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# A competitive ELISA for the anti-intercellular adhesion molecule-1 (anti-ICAM-1) binding activity of monoclonal antibody R6.5 in serum

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**Abstract**: A competitive ELISA was developed to measure serum concentrations of immunoreactive R6.5, a mouse $IgG_{2a}$ monoclonal antibody which binds to and inhibits the interactions of human intercellular adhesion molecule-1 with glycoproteins of the CD18 complex and with rhinoviruses. The assay design permitted quantitative measurement of the functional activity of the antibody in serum. The working range of the assay was from 2.15 to 215 ng $ml^{-1}$, and intra-assay and inter-assay relative standard deviations averaged 13 and 20%, respectively. Serum caused interferences with the assay when spiked *in vitro*, but authentic serum samples obtained from intravenously dosed animals did not exhibit these interferences. Characteristics of the assay, as well as results of a pharmacokinetic study in rabbits, are presented.

**Keywords**: *Competitive ELISA; intercellular adhesion molecule-1; R6.5 mouse monoclonal antibody; serum effects; rabbit pharmacokinetics.*

## Introduction

R6.5, a mouse $IgG_{2a}$ monoclonal antibody directed against human intercellular adhesion molecule-1 (ICAM-1), has been shown to block ICAM-1 dependent immunological processes including leukocyte adhesion and migration through endothelial cell monolayers and the cell proliferation seen in human mixed lymphocyte reactions [1, 2]. In rabbits, R6.5 was found to inhibit the migration of neutrophils into lungs during pulmonary inflammation [3]. In monkeys, prophylactic administration of R6.5 significantly delayed rejection of transplanted kidneys, and therapeutic administration caused a reversal of acute kidney rejection following kidney transplantation [4].

A common approach to measuring serum levels of intravenously administered monoclonal antibodies or of other biological substances has been the sandwich ELISA or similar methods [5–8]. However, the conventional sandwich ELISA does not provide any direct inferences concerning the functional activity of the antibody or other biological substance being assayed. Therefore, a competitive ELISA method using ICAM-1 as the trapping antigen was developed to measure serum concentrations of immunoreactive R6.5 (i.e. serum levels of anti-ICAM-1 binding activity).

## Experimental

### *Cell lines and antibodies*

The JY cell line, an Epstein Barr Virus-transformed B-lymphoblastoid cell line, was the kind gift of Dr Timothy Springer (Dana Farber Cancer Inst.). These cells constitutively express ICAM-1 [9]. Cells, cultured in RPMI-1640 (Gibco) containing 10% fetal bovine serum supplemented with 2 mM L-glutamine, penicillin (100 units $ml^{-1}$), and streptomycin (100 $\mu g$ $ml^{-1}$), grew to a density of approximately $1 \times 10^6$ cells $ml^{-1}$ with a viability of 95–97%. The anti-ICAM-1 antibody, R6.5 (mouse $IgG_{2a}$, kappa light chain), was derived from fusing spleen cells of mice, which had been immunized against cell lines bearing ICAM-1, with the mouse myeloma P3 × 63Ag8.653 [1]. R6.5 MAb was produced in culture, purified, and lyophilized at Dr Karl Thomae (an operating unit of Boehringer Ingelheim GmbH), Biberach, FRG. Purification steps were ultrafiltration, anion and cation exchange chromatography, diafiltration, and gel permeation chromatography. Biotinyl-

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ation of R6.5 was performed using biotin-*N*-hydroxysuccinimide ester (Bethesda Research Labs) and methods described by Goding [10]. The concentration of stock biotinylated R6.5 from which dilutions were made was approximately 1 mg $ml^{-1}$. Two lots of $^{125}I$-labelled R6.5 were prepared (iodogen procedure) for pharmacokinetic studies, and the specific activities were 0.9 μCi $\mu g^{-1}$ (rabbit study) and 1.8 μCi $\mu g^{-1}$ (monkey study) with radiochemical purities of 98 and 97%, respectively.

