Am J Physiol Heart Circ Physiol 329: H1174–H1179, 2025.
First published October 10, 2025; doi:10.1152/ajpheart.00428.2025

american physiological society

AMERICAN JOURNAL OF PHYSIOLOGY
HEART AND CIRCULATORY PHYSIOLOGY

METHODS AND RESOURCES

Vascular Biology and Microcirculation

Method for early and repeated measurement of blood volume in newborn piglets

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Abstract

There are limited studies of changes in blood volume after birth in preterm infants. This study aimed to develop a method to measure blood volume in preterm piglets that could potentially be adapted for use in a clinical setting. A tracer dilution method using fluorescent-labeled dextran was optimized and tested in vitro and in vivo in preterm (98/115 days gestation) and term piglets. In vitro, the dextran method had an error of individual samples ranging from −14.2% to +15.9% with a mean bias of +0.1%. Smaller tracer size (150 kDa) significantly overestimated volume with a bias of >20%, compared with the 500 kDa tracer. Using three or four sampling time points fitted with a linear or log-linear curve had only small bias for all groups (<2.3 mL/kg), despite significant differences compared with using a one-phase decay curve fitted to 2 h of sampling (14 time points). Single samples at 10 or 20 min after injection had biases of +4 ± 4mL/kg (range: −7 to +14 mL/kg) and +8 ± 4 mL/kg (range: −5 to +19 mL/kg), respectively. Repeated measures are feasible from birth with minimal blood sample losses (0.5 mL). In neonates, the dextran tracer method requires use of a large tracer size (500 kDa) with multiple, timed samples (minimum 3 samples over the first 40 min after injection). This simple fluorescent-labeled dextran method prevents overestimation of blood volume in neonates and allows for repeat measurements from birth. This method can be adapted for use in preterm infants, which is critical to testing the efficacy of interventions designed to support preterm blood volume.

NEW & NOTEWORTHY There are very limited studies on blood volume in preterm babies. Early, repeated measurements of blood volume are essential to understand rapid changes that occur after birth and test interventions to manage cardiovascular function. This study found that blood volume in neonates is overestimated unless large tracers with multiple, timed samples are used to overcome rapid tracer loss from the circulation. This simple method can be adapted for use in preterm infants.

blood volume; cardiovascular; preterm

INTRODUCTION

Although it is widely accepted that blood volume in preterm infants is higher than at term, and thus hypovolemia is unlikely to contribute to cardiovascular instability, there is limited evidence for this. Only three publications report blood volume in very preterm infants within one day of birth. The reported values vary widely, and none are higher than values reported at term (1).

Measurement of blood volume in preterm infants is particularly challenging. Development of such a method is complicated by the lack of a gold standard for measurement of blood volume, making validation of any method difficult. Historically, dye dilution methods for measuring blood volume were developed using adults and relied on small plasma tracers or dyes. It was assumed that these tracers are not lost from circulation within the time frame of the measurement. If the tracers are lost, they will overestimate blood volume. This is a particular problem for preterm infants where there is evidence of high loss from capillaries, even for albumin (2, 3). Thus, estimates of blood volume based on these small tracers

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Submitted 9 June 2025 / Revised 25 June 2025 / Accepted 7 October 2025

may be erroneously reporting higher values for preterm than term infants (4, 5).

More recently, red blood cell (RBC) tracers were developed for use in preterm infants, including the fetal:adult hemoglobin method and biotin-labeled RBC. As these studies reported similar average values for preterm and term infants (1), this again raises questions about whether preterm blood volume is higher than in term infants and highlights the need for a simple and reliable method for measuring blood volume in neonates.

The aims of this study were: *1*) to develop a method to measure blood volume in preterm piglets, and potentially in preterm infants, using a large molecular weight fluorescently labeled dye that can be measured in small sample volumes; *2*) to validate this method in vitro; *3*) to apply this method in vivo, determine if the tracer is lost, and develop a method to correct for loss; *4*) to determine if accurate measurements of blood volume could be obtained using only three to four samples; and *5*) to model the effects of incorrect assumptions about lack of tracer loss on historical volume measurements.

