Original Communication

Evaluation of the "Olson Equation", an Isotope Dilution Method for Estimating Vitamin A Stores

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Abstract: Isotope dilution methods have been successfully used to estimate vitamin A status in human populations as well as to evaluate the impact of vitamin A interventions. The most commonly applied isotope dilution method is the retinol isotope dilution technique, which is based on the 1989 "Olson equation" for estimating total body vitamin A stores (sometimes equated to liver vitamin A) after an oral dose of labeled vitamin A. The equation relies on several factors related to absorption and retention of the dose, the equilibration of label in plasma vs. liver, and timing of a blood sample for measurement of labeled vitamin A. Here, the assumptions underlying these factors are discussed, and new results based on applying model-based compartmental analysis [specifically, the Simulation, Analysis and Modeling software (WinSAAM)] to data on retinol kinetics in humans are summarized. A simplification of the Olson equation, in which plasma tracer is measured 3 days after administration of the oral dose and several factors are eliminated, is presented. The potential usefulness of the retinol isotope dilution technique for setting vitamin A requirements and assessing vitamin A status in children, as well as the confounding effects of inflammation and likely variability in vitamin A absorption, are also discussed.

Key words: deuterated retinol dilution technique, isotope dilution, model-based compartmental analysis, retinol, vitamin A kinetics, vitamin A status, WinSAAM

Introduction

The ability to accurately assess vitamin A status is critical, given the extent and consequences of vitamin A deficiency worldwide and the need to evaluate supplementation programs. As reviewed by Furr et al. [1], many methods have been proposed for estimating vitamin A status. Among the most actively investigated and potentially most useful are isotope dilution techniques which provide an indirect estimate of total body stores of vitamin A. The currently-used method will be reviewed, followed by a discussion of the assumptions upon which it is based as well as some limitations, cautions and potential simplifications derived from model-based compartmental analysis.

The Isotope Dilution Equation: Current Version and Underlying Assumptions

In 1989, Furr and colleagues in Olson's lab presented an isotope dilution equation (see below) for assessing vitamin A status in humans [2]. Since then, this "Olson equation" or "deuterated retinol dilution (DRD) equation" has been fruitfully used to estimate stores or to evaluate interventions in a number of human studies (for example, see references [3–5]). More details on the development and application of the retinol isotope dilution (RID) method have been presented by Furr et al. [1] and Haskell et al. [6].

Background

Before presenting and discussing the Olson equation, several important points should be emphasized. These were touched on in the original publication by Furr et al. [2] but there is still confusion about what is actually being estimated (liver vitamin A reserves vs. total body vitamin A stores) and when these two values are the same (or close to the same). Regarding the first point, since the early work of Bausch and Rietz [7] (see below), it has been assumed that vitamin A throughout the body is dynamically exchangeable or metabolically active. (Note: this may not be true when approaching vitamin A toxicity.) When an isotopic dose is administered, there will be interfusion (mixing) of the tracer (a stable or radioactive isotope of vitamin A) with the tracee (unlabeled vitamin A) in all of the exchangeable pools, and isotope dilution methods will therefore predict total body vitamin A, not just liver vitamin A. Since conventional wisdom in the vitamin A field has said that 90 % or more of total body vitamin A is stored in the liver, hepatic vitamin A levels have been considered the "gold standard" measurement for vitamin A status. Indeed, when stores are high, the liver is undoubtedly the major (albeit not the only) site of vitamin A storage. In contrast, in rats with low liver vitamin A stores, more than 90 % of total body vitamin A is actually present in extrahepatic tissues [8]. This distinction is important because many of the human populations of interest in field studies have low liver vitamin A stores. Fortunately, the biologically important parameter is total body stores – it is simply a matter of realizing that the historic focus on liver vitamin A concentration or content should be supplanted by the more useful parameter: total body vitamin A stores (TBS).

