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# Comparison of non-linear and linear models for estimating haemoglobin adduct stability

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According to the kinetic theory for the build-up and elimination of haemoglobin (Hb) adducts, unstable Hb adducts are simultaneously eliminated by zero-order Hb turnover and first-order chemical instability. Thus, the elimination of unstable Hb adducts is non-linear with respect to time. Nonetheless, many studies of Hb adduct stability have characterized the elimination of Hb adducts using linear zero-order or linear first-order models. This paper demonstrates the use of non-linear regression to estimate the first-order rate constant of Hb adduct instability ($k$) using data on the elimination of Hb adducts in rats dosed with benzene or *ortho*-toluidine. Results obtained using non-linear regression models are compared with results from the more commonly employed zero- and first-order linear models. It is shown that exposure estimates based on measured levels of unstable Hb adducts can be severely biased if zero-order turnover is assumed. Furthermore, based on published data, estimates of $k$ are subject to estimated relative biases in the range of −4% to 96% when first-order linear models are used to characterize Hb adduct instability.

*Keywords*: haemoglobin adducts, adduct stability, mathematical models, non-linear regression

*Abbreviations*: α, daily increment in adduct concentration in nmol $g^{-1}$ $d^{-1}$ or ng $mg^{-1}$ $d^{-1}$, $A(t)$, concentration of haemoglobin adducts at time $t$, in nmol $g^{-1}$; $A_0$, haemoglobin adduct concentration at $t=0$; d, days; Hb, haemoglobin; $k$, first-order rate constant for adduct instability; $t_{er}$, lifetime of the erythrocyte.



## Introduction

The use of protein adducts to assess the internal dose of electrophilic chemicals and metabolites was first proposed by Ehrenberg *et al.* (1974). Haemoglobin (Hb) adducts have since been widely used for dosimetry because Hb is abundant and long-lived *in vivo* (lifetimes of weeks or months). Long-lived biomarkers tend to damp exposure variability by reacting more slowly to exposure fiuctuations than short-lived biomarkers such as urinary metabolites (half lives in hours) (Rappaport 1985, 1991, Rappaport and Spear 1988, Droz and Wu 1991). This damping of exposure variability reduces the number of measurements required to obtain valid estimates of long-term mean exposure, which is of primary importance in risk assessment (Rappaport 1985, 1991).

The longevity of Hb adducts depends upon their chemical stability *in vivo*.

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Stable Hb adducts are eliminated by linear, zero-order kinetics determined by the constant lifespan of the red blood cell ($t_{er}$), which is approximately 120 days in humans, 60 days in rats, and 40 days in mice. Unstable Hb adducts are eliminated more rapidly than would be expected based on $t_{er}$, because they are simultaneously lost to zero-order Hb turnover and first-order chemical instability. While numerous Hb adducts appear to be stable *in vivo*, including those from ethylene oxide (Osterman-Golkar *et al*. 1976), butadiene (Sun *et al*. 1989), and fluoranthene (Gorelick *et al*. 1989), others appear to be unstable, notably those from 4-aminobiphenyl (Maclure *et al*. 1990), *ortho*-toluidine (DeBord *et al*. 1992), 4,4′-methylene-bis(2-chloroaniline) (Cheever *et al*. 1990), naphthalene (Cho *et al*. 1994), and benzo(*a*)pyrenediolepoxide (Viau *et al*. 1993). Appropriate statistical methods for estimating the first-order rate constant of Hb adduct instability ($k$) have not been described.

In this paper, we review the available mathematical models describing the kinetics of unstable Hb adduct build-up and elimination (Osterman-Golkar *et al*. 1976, Segerback *et al*. 1978, Bergmark *et al*. 1990, Fennell *et al*. 1992, Granath *et al*. 1992) and describe statistical methods for estimating $k$. While the zero-order elimination of stable Hb adducts is linear with respect to time, the mixed zero- and first-order elimination of unstable Hb adducts is non-linear. As a result, we use non-linear regression methods to estimate $k$ for benzene-derived Hb adducts (Troester *et al*. 2000) and *ortho*-toluidine-derived Hb adducts (DeBord *et al*. 1992).



## Methods

### *Data*

In a previous study (Troester *et al*. 2000), 36 male F-344 rates received a single oral dose of 400 mg [U-$^{14}$C]/[$^{13}C_6$]benzene per kg body weight. Four animals were sacrificed at 4 h, 8 h, 12 h, 16 h, 1, 2, 7, 14, and 21 d after dosing. The concentrations of benzene oxide adducts with cysteinyl residues in Hb (BO–Hb) were determined using gas chromatography–mass spectrometry (GCMS) (Waidyanatha *et al*. 1998). Concentrations of benzene-derived radiobound adducts with Hb ([$^{14}$C]B–Hb) were determined by scintillation counting.