*Competitive ELISA for anti-ICAM-1 binding activity in serum*

The principle of this assay was to measure the competition between R6.5 and biotinylated R6.5 for binding to ICAM-1 expressed on JY cells. Wells of microtiter plates (Costar No. 3596) were coated with 100 μl of 0.1 mg $ml^{-1}$ poly-L-lysine (Sigma) in pH 7.4 phosphate-buffered saline (PBS) overnight at 4°C. After washing with 0.9% NaCl, wells were loaded with 100 μl of JY cell suspension (approx. $2 \times 10^6$ cells $ml^{-1}$ in PBS) and centrifuged (approx. 15*g*, 3 min) to form a monolayer. Next, 100 μl of 2% paraformaldehyde (Fisher) in PBS was added gently to each well for a 60-min incubation at room temperature (RT). After this fixation step, the plates were washed (0.1% $NaN_3$ in PBS; PBS-AZ) and stored at 4°C for up to 1 week. Wells were blocked with 250 μl of 2% bovine serum albumin (Sigma) in PBS-AZ (PBS-AZ–BSA) at RT for 60 min. After washing plates with PBS-AZ, 100-μl aliquots of standard R6.5 solutions, quality control samples, and diluted test samples were added to wells in triplicate. Standard R6.5 solutions contained known concentrations (ranging from 1 to 1000 ng $ml^{-1}$) of R6.5 in PBS-AZ–BSA. Quality control samples of R6.5 in PBS-AZ–BSA were prepared in advance and aliquoted at concentrations of 10.0, 21.5 and 46.4 ng $ml^{-1}$. Aliquots were stored frozen (below −20°C) until used. Test samples (e.g. serum samples) were diluted in PBS-AZ–BSA, except where noted in the Results section. After these additions, the plate was stored overnight at 4°C. On the next day, without removing the samples already in the wells, 100 μl of biotinylated R6.5 (diluted with PBS-AZ–BSA, 1:50) was added to each well, and the plate was agitated gently for 10 min. After overnight storage at 4°C, the plates were washed, and 100 μl of streptavidin-β-galactosidase (Bethesda Research Labs, 1:500 dil. in PBS-AZ–BSA) was added to each well for a 90 min incubation at RT. After washing the plate, *p*-nitrophenyl-β-D-galactoside (Bethesda Research Labs, 100 μl at 0.5 mg $ml^{-1}$ in PBS-AZ, no BSA) was added to each well. The optical density at 405 nm ($OD_{405}$) of the reaction mixture was measured after approximately 90 min of incubation at RT.

*Data analysis*

Mean values of $OD_{405}$ obtained for standard concentrations of R6.5 assayed in the competitive ELISA were used to produce a standard curve through least-squares non-linear regression [11]. The four-parameter logistic function [12] was the model for the regression, and unweighted mean values of $OD_{405}$ were used. This approach routinely provided a mathematical equation which adequately described the standard curve. The concentration of R6.5 in test samples could be calculated by using a transformed and rearranged version of the equation and the parameter estimates derived from the regression analysis.

*Serum samples*

Blank serum samples were obtained from rabbits and cynomolgus monkeys who had not previously been exposed to mouse immunoglobulin experimentally. Blood samples were collected via indwelling venous catheters into serum Monovettes (Sarstadt). Serum samples from pharmacokinetic studies of $^{125}I$-labelled R6.5 in rabbits and monkeys (intravenous dosing, 1 mg $kg^{-1}$) were collected in a similar manner. Quantitation of total $^{125}I$ and of trichloroacetic acid (TCA) soluble $^{125}I$ in a gamma counter allowed the calculation of TCA-precipitable $^{125}I$, and the latter was used as an alternative method of estimating the serum concentration of R6.5. All serum samples were prepared promptly after blood collection and stored frozen at approximately −20°C until thawed for use.

## Results

*Linearization of the standard curve*

Over a range of R6.5 concentrations from 1 to 1000 ng $ml^{-1}$ the assay response resulting from the competition between R6.5 and biotinylated R6.5 was sigmoidal [Fig. 1(a)]. Figure 1(b) illustrates that the logit transformed data were reasonably linear over a wide range of

