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SEBACIC ACID BINDING TO HUMAN PLASMA ALBUMIN

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Abstract—Sebacic (decanedioic) acid is a dicarboxylic acid proposed recently as an alternative energy substrate in total parenteral nutrition. In this paper, binding of sebacic acid to defatted human plasma albumin, also in the presence of decanoic acid, was studied by means of equilibrium dialysis. In addition, the binding of sebacic acid in human serum was investigated. Binding to defatted albumin was analysed by a model with two independent classes of sites with different affinity constants. The fitting procedure took into account some of the measurement errors that are likely to affect the equilibrium dialysis technique. We found for sebacic acid one binding site with affinity constant $3.69 \times 10^4\ M^{-1}$ and four to five sites with affinity constant $7.14 \times 10^2\ M^{-1}$. Association constants for decanoic acid are 3–4-fold larger than those of sebacic acid. Data of binding of sebacic acid in human serum suggested that only three to five of the low affinity sites are available for binding. When disodium sebacate is administered i.v. for total parenteral nutrition, a substantial fraction of sebacic anions is likely to be bound in serum.

The disodic salts of dicarboxylic acids were proposed recently as an energy substrate in total parenteral nutrition [1, 2]. These diacids can be useful as an alternate fuel source under critical conditions such as an advanced state of sepsis, when glucose intolerance, increased gluconeogenesis and oxidative defects of intermediary metabolism ensue [3]; an impairment of lipoprotein lipase activity [4] and carnitine deficiency [5] were also demonstrated in sepsis. Dicarboxylic acids are oxidized at the level of both mitochondria and peroxisomes [6, 7], and their transport through the mitochondria membrane is a carnitine-independent process [8, 9]. In particular, sebacic acid (decanedioic acid, $HOOC\text{-}(CH_2)_8\text{-}COOH$) appears to be advantageous in total parenteral nutrition since it can be completely oxidized to CO_2 and H_2O and shows low levels of urinary excretion [2]. Moreover, sebacic acid has neither toxic nor teratogenic effects on laboratory animals [10] and can be directly administered through a peripheral vein in salt form.

A study of the binding of sebacic acid to human plasma albumin (HPA‡) is necessary for investigating the pharmacokinetics and bioavailability of sebacic acid. It appears also of interest to study the binding of sebacic acid in the presence of the monocarboxylic acid of the same chain length (decanoic acid). Decanoic acid forms in fact about 57% of the content of fatty acids in medium-chain triglyceride emulsions, used as a lipidic energy substrate in total parenteral nutrition [11, 12]. Binding of sebacic acid and other dicarboxylic acids to bovine serum albumin (BSA) was analysed by Tonsgard *et al.* [13, 14], and binding of decanoic acid to human serum albumin by Ashbrook *et al.* [15].

In the present work, the binding of sebacic acid to defatted HPA, also in the presence of decanoic acid at low concentrations, was investigated by equilibrium dialysis. The data were analysed by a model with two independent classes of high and low affinity sites, assuming competitive binding of sebacic and decanoic acid. Estimates of model parameters were obtained taking into account various sources of measurement error in the equilibrium dialysis technique. Additional data of sebacic acid binding in human serum appeared to be consistent with a model containing only the low affinity sites.

MATERIALS AND METHODS

Dicarboxylic and monocarboxylic acids. Unlabeled decanedioic acid (sebacic acid) and decanoic acid (capric acid) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The purity of the unlabeled acids was assessed by gas–liquid chromatography and was found to be at least 99%. [1,10-^{14}C]Decanedioic acid purchased from Amersham Corp. (Amersham, U.K.) was prepared by the dicyanation of 1,8-dibromooctane with [^{14}C]-potassium cyanide followed by alkaline hydrolysis. The chemical purity of the labeled compound was assessed by analytical HPLC and found to be at least 99%. The column employed was Techsphere ODS; solvent A was methanol:dioxan:tetraethylammonium formate (65:5:33, by vol.) and solvent B was methanol:dioxan:tetraethylammonium formate (87:5:8, by vol.). A gradient was performed from 100% A to 100% B over 10 min. The purity of labeled decanedioic acid was assessed by Amersham. The specific activity was 102 mCi/mmol.