It is also worth mentioning that any isotopic form of vitamin A can be used for an isotope dilution test and / or for other types of studies that require a tracer of vitamin A (e. g., kinetic studies). These forms include stable isotopes such as deuterium (for examples, see [2–6]) and ^{13}C (e. g., [9]), and radiolabeled forms (e. g., see [7, 8]). Because of the use of deuterium in the original Furr et al. work in Olson's lab [2] and by many subsequent users of the isotope dilution method (e. g., [3–6]), the method is sometimes called the deuterated retinol dilution (DRD) technique or the Olson equation. Here we use the more general term: retinol isotope dilution (RID).

"Olson Equation" and Underlying Assumptions

Below is the retinol isotope dilution equation proposed by Furr et al. [2]:

$$TLR = F * dose * (S * a * [(H:D) - 1]) \quad \text{Eqn [1]}$$

where TLR = total liver vitamin A; F = fraction of an orally administered isotopic dose that is absorbed and retained; S = ratio of specific activity of retinol in plasma to that in liver after equilibration; a = correction for fraction of dose lost via catabolism; H:D = post-equilibration hydrogen-to-deuterium ratio in plasma retinol after administration of the oral dose of deuterated vitamin A; and – 1 = correction for the contribution of the dose to vitamin A stores.

The theoretical basis of the isotope dilution technique rests on the fact that, following administration of a tracer dose of labeled vitamin A to a subject who is not consuming vitamin A, the specific activity of vitamin A in plasma will be equal to that in the exchangeable storage pools once the dose has mixed with these pools. Thus, by determining plasma vitamin A specific activity and knowing the amount of isotope administered, the mass of vitamin A in body storage pools can be estimated.

Isotope dilution was first applied to vitamin A by Bausch and Rietz [7] who used it to estimate liver vitamin A content in rats. Animals were given a known amount of tritiated retinyl acetate intravenously, and plasma retinol specific activity was determined 3 days later, after the dose had thoroughly mixed with body vitamin A pools. During this time, the rats consumed no vitamin A. The researchers found that, at equilibrium (i. e., after mixing, when the vitamin A specific activity in plasma and liver were equal), about 50 % of the test dose was recovered in the liver [10]. Thus, they proposed [7] that liver vitamin A content (IU) could be estimated as 0.5 * test dose (dpm) / plasma vitamin A specific activity (dpm/IU).

Several considerations impacted the modification of this technique for application in humans. First, since test subjects would typically be consuming vitamin A, the equilibrium condition does not always hold, and specific activity in plasma and liver will never be equal except at the moment in time when the values cross over. In addition, because of previous limits in analytical sensitivity, the amount of stable isotope that has been administered has typically been more than a trace amount (ideally, the dose should be < 1 % of total body stores). Smaller doses of ^{13}C can be used, with analysis by LC-MS-MS, an analytical tool that

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is very sensitive but also costly [9]. Finally, in human studies, the dose is generally given orally rather than intravenously, introducing the potentially highly variable process of vitamin A absorption.

Furr et al. [2] addressed some of these problems with the equation proposed in 1989 (Eqn [1]). Specifically, they introduced the factor *F* (fraction of the dose absorbed and retained in stores at the time of sampling) and assigned it a value of 0.5 based on the work of Rietz et al. discussed above (Fig. 2 in [10]). Furr et al. included the factor *S* (the ratio of vitamin A specific activity in plasma vs. liver), estimated as 0.65, to account for the dilution of the tracer/tracee ratio as unlabeled vitamin A (tracee) enters the system from the diet, and tracer and tracee are lost by catabolism. The value for *S* was based on earlier studies in Olson's lab on the metabolism of vitamin A in rats as a function of liver vitamin A stores [11]. Also related to catabolism, and thus loss of tracer, the factor *a* was introduced as a correction. This factor was calculated as

$$a = e^{-kt} \qquad \text{Eqn [2]}$$

where $k = (\ln 2)/140$ days based on the estimated $t_{1/2}$ of liver vitamin [12] and t = days since the dose was administered. Finally, H : D was measured in plasma retinol 19–47 days after oral administration of the dose, and the correction –1 was added to account for the contribution of the mass of vitamin A in the dose (that is, the dose was not a trace amount). Twenty-one days is often chosen as the sampling time based on the time it takes for the tracer to thoroughly mix with body vitamin A pools in humans [3, 7].