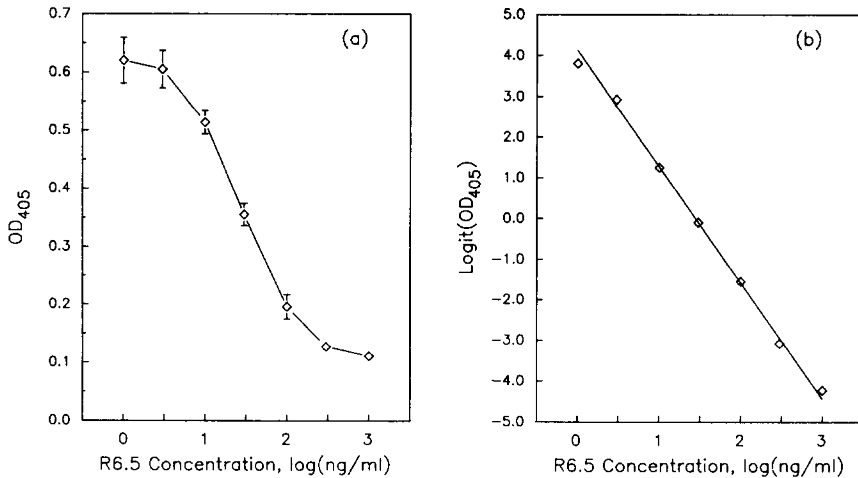


**Figure 1**
Standard curve of R6.5 in a competitive ELISA. Plot (a) shows the assay response, $OD_{405}$ (mean ±SD), and plot (b) shows the linearized assay response after the $OD_{405}$ values were transformed by the logit function. The line through the data in (b) is the calculated line of best fit determined by non-linear least-squares regression (see Experimental).

concentrations, and a correlation coefficient of 0.999 was calculated. However, with experience, it was found that responses for the extreme concentrations (i.e. 1 and 1000 ng $ml^{-1}$) could not always be adequately fit by non-linear regression. Therefore, a narrower range of concentrations (2.15 to 215 ng $ml^{-1}$) was routinely used for the standard curve.

*Serum effects*

To study the effects of serum on the assay, standard concentrations of R6.5 were assayed in the presence and absence of 1% rabbit or monkey serum. A characteristic serum interference was observed, but only with certain individual rabbits or monkeys. When a serum interference was observed, the assay response was generally found to be enhanced in the presence of serum, particularly at the lower concentrations of R6.5 and in the blanks to which no R6.5 was added. Results presented in Fig. 2 illustrate this effect for both rabbit and monkey sera. The net effect of this type of interference was that the dilution curve for R6.5 added to 1% serum *in vitro* was not always parallel to the dilution curve for R6.5 in the absence of serum.

Another type of serum effect was observed when whole monkey sera were spiked *in vitro* with known concentrations of R6.5. Sera,

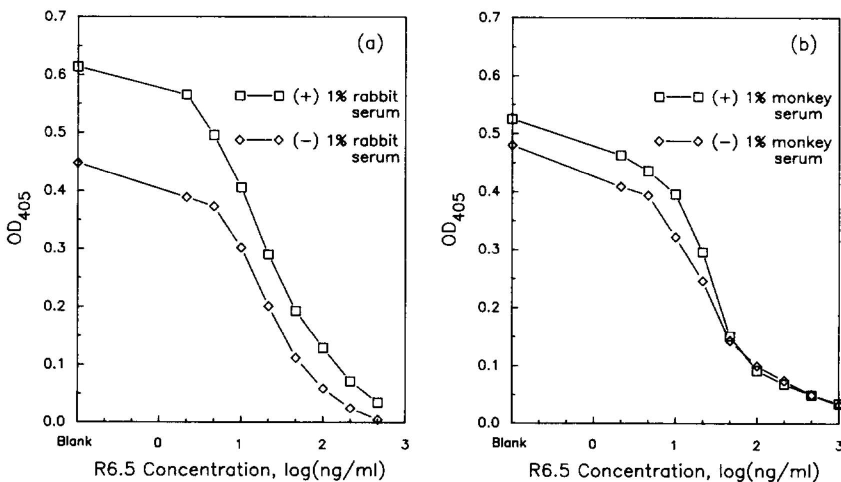


**Figure 2**
Effect of serum on the R6.5 standard curve in the competitive ELISA. Rabbit (a) or monkey (b) serum was either included at a concentration of 1% (squares) or omitted from (diamonds) the dilution buffer used to make the R6.5 standards.

obtained from five monkeys and which did not show the kind of non-parallel behaviour described above, were spiked with R6.5 at concentrations in the range observed *in vivo* after intravenous dosing (see below). Sera from three of the monkeys were pooled prior to spiking with R6.5, whereas sera from the other two monkeys were spiked individually with R6.5 (see Fig. 3 legend). Each spiked