Albumin. HPA essentially fatty acid free was purchased from Sigma.

Equilibrium dialysis. Binding of sebacic acid to defatted HPA was determined by equilibrium dialysis

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‡ Abbreviations: HPA, human plasma albumin; BSA, bovine serum albumin; *CV*, coefficient of variation.

as described by Ashbrook *et al.* [15]. We used a 5-cell Spectrum Equilibrium Dialyzer (Spectrum Medical Industries, Los Angeles, CA, U.S.A.). The teflon cells contained 1 mL compartments separated by a dialysis membrane (Spectra/Por, 47 mm diameter, M_r cutoff 12,000–14,000, Spectrum Medical Industries). Albumin does not cross the dialysis membrane. The dicarboxylic acid reached equilibrium within 3 hr at 37°. We chose a 6 hr incubation period to ensure equilibrium of both mono- and dicarboxylic acids.

Binding was assessed at varying concentrations of sebacic acid (from 1.25×10^{-5} to 4.9×10^{-3} M) in phosphate buffer, pH 7.4. Sebacic acid was added to one side of the chamber, and defatted HPA in the same salt solution at 0.1 mM concentration was added to the other side. Rotation speed of the dialysis cells was 30 rpm and solution temperature was kept at 37° by a thermostatic bath (Haakes, Karlsruhe, Germany). At the end of the incubation period, aliquots of 500 μL (measurement volumes) were removed from each side of the equilibration chamber, and the radioactivity was measured by a Beta scintillation counter (Packard Tricarb). The recovery of radioactivity ranged from 89% to 100%.

Monocarboxylic acid competition. The competition experiments were performed as described above except that either 0.4×10^{-4}, 1.0×10^{-4} or 3.0×10^{-4} M of decanoic acid was added to the compartment of the equilibrium chamber containing [1,10-^{14}C]sebacic acid.

Binding of sebacic acid in serum. Ten samples of human serum were obtained from healthy volunteers. Binding was assessed, for each sample, at four to five different concentrations of sebacic acid. Concentration of total sebacic acid added to samples ranged from 1.25×10^{-5} to 9.0×10^{-3} M. Binding was measured at 37°, pH 7.4, using the above described method. Albumin concentration in the samples ranged from 37 to 50 g/L.

Binding model. Binding data of sebacic acid to defatted albumin, measured both in the absence and in the presence of decanoic acid, were preliminarily reported in a double-reciprocal plot. The plot suggested that the interaction of sebacic acid and decanoic acid with HPA could follow a competitive scheme, and that the number of albumin sites available for sebacic acid binding is in the range four to six. Data by Ashbrook *et al.* [15] show, on the other hand, that a larger number of binding sites is available for decanoic acid on the albumin molecule (up to 14 sites). Assuming that the additional sites available for decanoic acid are low affinity sites, and considering that the maximal concentration used in the present work for decanoic acid (0.3 mM) is less than 1/10 of the maximal concentration of sebacic acid, we neglected in the binding model the contribution of these sites when computing the bound competitor concentration.

We fitted the data of sebacic acid binding to defatted albumin by a model in which both ligands compete for two classes of independent binding sites on HPA (n_1 plus n_2 model, see Meisner *et al.* [16]):

$$\bar{\nu} = \frac{n_1 k_1 c}{1 + k_1 c + k_1' c'} + \frac{n_2 k_2 c}{1 + k_2 c + k_2' c'} \qquad (1)$$

$$\bar{\nu}' = \frac{n_1 k_1' c'}{1 + k_1' c' + k_1 c} + \frac{n_2 k_2' c'}{1 + k_2' c' + k_2 c} \qquad (2)$$

where: $\bar{\nu}$ is the molar ratio of bound sebacic acid to albumin; $\bar{\nu}'$ is the molar ratio of bound competitor (decanoic acid) to albumin; c is the concentration (M) of free sebacic acid; c' is the concentration (M) of free competitor; n_1 and n_2 (mol/mol HPA) denote the number of binding sites in the high and low affinity class, respectively; k_1 and k_2 (M^{-1}) are the association constants of sebacic acid for the sites in the first and the second class, respectively; k_1' and k_2' (M^{-1}) are the association constants of the competitor for the sites in the first and the second class, respectively. In the absence of competition, the model reduces to Eqn 1 with $c' = 0$.

