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The Graduate School
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MODIFICATION OF A THREE DAY DEUTERATED RETINOL DILUTION EQUATION FOR THE ESTIMATION OF VITAMIN A STORES IN HUMANS

A Thesis in
Nutrition
by
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Isotope dilution analysis has been successfully used to estimate vitamin A stores in humans via the Furr-Olson deuterated retinol dilution (DRD) equation: total body vitamin A stores = $F \times \text{dose} \times \{S \times a \times [(1/D:H) - 1]\}$. The method requires a blood sample 21 days after isotope administration to determine D:H (deuterium:hydrogen ratio) in plasma; the equation assumes that $S$, $F$, and $a$ are constants. Because of the long wait time involved in using the current equation, the goal of this study was to create a modified DRD equation that would use a sample obtained 3 days after dose administration. This would be not only more cost effective, but it would limit the possibility of acute infections disrupting normal retinol levels. Compartmental analysis was used to simulate the $S$, $F$, and $a$ values at 3 days in both American and Chinese subjects, which have high and marginal vitamin A stores, respectively. From this analysis, $S$ and $a$ were both found to differ between subject groups showing that the values are affected by vitamin A stores at 3 days. Using these data, a regression equation was developed for factor $S$: $S = 0.05668 \times (D:H/\text{dose})^2 + 0.777 \times (D:H/\text{dose}) + 4.696$. Next, the $F$ and $a$ values were combined and an equation was developed for the newly created $Fa$ factor: $Fa = 0.7705e^{-0.0671 \times D:H/\text{dose}}$. Finally, these two factors were used in the new 3 day DRD equation: total body vitamin A stores = $Fa \times \text{dose} \times \{S \times [(1/D:H) - 1]\}$, and total reserves of vitamin A were estimated from this equation. For published data from humans of varying age, gender and ethnicity, vitamin A stores predicted by the new equation agree better with estimates from the original 21 day Furr-Olson equation than do values obtained using other published 3 day DRD equations. Thus the new equation provides an improved tool to determine vitamin A stores in humans.
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ABBREVIATION LIST

**VA**: Vitamin A

**VAD**: Vitamin A Deficiency

**RBP**: Retinol Binding Protein

**RDR**: Relative Dose Response

**MRDR**: Modified Relative Dose Response

**DRD**: Deuterated Retinol Dilution

**SA**: Specific Activity
Chapter 1

Introduction

The ability to accurately predict total vitamin A stores is very important for recognizing and correcting deficiencies before clinical manifestations develop, especially since most vitamin A-deficient people are within this sub-clinical deficiency range. In addition, the ability to determine the success of vitamin A interventions is imperative for creating effective strategies for decreasing the number of individuals with vitamin A deficiencies worldwide. Since vitamin A deficiency is a major problem in developing countries, a quick and cost effective method for estimating total vitamin A stores in humans is essential. The current Furr-Olson method requires a 21 day waiting period for sample equilibration, which is much too long. In addition to the long wait time, acute infections and diarrhea could alter plasma retinol levels, confounding results. In this study, a much shorter, 3 day prediction equation was developed that can estimate total vitamin A stores just as accurately as the Furr-Olson 21 day equation.
Chapter 2

Review of the Literature

Vitamin A status and measurements

Vitamin A is essential for growth, reproduction, immunity, vision, and cell differentiation [1-4]. Vitamin A deficiency (VAD) is one of the most prevalent health problems in developing countries, only behind iron deficiency anemia. It affects about 140 million children and 20 million per year in these countries [5]. The effects of VAD can be devastating and can result in xerophthalmia, which can lead to blindness, and possibly to death. It can also result in anemia and an increased risk of infection and illness, like measles or diarrhea [6-8]. Maternal night blindness can also increase the mortality risk for infants and leave them susceptible to their own VAD, if breastfed [9]. Because of these risk factors, decreasing VAD is of great importance.

With many supplementation programs aimed at increasing the amount of vitamin A in the body, an accurate measurement of vitamin A status and stores is extremely important.

In vitamin A-sufficient adults, the liver is the main storage site for vitamin A, accounting for about 90% of whole-body stores [10]. Thus, to find the best estimate of total vitamin A stores, a hepatic biopsy would be ideal. Several groups have analyzed liver samples from an autopsy or those taken at the time of surgery [11-15]. Although vitamin A is heterogeneously distributed throughout the liver [13, 15], a biopsy of the central portion of the right lobe can accurately predict total hepatic vitamin A concentration to within 15% [14]. But biopsies are rarely justifiable for field use, because of the risk and expense in obtaining them. Naturally, non-invasive physiological and clinical signs would be a useful tool in predicting vitamin A status.

In 1976, the International Vitamin A Consultative Group recommended that vitamin A deficiency be determined by the clinical signs of xerophthalmia [16]. Other physiological
indicators like night blindness, impression cytology, and dark adaptometry can also be used, but they can only be identified in severe vitamin A depletion. With only about 2% of VAD children (3.3 million) exhibiting clinical signs of vitamin A deficiency [7], sub-clinical vitamin A deficiency seems to be much more prevalent. Also, changes in total vitamin A levels cannot be measured or quantified by simply using physiological indicators.

Biochemical indicators serve as the best way to measure not only vitamin A status but also changes in the amount of total vitamin A. Serum retinol concentration is one of the most common ways to determine VAD [17], but it has several drawbacks. It is homeostatically controlled within a tight range. A decrease in serum retinol is not seen until liver reserves are almost non-existent [16]. In addition, increases are not always seen in supplementation interventions in low income groups because of a high risk for infection, which can alter serum retinol levels [18]. The status of other nutrients, especially iron deficiency [19, 20], may also alter plasma retinol levels.