Furr et al. [2] compared TLR calculated using Equation [1] with direct measurement of liver vitamin A concentrations (in other words, these authors assumed that, as stated in their publication, "the bulk of total body vitamin A in the well-nourished individual [perhaps 90 % of the total] is stored in the liver as retinyl esters"). When vitamin A concentrations (and extrapolated content) were determined by liver biopsy of well-nourished adult surgical patients, the correlation between the two estimates was quite good (r = 0.88). Later, Haskell et al. [3] did a similar comparison in 31 Bangladeshi surgical patients with lower vitamin A reserves than the subjects studied by Furr et al. Haskell and colleagues reported that the RID technique provided a good estimate of hepatic vitamin A content in this population but the relationship did not hold for individual subjects. This important observation deserves emphasis: it makes sense that using population means for factors *F* and *S* might give a good estimate of stores in a given population, but there is no reason to expect good prediction of a specific individual's TBS since *F* and *S* vary among individuals. Large inter-individual variation has also been found in kinetic parameters when vitamin A kinetic data from studies in both rats [8] and humans [13] were analyzed by model-based compartmental analysis. The issue of defining TBS for individuals will require further refinement of the technique if this is deemed a needed measurement. Perhaps more useful would be to explore the application of interval analysis [14] and statistical methods to estimate TBS, thus defining the 95 % confidence interval that stores range between X and Y. That is, rather than seeking an absolute value for a given individual, one would report an interval within which there is confidence that the value falls.

A Simplified 3-Day RID Equation

There is intense interest among vitamin A researchers in simplifying the Olson equation and in particular, in adapting the original equation so it would rely on fewer assumptions and be applicable for a blood sample taken at an earlier time (e. g., 3 days after dose administration), when it is more feasible to still have access to subjects in the field. One approach has been to estimate what the 3-day D : H ratio would have been based on the measured ratio at 21 days, after interfusion of tracer and tracee [15, 16]. Recent work summarized below indicates that we may be able to modify the RID equation to both eliminate some of the assumptions and to use data actually collected 3 days after dosing.

In order to explore the factors that influence the values of *F*, *S*, *a* and D : H in the Olson equation and to evaluate whether any might be eliminated, we used data obtained by Lietz and collaborators at Newcastle University (manuscript in preparation; see also [9]). Lietz et al. are studying the conversion of β-carotene to retinol in humans. We used a subset of their data on plasma retinol kinetics during 14 days after oral administration of a labeled dose of [$^{13}C_{10}$]retinyl acetate (3 μmol) to 33 healthy young adults (17 females, 16 males; age range, 20–36 years; average weight, 63 ± 10 kg) who were assumed to have moderate liver vitamin A levels based on dietary intake data. Model-based compartmental analysis was applied to the plasma isotope data using WinSAAM, the Windows version of the Simulation, Analysis and Modeling software [17 (Chapter 6), 18, 19] to obtain kinetic parameters describing vitamin A metabolism in these subjects. This technique has been fruitfully applied in numerous studies of vitamin A metabolism in rats and humans (see [18] for review). As a side note related to future vitamin A kinetic studies in

humans, the kinetic parameters will be more accurately identified if experiments are carried out longer than 14 days. Based on the rat model, we know that the higher the liver stores, the longer the time required to accurately estimate certain model parameters.

We found that kinetic data for the Newcastle subjects fit well to the compartmental model previously developed by Cifelli et al. [13]. That model (Fig. 2 in [13]) includes compartments related to the digestion and absorption of vitamin A, chylomicron production and metabolism, liver uptake of chylomicron remnants (which contain most of the absorbed vitamin A), and hepatic processing of newly absorbed retinol. In the model, retinol is secreted from liver into plasma bound to retinol-binding protein (RBP); plasma retinol exchanges with vitamin A in one extravascular pool, which is also the site of irreversible loss of vitamin A. After the data for each subject were fit to this model and model parameters (fractional transfer coefficients) were generated by WinSAAM, the estimated plasma retinol pool size (concentration * estimated plasma volume) was used in a steady-state solution to predict compartment pool sizes and rates of transfer. For purposes of our discussion of the RID, we will focus here on the model-predicted size of the extravascular pool (that is, TBS, which was 114 ± 72 μmol [mean ± SD] in these subjects).