We used the model of Eqns 1 and 2 to fit the binding data in the Scatchard plane. The concentrations c and c', in the absence of measurements of the concentration c' of the free competitor, were found following the procedure in [16]. To Eqns 1 and 2 we added the equation of conservation of the competitor, whose total concentration c_t' was known. Then, for each given pair of c_t' and $\bar{\nu}$ values, the model equations were solved numerically with respect to c and c' and the binding curves in the Scatchard plane were found.

The model of Eqn 1 with $c' = 0$ was tested, on the basis of the data measured in the absence of the competitor only, against the stepwise equilibrium model [17]. This model, which has been used for the analysis of binding of medium- and long-chain fatty acids to HPA [15, 18], has the form:

$$\bar{\nu} = \frac{K_1 c + 2K_1 K_2 c^2 + \cdots + pK_1 \cdots K_p c^p}{1 + K_1 c + K_1 K_2 c^2 + \cdots + K_1 \cdots K_p c^p} \qquad (3)$$

where the parameters $K_1, \cdots, K_p$ are the equilibrium constants for sebacic acid binding to albumin. We tried the model of Eqn 3 with $p = 4$, 5 and 6.

Estimation of binding parameters. The unknown binding parameters were estimated according to the method of maximum likelihood [19]. The maximum likelihood function was written assuming normal errors and taking into account, at least approximately, the experimental uncertainties in both independent and dependent variables (abscissas and ordinates of the Scatchard plot) and the correlation between them [20]. Variances and covariance of data measured by equilibrium dialysis and reported in the Scatchard plane were obtained from a statistical analysis of measurement errors in the equilibrium dialysis technique (see Appendix). The variances and covariance are expressed in terms of three coefficients of variation (CV, the ratio of standard deviation to mean): the coefficient of variation of total ligand concentration (CV_t), of albumin concentration (CV_A), and of measurement volume (CV_v). These statistical parameters are also unknown, and have to be estimated from the data together with the parameters of the binding model.

The likelihood function was maximized by a quasi-Newton algorithm on a VAX-780 computer. Standard errors of the estimates of unknown parameters were evaluated from the inverse Hessian matrix [19], computed numerically at the optimum.