A better estimate of the efficacy of interventions is the relative dose response (RDR) and the modified relative dose response (MRDR) tests [21]. As dietary retinol drops, the body deploys mechanisms to increase the conservation of the remaining retinol, and the rate of release of liver vitamin A reserves decreases. This creates an accumulation of retinol-binding protein (RBP) in the liver that will be released with vitamin A intake [22]. The RDR test involves taking a fasting blood sample and then obtaining a subsequent sample five hours after administration of retinyl ester. RBP/vitamin A (holo-RBP) will then be released from the liver. The more recent MRDR test uses 3,4 didehydroretinyl acetate, which is found at very low levels in human plasma [23], rather than retinyl ester, and therefore only one blood sample is needed. These methods have been used in many studies to diagnose sub-clinical VAD in various populations [16], and they are also useful in assessing supplementation interventions [24]. The main drawback, however, is the inability of these tests to quantify total-body reserves of vitamin A.
Isotope Dilution Technique

Isotope dilution techniques can provide more information than the MRDR test, while still only requiring one blood sample. The procedure involves administering an oral dose of tracer to subjects. The dose is usually 5-10 retinol equivalents of deuterium-labeled retinyl acetate (either D4 or D8). After sufficient mixing of the tracer with endogenous vitamin A, a blood sample is collected, and the ratio of tracee: tracer (H:D) is measured. A prediction equation is then used to determine the total amount of vitamin A stores based on that ratio. The original equation developed by Bausch and Reitz [25] was:

Total liver reserves = \( F \times \text{dose} \times (\text{H:D} - 1) \)

In this equation, \( F \) is the storage efficiency of the oral dose, which Bausch and Reitz estimated to be 0.5 [25, 26]. The dose is expressed in millimoles. The factor H:D is the ratio of retinol tracee (hydrogen) to tracer (deuterium), with the -1 correcting for the mass of the tracer that contributes to total-body vitamin A. This original equation was modified by Furr et al. to the currently-accepted deuterated retinol dilution (DRD) technique, also known as the Furr-Olson equation:

Total-body vitamin A pool size = \( F \times \text{dose} \times (S \times a \times [(\text{H:D})-1]) \)

In their research, Furr et al. determined that the plasma H:D ratio is affected by several factors and thus they modified the equation. First, the plasma retinol concentration is influenced by both dietary vitamin A and reserves. Thus the ratio of the specific activities of retinol in plasma versus liver was added, seen as factor \( S \). Hicks et al. estimated the value of \( S \) to be 0.65 over a range of total vitamin A levels in rats [27]. Also, because the tracer is being replaced by newly ingested vitamin A, the factor \( a \) was added to correct for catabolism. The factor \( a = e^{-kt} \) where \( k \) is \( \ln 2 / t_{1/2} \), the half-life of retinol is 140 days [10, 26] and \( t \) is the days since the dose.
In the original Bausch-Reitz paper, it was noted that the equilibration time for the administered dose would be 26 days in humans [25] but in the Furr-Olson work [12], it was estimated to be around 20-21 days. Haskell et al. [28] showed that vitamin A pool size does not affect the equilibration time. In the studies by Haskell et al., deuterium-labeled retinyl acetate was given to both Bangladeshi and United States adults, and fraction of dose in plasma was measured for over 90 days. Even though vitamin A pool sizes varied, averaging 1.03 and 0.10 mmol for the Americans and Bangladeshi, respectively, equilibration times were very similar: 17.5 for Americans and 16.3 days for Bangladeshi (P=0.69). A similar study was done with Guatemalan elders, further indicating that 20 days provided sufficient mixing [29]. Haskell et al. determined that only eight days are needed for sufficient mixing in 12-24 month old Peruvian children [30]. Measurements at day eight were then used in the DRD equation, with the estimated mean liver vitamin A concentration within the range of several measured autopsy liver vitamin A levels.

To investigate the validity of the DRD equation, two studies compared predicted vitamin A stores with liver biopsy results. In the original DRD paper, Furr et al. analyzed liver samples from eleven subjects and compared the results to the newly developed DRD equation [12]. The results gave a correlation coefficient of 0.88, even though the sample included patients at several different levels of vitamin A status. In a later experiment, Haskell et al. studied 31 Bangladeshi patients by administering an isotope dose 9-11 days before surgery [11]. Plasma samples were collected 18-25 days after isotope administration (depending on patient availability), and the estimates calculated using the DRD equation were compared with the biopsy results. A strong linear relationship was found (r=0.75, P=0.0001), with an even higher correlation (r=0.88) when several outliers were omitted. Interestingly, the study also showed that the calculated values of $F$ and $S$ (0.38 and 0.8, respectively) differed from the values used in earlier work (0.5 and 0.65). These different values did not result in significant differences in predicted vitamin A stores and
were thus not used in the equation. But with a calculated liver reserve of 0.110 mmol vs. an observed value of 0.100 mmol (P=0.27), Haskell et al. showed the effectiveness of the DRD equation in determining total hepatic reserves. This effectiveness only held when comparing the whole group of subjects, since individual subject predictions were not very accurate.

There have been numerous studies using the DRD equation to assess vitamin A status. Ribaya-Mercado [29, 31] utilized it to determine status in older adults in both Guatemala and the Philippines. Tang and colleagues have done several studies determining not only the vitamin A status of Chinese children [32] but also the effectiveness of vegetable interventions. Recently, Cifelli et al. [33] compared the 21 day estimates for American and Chinese adults with predictions based on a compartmental model.

Three Day Isotope Dilution Technique

There are several problems with a 21 day equilibration period. A major problem is the effect infections and illnesses can have on retinol levels. Retinol has been shown to be excreted both during acute infections [34] and by children suffering from acute diarrhea [35]; these are both very common problems in developing countries. Because VAD is a major problem in developing nations, long distance travel may be required to collect blood samples. Having to spend 21 days in a foreign country can be very costly. Lastly, it may be difficult to locate a subject and obtain a sample 21 days later. Therefore, a shorter time period would be ideal for not only a cost benefit, but also for lessening changes in vitamin A levels due to intervening illness.

The first indication that a shorter experiment might give reliable results using the DRD method was obtained in several rat studies. First, Green et al. showed that, at only 5 days after an intravenous injection of radio-labeled retinol bound to the RBP / transthyretin (TTR) complex, a predictive one component exponential equation for liver vitamin A could be established [36].
This equation was shown to be accurate for vitamin A store levels of 1.4 - 5200 nmol. In another experiment, a two component exponential equation was developed that could predict a range of 1.4-23000 nmol using data collected at 4.4 days [37]. The equation is:

$$\text{Liver vitamin A (nmol) = 88,928*exp(-1347*FDp) +5606*exp(-120*FDp)}$$

where FDp is fraction of the labeled dose in plasma at 4.4 days. Prediction equations could also be created from measurements taken at 4, 5, and 5.4 days. To better model a human DRD study, Adams et al. investigated the ability of an oral dose of radio-labeled retinol to predict liver vitamin A stores [38]. They found that, at only 3 days, an extremely accurate equation could be developed to estimate vitamin A stores:

$$\text{Liver vitamin A (nmol) = 58,577*exp(-2715*FDp) + 1810*exp(-127 *FDp)}$$

These results implied that it might also be possible to accurately predict total vitamin A stores in humans using data collected at 3 rather than 21 days after dosing.