In a separate study (DeBord *et al*. 1992), 36 Sprague–Dawley rats received a single intraperitoneal dose of 100 mg [$^{14}$C]*ortho*-toluidine per kg body weight. At least six animals were sacrificed at 4 h, 24 h, 1 week, 2 weeks, and 4 weeks after dosing. Total *ortho*-toluidine-derived radiobound Hb adducts ([$^{14}$C]OT–Hb) were measured by scintillation counting. (These data for the *in vivo* elimination of [$^{14}$C]*ortho*-toluidine Hb adducts over time were kindly provided by Dr D. Gayle DeBord.) Data on body weight at sacrifice were not available for these animals.

### *Mathematical relationships: elimination of Hb adducts following acute exposure*

Following acute exposure, the concentration of Hb adducts decreases over time due to zero-order Hb turnover and first-order chemical instability. The overall rate of disappearance of Hb adducts can be described by the general differential equation

$$\frac{dA(t)}{dt} = -\frac{A_0}{t_{er}}e^{-kt} - kA(t) \quad (1)$$

where $A(t)$ is the concentation of adducts (nmol adduct per g of Hb or ng of adduct per mg of Hb) at time $t$ (in days, d), $A_0$ is the initial adduct concentration at $t = 0$, $t_{er}$ represents the lifetime of the erythrocyte (in d), and $k$ (in d$^{-1}$) is the first-order rate constant for adduct instability. Equation (1) differs slightly from a previously reported differential equation for unstable Hb adduct turnover (Granath *et al*. 1992); however, its solution has been reported previously (Bergmark *et al*. 1990, Fennell *et al*. 1992, Granath *et al*. 1992):

$$A(t) = A_0\left(1 - \frac{t}{t_{er}}\right)e^{-kt}, \text{ for } 0 < t < t_{er} \quad (2)$$

Equation (2) is non-linear with respect to time when $k > 0$; but when Hb adducts are stable ($k = 0$), equation (2) reduces to the linear, zero-order (with respect to $k$) equation

$$A(t) = A_0\left(1 - \frac{t}{t_{er}}\right), \text{ for } 0 < t < t_{er} \text{ (Granath } et\ al. \text{ 1992)} \quad (3)$$

Many studies have applied equation (3) or the following first-order (with respect to $k'$) log-linear model to describe Hb adduct elimination (Cheever *et al.* 1990, DeBord *et al.* 1992, Viau *et al.* 1993, Cho *et al.* 1994):

$$\ln\ A(t) = \ln\ A_0 - k't \quad (4)$$

where $k'$ represents the rate constant for overall first-order adduct elimination. Equation (4) is generally not a good approximation for equation (2).

*Mathematical relationships: buildup of Hb adducts following continuous exposure*

If exposure is chronic or continuous, there is a daily increase in adduct concentration (denoted $\alpha$, with units nmol $g^{-1}$ $d^{-1}$). The rate of build-up of adducts subject to mixed zero- and first-order elimination can be described by the following differential equation:

$$\frac{dA(t)}{dt} = \alpha\left(1 - \frac{t}{t_{er}}\right)e^{-kt} \quad (5)$$

which has the solution:

$$A(t) = \frac{-\alpha}{k^2 t_{er}}(1 - kte^{-kt} - e^{-kt}) + \frac{\alpha}{k}(1 - e^{-kt}), \text{ for } 0 < t < t_{er} \quad (6)$$

From equation (6), the concentration of Hb adducts increases over time to a steady state ($A_{s.s.}$), where the rates of production and elimination of adducts are equal. Hb adducts reach steady state concentration after $t_{er}$ days of exposure (since $\frac{dA(t_{er})}{dt} = 0$); thus, by substituting $t = t_{er}$ into equation (6),we have

$$A(t_{er}) = A_{s.s} = \frac{\alpha}{k}\left(1 - \frac{1 - e^{-kt_{er}}}{kt_{er}}\right) \quad (7)$$

Equation (7) is similar to a previously reported equation for estimating $A_{s.s.}$ given unstable Hb adducts and continuous exposure (Granath *et al.* 1992).