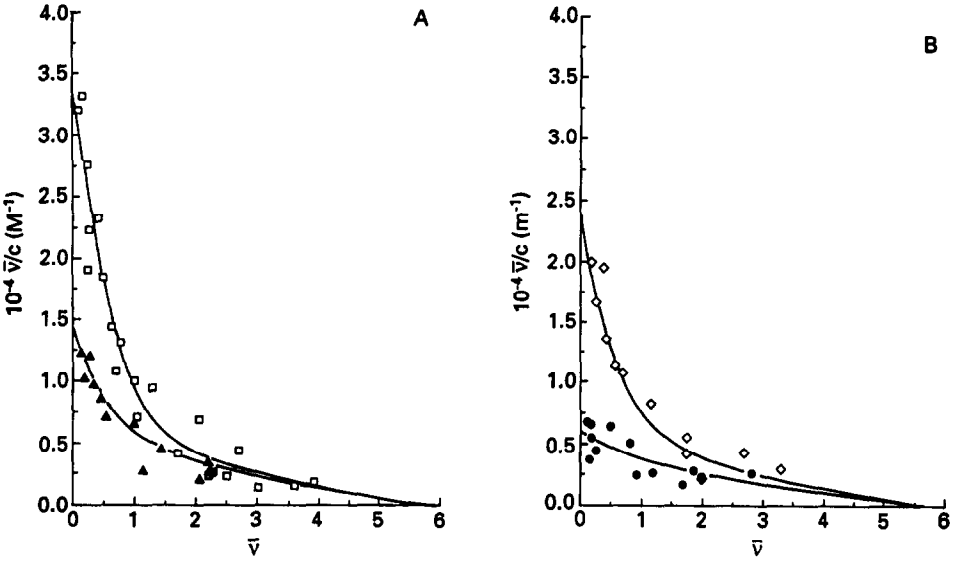

Fig. 1. Binding data of sebacic acid to defatted HPA reported in the Scatchard plot. Panel A: decanoic acid absent (□), 0.1 mM decanoic acid (▲). Panel B: 0.04 mM decanoic acid (◇), 0.3 mM decanoic acid (●). Continuous lines represent in both panels the binding curves predicted by the model of Eqns 1 and 2 with the parameter estimates of Table 1.

Table 1. Estimates (±SE) of binding parameters of sebacic acid to defatted HPA

n_1 (mol/mol HPA)	0.81 ± 0.15
k_1 (M^{-1})	$3.69 \pm 0.78 \times 10^4$
k_1' (M^{-1})	$1.21 \pm 1.02 \times 10^5$
n_2 (mol/mol HPA)	4.86 ± 3.02
k_2 (M^{-1})	$7.14 \pm 5.52 \times 10^2$
k_2' (M^{-1})	$3.00 \pm 4.51 \times 10^3$

Binding parameters assessed for 0.1 mM defatted albumin at pH 7.4, and in the presence of decanoic acid at various concentrations as a competitor (59 data points).

RESULTS

Figure 1 shows the experimental data of binding to defatted albumin plotted on the Scatchard plane. In panel A the binding data in the absence of competition and at a total competitor concentration of 1.0×10^{-4} M are reported; panel B shows the data at total competitor concentrations of 0.4×10^{-4} and 3.0×10^{-4} M. In both panels the continuous line represents the best fit of the data obtained by the independent site model of Eqns 1 and 2, using all the measurements simultaneously (59 data points). Table 1 reports the estimates of binding parameters and the standard errors (SE) of the estimates. Significantly, the value here obtained for the quantity $n_1k_1' + n_2k_2' = 1.13 \times 10^5\,M^{-1}$ closely agrees with the value, $1.03 \times 10^5\,M^{-1}$, of the largest stoichiometric constant found by Ashbrook *et al.* [15] for decanoic acid using the stepwise equilibrium model. The value of CV_A (set *a priori* equal to $2CV_t$ to take into account the greater difficulty in manipulating the albumin solution) was estimated to be 7.27%. CV_v was estimated to be 2.16%, a value that agrees with the *a priori* evaluation of an uncertainty of 10 μL affecting the measurement volumes.

When only the data measured in the absence of competition were used (22 data points), the independent site model gave the following parameter estimates: $n_1 = 0.69$, $k_1 = 4.47 \times 10^4\,M^{-1}$, $n_2 = 4.22$, $k_2 = 1.05 \times 10^3\,M^{-1}$. These values are in good agreement with those of Table 1, obtained using all the data, and fall within the range estimate ± SE. The stepwise equilibrium model with $p = 5$ gave the following stoichiometric constants (M^{-1}): $K_1 = 3.24 \times 10^4$, $K_2 = 1.69 \times 10^3$, $K_3 = 2.98 \times 10^3$, $K_4 = 0.67 \times 10^{-1}$, $K_5 = 0.17 \times 10^7$. The value of log-likelihood at the optimum for the stepwise equilibrium model was slightly larger than the value obtained with the independent site model. However, the difference of goodness of fit is too small to be conclusive for model choice.