These studies also documented differences in the utilization of vitamin A based on status. Data from the 1987 Green et al. paper showed that, over a wide range of marginal status, disposal rate was fairly constant, but when plasma retinol levels started to drop, conservation mechanisms increased and disposal rate dropped [36]. Also, other studies demonstrated that, when vitamin A status was low, the fraction of dose remaining in plasma was higher over time compared to rats with high vitamin A status. This could be due to the fact that, when status is low, there is only a small amount of extravascular retinol for the dose to mix with. Thus, as recycled plasma retinol is brought back into plasma, most of it is still the radio-labeled dose. The fraction of dose will not truly decrease until label is removed by irreversible loss or replaced by new dietary retinol. The opposite holds true for high vitamin A status, when there are larger extravascular retinol pools to mix with, thus decreasing the amount of dose recycled back into plasma. But at extremely high vitamin A status, a low fraction of dose is not observed. This may be due to a very slow turning over storage pool of vitamin A that does not readily mix with the dose.
Following up on the studies in rats that suggested that an earlier sampling time might provide a reliable estimate of vitamin A stores, several groups have looked at this possibility in humans. While studying the bioconversion of plant carotenoids in an intervention study in Filipino children, Ribaya-Mercado et al. observed that the 3 day D:H retinol ratio was a good predictor of the group’s response to carotenoid intervention [39]. There was a strong linear relationship (r= -0.99, P=0.0001) between the amount of change in D:H retinol in relation to the baseline D:H retinol ratio (the larger D:H ratio at baseline [the lower the vitamin A status], the greater the reduction [or vitamin A status improvement]). This relationship suggested that the D:H retinol ratio at 3 days could be an effective predictor of total body vitamin A status and change thereof.

In an earlier study, Ribaya-Mercado et al. found that, at 3 days, there is a negative correlation between total body stores of vitamin A and the D:H ratio (r= -0.81, P=0.004 with one outlier) in Guatemalan elders [40]. Interestingly, this relationship was stronger than the correlation found at six days. In a subsequent experiment, Ribaya-Mercado et al. showed that there was a linear correlation between the D:H ratios at 3 days and 21 days in Guatemalan elders (r=0.94, P=0.0001). A similar linear correlation was found when a group of Filipino elders was studied, even though they had much higher D:H values (indicating lower vitamin A status) [29]. There was a non-linear relationship between D:H retinol at 3 days and total body vitamin A stores in both the Filipino and Guatemalan elders. By logarithmically transforming the data, the authors were able to fit linear regression models to both sets of data. By combining the models, an equation for determining total body stores from a 3 day D:H ratio was developed. The equation was simplified to:

\[
\text{Total body stores of vitamin A (mmol retinol)} = 0.00468 \times 10^{37} \frac{[\text{isotope dose (in mmol)}]}{\text{D:H retinol in serum 3 d after the isotope dose}}
\]
The equation was derived by using the 21 day estimates of total vitamin A rather than liver biopsy measurements because of the difficulty in obtaining the latter data. The authors do note that this equation is limited to the two data sets for which it was developed until it can be verified against another cohort of elders. An additional drawback is that, because all utilization and absorption factors were taken out of the equation, it may be limited to elders and not be applicable to children and younger adults.

An additional 3 day equation was derived by Tang et al. to determine total vitamin A stores in Chinese children (age 10-11) whose vitamin A status ranged from marginal to normal [32]. In this paper, the authors found no correlation between percentage enrichment (D/(D+H)) at six hours vs. 21 days, but they did find a correlation at 3 days (r=0.74, P<0.001). This is consistent with the Filipino study that also found a strong correlation at 3 days. By using this linear correlation, Tang et al. created an equation that converted the percent enrichment at 3 days to its corresponding 21 day enrichment value which could then be used in the DRD equation. The equation is:

\[
\frac{D}{(D + H)}_{21d} = -0.061 + 0.243 \left( \frac{D}{(D + H)} \right)_{3d}
\]

While performing a ten week vegetable intervention study, Tang et al. took before and after samples at 3 days for one group and at 21 days for another [32]. They found no difference between the calculated 21 day enrichment (by using the 3 day equation) and the measured 21 day enrichment groups.

More recently, Cifelli et al. used kinetic data from both Chinese and American adults to develop a compartmental model that, among other things, could estimate total vitamin A stores [33]. The authors also used 3 day D:H ratio data along with calculated S values to determine total body vitamin A estimates from the original DRD equation. Both the compartmental model and the modified 3 day DRD estimates of stores were very similar to those calculated from the 21 day DRD equation. The calculated value for S differed greatly from the 0.65 reported by Hicks et al.
Cifelli et al. calculated $S$ values of 3.4 for Americans and 2.4 for Chinese adults [unpublished data], suggesting that, at 3 days, the specific activity of plasma retinol is greater than that of liver retinol. Also, unlike in the Hicks et al. study, the specific activity values varied for groups with different vitamin A status, with high vitamin A status groups having a higher $S$ value.

Finally, Haskell et al. developed an equation based on data from Peruvian children (age 1-2) to determine total body stores of vitamin A [30]:

Estimated total body stores of vitamin A = \frac{1}{(6.08 + 44.88 \times D:H \text{ on day 3})}

They evaluated this equation by comparing the D:H ratio at 3 days with the estimated total body vitamin A stores predicted by the Furr-Olson equation. The unique difference was that they showed sufficient equilibration of the dose with plasma retinol in only eight days, as opposed to twenty in adults. Their calculated 3 day total vitamin A stores were not significantly different from the Furr-Olson estimates.

In conclusion, the stable isotope dilution technique can be a very valuable tool in accessing total vitamin A stores in humans. But the current 21 day equation would be even more useful if it was modified to a 3 day equation. Current 3 day equations are very population specific and have only been tested against the data that were used to create the equation. A more universal 3 day equation is needed: one that can estimate stores for a wide range of subjects and vitamin A statuses.
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Chapter 3

Objectives and Hypotheses

The primary goal of this research was to modify the 21 day Furr-Olson equation into a 3 day equation to accurately estimate total vitamin A stores. The Furr-Olson equation:

\[ \text{Total reserves} = F \times \text{dose} \times (S \times a \times [(1/D:H) - 1]) \]

includes many assumptions, including fixed values for \( F, S, \) and \( a \) at 21 days. The objective of the study was to gather all available published human data containing both 3 and 21 day serum retinol D:H ratios and to use those data to determine a prediction equation for total vitamin A reserves.