How can these results shed light on the RID and especially on the factors used in the Olson equation? When we plotted the fraction of dose in plasma at 3 days (equivalent to D : H) vs. model-predicted TBS for the Newcastle subjects, we found that, as expected based on the theory of isotope dilution, the larger the store, the smaller the fraction of dose.

The Newcastle data also provide some information related to the Olson equation's factor *F*, the fraction of the oral dose that is absorbed and retained. The generally used value of 0.5 was based on the middle range of liver vitamin A concentrations in rats studied by Rietz et al. [10 (Fig. 2)]. However, outside the mid-range, Rietz' data indicates that the fraction of dose recovered in liver is dependent on liver stores, as studies in my laboratory have also found [18 (Fig. 6)] – the lower the stores, the lower the recovery of the dose in liver. When we plotted the model-simulated fraction of the dose in the extravascular storage compartment at 3 days vs. the mass of vitamin A in that compartment for the Newcastle subjects, we found the same relationship: as the mass of vitamin A in stores went up, so did the fraction of dose in "liver" (actually, in the model-predicted TBS). That is, all of these studies indicate that *F* is dependent on stores and thus, as we refine the Olson equation, we will need to keep that in mind. Plotting fraction of dose in plasma at 3 days for the Newcastle subjects vs. the model-predicted fraction of dose in TBS at that time, we found that 50 – 70 % of the oral dose was predicted to be in stores at 3 days. Although the slope was small and inter-individual variation was high, there was a significant relationship (P = 0.004) between the variables, with a mean value for *F* of 0.61. For subjects with moderate vitamin A status, this value is an improved factor to use in the RID equation.

The factor *S* in the Olson equation (the ratio of retinol specific activity in plasma to that in liver) has been assumed to be 0.65. For the Newcastle subjects, we converted model-simulated data on fraction of dose to specific activity (fraction of dose/mass) for the plasma and extravascular storage compartment vs. time. On average, the individual subject curves for stores crossed over those for plasma at 2.74 days after dosing, resulting in a value for *S* of 0.94 at 3 days. After that, the curves finally became parallel, giving a value for *S* of 0.7 at 14 days. The fact that the curves cross over indicates that plasma retinol is the precursor of extravascular vitamin A, based on the precursor-product relationship proposed by Zilversmit et al. in 1943 [20]. The data for "liver" vitamin A specific activity will cross over that for plasma if there is input of dietary (i. e., unlabeled) vitamin A. If no vitamin A is ingested during the interfusion period, then isotopic equilibrium will be reached, and the specific activity in plasma and stores will be equal from then on. When we plotted the model-predicted size of the extravascular pool vs. cross-over day for the Newcastle subjects, we observed that, although variability was high, the cross-over day increased to an apparent plateau as stores went up. Cross-over time ranged from 1.5 – 4.1 days, with a mean of 2.74 days. Assuming this relationship is confirmed in future studies, it indicates that, if plasma is sampled 3 days after dose administration, we should be able to eliminate the factor *S* in the Olson equation since, at that time, the retinol specific activities in plasma and stores should be about the same (*S* = 1).

Putting this all together, we come up with the following simplified equation for estimating total body vitamin A stores:

$$TBS = F * Dose * H:D \qquad \text{Eqn [3]}$$

or:

$$TBS = F * (1/SA) \qquad \text{Eqn [4]}$$

where SA = fraction of dose in plasma retinol / mass of plasma retinol. For both of these equations, *F* = 0.61

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and the blood sample for measuring SA or H : D is collected 3 days after dose administration. Thus, in summary, we were able to eliminate the factor *S* from Eqn [1] since the ratio of plasma / "liver" specific activities at 3 days is about 1. We can also eliminate the factor *a* because a careful review of the work of Bausch and Rietz [7] indicates that *a* is actually incorporated into *F*. And, if the dose is indeed a tracer (i.e., the mass is much less than body stores), we can also eliminate the factor –1, leaving us with Eqn [4].