MATERIALS AND METHODS

This study was covered by animal ethics approvals from The University of Queensland (UQ) (UQCCR/HMRC/592/16 and 2019/AE000534). All pigs were Large white × Landrace and were sourced from UQ's piggery at Gatton (Queensland, Australia), where pigs were provided standard care and had access to food and water, ad libitum. All pigs were euthanized using phenobarbitone sodium (162 mg/kg; Virbac Australia; piglets: 0.5 mL ip).

Method for Measuring Blood Volume

The plasma tracer chosen for this study was dextran, as it is stable, nontoxic, and not biologically reactive. Initially, a very high molecular weight dextran (500 kDa) was selected to limit the rate of tracer loss from the circulation and conjugated with either Fluorescein isothiocyanate (FITC) or tetramethylrhodamine (TRITC) (Sigma-Aldrich, St Louis). Then a lower molecular weight dextran (150 kDa) that was conjugated to CF680 (Biotium) was tested to determine if it was as accurate as the larger tracers.

A known amount of labeled dextran was added to the blood volume to be measured to target a final concentration of 20–40 μg/mL blood. This concentration minimizes the dose of dextran while still using the linear section of the standard curve. Blood samples (100–200 μL) were collected before addition of dextran (plasma blank) and after addition, allowing time for mixing. The concentration of dextran in the samples was determined by comparison with a standard curve. Standards were made using plasma collected from animals of the same gestational age. Plasma from samples (40 μL) and standards were diluted with phosphate-buffered saline (PBS) (160 μL) to minimize variations in pH that affect fluorescence levels. Fluorescence intensity was then measured in duplicate (2 × 80 μL; Spark 10 M multimode reader; Tecan, Switzerland). Following subtraction of the fluorescent intensity of baseline plasma samples, the dextran concentration of each sample was calculated using the linear equation from the standard curve. Duplicate values were excluded if >2 SD difference between fluorescence intensity readings. Plasma and blood volumes were calculated as below:

$$\text{Plasma volume (mL)} = \text{amount of dextran injected (μg)} / \text{dextran concentration of plasma sample (μg/mL)}.$$

$$\text{Blood volume} = \text{plasma volume}/(1 - \text{Hct}).$$

$$\text{Hct} = \text{hematocrit}.$$

In Vitro Validation

In newborn piglets (10–20 h old), anesthesia was induced with 2% isoflurane before administration of propofol (5 mg/kg) via an ear vein. Piglet blood (10–30 mL) was collected from umbilical artery and heparinized (10 IU heparin/mL blood).

The volume of the blood sample was determined accurately using an electronic pipette and confirmed gravimetrically. Dextran tracer solution (known concentration) was added as above, and the volume of tracer solution added was confirmed gravimetrically. Tracer was mixed with the sample volume by gently inverting the tube. A single blood sample was collected, and volume was calculated as above *method for measuring blood volume*. The accuracy of the method in vitro was assessed using a one sample *t* test to determine whether the difference between the known and the measured value was different to zero.

In Vivo Application and Tracer Loss

Preterm ($n = 12$) or term ($n = 15$) piglets were delivered by cesarean section at 98 days or 114 days of gestation, respectively (full term is 115 days). Piglets at 98 days gestation are developmentally similar to a 28 wk infant and are assumed to replicate vascular biology and leak. All piglets were delivered by cesarean section and had cords milked before delivery (see the study by Nguyen et al. 6 for details on sow anesthesia, piglet resuscitation, and blood volume values after birth). Piglets were intubated, ventilated, and maintained in intensive care. Sedation immediately commenced via the umbilical vein using a loading protocol, where dosage was reduced across the first hour to reach a target maintenance dose range (4–6 mg/kg/h propofol and 0.89–1.33 μg/kg/h fentanyl). Both umbilical arteries were catheterized; one to allow tracer injection and glucose (10%) infusion (3 mL/kg/h), the other for blood sample collection to prevent cross contamination, and heparinized saline infusion (0.5 mL/h). All animals included in this study were appropriate for gestational age.