Figure 2 shows the data of binding of sebacic acid in serum, reported in the plane $\bar{v}$ versus c. The normalization of bound ligand concentration to albumin concentration of the sample allowed us to report together all the measurements from the 10 serum samples. The figure shows that, as expected, these data lie well below the binding curve found in the case of sebacic acid binding to defatted albumin in the absence of competition. Moreover, the data fall within or in the proximity of a region of the (c, $\bar{v}$) plane, bounded by the binding curves generated by a model with a single class of low affinity sites (association constant equal to $7.14 \times 10^2\,M^{-1}$, the constant k_2 of Table 1) and three or five binding sites. In comparison with the binding to defatted albumin, sebacic acid binding in serum shows inhibition of the binding to the high affinity site.

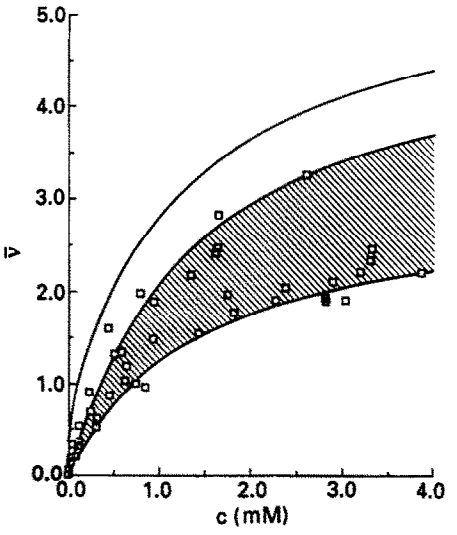

Fig. 2. Binding data of sebacic acid in human serum reported in a plot $\bar{\nu}$ vs c (open symbols). The continuous lines enclosing the dashed area are the binding curves predicted by the model $\bar{\nu} = nkc/(1 + kc)$, with $k = 7.14 \times 10^2\ M^{-1}$ and $n = 3$ (lower line) or $n = 5$ (upper line). The bolder line represents the binding curve of sebacic acid to defatted albumin in the absence of competition (the same curve is shown in Scatchard coordinates in Fig. 1A, upper line).

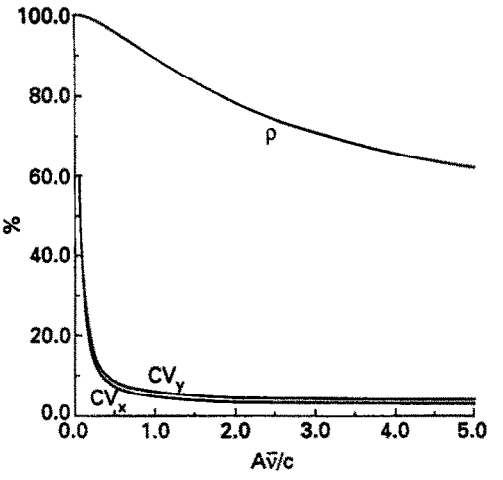

Fig. 3. Behavior of the coefficients of variation (CV_x, CV_y) and of the correlation coefficient (ρ) of data in the abscissa and ordinate of the Scatchard plot vs $A\bar{\nu}/c$ (A denoting albumin concentration). CV_x, CV_y and ρ are computed on the basis of Eqns A.3–A.5 of the Appendix with $\Delta = 0$ and $CV_t = CV_A = CV_v = 2\%$.

The statistical analysis of measurement errors in the equilibrium dialysis technique (see Appendix) allowed expression of the coefficient of variation of the data in the abscissa and ordinate of the Scatchard plot (CV_x and CV_y, respectively) and the correlation coefficient (ρ) as a function of the quantity $A\bar{\nu}/c = c_b/c$. It can be seen that CV_x, CV_y and ρ increase at low values of $A\bar{\nu}/c$, i.e. for the data measured at large values of the concentration c_t of total ligand. To give some insight into their behavior, CV_x, CV_y and ρ are plotted in Fig. 3 against $A\bar{\nu}/c$ (we have set here $CV_t = CV_A = CV_v = 2\%$ and $\Delta = 0$). The coefficients of variation CV_x and CV_y remain rather constant within a large range of $A\bar{\nu}/c$ values, CV_x being slightly smaller than CV_y. The large values of ρ show, as expected, that the data in the abscissa and ordinate of the Scatchard plot are highly correlated.