The mean daily blood concentration of electrophile (denoted $\mu_{RX}$ with units nmol $l^{-1}$) is defined as the area under the time–concentration curve for 1 day. Equation (7) can be used to estimate $\mu_{RX}$ by acknowledging the following relationship (Ehrenberg *et al.* 1974):

$$\alpha = \frac{[R - Hb]}{[Hb]} = k_{R-Hb}\mu_{RX} \quad (8)$$

Table 1. Estimated lifetime of the erythrocyte ($\hat{t}_{er}$) for various rat strains (summarized from Derelanko *et al.* 1989).

| Rat Strain | Estimated $t_{er}$ | 95 % Confidence limits for $t_{er}$ |
|---|---|---|
| F-344 | 66.3 | (62.4, 70.2) |
| Wistar | 59.8 | (54.6, 64.9) |
| Sprague–Dawley | 61.0 | (57.9, 64.1) |



where $k_{R\text{-}Hb}$ represents the second-order reaction rate constant for reaction of electrophile RX with nucleophile site on Hb [with units l(g of Hb)$^{-1}$ (d$^{-1}$)]. Substituting $\alpha = k_{R\text{–}Hb} \cdot \mu_{RX}$ into equation (7) leads to the following equation for $\mu_{RX,unstable}$:

$$\mu_{RX,unstable} = \frac{kA_{s.s.}}{k_{R\text{–}Hb}} \left(1 - \frac{1 - e^{-kt_{er}}}{kt_{er}}\right)^{-1} \quad (9)$$

Equation (9) differs substantially from the analogous dosimetric model for stable adducts:

$$\mu_{RX,stable} = \frac{2A_{s.s.}}{k_{R\text{–}Hb}\, t_{er}} \text{ (Osterman-Golkar } et\ al. \text{ 1976)} \quad (10)$$

*Statistical analysis*

For all data sets involving acute exposure, only those time points including and subsequent to the peak adduct measurement were included in statistical analyses, because prior points are not relevant to determination of adduct stability. Non-linear least-squares regression using equation (2) was conducted using SAS statistical software (Cary, NC) to obtain estimates $\hat{A}_0$ and $\hat{k}$. [For discussion of non-linear regression in SAS, see Gallant (1987).]

Non-linear regression requires that initial guesses of parameter values be supplied, so starting values of $A_0$ and $k$ were chosen by visual inspection of a plot of adduct concentration vs time. While it is theoretically possible to estimate $t_{er}$ using non-linear regression (equation (2)), Hb adduct stability studies generally involve multiple measurements at only a few (< 10) time points. Non-linear regression can yield uninformative parameter estimates, particularly when the number of unknown parameters is large compared with the size of the data set (Gallant 1987). Furthermore, treating $t_{er}$ as a known constant is consistent with current practice in protein adduct dosimetry. Thus, for non-linear regression using equation (2), $t_{er}$ was specified *a priori*. However, since the estimate of $t_{er}$ in each strain of rat (table 1, Derelanko 1987) is associated with some uncertainty, non-linear regression was conducted using the point estimate of $t_{er}$ for the strain as well as both the upper and lower 95 % confidence interval values for $t_{er}$. This approach allows assessment of the sensitivity of $\hat{k}$ to the value used for $t_{er}$.

We also analysed all data sets using the linear zero-order model (equation (3)) which assumes adduct stability. Using this model, the estimated intercept $\hat{A}_0$ and slope $\hat{\beta}$ obtained by linear least-squares regression of adduct level on time can be used to calculate $\hat{t}_{er}$ according to the following equation:

$$\hat{t}_{er} = -\frac{\hat{A}_0}{\hat{\beta}} \quad (11)$$

Using equation (11), an approximate 95 % confidence interval for $t_{er}$ is calculated as:

$$\exp\{\text{In}(\hat{t}_{er}) \pm 1.96\ \widehat{\text{SE}}[\ln(\hat{t}_{er})]\} \quad (12)$$

Table 2. Estimated parameters for non-linear regression (equation (2)) of benzene-derived hemoglobin adduct level on time[a,b].

| | | No body weight adjustment | | Body weight adjustment[c] | |
|---|---|---|---|---|---|
| | $t_{er}$ (d) | $\hat{A}_0$ (nmol g$^{-1}$) | $\hat{k} \times 10^3$ (d$^{-1}$) | $\hat{A}_0$ (nmol g$^{-1}$) | $\hat{k} \times 10^3$ (d$^{-1}$) |
| [$^{14}$C]B–Hb | 70 | 135.4 (112.7, 158.1) | 11.8 (–4.1, 28) | 134.2 (110.1, 158.3) | 9.4 (–7.3, 26) |
| | 66 | 135.2 (112.5, 157.9) | 10.6 (–5.4, 27) | 134.0 (110.0, 158.1) | 8.1 (–8.6, 25) |
| | 62 | 135.0 (112.3, 157.7) | 9.1 (–6.9, 25) | 133.8 (109.8, 157.9) | 6.7 (–10, 23) |
| BO–Hb | 70 | 41.9 (35.8, 48.1) | 6.6 (–9.0, 22) | 41.5 (35.0, 47.9) | 3.8 (–12, 20) |
| | 66 | 41.9 (35.7, 48.0) | 5.4 (–10, 21) | 41.4 (35.0, 47.9) | 2.6 (–14, 19) |
| | 62 | 41.8 (35.7, 48.0) | 4.0 (–12, 20) | 41.4 (35.0, 47.8) | 1.2 (–15, 17) |