An estimated blood volume of 80 mL/kg was used to calculate the amount of labeled dextran solution (known concentration) required to achieve the target final concentration of 20–40 μg/mL blood. Dextran solution was injected via an umbilical artery catheter and flushed with 1 mL of saline. The volume injected was confirmed gravimetrically. For the one-phase decay curve, timed blood samples (0.1–0.2 mL SST) were collected from the second arterial catheter over a 2 h period (5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 min after injection). In a different cohort (preterm, $n = 18$; term, $n = 18$), a 150 kDa dextran was injected alongside the 500 kDa to compare blood volumes generated using a smaller dextran. For this experiment, timed blood samples were collected over the same sampling time points. In all

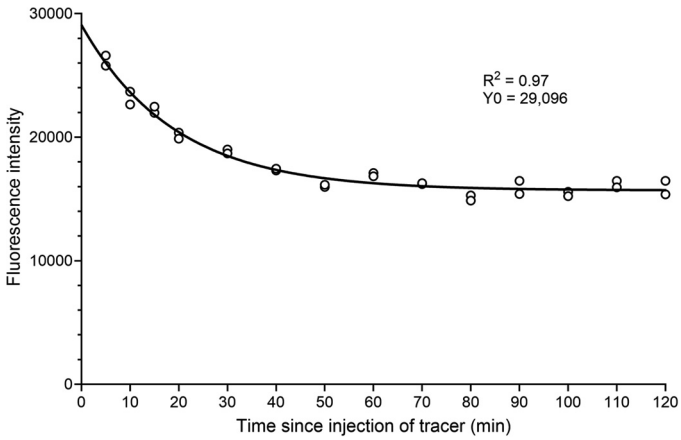

Figure 1. Representative figure of the fluorescence intensity of multiple-timed plasma samples over 2 h after injection of a 500 kDa dextran tracer with back extrapolation to the time of injection fitted using a one-phase decay curve. In this example, $R^2 = 0.97$.

piglets, blood volume was measured twice, at least 8 h apart to ensure that tracer levels had plateaued. Residual levels of the tracer from the first blood volume measurement were accounted for by taking a baseline blood sample before the second injection.

Correction for Tracer Loss

There was significant loss of the tracer out of the circulation over the 2 h experimental period. Tracer concentration decreased by 9% (SD 7) between 5 and 30 min after injection for 500 kDa dextran, then reached a plateau at 29% (SD 13) loss (e.g., Fig. 1). To correct for this tracer loss a one-phase decay curve was fitted to fluorescence values of plasma samples, using GraphPad Prism (Version 9.4.1, San Diego, CA). The 2 h period with multiple early samples ensured that the curve and plateau could be determined accurately. This type of curve was observed to have the best R^2 value of curve fitting methods trialed (including linear and log-linear) (Table 1). Back extrapolation was used to calculate the fluorescence intensity at the time of injection (I_0). Blood volume was calculated as above using I_0. Animals were excluded if data, such as Hct, was not available to calculate an accurate blood volume or if body weight was <10th centile.

Can Blood Volume be Determined with Only 3 or 4 Samples?

We also calculated blood volume using only three or four samples (GraphPad Prism). Two subsets of four samples were tested: *1*) "fast 4" using samples collected 5, 10, 15, and 20 min after injection, and *2*) "slow 4" using samples collected 10, 20, 30, and 40 min after injection. Two subsets of three samples were also tested: *1*) "fast 3" using samples collected 5, 10, and 20 min after injection, and *2*) "slow 3" using samples collected 10, 20, and 40 min after injection. For each sampling subset, both log-linear and linear curves were fitted, and back extrapolation was used to determine I_0. Blood volume values generated by minimum sampling was compared with those calculated by fitting a one-phase decay curve. One sample t tests were used to determine if the difference between the values obtained using the minimal sampling methods and one-phase decay curves were different to zero.

Possible Historical Errors

To investigate the effect of failure to account for tracer loss, we calculated blood volume using a single sample collected at either 10 min or 20 min after injection, similar to previous studies. These values were then compared with blood volumes calculated using the one-phase decay curve fitting. One sample t test was used to determine whether the difference between the values obtained using the single sample methods and one-phase decay curves were different to zero.

RESULTS

In Vitro Validation

The difference between the known volume of the sample and the measurement of blood volume obtained using fluorescently labeled dextran in vitro was not significant ($P = 0.93$, $n = 23$ samples). In individual experiments, the difference between the measured and the known volume ranged from −14.2% to +15.9% with a mean bias of +0.1%.