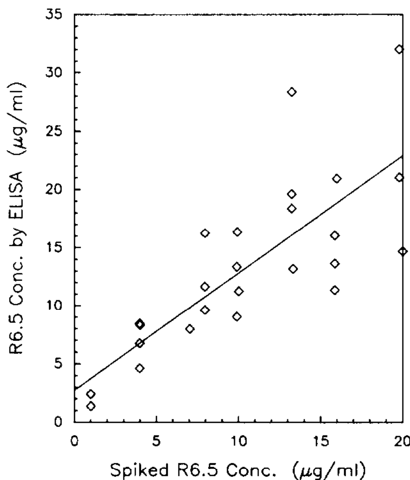


**Figure 3**
R6.5 concentrations estimated by a competitive ELISA for monkey serum samples spiked *in vitro*. Of the 26 spiked samples, seven used pooled sera from three individual monkeys, seven used serum from a fourth monkey, and 12 used serum from a fifth monkey. The line in the figure was calculated by least-squares linear regression. The slope and intercept (±SE) were 1.01 ± 0.16 and 2.75 ± 1.83, respectively.

sample was diluted with buffer to give a series of five dilutions (1:100, 1:215, 1:464, 1:1000 and 1:2150), and each dilution was assayed in triplicate wells. Mean $OD_{405}$ values which fell within the middle 60% of the standard curve (a criterion intended to avoid the excessive, expected variability on the plateau regions of the curve) were used for calculating R6.5 concentrations for each dilution. After correction for dilution, the results were averaged to give a single concentration estimate for each spiked sample. Figure 3 illustrates the results for 26 spiked serum samples. It is apparent that the level of variability was quite large. The average measured serum R6.5 concentration was 13 μg $ml^{-1}$, and the standard deviation about the line was calculated to be 4.6 μg $ml^{-1}$, which corresponds to an RSD of 35%. This level of variability was considered to reflect serum interference, because intra-assay and inter-assay variability for serum-free samples were much lower (see below).

When authentic serum samples obtained after intravenous administration of $^{125}$I-R6.5 (1 mg $kg^{-1}$) were assayed, the two types of serum interferences discussed above were not observed. Authentic serum samples were diluted (1:100, 1:215, 1:464, 1:1000 and 1:2150) with PBS-AZ–BSA buffer, and dilutions were assayed in triplicate. Figure 4 illustrates, in a representative manner, the parallelism which was routinely observed between the standard curve and dilutions of authentic rabbit and monkey serum samples.

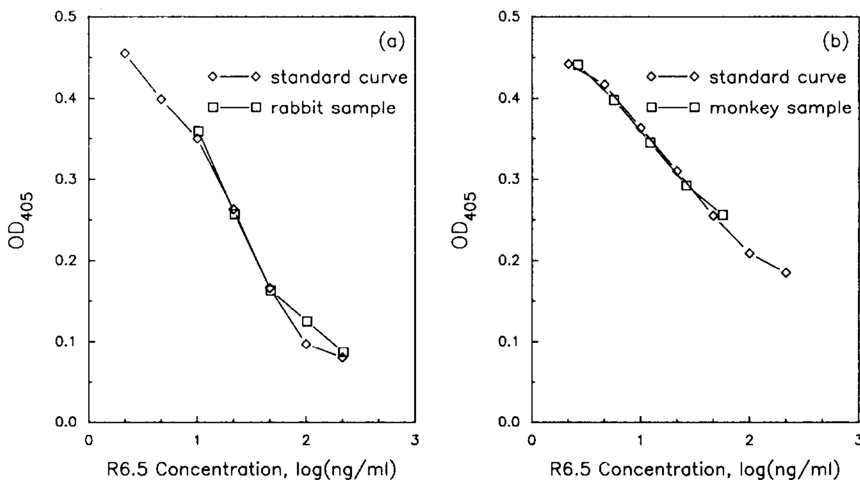


**Figure 4**
Comparison between R6.5 standard curves and dilution curves for authentic rabbit and monkey serum samples. The plots show the assay response for two R6.5 standard curves (diamonds) and for dilutions (1:100, 1:215, 1:464, 1:1000 and 1:2150) of authentic serum samples (squares) from a rabbit (a) and a monkey (b). Serum samples were obtained from animals who had received an intravenous dose (1 mg $kg^{-1}$) of $^{125}$I-R6.5.