[a] $t_{er}$ = lifetime of erythrocyte, $\hat{A}_0$ = estimated adduct concentration at $t = 0$, $\hat{k}$ = estimated rate constant of Hb adduct instability.

[b] Parameter estimate (approximate 95 % confidence limits).

[c] Body weight adjustment was conducted using equation (14).

and the estimated standard error ($\widehat{SE}$) of $\ln(\hat{t}_{er})$ is calculated as:

$$\widehat{SE}[\ln(\hat{t}_{er})] \approx \sqrt{\frac{\hat{V}(\hat{A}_0)}{(\hat{A}_0)^2} + \frac{\hat{V}(\hat{\beta})}{(\hat{\beta})^2} - \frac{2\ \hat{Cov}(\hat{A}_0, \hat{\beta})}{(\hat{A}_0)(\hat{\beta})}} \tag{13}$$

where $\hat{V}$ and $C\ v$ denote, respectively, the estimated variances of, and the estimated covariance between, the regression parameter estimates $\hat{A}_0$ and $\hat{\beta}$ (Troester *et al.* 2000).

*Adjusting for body weight change in time course adduct stability studies*

For our analysis of benzene-derived adduct stability, calculations similar to those described in Osterman-Golkar *et al.* (1999) were conducted to assess the impact of changes in the body weight of animals on the estimates of $k$. For example, 21 days after exposure to [$^{14}$C]/[$^{13}C_6$]benzene (Troester *et al.* 2000), animals had increased in body weight from 251.8 – 2.1 g (SE) to 272.0 – 3.2 g (SE). For each animal, the body-weight (b.w.) adjusted adduct level [$A(t)_{adj}$ in nmol g$^{-1}$] was calculated from measured adduct levels [$A(t)$ in nmol g$^{-1}$] according to the following:

$$A(t)_{adj} = A(t)\frac{b.w._{(sacrifice)}}{b.w._{(dosing)}} \tag{14}$$

Non-linear regression of $A(t)_{adj}$ on days was conducted as described above.

## Results

### *Non-linear model for Hb adduct stability*

For [$^{14}$C]B–Hb and BO–Hb, the parameter estimates $\hat{A}_0$ and $\hat{k}$ obtained by non-linear least-squares regression (equation (2)) are shown in table 2. For the range of plausible values of $t_{er}$ in the F-344 rat ($62 \le t_{er} \le 70$ d), the value of $\hat{k}$ for [$^{14}$C]B–Hb ranged from $9.1 \times 10^{-3}$ d$^{-1}$ (given $t_{er} = 62$ d) to $11.8 \times 10^{-3}$ d$^{-1}$ (given $t_{er} = 70$ d). For all values of $t_{er}$, the 95 % confidence interval for $k$ includes zero. Similarly, the value of

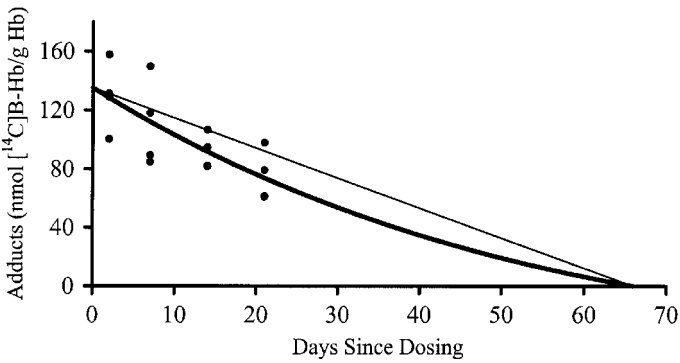


Figure 1. Elimination of [$^{14}$C]B–Hb adducts following administration of 400 mg [$^{14}$C]/[$^{13}C_6$]benzene per kg body weight to male F–344 rats. Each data point represents the measured concentration of adducts (nmol [$^{14}$C]B–Hb per g Hb) for a single animal. The heavy line represents the fitted non-linear model (Equation (2)) where $t_{er} = 66$ days, $\hat{k} = 10.6 \times 10^{-3}$ d$^{-1}$, and $\hat{A}_0 = 135.2$ nmol g$^{-1}$. The lighter line represents a theoretical linear, zero-order (in $k$) model where $t_{er} = 66$ days, $k = 0$ d$^{-1}$, and $A_0 = 135.2$ nmol g$^{-1}$.