The hypothesis to be tested in this research was that, at 3 days, the \( F, S, \) and \( a \) values would all fluctuate and be dependent on vitamin A status, which is directly related to the serum retinol D:H ratio. Furthermore, these fluctuating values could be used to develop an equation that would accurately estimate total vitamin A stores.
Chapter 4

Modification of a Three Day Deuterated Retinol Dilution Equation for the Estimation of Vitamin A Stores in Humans

Introduction

Vitamin A deficiency (VAD) is one of the most prevalent health problems in developing countries, affecting up to 140 million children [1]. With over 4 million children per year developing xerophthalmia [2], interventions aimed at improving vitamin A status are of great importance. While many tests have been developed to aid in determining vitamin A status, the deuterated retinol dilution (DRD) technique is one of the only assessment tools that can quantify total liver reserves of vitamin A. Originally developed by Bausch and Rietz to determine vitamin A stores in rats [3], the DRD was later modified for use in humans by Furr et al. [4]. The method requires the administration of an oral dose of deuterated retinyl acetate, followed by the measurement in plasma of the ratio of deuterated retinol to non-deuterated retinol (D:H) after adequate equilibration of the isotope with endogenous vitamin A, which is usually 21 days (although 17 days may be adequate [5]). This ratio is then used in the Furr-Olson equation (see later) to estimate total liver vitamin A [4]. The technique has been validated by comparison of the DRD test results to direct measurement of retinol in liver biopsies [4, 6], and it has been used to determine vitamin A stores in adults [4, 6, 7], the elderly [8, 9], and children [10-12] in the field.

One of the drawbacks for use of the DRD test in the field is the long time between dose administration and blood sampling, since it is not always feasible to reconnect with subjects several weeks after dosing. In addition, retinol has been shown to be excreted during both acute infections [13] and acute diarrhea [14], thus also supporting the need for a test that could be done in a shorter time period. Since an accurate equation had been developed to predict vitamin A
stores in rats based on data obtained 3 days after an orally administered dose of radiolabeled retinol [15], it seemed likely that an earlier time might also work in humans.

In fact, excellent correlations between 3 day D:H ratios and total liver vitamin A stores predicted by a 21 day equation have been found in several human studies [7, 8, 11, 12, 16, 17]. For example, in a study of Guatemalan elders, Ribaya-Mercado et al. found an inverse relationship between the D:H ratio at 3 days and total body vitamin A levels calculated by the 21 day DRD technique [8]. Follow-up studies showed a linear correlation between 3 day D:H ratios and 21 day D:H ratios in both Guatemalan and Filipino elders, from which a prediction equation using only the dose and the D:H ratio was developed [16]. A similar approach was taken in children, in which percent isotope enrichment at 3 days correlated well with the percent isotope enrichment at 21 days [12]. A prediction equation was developed that could determine the corresponding 21 day enrichment from a 3 day enrichment sample. Finally, Haskell et al. created a prediction equation for pre-school aged children by comparing total body stores calculated by the 21 day DRD with 3 day D:H ratios [11]. More recently, Cifelli et al. showed no difference in total vitamin A stores estimated with the 21 day DRD equation and a modified 3 day equation [7]. This modified equation was similar to the 21 day equation but contained different values for several factors. While each of these equations is accurate for predicting total vitamin A in a specific group of subjects, the use of each equation is limited to that specific group.

The aim of this study was to improve the modified 3 day equation developed by Cifelli et al. [7] for use in many subject populations and many age groups. This new 3 day equation would have similar modifications as did the Cifelli et al. equation, especially in factors related to absorption and retention of the dose and specific activities of retinol in plasma and liver. While the factors for absorption and retention and specific activity are fairly constant after equilibration [3, 18], they might vary greatly at earlier times (e.g., 3 days). Also, the Furr-Olson equation for estimating total vitamin A is based on liver reserves, using the assumption that most vitamin A is
stored in the liver [4]. However, in vitamin A-deficient states, that is not always the case [19].

Using all available published human data that included a 3 day serum retinol D:H ratio, we were able to develop a new prediction equation that could determine total body vitamin A stores in many subjects of varying age and vitamin A status.

**Research Design and Methods**

**Subjects**

All of the eight groups analyzed represent the total number of subjects for which both a 3 day and 21 day serum retinol D:H measurement had been obtained. The subjects consisted of two elderly groups, one from Guatemala (n=15) [8] and the other from the Philippines (n=58) [16]. The procedures for subject selection and data collection are found in the earlier publications. Middle-aged Chinese (n=14) and American (n=12) subjects were used as well. The protocol for these subjects can be found in reference [22, 23]. In addition, two groups of children were used, one of ages 10-11 from China (n=34), and a group of preschool children from Peru (n=12). The procedures for subject selection, ethical issues, and protocol for data collection can be found in earlier publications [11, 12]. Finally, data from two smaller groups (n=4 each) of middle aged Bangladeshi and Americans were also included, with the data collection procedures and subject selection described earlier [5]. The 3 day and 21 day D:H measurements for the elderly Guatemalan and Filipinos, and for Chinese and Peruvian children, were extrapolated from the published figures. Three subjects were excluded from our analyses because of possible errors in data collection or analysis: one from the elderly American subjects, and one from each of the two groups of children (Peruvian and Chinese). Their 3 day D:H retinol measurements were either extremely low or extremely high, compared to other similar 21 day measurements.