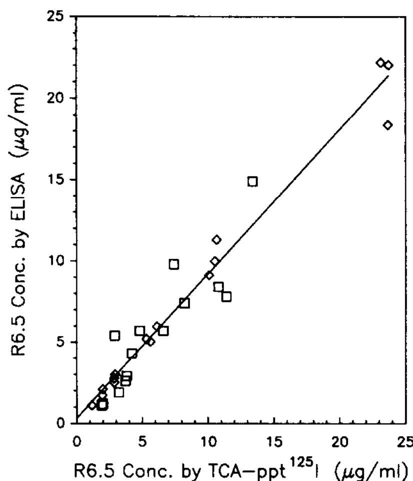


**Figure 5**
Comparison of R6.5 concentrations in authentic serum samples estimated either by competitive ELISA or by the TCA-precipitable $^{125}$I method (see text). Serum samples were obtained from three rabbits (diamonds) and from three monkeys (squares) at various times after intravenous dosing with $^{125}$I-R6.5 (1 mg kg$^{-1}$). The line in the figure was calculated by least-squares linear regression. The slope and intercept (±SE) were 0.89 ± 0.04 and 0.31 ± 0.39, respectively.

A total of 29 authentic serum samples (five from each of three rabbits and four or five from each of three monkeys) were assayed, both by the competitive ELISA and by TCA precipitation of $^{125}$I. Figure 5 presents a summary of the results by comparing the concentrations of R6.5 estimated by competitive ELISA with those estimated by the TCA-precipitable $^{125}$I method. These results show a good correlation between R6.5 concentrations estimated by the two methods; the standard deviation about the line was 1.3 μg ml$^{-1}$. For an average measured serum R6.5 concentration of 6.9 μg ml$^{-1}$, this corresponds to a RSD of 19%.

*Intra-assay and inter-assay variability*

Because of the effects of serum on the assay when spiked with R6.5 *in vitro*, assay variability was assessed using solutions of R6.5 prepared in the standard dilution buffer, PBS-AZ-BSA. Intra-assay variability was assessed using six microtiter plates, each of which included multiple replicates (8–10 triplicates, i.e. 24–30 wells) of a single test concentration of R6.5. The concentrations (mean ±SD, $n$ = 8–10) estimated for the six test samples were 7.3 ± 1.1, 11.8 ± 2.1, 19.5 ± 2.4, 24.3 ± 2.4, 36.3 ± 3.5 and 50.2 ± 7.1 ng ml$^{-1}$, and RSDs ranged from 9.6 to 17.8%. Inter-assay variability was assessed using quality control samples (10.0, 21.5 and 46.4 ng ml$^{-1}$ R6.5 in PBS-AZ-BSA) prepared in advance, aliquoted, and frozen until assayed. The concentrations (mean ±SD, $n$ = 28 or 29 assays) estimated for the three levels of quality control samples were 10.8 ± 2.6, 18.6 ± 3.5 and 45.3 ± 7.7 ng ml$^{-1}$, respectively. RSDs were 24.1, 18.8 and 17.0%, respectively.

*Pharmacokinetics of R6.5 in rabbits*

Figure 6 illustrates the time course of R6.5 serum concentrations following intravenous administration (1 mg kg$^{-1}$). The average elimination phase half-life for R6.5 in three rabbits, estimated by compartmental analysis, was 27 h (range 24–29 h). The steady-state volume of distribution of R6.5 was estimated to be 50 ml kg$^{-1}$ (range 47–52 ml kg$^{-1}$). The average plasma clearance was 22 μl min$^{-1}$ kg$^{-1}$ (range 21–23 μl min$^{-1}$ kg$^{-1}$).

## Discussion

A competitive ELISA was developed to measure the levels of anti-ICAM-1 binding activity in serum following intravenous administration of R6.5, a mouse $IgG_{2a}$ MAb directed against human ICAM-1. The principle of the assay was that R6.5 in buffer or in diluted serum competed with biotinylated R6.5, the detecting antibody, for binding to

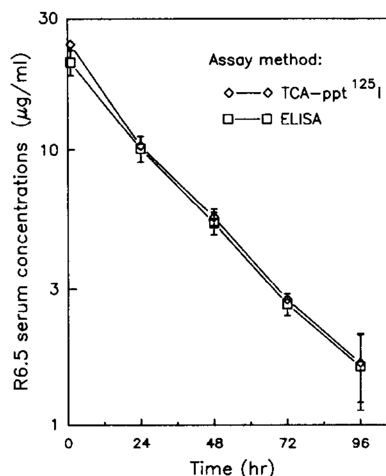


**Figure 6**
Serum levels of R6.5 following intravenous dosing in rabbits estimated using two different assay methods. Levels were estimated either from measurements of TCA-precipitable (TCA-ppt) $^{125}$I (diamonds) or with the competitive ELISA (squares). The values are the mean ±SD for three rabbits. The intravenous dose was 1 mg of R6.5 per kg body weight and included tracer amounts of $^{125}$I-R6.5.