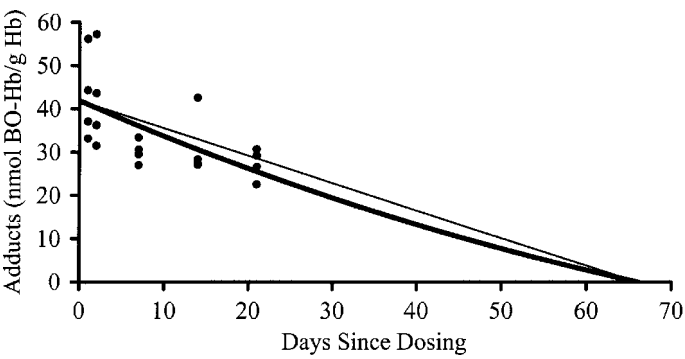


Figure 2. Elimination of BO–Hb adducts following administration of 400 mg [$^{14}$C]/[$^{13}C_6$]benzene per kg body weight to male F–344 rats. Each data point represents the measured concentration of adducts (nmol BO–Hb per g Hb) for a single animal. The heavy line represents the fitted non-linear model (Equation (2)) where $t_{er} = 66$ days, $\hat{k} = 5.4 \times 10^{-3}$ d$^{-1}$, and $\hat{A}_0 = 41.9$ nmol g$^{-1}$. The lighter line represents a theoretical linear, zero-order (in $k$) model where $t_{er} = 66$ days, $k = 0$ d$^{-1}$, and $A_0 = 41.9$ nmol g$^{-1}$.

$\hat{k}$ for BO–Hb ranges from $4.0 \times 10^{-3}$ d$^{-1}$ (given $t_{er} = 62$ d) to $6.6 \times 10^{-3}$ d$^{-1}$ (given $t_{er} = 70$ d), with all 95 % confidence intervals including zero. Figure 1 and figure 2 illustrate the fitted non-linear curves (equation (2)) for [$^{14}$C]B–Hb (given $t_{er} = 66$ days, $\hat{k} = 10.6 \times 10^{-3}$ d$^{-1}$, $\hat{A}_0 = 135.2$ nmol g$^{-1}$) and BO–Hb (given $t_{er} = 66$ days, $\hat{k} = 5.4 \times 10^{-3}$ d$^{-1}$, $\hat{A}_0 = 41.9$ nmol g$^{-1}$) and a hypothetical straight-line model (equation (3)) where $t_{er} = 66$, $k = 0$, and $A_0 = 135.2$ for [$^{14}$C]B–Hb, and $t_{er} = 66$, $k = 0$, and $A_0 = 41.9$ nmol g$^{-1}$ for BO–Hb. The hypothetical model is presented to allow visual comparison between the cases where $k = 0$ (stable adducts) and $k > 0$ (unstable adducts).

Table 3 presents estimates for the rate constant $k$ of [$^{14}$C]OT–Hb instability based on non-linear regression. In this case, the estimates of $k$ are positive throughout the plausible range of $t_{er}$, with $\hat{k}$ ranging from $42 \times 10^{-3}$ d$^{-1}$ (given

Table 3. Estimated parameters for non-linear regression (equation (2)) of *ortho*-toluidine-derived haemoglobin adduct level ([$^{14}$C]OT–Hb) on time[a,b].

| $t_{er}$ (d) | $\hat{A}_0$ (ng mg$^{-1}$) | $\hat{k} \times 10^3$ (d$^{-1}$) |
|---|---|---|
| 64 | 23.8 (20.8, 26.9) | 44 (27, 62) |
| 61 | 23.8 (20.8, 26.9) | 43 (25, 61) |
| 58 | 23.8 (20.8, 26.8) | 42 (24, 60) |

[a] $t_{er}$ = lifetime of erythrocyte, $\hat{A}_0$ = estimated adduct concentration at $t = 0$, $\hat{k}$ = estimated rate constant of Hb adduct instability.

[b] Parameter estimate (approximate 95 % confidence limits).