**Table 1** details average subject characteristics, D:H retinol in plasma on day 3 and calculated D:H/dose, as well as total vitamin A stores based on the 21 day Furr-Olson equation
Table 1

*Characteristics of the eight groups used in the study, including total vitamin A levels and serum retinol D:H*

<table>
<thead>
<tr>
<th></th>
<th>American (n=11)</th>
<th>Chinese (n=14)</th>
<th>Guatemalan (n=15)</th>
<th>Filipino (n=68)</th>
<th>Peruvian (n=12)</th>
<th>Chinese Pre-Teen (n=34)</th>
<th>American (n=4)</th>
<th>Bangladeshi (n=4)</th>
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<td><strong>Gender (M/F)</strong></td>
<td>8/6</td>
<td>4/8</td>
<td>7/8</td>
<td>35/33</td>
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<td>20/14</td>
<td>2/2</td>
<td>0/4</td>
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<tr>
<td><strong>Age (yrs)</strong></td>
<td>54.5 ± 3.73</td>
<td>58.8 ± 8.99</td>
<td>70 ± 7.7</td>
<td>69 ± 8.2</td>
<td>1.5 ± 0.34</td>
<td>10-11</td>
<td>35.5 ± 5.9</td>
<td>28.8 ± 5.5</td>
</tr>
<tr>
<td><strong>Body Weight (kg)</strong></td>
<td>65.2 ± 7.87</td>
<td>67.2 ± 10.2</td>
<td>45.9 ± 6.6</td>
<td>47.8 ± 9.9</td>
<td>9.4 ± 1.4</td>
<td>35.0 ± 7.5</td>
<td>70.9 ± 5.2</td>
<td>47.9 ± 7.7</td>
</tr>
<tr>
<td><strong>Serum Retinol (µmol/L)</strong></td>
<td>1.24 ± 0.37</td>
<td>1.70 ± 0.32</td>
<td>1.68 ± 0.50</td>
<td>1.85 ± 0.58</td>
<td>0.80 ± 0.28</td>
<td>0.989 ± 0.244</td>
<td>1.85 ± 0.40</td>
<td>1.23 ± 0.51</td>
</tr>
<tr>
<td><strong>3 d D:H</strong></td>
<td>0.026 ± 0.014</td>
<td>0.065 ± 0.030</td>
<td>0.096 ± 0.067</td>
<td>0.12 ± 0.093</td>
<td>0.17 ± 0.074</td>
<td>0.047 ± 0.023</td>
<td>0.101 ± 0.048</td>
<td>0.34 ± 0.12</td>
</tr>
<tr>
<td><strong>Dose (µmol)</strong></td>
<td>8.92</td>
<td>8.92</td>
<td>30</td>
<td>15</td>
<td>14</td>
<td>7.4</td>
<td>60.2</td>
<td>0.78–0.92</td>
</tr>
<tr>
<td><strong>3 d D:H/dose</strong></td>
<td>2.92 ± 1.52</td>
<td>7.41 ± 3.21</td>
<td>3.20 ± 2.21</td>
<td>8.23 ± 6.2</td>
<td>12.30 ± 5.27</td>
<td>5.18 ± 2.51</td>
<td>1.68 ± 0.80</td>
<td>9.40 ± 3.78</td>
</tr>
<tr>
<td><strong>Total Body Vitamin A (mmol)</strong></td>
<td>1.07 ± 0.64</td>
<td>0.24 ± 0.12</td>
<td>0.79 ± 0.36</td>
<td>0.21 ± 0.14</td>
<td>0.076 ± 0.040</td>
<td>0.34 ± 0.20</td>
<td>1.03 ± 0.45</td>
<td>0.10 ± 0.11</td>
</tr>
</tbody>
</table>

1 Data are from references [7, 7, 8, 16, 12, 11, 5, 5], respectively.

2 Values are based on extrapolation of data from published papers (except in the larger group of Americans and the Chinese).
for the 8 groups used in this study. The subject characteristics are for all subjects in the group, whereas the values for D:H, D:H/dose, and total vitamin A stores exclude the outliers (see above) and therefore include only the subjects that were used in the development of the 3 day equation.

**Estimation of Total Body Vitamin A Stores**

The administration of deuterated retinol and subsequent blood sampling is described in the earlier publications and was similar in all of the studies analyzed [8, 16]. Briefly, a known amount of labeled retinol, usually tetradeuterated retinyl acetate (all-trans-retinyl-10,19,19,19-[^2]H₄]acetate) in corn oil, was given orally at day zero. This was followed by a meal containing low vitamin A and high fat, to maximize vitamin A absorption. Blood samples were then drawn at 3 days, and sometime around 21 days. The D:H ratio was then determined by gas chromatography and mass spectroscopy. For determination of total vitamin A stores, the 21 day serum retinol D:H ratio is used in the Furr-Olson equation [4]:

\[
\text{Total reserves} = F \times \text{dose} \times (S \times a \times (\frac{1}{D:H} - 1))
\]

where \(F\) is a factor describing the retention and efficiency of storage of an orally administered dose of vitamin A and is assumed to be 0.5 based on the work of Bausch and Rietz [3]; “dose” is the amount (umol) of labeled retinol administered; \(S\) represents the ratio of the specific activity of retinol in plasma versus liver and is taken to be 0.65 from previous work by Hicks et al. [18]; \(a\) corrects for irreversible loss of vitamin A and is calculated as \(a = e^{-kt}\), where \(k = 0.693/140\) (0.693 = ln 2), with 140 days being the half life of vitamin A in healthy adults [19] and \(t\) is the amount of time since the dose was administered; and the “-1” term corrects for the contribution of the dose of labeled vitamin A to the total vitamin A pool. Factors \(S\) and \(a\) correct for the fact that the labeled vitamin A does not truly equilibrate with the body vitamin A pool, because as unlabeled vitamin A is ingested and absorbed, it will preferentially contribute to the plasma pool.
Regression analyses were used to compare the relationship between estimates of total body vitamin A stores as calculated by the DRD equation using the 3 day D:H/dose of retinol vs. the 21 day data for all individual subjects, as well as the eight groups. In addition, the data were logarithmically transformed for further regression analyses. An important note here is that subjects were given different doses of labeled retinol. To correct for this, the D:H in retinol was divided by dose. So all comparisons of D:H at 3 days are dose corrected.

Determination of 3 Day Equation

To determine the numerical values of factors $S$, $F$, and $a$ for the new 3 day equation, the values were compared between the Chinese and American subjects from the Cifelli et al. paper [7] by one way ANOVA. The individual subject $S$, $F$, and $a$ values were calculated from the compartmental analysis presented in the paper [7]. The developed model has four compartments representing the physiological digestion and absorption of vitamin A, and two other main compartments, one for plasma retinol and the other for all extravascular storage of vitamin A. By simulating the model at three days, we estimated the fraction of dose in both plasma and in the extravascular compartment, as well as the vitamin A masses in those compartments. The fraction of dose in the extravascular compartment was equivalent to $F$, the absorption and efficiency of storage of vitamin A. We then took the fraction of doses in both compartments and divided them by their respective masses, giving us the specific activities of vitamin A in plasma and extravascular tissues. The ratio of those specific activities represents factor $S$. Lastly, compartmental analysis allows for the estimation of irreversible loss of vitamin A, which is factor $a$.