In Vivo Measurements and Tracer Loss

Using fluorescently labeled dextran and one-phase decay curve fitting (Fig. 1), blood volume was measured in 12 preterm and 15 term piglets. Differences in body weight, sex ratios, and blood volume measurements between groups were analyzed using Student's t tests are reported in Table 1.

Minimal Sampling Optimization

The differences between blood volumes obtained using one-phase decay curve and the three or four sample methods were significant; however, the bias was small in all groups (<2.3 mL/kg) (Fig. 2). For preterm piglets, the faster sampling methods had lower bias than the slower method, but the opposite was true for term piglets.

The best curve fitting for the 500 kDa dextran method using fast and slow sampling with both log-linear and linear curve fitting was determined by assessing the R^2 values. The R^2 values demonstrate that all minimum sampling methods had similar R^2 values to each other and that all were lower than the one-phase decay curve fitting (Table 2).

Comparison with Historical Methods

Blood volume calculated from a single at 10 or 20 min after injection was significantly higher than blood volume calculated using results from the one-phase decay curve. The

Table 1. *Body weight, sex ratios, and blood volume of term and preterm piglets*

	Preterm	Term	*P* Values
n, %male	12 (50)	15 (60)	
Body weight, g	933 ± 120	1,439 ± 159	<0.0001
Blood volume, mL/kg	78 ± 18	92 ± 7	0.026

Values are represented as means ± SD. n = number of animals.

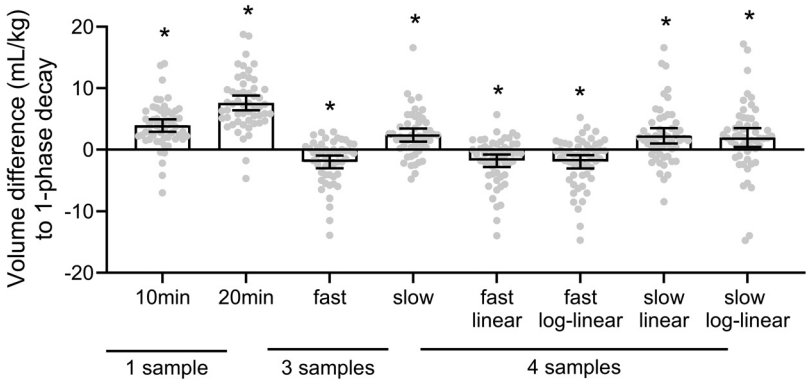

Figure 2. Minimum of three samples is required for accurate blood volume measurement. Discrepancies in blood volume measurements using 500 kDa dextran between the minimum sampling methods (a single sample at 10 min or 20 min, and fast or slow sampling using 3 or 4 samples with log-linear or linear curve fitting) vs. fitting a one-phase decay curve. Data are represented as means ± 95% CI. $n = 54$ blood volume measurements in preterm and term piglets. *Significant difference to the one-phase decay curve method ($P < 0.02$).

difference in methods was +4 ± 4 mL/kg (range: −7 to +14 mL/kg) for a 10 min sample and +8 ± 4 mL/kg (range: −5 to +19 mL/kg) for a 20 min sample (both $P < 0.0001$; Fig. 2).

Large Tracers Are Necessary for Accurate Blood Volume Measurement

Blood volume was significantly higher when calculated using a smaller 150 kDa tracer compared with a 500 kDa tracer, regardless of the method used (Fig. 3). Blood volume was overestimated by 20 ± 10 mL/kg (range: −14 to +35 mL/kg) when using the fast sampling log-linear method and 30 ± 14 mL/kg (range: −13 to +54 mL/kg) when using the slow sampling linear method.

DISCUSSION

This paper describes a new method for measuring blood volume in preterm piglets using fluorescently labeled dextran, a method that could be adapted for use in preterm infants. This method uses a large tracer (500 kDa) with multiple samples (minimum 3) to overcome errors associated with loss of the tracer from the circulation and avoids the likely overestimation in earlier studies in preterm infants.