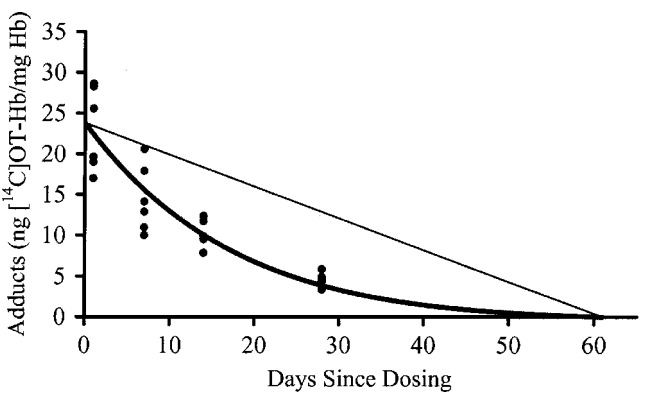


Figure 3. Elimination of [$^{14}$C]OT–Hb adducts following administration of 100 mg [$^{14}$C]*ortho*-toluidine per kg body weight to male Sprague–Dawley rats. Each data point represents the measured concentration of adducts (ng [$^{14}$C]OT–Hb per g Hb) for a single animal. The heavy line represents the fitted non-linear model (Equation (2)) where $t_{er} = 61$ days, $\hat{k} = 4.3 \times 10^{-2}$ d$^{-1}$, and $\hat{A}_0 = 23.8$ ng mg$^{-1}$. The lighter line represents a theoretical linear, zero-order (in $k$) model where $t_{er} = 61$ days, $k = 0$ d$^{-1}$, and $A_0 = 23.8$ ng mg$^{-1}$.

$t_{er} = 58$ d) to $44 \times 10^{-3}$ d$^{-1}$ (given $t_{er} = 64$ d). In all cases, the 95 % confidence intervals for $k$ exclude zero. Figure 3 illustrates the fitted non-linear curves (equation (2)) for [$^{14}$C]OT–Hb (given $t_{er} = 61$ days, $\hat{k} = 4.3 \times 10^{-2}$ d$^{-1}$, $\hat{A}_0 = 23.8$ ng mg$^{-1}$) and a hypothetical straight-line (equation (3)) model where $t_{er} = 61$, $k = 0$, and $A_0 = 23.8$ ng mg$^{-1}$. Again, the hypothetical straight-line model is presented to allow visual comparison between the situations when $k = 0$ and $k > 0$.

### *Linear model for Hb adduct stability*

Based upon linear regression (equation (3)), the parameter estimates $\hat{A}_0$ and $\hat{\beta}$ were obtained for [$^{14}$C]B–Hb, BO–Hb, and [$^{14}$C]OT–Hb (table 4). From $\hat{A}_0$ (in nmol g$^{-1}$) and $\hat{\beta}$ (in nmol g$^{-1}$ d$^{-1}$) for [$^{14}$C]B–Hb, $\hat{t}_{er}$ (d) was estimated (equation (11)) to be 46.8 d (with 95 % confidence interval of 32.0 to 68.4 d). From $\hat{A}_0$ (in nmol g$^{-1}$) and $\hat{\beta}$ (in nmol g$^{-1}$ d$^{-1}$) for BO–Hb, $\hat{t}_{er}$ was estimated as 55.6 d (with 95 % confidence interval of 33.1 to 93.5 d). From $\hat{A}_0$ (in ng mg$^{-1}$) and $\hat{\beta}$ (in ng mg$^{-1}$ d$^{-1}$) for [$^{14}$C]OT–Hb, $\hat{t}_{er}$ was estimated as 33.1 d (with 95 % confidence interval of 27.9 to 39.2 d).

Table 4. Estimated parameters for linear regression (equation (3)) of benzene- and *ortho*-toluidine-derived haemoglobin adduct level on time[a,b].

| | $\hat{A}_0$ (nmol g$^{-1}$) | $\hat{\beta}$ [nmol/(g$^{-1}$d$^{-1}$)] | $\hat{t}_{er}$ (d) |
|---|---|---|---|
| [$^{14}$C]B–Hb[c] | 133 (115, 151) | –2.84 (–4.23, –1.45) | 46.8 (32.0, 68.4) |
| BO–Hb[c] | 41.4 (36.1, 46.7) | –0.74 (–1.19, –0.29) | 55.6 (33.1, 93.5) |
| OT–Hb | 21.0 (18.5, 23.5) | –0.63 (–0.79, –0.48) | 33.1 (27.9, 39.2) |

[a] $\hat{A}_0$ = estimated intercept, $\hat{\beta}$ = estimated slope, $\hat{t}_{er}$ = estimated lifetime of erythrocyte.
[b] Parameter estimate (95 % confidence limits).
[c] These parameter estimates were originally reported in Troester *et al.* (2000).