Then regression analyses were done on the $F$, $S$, and $a$ values for all subjects ($n=25$) from Cifelli et al. [7] vs. their serum retinol D:H/dose at three days. The reason for using
D:H/dose is to correct for the fact that the same dose was not given to all subjects. While it was the same in the case of these two groups, a universal equation must be applicable for all doses.

After a quadratic regression equation between the $S$ value and D:H/dose was determined, an equation for a value describing both $F$ and $a$ ($Fa$) was developed. Factors $F$ and $a$ have been combined because we feel that they represent the same concept physiologically. While $F$ corrects for absorption and retention, factor $a$ represents all that is absorbed then irreversibly lost (i.e., is not stored, or was stored but then lost before sampling), and thus influences the value of $F$. Thus we decided to combine $F$ and $a$ into a single factor. The 3 day estimation of body stores was done by taking the 3 day D:H ratio and inserting it in the unfinished equation:

$$\text{Total body stores of vitamin A} = S \times \text{dose} \times ((1/D:H)-1)$$

where the factors $F$ and $a$ have been left out and $S$ is the quadratic equation developed. Next, each subject’s total store estimation from the 21 day DRD equation was divided by this unfinished 3 day estimate, giving an estimate of the $F$ and $a$ combined value ($Fa$) that could be used to determine total body stores in the 3 day equation. These calculated $Fa$ values were then correlated with their corresponding D:H/dose values. Because the relationship was nonlinear, an exponential relationship was developed. This value for $Fa$ was used in the equation.

Next, all values for 3 day serum retinol D:H were used in the new 3 day equation to estimate total vitamin A stores. Finally, the estimates from the 3 day and 21 day DRD equation for each subject group were compared by a one way ANOVA. The statistical software Minitab was used for all data analyses.

**Results**

A strong non-linear relationship can be seen between all subjects’ total vitamin A stores, as calculated by the Furr-Olson equation, and their corresponding 3 day serum retinol D:H/dose (Figure 1). When the data are logarithmically transformed, and linear regression is applied, a
very strong relationship exists (p<0.001, r = 0.88; Figure 2). The same is true when each group’s average total vitamin A stores and serum retinol D:H/dose are plotted: there is a strong non-linear relationship as seen in Figure 3. When the data are logarithmically transformed, a very strong linear relationship is present (p<0.001, r=0.95; Figure 4). This relationship among groups is consistent with the same relationships within the Guatemalan, Filipino, and Peruvian groups [11, 16]. Finding these common relationships indicates that dividing the serum D:H retinol by the dose of labeled retinol is an effective way to control for the varying dose administered to different groups in different studies.

In the Cifelli et al. paper [7], compartmental analysis was done to accurately describe vitamin A kinetics in Chinese and American subjects with varying vitamin A status (Americans had ~4.7 times larger stores). Using this model, we were able to compare the specific activities of plasma retinol versus the total extravascular vitamin A pool at three days between groups, because unlike people with high stores, subjects with low vitamin A status may store less than 90% of vitamin A in the liver [19, 20]. Figure 5a shows the significant difference (p=0.04) in the calculated S value (related to the vitamin A specific activities in plasma vs. liver) between the Americans and Chinese, with the Americans having an S value of 3.27 ± 0.9 and the Chinese a value of 2.4 ± 1.0. Figure 5b, on the other hand, shows no difference in the F value (related to the efficiency of absorption and storage of the oral dose) between groups. Lastly, there is a difference seen between the a value, or irreversible loss, with the Chinese group having a higher value than the Americans (p=0.007; Figure 5c)

Regression analyses were then run on these F, S, and a values. The analyses for F and S based on D:H/dose yielded a significant quadratic relationship for S (r=0.48, p=0.029; Figure 6a) but no relationship for F (r=0.3, p=0.083; not shown). The mean F value for all subjects was 0.61 ± 0.035. Factor a (Figure 6b) shows a linear relationship, with an increase in D:H/dose yielding a higher irreversible loss value (r=0.43, p=0.03). The quadratic relationship for the factor S
Figure 1. Comparison of subjects’ vitamin A stores, as calculated by the 21 day Furr-Olson equation, and their respective 3 day serum retinol D:H corrected for each subject’s dose.
Figure 2. Logarithmic comparison of each subject’s total vitamin A, as measured by the 21 day Furr-Olson equation, and their respective 3 day serum retinol D:H/dose (p<0.001, r=0.88).
**Figure 3.** Relationship between D:H/dose for the eight groups of subjects vs. total vitamin A stores, as measured by the 21 day Furr-Olson equation (p=0.064, r=0.97).
Figure 4. Relationship between D:H/dose for the eight groups of subjects vs. respective total vitamin A stores using log transformations (p<.001, r=0.95).
Figure 5. Differences in values for $S$, $F$, and $a$ from the Furr-Olson equation applied to data from Chinese and American subjects. Americans are represented by the black bars and Chinese by the grey bars. Numbers are estimates from compartmental analysis. Significant differences were found in panel A between $S$ values ($p=0.04$) and in panel C between $a$ values ($p=0.007$), but not between $F$ values ($p=0.353$; panel B).
Figure 6. Estimated $S$ and $a$ values (symbols) and equations (lines) for both the $S$ and $a$ values of each American and Chinese subject.  

A. Relationship between $S$ and D:H/dose ($r=0.47$, $p=0.029$).

B. Relationship between $a$ and D:H/dose ($r=0.43$, $p=0.03$).
shows that, with varying vitamin A status, the specific activities of vitamin A in plasma vs. extravascular tissue also vary. The newly derived quadratic equation:

\[ S = 0.05668 \times (D:H/dose)^2 + 0.777 \times (D:H/dose) + 4.696 \]  

(2)

may be used to calculate \( S \) for any vitamin A status, and it can be used for any dose. The quadratic equation was then used to determine \( S \) values for the Chinese and American subjects. **Figure 7** shows that there was no difference between the values predicted by the model and the values calculated using the equation (American \( p=0.455 \); Chinese \( p=0.532 \)), but there was a significant difference between subjects (\( p=0.031 \)), similar to what was seen in **Figure 5a**.