The fluorescently labeled dextran method performed very well in vitro with a very low bias (0.1%). The maximum error was ~16% but most errors were less than 1%. This means that although a small number of individual measurements could have some error, there is negligible systematic bias and the mean of group measurements is likely to be very accurate.

Ideally, we would have also validated the blood volume measurement method in vivo. However, in vivo validation is very difficult as there is no gold standard method for measurement of blood volume. An alternative would have been to conduct accurately known manipulations of blood volume and assess the ability of the method to correctly measure the changes. There are several problems with this approach. Preterm piglets are extremely sensitive to removal of blood volume (7) and therefore only a small volume (<8%) could be removed. It is very difficult to accurately measure these small changes, and this would not provide a good indication of the performance of the method when measuring the larger total blood volume. Alternatively, we could increase blood volume by injecting a known amount of blood. However, the capillaries of the preterm piglet are very permeable, and it is likely that when blood volume is expanded by a large enough amount to measure, the increase in blood pressure will lead to a significant plasma loss (8) and thus the actual volume increase will be unknown.

Our results indicate that even though we used a very large tracer, there was a significant loss of the tracer from the circulation. This tracer loss, if not accounted for, will result in reduced tracer levels at the time of sampling and overestimation of blood volume. This is accounted for in our method by fitting a one-phase decay curve and back extrapolation to the time of injection. This method was treated as our "best estimate" against which other sampling protocols were assessed.

We compared results using this method with those obtained using only three or four samples, and values were significantly different; however, the bias was very small (<2.3 mL). This small error is clinically insignificant. Total sample volume collected for three or four sample calculations was 0.8–1.0 mL. This volume could be reduced by using fluorescence enhancers, increasing its suitability for use in preterm neonates. In preterm piglets, a shorter sampling period (5–20 min) had small bias than the longer sampling period (10–40 min), but the reverse was true in term piglets. As the magnitude of bias was <2.3 mL/kg across all groups, this difference in bias between preterm and term piglets is not clinically relevant allowing the application of a standard methodology across gestational ages.

Fluorescently labeled dextran has been used for measurement of blood volume in human adults and is safe (9). Testing of a carboxy-methylated 150 kDa conjugated to FITC in 89 adults found no serious side effects, likely because the carboxymethyl substitution has been demonstrated to

Table 2. *R^2 values for different curves fitted to slow and fast sampling methods*

		Three Samples		Four Samples			
R^2	One-Phase Decay	Fast Linear	Slow Linear	Fast Log-lin	Fast Linear	Slow Log-lin	Slow Linear
Mean (SD) $n = 43$	0.82 (0.13)	0.64 (0.27)	0.65 (0.30)	0.59 (0.31)	0.54 (0.28)	0.57 (0.30)	0.55 (0.28)

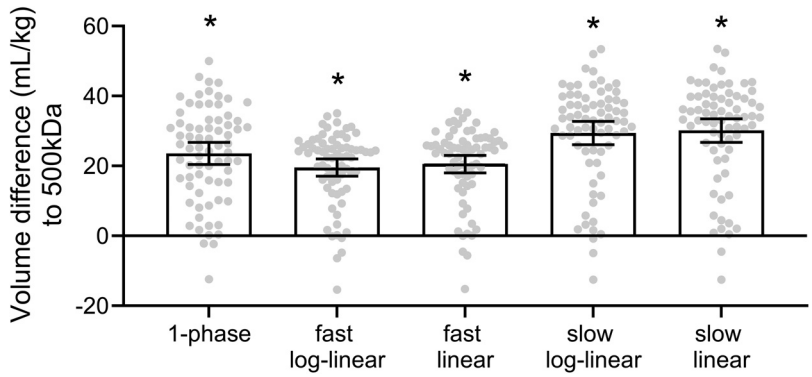

Figure 3. Large tracer necessary for accurate blood volume measurement. Discrepancies in blood volume measurements using 150 kDa dextran (4 samples, slow or fast sampling, linear and log-linear curve fit) compared with 500 kDa dextran (4 samples, fast sampling with linear curve fit). Data are represented as means ± 95% CI. $n = 72$ blood volume measurements in preterm and terms piglets. *Significant difference ($P < 0.02$).

markedly improve immunological tolerance (10). The dose given to adults was higher than that used in the current study (2 vs. 20–40 μg/mL blood) due to the treatment of the plasma sample with a fluorescence-enhancing reagent. So, our method using a 500 kDa with repeated sampling is likely to be safe for use in babies by the addition of these steps; using a carboxymethyl substitution at a lower dose coupled with a fluorescence-enhancing reagent.