ICAM-1 on the surface of JY cells. Through non-linear regression using the four-parameter logistic function, the sigmoidal relationship between $OD_{405}$ and the logarithm of R6.5 concentration was linearized, and the parameter estimates provided a simple method for calculating R6.5 concentration in quality control samples and diluted serum samples.

Intra-assay variability as measured by the RSD at different R6.5 concentrations ranged from 9.6 to 17.8%, and inter-assay variability ranged from 17.0 to 24.1%. However, because the assay procedure calls for authentic serum samples to be assayed at more than one dilution in the useful range of the standard curve, the average RSDs for intra-assay and inter-assay variability (i.e. roughly 13 and 20%, respectively) over the concentration range studied were considered to provide an appropriate representation of the assay variability.

Serum interfered with the assay when it was spiked with R6.5. For some sera, inclusion in the dilution buffer at a concentration of 1% (v/v) resulted in a dilution curve for R6.5 which did not parallel that of the standard (serum-free) curve. Parallelism between the dilution curves was considered essential for meaningful quantitation. This non-parallel relationship was specific to sera taken from certain individual animals. Upon screening several sera within a single assay, some sera would show the interference while others would not, and this occurred on numerous occasions (data not shown).

A second type of serum interference was also observed in which the assay variability was enhanced in serum samples spiked with R6.5 *in vitro* relative to serum-free samples. This effect was observed even when controlling for the non-parallel dilution curve effect discussed above.

Authentic serum samples obtained from rabbits and monkeys dosed intravenously with $^{125}$I-R6.5 did not appear to exhibit these types of serum interference. The dilution curves for authentic serum samples had slopes which paralleled the standard curves. Also, R6.5 concentrations in authentic serum samples estimated by competitive ELISA correlated quite well with R6.5 concentrations estimated by the TCA-precipitable $^{125}$I method. The latter finding suggests that the second type of serum effect described above, that of enhancing assay variability, may not have been a serious problem when assaying authentic serum samples.

The cause or causes of the serum interferences are not known. Anti-mouse IgG antibodies, which may exist in control sera of non-immunized animals [13], were considered to be a possible source of interference. However, no such antibodies were detected using conventional ELISA methods (data not shown). Attempts to identify the source or sources of interference have not been successful.

Another characteristic of the assay was the slow rate of colour development during the substrate incubation step. Over a 90-min incubation, the assay response only reached maximal $OD_{405}$ values in the range from 0.45 to 0.65, suggesting a low density of solid phase ICAM-1 or a weak affinity of R6.5 for fixed ICAM-1. This was probably a necessary result due to the procedures used to prepare the solid phase ICAM-1. Sufficient numbers of JY cells were added to wells to form monolayers, but the fixation step had to be severe enough to hold the cells in place. Therefore, there was a compromise in which R6.5 binding sites on the surface of JY cells were presumably lost or altered due to prolonged fixation in exchange for a well controlled adherence of JY cells to the microtiter plate wells.

A study of the pharmacokinetics of R6.5 in rabbits showed that the time course of serum R6.5 measured by competitive ELISA was very similar to that measured as TCA-precipitable $^{125}$I-R6.5. The elimination phase half-life of R6.5 averaged 27 h, and the steady-state volume of distribution averaged 50 ml $kg^{-1}$ which is slightly larger than the estimated plasma volume of the rabbits [14].

The characteristics of this competitive ELISA make it a useful adjunct to the simpler approach of the sandwich ELISA for pharmacokinetic studies of R6.5. It provides information regarding serum levels of anti-ICAM-1 binding activity, and in that sense it is a functional assay. The ability to quantitatively measure serum levels using a functional assay is an important consideration for pharmacokinetic studies in the context of the development of biological agents as therapeutics.

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