A SIMPLE METHOD FOR SIMULTANEOUS DETERMINATION OF PLASMA AND RED CELL VOLUME
BY
M. L. HEATH, M. D. VICKERS AND D. DUNLAP

SUMMARY

Several aspects of the methodology and interpretation of dual tracer measurements of blood volume have been investigated. A simple technique using autologous $^{51}$Cr chromated red cells and $^{125}$I RIHSA is presented in detail. Accuracy and reproducibility have been assessed in duplicate studies in volunteers. Measured volumes have been compared with the volumes computed from standard prediction formulae.

For many clinical purposes, a single-tracer dilution volumes using either labelled red cells or a plasma label can be used to estimate blood volume. The accuracy is improved by measuring the venous haematocrit and applying a correction assuming a normal whole body/venous haematocrit ratio (Heath and Vickers, 1968). In some circumstances this assumption may not be permissible (Smith and Moore, 1962; Remington and Baker, 1961; Heath, Vickers and Dunlap, 1969) and both red cell and plasma volumes must be measured independently. Various techniques have been employed, either using two tracers of different physical natures, such as one dye and one radioactive substance (Chaplin, Mollison and Vetter, 1953), or techniques using two radioactive tracers. These have usually involved separating cells from plasma before counting, e.g., Hart and Metz (1962). $^{131}$I labelled albumin has been available for some time but because its emission energy is very similar to that of $^{51}$Cr, it is not possible to assay the two isotopes in an unseparated sample. $^{125}$I, however, can be assayed in the presence of $^{51}$Cr, and has the further advantages of a longer half-life and a slightly lower dose of radiation to the patient. Weinstein (1964) and Wood and Levitt (1965) used this pair of isotopes, but their techniques involved counting samples of separated plasma. Brozovic and associates (1966) also used $^{125}$I and $^{51}$Cr, recounting the samples using a brass shield to eliminate counts due to the $^{125}$I. These isotopes can, however, be assayed independently in an unseparated sample by counting only those emissions whose energies fall within ranges which are relatively specific for each isotope. If two independent counting channels are available, both counts can take place simultaneously. There is some overlap of $^{51}$Cr emissions in the $^{125}$I energy band, but this can be allowed for in the calculation.

The basis of the technique described in this paper is an indicator-dilution measurement of red cell volume using red cells labelled with $^{51}$Cr as sodium chromate, and plasma volume using $^{125}$I radio-iodinated human serum albumin (RIHSA). Dilution specimens of whole blood from the patients are compared with standards of approximately similar activity, prepared by volumetric dilution of accurately weighed aliquots of the injected isotopes. Specimens and standards are assayed under two sets of counting conditions simultaneously. Some initial experiments have also been conducted with a view to simplification of currently recommended methodology and these are described first.

Red cell labelling techniques.

We have been unable to trace experimental justification for the various red cell labelling techniques advocated by different authors. The effects


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of three variations in technique were therefore investigated. Cells were labelled with and without prior centrifugation, incubated either at 37°C or at room temperature (21°C), and either 3.8 per cent sodium citrate or standard ACD* used as anticoagulant. Every combination of these variables was tried, making eight alternatives in all (table I). The blood was all from the same subject and 20 μC of $^{51}Cr$ as sodium chromate was added to 10 ml blood and 2.5 ml of anticoagulant. After a ten minute incubation, cells were washed with saline three times, and the activity of the red cell suspension and the final supernatant then assayed. The results are shown in table I. The lowest activity in the supernatant and the highest in the red cell suspension was obtained by adding $^{51}Cr$ before centrifugation, incubating at 37°C and using 3.8 per cent citrate as anticoagulant (table I). Using this technique estimation of activity injected as free $^{51}Cr$ was always less than 0.1 per cent of the dose.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts</th>
<th>Final supernatant</th>
<th>Red cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp (°C)</td>
<td>Anticoag.</td>
<td>Prior spin</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>3.8% cit.</td>
<td>Yes</td>
<td>4,792</td>
</tr>
<tr>
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<td>3.8% cit.</td>
<td>Yes</td>
<td>5,499</td>
</tr>
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<td>3.8% cit.</td>
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<td>2,776</td>
</tr>
<tr>
<td>20</td>
<td>3.8% cit.</td>
<td>No</td>
<td>4,137</td>
</tr>
<tr>
<td>37</td>
<td>A.C.D.</td>
<td>Yes</td>
<td>3,912</td>
</tr>
<tr>
<td>20</td>
<td>A.C.D.</td>
<td>Yes</td>
<td>6,714</td>
</tr>
<tr>
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<td>A.C.D.</td>
<td>No</td>
<td>3,607</td>
</tr>
<tr>
<td>20</td>
<td>A.C.D.</td>
<td>No</td>
<td>5,335</td>
</tr>
</tbody>
</table>