Another advantage of the dextran method is that repeated measurement of blood volume is possible using dextran-labeled with different fluorophores. Also, repeat dosing with the same color is possible with at least a 3-h gap between measurements to allow the tracer levels to plateau so that background levels can be subtracted (Fig. 1). As there are substantial changes in blood volume after birth, this new method that allows measurement of blood volume at multiple time points across the first hours and days of life is essential to characterizing the dynamic changes that are likely occurring and for testing interventions to support newborn blood volume. This new information is now available for a preclinical piglet model (6).

An alternative method involves using a red blood cell tracer rather than a plasma tracer. In this case, the tracer is attached to red blood cells and so loss is negligible. Several options are available including biotin labeling and ^{51}Cr labeling. Ideally, autologous cells would be used; however, this has several disadvantages. The volume of blood that needs to be removed for labeling is prohibitive for neonates. The time required to label the cells prevents early assessment of blood volume. The dextran method can be used as soon as vascular access is secured allowing measurements very close to birth. The use of donor cells or the fetal:adult hemoglobin method, which can be used at birth, carries all the standard risks associated with transfusion.

We also calculated the likely error when volume is assessed using a single sample taken 10 or 20 min after tracer administration. This protocol partially mimics many of the early studies of blood volume in preterm and term neonates, where small tracers were used and only one sample was collected to measure tracer concentration. In fact, the early studies used smaller tracers than the current study and thus the error was likely to be even greater than we have estimated. Results were significantly higher than those obtained using one-phase decay curves. The average error was +4 mL/kg for 10 min samples and +8 mL/kg for 20 min samples. These high values may explain why early estimates of newborn blood volume are higher than those obtained in more recent studies where tracer loss is corrected for.

Volume measurements obtained by this method in preterm and term piglets averaged 69 and 81 mL/kg, respectively. These values are lower than the 90–100 mL/kg estimated in clinical guidelines for preterm infants (4, 5), this underlines the critical importance of further studies assessing early blood volume in preterm infants. More recent studies in preterm neonates (<24 h after birth) that account for tracer loss report very variable volumes (45–103 mL/kg) (11–13). The current study and others in preterm piglets also report variable volumes (37–101 mL/kg) (6) and substantial changes in volume in the hours after birth suggesting that large differences exist between individuals. If this also occurs in preterm infants, then some preterm infants may become hypovolemic soon after birth.

Conclusions

This study provides a simple new method for measuring blood volume in piglets. The method accounts for loss of tracer from the circulation and therefore avoids the overestimation of blood volume that likely occurred in early studies in neonates. This method allows repeated measurements of blood volume from very soon after birth to capture rapid changes in blood volume that occur in the hours after birth. Importantly, this method can be adapted for use in infants. There is an urgent need to measure blood volume in preterm neonates to understand the dynamic changes in blood volume that occur in the hours after birth and how this contributes to cardiorespiratory instability.

DATA AVAILABILITY

The data set generated and analyzed during the current study is available in The University of Queensland repository, https://doi.org/10.48610/65b722d.

GRANTS

Financial support for this study was provided by the Health and Medical Research Council of Australia (APP1127142).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.E.L., I.M.R.W., and Y.A.E. conceived and designed research; H.K.M., T.M.T.N., B.E.L., B.A.B., I.M.R.W., and Y.A.E. performed experiments; H.K.M., T.M.T.N., B.E.L., B.A.B., I.M.R.W., and Y.A.E. analyzed data; H.K.M., T.M.T.N., B.E.L., B.A.B., I.M.R.W., and Y.A.E. interpreted results of experiments; B.A.B. and Y.A.E. prepared figures; Y.A.E. drafted manuscript; B.E.L., B.A.B., and Y.A.E. edited and revised manuscript; H.K.M., T.M.T.N., B.E.L., B.A.B., and Y.A.E. approved final version of manuscript.

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