Table 5. Impact of adduct instability on calculated values of mean daily blood concentration of electrophile ($\mu_{RX}$ with units nmol l$^{-1}$) in humans.

| $k$ (in d$^{-1}$) | $t_{er}$ (in d) | Ratio of $\mu_{RX,unstable}$ (equation (9)) to $\mu_{RX,stable}$ (equation (10)) |
|---|---|---|
| 0 | 120 | 1.0 |
| 0.01 | 120 | 1.4 |
| 0.03 | 120 | 2.5 |
| 0.06 | 120 | 4.2 |
| 0.10 | 120 | 6.5 |

### *Impact of body-weight adjustment*

Table 2 also illustrates the impact that adjusting for changes in the body weight of experimental animals has on estimates of $A_0$ and $k$. Using adjusted benzene-derived adduct values for body weight based on equation (14), non-linear regression yielded values of $\hat{k}$ for [$^{14}$C]B–Hb that ranged from $6.7 \times 10^{-3}$ d$^{-1}$ (given $t_{er} = 62$ days) to $9.4 \times 10^{-3}$ d$^{-1}$ (given $t_{er} = 70$ days). For BO–Hb, the values of $\hat{k}$ ranged from $1.2 \times 10^{-3}$ d$^{-1}$ (given $t_{er} = 62$ days) to $3.8 \times 10^{-3}$ d$^{-1}$ (given $t_{er} = 70$ days).

## Discussion

A major impetus for use of Hb adducts is to predict mean daily blood concentrations of reactive electrophiles ($\mu_{RX}$) in exposed humans. However, $\mu_{RX}$ can be substantially underestimated if Hb adducts are incorrectly assumed to be stable, even when the true rate constant of Hb adduct instability is small. This is illustrated in table 5, which shows the ratios of $\mu_{RX,unstable}$ to $\mu_{RX,stable}$ in humans (where $t_{er}$ is approximately 120 days) for hypothetical rates of adduct instability represented by $k = 0$, 0.01, 0.03, 0.06, and 0.10 d$^{-1}$. Within this modest range of $k$ values, $\mu_{RX,unstable}$ (equation (9)) can be up to 6.5 times larger than $\mu_{RX,stable}$ (equation (10)).

The mathematical theory of unstable Hb adducts dictates that, following acute exposure, adduct loss should follow a curve that is non-linear with respect to time (equation (2)). However, many studies of Hb adducts have used linear regression to evaluate Hb adduct stability. If the fit of the linear zero-order model (equation (3)) was inadequate for describing elimination of the adduct, then simple first-order elimination (equation (4)) was assumed (Cheever *et al.* 1990, DeBord *et al.*, 1992, Viau *et al.* 1993, Cho *et al.* 1994). However, equation (4) can lead to biased

Table 6. Estimates of the rate constant for adduct instability based on equation (2) and equation (4).

| Chemical administered (Reference) | Species[a] | $\hat{k}$ ($d^{-1}$)[b,c] (equation (2)) | Reported $\hat{k}'$ ($d^{-1}$) (equation (4)) | Estimated relative bias $\left(\frac{\hat{k}' - \hat{k}}{\hat{k}}\right)$ |
|---|---|---|---|---|
| Naphthalene (Cho *et al.* 1994) | Mouse ($n = 7$) | 0.059 (–0.028, 0.147) | 0.060 | 0.02 |
| *Ortho*-toluidine (DeBord *et al.* 1992) | Rat ($n = 24$) | 0.043 (0.025, 0.061) | 0.056 | 0.30 |
| Benzo(*a*)pyrenediolepoxide (Viau *et al.* 1993) | Rat ($n = 6$) | 0.068 (0.042, 0.094) | 0.065 | –0.04 |
| 4,4´-Methylene-bis(2-chloroaniline) (Cheever *et al.* 1990) | Rat ($n = 6$) | 0.025 (–0.001, 0.050) | 0.049 | 0.96 |

[a] To estimate $k$, $t_{er}$ was set at 40 days for the mouse study and 61 days for all three Sprague–Dawley rat studies.

[b] Non-linear regression was conducted using the full data set for *ortho*-toluidine only. Non-linear regressions for the remaining data sets were based on mean values at each time point; hence confidence intervals tended to be wider due to lower data.

[c] Given as: estimate, (approximate 95 % confidence limits).



estimates of $k$. Based on four published data sets, estimated relative biases in the range of –4 % to 96 % resulted when $k'$ was estimated rather than $k$ (table 6).

