation vehicle are important factors to consider with nitroglycerin ointment.

This communication describes the effect of the ointment application site on nitroglycerin absorption in the rat. The results may have important bearing not only on the most effective use of nitroglycerin ointment for systemic effects but also on the choice of proper animal models for screening topically delivered drugs.

Male Sprague-Dawley rats[1], 280–390 g, were used. Fifteen to eighteen hours prior to an experiment, the intended dosing site was clipped and shaved with an electric razor[2]. The animal was returned to a cage with free access to water only. The skin was examined under low power magnification for damage resulting from shaving or scratching, and the animal was not used if the skin barrier was disrupted.

A cannula[3] was implanted in the right jugular vein under ether anesthesia and kept patent with heparin sodium (20 U/ml) in normal saline. The animal was loosely restrained to expose the dosing site. Light ether anesthesia was maintained for the remainder of the 4-hr experiment to preclude movements that could result in removal or contamination of the applied dose. Experiments were conducted at ambient temperatures (21–24°). Doses were applied to a 3 × 3-cm area centered midline and midway between the sternum and penis (abdominal site) or midline and 6 cm up from the tail connection (back site).

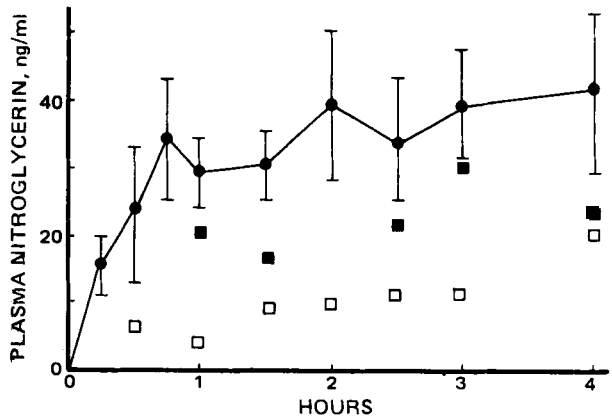


**Figure 1**—*Plasma nitroglycerin concentrations in rats after application of 2% nitroglycerin ointment. Key: ●, shaved abdominal surface (mean ± SEM, n = 6, 20 mg of nitroglycerin/kg); and ■ and □, back area after stripping with adhesive tape (results from two animals, 14 mg of nitroglycerin/kg).*

At appropriate intervals, 0.5 ml of blood was sampled *via* the implanted cannula. Plasma (200 μl) was stabilized against rapid degradation of nitroglycerin by the addition of 10 μl of 1.0 *N* $AgNO_3$ and assayed for intact nitroglycerin by the specific electron-capture GLC procedure of Yap *et al.* (7). The lower limit of quantitation in this experiment was 1.0 ng of nitroglycerin/ml of plasma.

Nitroglycerin was applied to the shaved back in a variety of doses and dosage forms. Plasma nitroglycerin levels could not be detected after topical application of a 2% nitroglycerin commercial ointment[4] (7 and 14 mg/kg) or a 6.9% nitroglycerin alcohol solution (8, 15, and 20 mg/kg). Each test group consisted of at least two animals. Oral dosing of rats with nitroglycerin at 7 mg/kg gives peak plasma concentrations up to about 10 ng/ml (8).

The tissue on the underside of the animal was of distinctly different character and strength than the dorsal region. Application of 2% nitroglycerin ointment to the shaved abdominal surface showed rapid drug absorption into the systemic circulation, with peak plasma levels ranging between 30 and 40 ng/ml (Fig. 1).

Considerable documentation is available describing the role of the stratum corneum as a drug penetration barrier (9). Comparative photomicrographs of histologic preparations[5] of back and abdominal tissue sections from a rat show a marked difference in the epidermis between the two sites (Fig. 2). The number of layers and relative depth of cornified tissue on the back area are significantly greater than on the abdomen.

If the stratum corneum represents a barrier to nitro-

[1] Blue Spruce Farms, Altamont, N.Y.
[2] Lady Remington MS-120, Sperry Remington, Bridgeport, CT 06602.
[3] Intramedic 7410, Clay-Adam, Parsippany, NJ 07054.
[4] Lot T4808 Nitro-bid Ointment, Marion Laboratories, Kansas City, MO 64137.
[5] Sections taken from a 295-g animal, fixed in formalin, and stained with eosin.

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