DISCUSSION

Blood plasma proteins, and albumin in particular, bind significant fractions of many drugs [21]. Since the bound fraction is physiologically ineffective, an assay of the level of free drug in the blood becomes imperative in order to determine the proper dosage. In the present paper the affinity and capacity of HPA for sebacate, a substance proposed recently as energy substrate in total parenteral nutrition, was investigated.

The non-linear pattern of binding data on the Scatchard plane (Fig. 1) indicates that defatted HPA contains at least two classes of binding sites with different affinity constants for sebacic acid. Moreover, as the concentration of competing monocarboxylic acid is raised, the bound sebacic acid concentration decreases markedly. The model of Eqns 1 and 2, although of a simplified form, appears to fit all the data reasonably well, and reveals the presence of a single site with high affinity plus several low affinity sites, as also found for many other drugs [21]. Number and affinity constants of low affinity sites were estimated with greater uncertainty, as seen from the SE of the estimates. Association constants of decanoic acid, k_1' and k_2', are 3–4-fold larger than the corresponding constants of decanedioic acid. The stepwise equilibrium model and the independent site model gave a comparable fitting on the present data.

Recently, Tonsgard *et al.* [13] reported the values of dissociation constants for the binding of some saturated straight-chain dicarboxylic acids to BSA. In particular, octadecanedioic, hexadecanedioic, tetradecanedioic, dodecanedioic and decanedioic acids were investigated. These authors found for decanedioic acid, in the absence of competition, a single binding site (0.8 mol/mol BSA) with a dissociation constant of 31.5×10^{-6} M. Our data revealed two classes of binding site in HPA, the dissociation constant of the high affinity site being $1/k_1 = 27.1 \times 10^{-6}$ M, with $n_1 = 0.81$ mol/mol HPA. The values of k_1' and k_2' estimated by us are consistent with the value of the largest stoichiometric constant K_1 found in Ref. 15 for the binding of decanoic acid to HPA in the absence of competition.

Binding of sebacic acid in serum appears to be adequately represented by a simple model with a single class of low affinity sites. It is likely that the competitors of sebacic acid present in serum (e.g. fatty acids and bilirubin) prevent binding to the high affinity site found in defatted albumin, so that only

the low affinity sites remain available for sebacic acid binding in serum. Also for dodecanedioic acid, it was found that bilirubin inhibits binding to the high affinity site of defatted albumin [14]. The spread of the data in Fig. 2 is probably due to the different concentrations of sebacic acid competitors present in the samples examined. Thus, the number of available low affinity sites appears to be in the range of three to five.

The statistical analysis in the Appendix shows that the quantities reported in the abscissa and ordinate of the Scatchard plot are strongly correlated and have a variable coefficient of variation (Fig. 3), so that the usual assumption of constant CV in binding data [22] should be considered with caution. We note also that error sources not considered in the present analysis (e.g. an increase in CV_t at low concentrations of total ligand) could lead to an increase in CV_x and CV_y at large $A\bar{v}/c$ values. As observed by Johnson [23], the maximum likelihood estimation of binding parameters in the presence of experimental uncertainties in the independent variables and of correlations would require the determination of "optimal" values for the independent variable. The estimation procedure here adopted [20] is an approximated version of maximum likelihood that avoids the determination of these values.

In conclusion, only one high affinity site is present on the HPA molecule for sebacic acid, other sites having a much lower affinity. Affinity and number of available sites for sebacic acid appear to be smaller than for decanoic acid. Thus, it seems likely that sebacic acid is vehicled by HPA to a lesser extent than decanoic acid, which is contained in medium-chain triglyceride emulsions used in total parenteral nutrition. However, the results obtained from the study of sebacic acid binding in serum suggest that the ratio of bound to total sebacic acid in serum can still be high at concentrations suitable for total parentral nutrition (it could be found, on average, a ratio bound/total $\simeq 30\%$ at a concentration of free sebacic acid in serum of 5 mM).