The stability of Hb adducts should be evaluated by applying the non-linear model given by equation (2), as illustrated for benzene-derived Hb adducts (table 2) and *ortho*-toluidine-derived Hb adducts (table 3). Benzene-derived adducts displayed only slight evidence of instability ($\hat{k} \leq 0.01$; 95 % C.I.s included 0), while the *ortho*-toluidine-derived adducts [$^{14}$C]OT–Hb displayed strong evidence of instability ($\hat{k} > 0.04$; 95 % C.I.s excluded 0). Figures 1 and 2 illustrate that it is difficult to distinguish the case where $k = 0$ from the case where $k$ is small and non-zero because of interanimal variability in measured adduct concentration. It becomes easier to differentiate between stable and unstable adducts as the rate constant of Hb adduct instability ($k$) increases (figure 3).

Despite the lack of statistical significance that can result when small, positive estimates of $k$ are obtained by non-linear regression, the data in table 4 demonstrate that assuming $k = 0$ and reverting to the linear model (equation (3)) is not advisable. The estimates of $t_{er}$ obtained using the linear model to describe the elimination of [$^{14}$C]B–Hb and BO–Hb (46.8 and 55.6 days, respectively) are unreasonable when compared with the literature values of $t_{er}$ for the F-344 rat (table 1).

The estimate $\hat{k}$ obtained by non-linear regression (equation (2)) is sensitive to the value of $t_{er}$ that is assumed. Thus, we recommend that authors report $\hat{k}$ as a range, bounded by values corresponding to the lower and upper 95 % confidence limits of $t_{er}$ for that strain or species (see table 1 for the rat). This more accurately reflects the uncertainty associated with estimation of $k$, an especially important consideration when $\hat{k}$ is extrapolated between species.

This study also examined the influence that body weight changes between the time of dosing and time of sacrifice have on estimating $k$. Over the course of a 4-week experiment, it is not uncommon for animals to gain between 10 and 20 % of their original body weight. This weight gain is accompanied by an increase in blood volume (and red blood cells) and can lead to dilution of the Hb adduct

concentration. Comparison of the estimates of $k$ reported in table 2 (with and without body weight adjustment) demonstrates that adjusting for body weight change can substantially alter the conclusions. The range of $\hat{k}$ values reported for BO–Hb before body weight adjustment ($4.0 \times 10^{-3}$ to $6.6 \times 10^{-3}$ $d^{-1}$) and after body weight adjustment ($1.2 \times 10^{-3}$ to $3.8 \times 10^{-3}$ $d^{-1}$) do not overlap. Similarly for [$^{14}$C]B–Hb, the ranges of $\hat{k}$ obtained before ($9.1 \times 10^{-3}$ to $11.8 \times 10^{-3}$ $d^{-1}$) and after $6.7 \times 10^{-3}$ to $9.4 \times 10^{-3}$ $d^{-1}$) body weight adjustment have very little overlap. We recommend that future adduct stability studies adjust for changes in the body weight of animals prior to conducting non-linear regression analysis.

Interpreting non-linear regression parameter estimates can be problematic when non-specific radiobinding has been used to measure all radiobound Hb adducts (Gorelick *et al.* 1989, Sun *et al.* 1989, Cheever *et al.* 1990, DeBord *et al.* 1992, Cho *et al.* 1994, Troester *et al.* 2000) rather than specific Hb adducts. This is because adduct stability has been shown to vary by amino acid and by metabolite (Osterman-Golkar *et al.* 1995, Troester *et al.* 2000). If the adduct population is non-homogeneous with respect to stability, the rate constant of adduct instability ($k$) can change over time as unstable adducts are eliminated and stable adducts persist. While the adduct instability rate constant of a heterogeneous adduct population can be estimated using equation (2), error and uncertainty are introduced by the fiuctuation of $k$ over time. Furthermore, since non-specific radiobinding is not commonly used to detect adducts in human populations, estimates of stability obtained by these methods are typically not useful for dosimetry.

Use of equation (9) for human exposure assessment depends upon the degree to which adduct instability can be extrapolated between animals and humans because direct estimates of $k$ in humans are seldom available. Future work should employ the quantitative methods presented here to compare the rate constants for Hb adduct instability ($k$) in humans and animals where data exists on both species. For example, the Hb adduct formed from 4-aminobiphenyl (4-ABP) demonstrated some instability in smokers enrolled in a withdrawal programme (Maclure *et al.* 1990), while a study of 4-ABP-Hb adduct stability in rats concluded that the adducts were stable (Green *et al.* 1984). The non-linear methods described here enable more precise quantitative comparisons of data in such studies.



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