Next, we aimed to combine the \( F \) and \( a \) factors into one varying "\( Fa \)" factor. We took the original DRD equation (equation 1) and replaced \( S \) with equation 2. We then removed factors, inserted the 3 day D:H measurements, and calculated a total stores value with absent \( F \) and \( a \) values. Next, each subject’s 21 day calculated vitamin A store was divided by their corresponding absent \( F \) and \( a \) value. This would then provide the “perfect” \( Fa \) value for each subject:

\[
\text{[Total stores calculated by 21 day DRD] / [S x dose x (H:D-1) - 3 d calc] = Fa}
\]

When the \( Fa \) values were plotted against the corresponding D:H/dose values, a non-linear exponential relationship was found (\( r=0.57, p<0.05; \) **Figure 8**). This equation was then used to calculate the \( Fa \) value for each subject:

\[
Fa = 0.7705e^{-0.0671 \times D:H/dose}
\]

(3)

The final equation for determining total vitamin A stores from the 3 day D:H measurement is now:

\[
\text{Total vitamin A stores (mmol) = S x Fa x dose x (H:D-1)}
\]

(4)

where \( S \) is equation 2 and \( Fa \) is equation 3.
Figure 7. Calculated $S$ values based on the regression equation developed in Figure 5a and observed values from compartmental analysis. Data from Americans are shown in black and Chinese in grey. Values calculated by the regression equation used in the 3 day DRD (3d Eqn) are indicated with solid colors, and calculated values from compartmental analysis (CA) are shown using stripes. There is no difference between the observed model analysis and calculated $S$ values for both American subjects ($p=0.455$) and Chinese subjects ($p=0.532$), but there is a significant difference in regression estimated values for the American vs. Chinese subjects ($p=0.031$).
Figure 8. Fa values for all subjects (symbols) calculated by finding a “perfect value,” that if used in the 3 day DRD, would yield the exact 21 day DRD estimate and best fit equation (line) (r=0.57).
Incorporating these, the new prediction equation is:

$$\text{Total vitamin A stores (mmol)} = (0.05668 \times (D:H/dose)^2 + 0.777 \times (D:H/dose) + 4.696) \times (0.7705e^{-0.0671 \times (D:H/dose)} \times \text{dose} \times (H:D-1))$$ (5)

**Figure 9** compares total vitamin A stores estimated using the 21 day Furr-Olson equation with values calculated using the new 3 day DRD equation. There are no significant differences between the two estimates for any subject group. Vitamin A stores for the Americans were estimated to be $0.920 \pm 0.62$ mmol (p= 0.466). Corresponding values for the other groups are: Chinese, $0.225 \pm 0.23$ mmol (p= 0.856); Guatemalans, $0.754 \pm 0.42$ mmol (p= 0.793); Filipinos, $0.254 \pm 0.24$ mmol (p= 0.277); Peruvian children, $0.100 \pm .29$ mmol (p= 0.094); Chinese pre-teens, $0.383 \pm 0.34$ mmol (p= 0.573); Bangladeshis, $0.122 \pm 0.12$ mmol (p= 0.793); and the group of 4 Americans, $1.69 \pm 1.0$ mmol (p= 0.282).

**Discussion**

While there are many methods to measure vitamin A stores in humans, the DRD equation is one of the few that actually gives a quantitative estimate of total vitamin A reserves. The DRD equation has been validated in several studies [4, 6] that compared estimates to liver biopsies, and it has been shown to be very useful in assessing the success of dietary interventions [10, 12, 21]. The main drawback to use of the Furr-Olson equation is the 21 day equilibration period specified after the dose is given. In these 21 days, acute infections could affect the level of retinol in plasma [13, 14]. In addition, a shorter wait time could be both easier and more cost effective for researchers. Adams and Green [15] showed that 3 days after dosing was enough to accurately estimate vitamin A stores in rats. Subsequently, several researchers developed 3 day equations to predict total vitamin A stores in humans [11, 12, 16], but each equation is limited to the subjects that were used to develop it. To develop an equation that could be used to predict vitamin A status for all types of study groups, the equation must be independent of the amount of isotope
Figure 9. Vitamin A stores estimated by using the newly developed 3 day DRD equation (grey bars) and the 21 day DRD equation (black bars). There were no significant differences between stores calculated using the two equations across all groups.
administered since the amount of labeled retinol given would alter the serum D: H in retinol. Thus we decided to correct for differences in dose by normalizing D: H (D: H/dose). We then compared the D: H/dose to the 21 day estimate predicted by the original Furr-Olson equation and saw similar relationships to those published in other studies relating D: H and total stores (Figures 1 and 2) [11, 16]. This similarity shows that dividing D:H by dose is an effective way to eliminate the discrepancies seen due to varying doses, and that this correction can therefore be used in developing an equation.

Given this correction, we were able to develop a more complete 3 day equation by incorporating many studies (n=8) that included subjects with varying vitamin A status and subject age as well as dose. In addition, Figures 3 and 4, which compare group D: H/dose vs. 21 day estimates, show similar relationships to those in Figures 1 and 2, or the individual subject relationships. Because the DRD equation is useful for determining population vitamin A levels (not individuals directly), we thought it was important to show that D: H/dose also is effective in that regard. In addition, both figures show that as D: H/dose increases, vitamin A levels decrease, which is the opposite when comparing D: H to vitamin A levels (low D: H represents a high total store).