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APPENDIX

Analysis of measurement errors

Let z_1 and z_2 denote the number of counts per minute (dpm) measured from the albumin-containing compartment of the equilibration chamber and from the other compartment, respectively; denote by c_t the initial total

concentration (M) of sebacic acid, added initially to one side of the chamber. Determine the specific activity by $\sigma = (z_1 + z_2)/c_t$ (dpm/M); the measured value of the free ligand concentration c (M) is obtained as z_2/σ and the measured value of the bound ligand concentration c_b (M) as $(z_1 - z_2)/\sigma$ (experimental design "C" in [24]). Thus, the measured $\bar{\nu}$ and the measured $\bar{\nu}/c$ of the Scatchard plot, denoted as z_x and z_y, respectively, are given by

$$z_x = \frac{z_1 - z_2}{z_1 + z_2}\frac{c_t}{A} \tag{A.1}$$

$$z_y = \frac{z_1 - z_2}{z_2}\frac{1}{A} \tag{A.2}$$

where A is the total albumin concentration. Incomplete filling of a compartment of the equilibration chamber contributes to errors on c_t and A.

In order to obtain approximate expressions for the variances of z_x and z_y and their covariance we linearize the right hand side of Eqns A.1 and A.2 around the true values, following the approach in Ref. 25. Let v_1 and v_2 be the volumes (measurement volumes) removed for counting from the albumin-containing compartment and from the other compartment, respectively. Then z_1 and z_2 can be written as $z_1 = kv_1(c + c_b) + \zeta_1$ and $z_2 = kv_2c + \zeta_2$, where k is a proportionality constant (related to the radioactivity level) and ζ_1, ζ_2 are independent zero mean random variables. Therefore, the data z_x and z_y are actually functions of the random quantities ζ_1, ζ_2, v_1, v_2, c_t and A.

The linearization of Eqns A.1 and A.2 is performed using the above expressions for z_1 and z_2. After some computations, the following expressions for variances and covariance of the data are obtained:

$$\mathrm{var}(z_x) = x^2(CV_t^2 + CV_A^2) + 4x^2(2CV_v^2 + \Delta)\left(\frac{Ay + 1}{Ay(Ay + 2)}\right)^2 \tag{A.3}$$

$$\mathrm{var}(z_y) = y^2CV_A^2 + y^2(2CV_v^2 + \Delta)\left(1 + \frac{1}{Ay}\right)^2 \tag{A.4}$$

$$\mathrm{cov}(z_x, z_y) = xyCV_A^2 + 2xy(2CV_v^2 + \Delta)\frac{(Ay + 1)_2}{(Ay)^2(Ay + 2)}. \tag{A.5}$$

In the above expressions, x, y denote the true values of z_x, z_y (i.e. x is $\bar{\nu}$ and y is $\bar{\nu}/c$); CV_t, CV_A and CV_v indicate, respectively, the coefficients of variation of c_t, A and v_1 (taken equal to the CV of v_2); Δ is the sum of the square of coefficient of variation of z_1 plus the square of coefficient of variation of z_2. It can be seen that, if the measured dpms exceed 10^4 (as in the present experiments) with adequate counting time, the quantity Δ can be neglected with respect to $2CV_v^2$ in Eqns A.3–A.5 for errors on volumes larger than 2–3%. Assuming that Δ is negligible and that CV_t, CV_A and CV_v are constant, the coefficients of variation of z_x ($CV_x = \sqrt{\mathrm{var}(z_x)}/x$) and of z_y ($CV_y = \sqrt{\mathrm{var}(z_y)}/y$), and the correlation coefficient between z_x and z_y [$(\rho = \mathrm{cov}(z_x, z_y)/\sqrt{\mathrm{var}(z_x)\mathrm{var}(z_y)})$], are thus expressed as a function of $Ay = c_b/c$ only (see Fig. 3).