Other Considerations

Is the RID useful for determining vitamin A requirements?

Haskell et al. have performed the RID twice in the same individuals ("repeated" RID) to evaluate the impact of interventions on TBS [21]. Using data for Bangladeshi subjects, these researchers looked at the change in the 3-day D : H ratio as an indicator of changes in vitamin A stores (i.e., as status goes up, there is a reduction in the plasma D : H ratio as the tracer gets diluted in stores). Haskell et al. concluded that the repeated RID can be used to estimate vitamin A EAR (estimated average requirement) for population subgroups for which there are no direct measures. The repeated RID would also be useful in evaluating intervention studies, when the apparent stores would be measured before supplementation or treatment and again after, to see how the size of the stores was changed by the intervention. This may be a better approach – to assess effects of treatment in a group – rather than trying to get an absolute value for TBS in an individual; see results of Haskell et al. [22].

Haskell et al. [21] also plotted the log of the change in vitamin A pool vs. the amount of supplement and used the cross-over point as an estimate of the intake required to maintain vitamin A balance. They found that about 400 μg/d was needed to maintain balance in subjects with low liver vitamin A stores vs. 700 μg/d in those who had been supplemented before the second RID test so that their liver stores were higher. This result shows that vitamin A utilization rate is dependent on stores, as has been shown in rats [23]: the higher the stores, the higher the utilization, and therefore the higher the amount needed to maintain balance. This indicates that, for human subjects with larger body stores, if the EAR for vitamin A is set below their levels of utilization, subjects may go into negative vitamin A balance until utilization again equals intake.

Is the RID equation valid for estimating TBS in children?

Currently, there is not much data on this point but, given the extent of vitamin A deficiency in children worldwide, it is something that should be studied. Experiments in animal models, as well as "superkid" studies [24], in which a limited number of blood samples are collected from each subject, with times varying between subjects except that all are sampled at 3 days after dosing, are recommended to determine the effect of stores on *F* in young subjects.

Is inflammation a confounding factor when applying the RID?

It is known that inflammation affects the steady state level of retinol in plasma. Specifically, both chronic and acute inflammation cause plasma retinol to fall (reviewed in [18]; see also [25]). In rats, acute inflammation leads to a decrease in plasma retinol specific activity – both tracer and tracee drop [26]. If the labeled dose is given orally and blood is sampled 3 days later, plasma retinol specific activity may actually increase but this needs to be determined experimentally. Based on what is currently known, it is very likely that inflammation will have an effect on the results of the RID technique.

What do we know about vitamin A absorption and its impact on the RID?

Surprisingly little is known about the efficiency of vitamin A absorption in humans. Knowing the extent of absorption is critical for developing accurate whole-body models because the prediction of stores is highly dependent on absorption efficiency. Absorption efficiency could be assessed using a dual isotope method, modeled on a test developed to determine cholesterol absorption in rats [27]. In the dual label method, subjects would be given an oral dose containing one stable isotope of vitamin A and an intravenous dose of artificial chylomicrons [28, 29] containing a different stable isotope. Once the labels have equilibrated, the ratio of the fraction of dose of the two isotopes in plasma would reflect absorption of the oral dose, under the assumption that the intravenous dose represents 100 % "absorption."

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Conclusion

In conclusion, much progress has been made in developing a feasible method for assessing vitamin A status in humans. With continued research to refine specific aspects of the ^{2}H or ^{13}C retinol isotope dilution technique and with careful attention to its limitations and capabilities, we should be able to accurately assess vitamin A status in a wide variety of settings and populations in future studies.

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Abbreviations

DRD	Deuterated retinol dilution
EAR	Estimated average requirement
H:D	hydrogen:deuterium
RBP	Retinol-binding protein
RID	Retinol isotope dilution
SA	Specific activity
TBS	Total body stores
TLR	Total liver reserves
WinSAAM	Windows version of the Simulation, Analysis and Modeling software

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