This paper is the first to present a 3 day equation that can accurately predict vitamin A stores across different vitamin A statuses, ages and gender. The equation was developed by using compartmental analysis to predict the fraction of an isotopic dose of vitamin A in both plasma and extravascular tissues at 3 days. The equation was applied to twenty five subjects with vitamin A status ranging from 0.12 mmol to 1.98 mmol (as estimated by the Furr-Olson equation). By predicting the fraction of dose and vitamin A mass in both the plasma and extravascular storage compartment, we were able to estimate what the $S$ factor would be for each subject. While it is assumed to be constant at 0.65 in the 21 day DRD equation [18], at 3 days it can vary greatly. Figure 5a shows that Chinese and American subjects [7], whose vitamin A
levels were significantly different (4.7 times larger in Americans), also had significantly different $S$ values ($p=0.040$). Using these estimates of factor $S$, we found that there is a quadratic relationship between $S$ and $D$: H/dose (**Figure 6a**), such that both low and high $D$: H/dose values (corresponding to high and low vitamin A status, respectively) produce large $S$ values. This is consistent with rat data from our lab that show a similar quadratic relationship between $S$ and $D$: H/dose (**Appendix**). The reason for the high ratio of specific activities of vitamin A in plasma vs. extravascular tissue for subjects with low vitamin A status (high D:H/dose) could be due to the fact that most of the retinol may be directly going into plasma to be circulated for use, thus giving a high specific activity for the plasma compartment. And since there is a small amount of stores, little mixing will occur, yielding a low specific activity of extravascular storage, and a high $S$ value. As stores start to increase, the specific activity of the storage will increase, creating a lower $S$ value. This will continue to a certain point, where stores will be so high that some may become un-exchangeable, which will lower the specific activity and create a larger $S$ value. It is important to note that $S$ is simply the ratio of specific activities, so while the specific activities in plasma and extravascular tissue in subjects with low vitamin A status may be higher than those with high vitamin A status subjects, the ratio between plasma and extravascular vitamin A could be equal in the two cases.

Equation 2 gives a good estimate of $S$, as seen by the accurate prediction in American ($p=0.455$) and Chinese subjects ($p=0.532$) and their difference ($p=0.031$; **Figure 7**). On the other hand, the factor $F$, which represents the fraction of dose in the extravascular compartment at day 3, did not vary between the Chinese and American groups (**Figure 5b**). And similarly, there was no relationship between $D$: H/dose and $F$ in those subjects. However, the value at 3 days (0.61) was higher than that observed at 21 days (0.5) [3]. And although the Furr-Olson equation assumed irreversible loss to be constant by 21 days [4], at 3 days, it is not. Subjects with low vitamin A status had higher rates of irreversible loss. This may be because of the fact that, with
lower status, more of the vitamin A absorbed could be going directly to physiological processes, while in high vitamin A status, the vitamin is not needed and thus is stored.

Next, we combined factors $F$ and $a$. Factor $F$ in the Furr-Olson equation represents the absorption and efficiency of storage, while $a$ represents irreversible loss. These factors represent related processes: that is, how much of a given amount of vitamin A is actually stored. Storage is affected both by absorption and by how much is used and/or lost. Thus we decided to make a factor $Fa$, which is the general term for all efficiency of storage and which incorporates how much is irreversibly lost. By using equation 2 and eliminating $F$ and $a$ from the Furr-Olson equation, a partially complete equation was developed. The estimates from the 21 day DRD equation were then divided by this partial equation, and a combined $F$ and $a$ factor is left, which would give a perfect estimate of those variables (assuming the 21 day estimate is perfect) for each subject. Figure 8 shows the relationship for all subjects from the eight studies. An exponential relationship can be seen, in which subjects with high stores have a very high $Fa$ value whereas those with low vitamin A status have a low $Fa$ value. This observation (Figure 8) corresponds with Figure 6b, which indicates that subjects with low vitamin A status had a higher amount of irreversibly lost vitamin A. This is also similar to other studies, when as vitamin A levels decreased, so did absorption and retention [3, 6]. Haskell et al. [6] showed that the dose retention was significantly lower in subjects with low vitamin A status (<0.07 μmol/g) than in subjects with high vitamin A status at 21 days (29.8% retention vs. 42.3%).

Using both equations 2 and 3, a new DRD equation was developed that accurately estimates total vitamin A reserves. While Ribaya-Mercado et al. [16], Tang et al. [12], and Haskell et al. [11] developed equations for 3 day measures, their equations were only useful to estimate vitamin A stores in their specific groups, and the equations lack the ability to estimate stores in other subject groups. Equation 5 can give estimates for groups with low, marginal, and high vitamin A status. There was no difference between the 3 and 21 day DRD equations for all
subject groups, as seen in Figure 9. The groups studied included subjects with very high stores (American groups) to marginal stores (Chinese and Filipino) to low stores (Peruvian). In addition, the equation effectively estimates stores for both young and old groups of subjects. There was no difference between estimates of stores from the 3 and 21 day equations in children in the Peruvian study (mean age, 1.5 years) or in the Chinese pre-teenage group. In addition, the equation worked well for groups of elderly Guatemalans and Filipinos.

The main drawback related to the newly developed 3 day equation is that it was developed by using the total reserves estimates from the 21 day Furr-Olson equation. While these have been proven valid [4, 6], using direct hepatic biopsy samples would have been better. But because this is not feasible, the Furr-Olson estimates represent the next best “gold standard” to quantify total reserves.

We conclude that this new 3 day DRD equation is an effective way to determine total vitamin A stores in humans. The equation was very accurate in predicting total body vitamin A reserves over a wide range of age groups and vitamin A levels. While the equation still needs to be validated by comparison to direct liver biopsies, its overall success in predicting vitamin A stores in all eight groups shows that it can be an effective tool for measuring vitamin A levels. Additionally, the new equation drastically shortens the amount of time at which a blood sample is obtained after dosing. Although the equation has combined the $F$ and $a$ factors, it is still a complex equation with many variables. A future aim could be to simplify the equation to only using D:H and dose factors, as has been done in the other 3 day equations [11, 12, 16]. Until then, we feel that this equation can provide an accurate estimate for the total amount of vitamin A stores in any human.
Literature Cited


22. Tang G, Qin J, Dolnikowski GG, Russell RM: *Short-term (intestinal) and long-term (postintestinal) conversion of beta-carotene to retinol in adults as*

Chapter 5

Future Studies

There are several ways upon which this equation can be improved. First of all, while it was created with many subjects of varying vitamin A status and age, it was not verified by liver biopsies like the original Furr-Olson equation. Also, the equation has yet to be tested on data that were not used to create it. In other words, it may not be effective for subjects other than the eight groups used to create it.

Additionally, simplifying the equation would create a more useful tool. While the current 3 day equation is accurate in its estimations, it is also very long, containing four factors, two of which have separate equations. A condensed equation with only one variable would be much easier to use.
Appendix

Additional Figures and Tables

**Figure 10.** Quadratic relationship between D:H/dose and S value for 7 groups of rats of varying vitamin A status.
Table 2

*D:H values for all subjects at 3 days*

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