Veall and Vetter (1958) advise that to avoid damage to cells, centrifugation during the cell washing procedure should be limited to 1000 r.p.m. and continued for ten minutes. This advice is of limited value unless the dimensions of the centrifuge are known and the force can be calculated. We found no haemolysis at 900 g (2000 r.p.m. on an MSE super medium centrifuge) for five minutes. This reduction in centrifuging time reduces overall cell preparation time by a third.

Initially, glass centrifuge tubes were used. When these tubes had been used two or three times it was noticed that labelled red cells appeared to be rapidly removed from the circulation. There was no excess activity in the final supernatant to suggest haemolysis prior to injection and cultures were sterile. Since sodium citrate is known to attack glass, autoclavable polypropylene tubes were subsequently used without further difficulty.

**Preparation of standards.**

RIHSA is known to be adsorbed on to glass (Reeve, 1957). Using several cartridges from the same batch, the time course and degree of adsorption were investigated. Twenty-four samples were prepared by ejecting a weighed amount of isotope directly into counter tubes, and making up the volume with water. Nine glass volumetric flasks were made up by dilution of weighed amounts of isotopes and three aliquots from each of these were dispensed after various intervals. The quantity of isotope was adjusted so that the volumes and activities of all samples were similar. No significant drop in counts occurred if the solution was dispensed within 30 minutes of preparation (mean difference $-0.6$ per cent; $P = 0.14$).

These counter tubes were retained. Nine days later further aliquots were dispensed from the flasks. The original samples were reassayed together with these. The samples which had been dispensed later showed a significantly lower count rate (mean difference $-2.2$ per cent; $P<0.01$). The technique for limiting adsorption recommended by Veall and Vetter (1958, p. 225) was investigated by comparing three aliquots drawn from each of six volumetric flasks, to three of which potassium iodide and sodium hydroxide had been added. Samples were dispensed immediately after dilution. Under these circumstances, this technique made no difference. (With KI and NaOH: net mean counts 81,114, SD 1267; without KI and NaOH: net mean counts 81,116, SD 1547).

**Methods of dispensing aliquots.**

Standard glass safety pipettes were compared with disposable plastic syringes. Nine 5 ml samples of lysed blood and nine of distilled water at room temperature were dispensed by safety pipettes. The amounts ejected were weighed. The specific gravity of the lysed blood was measured and the
weights of water corrected to give the equivalent volume. The experiment was repeated using disposable plastic syringes (Johnson and Johnson Ltd, 5 ml) adjusted by eye. Although the variability of pipettes was greater than that of syringes, the difference was not significant (variance ratio 1.78; \(P > 0.05\)). However, the volume of blood delivered by pipettes was significantly smaller than the volume of water (mean difference 1.3 per cent; \(P = 0.001\)). There was no significant difference between blood and water volumes using syringes (mean difference 0.1 per cent, \(P = 0.7\)).

Reeve (1957) has shown that significant adsorption of RIHSA on to glass pipettes and syringes can occur. Plastic syringes were therefore investigated for this effect by comparing the activity of samples dispensed by direct pouring of a RIHSA solution into counter tubes against that of samples dispensed using plastic syringes. Samples were weighed and activity per gram calculated. No significant difference was detected (mean difference 0.29 per cent; \(P = 0.6\)). Syringes were therefore more reliable as well as being considerably more convenient.

Variations in sampling technique.

Emphasis has been placed on the avoidance of stasis during venous sampling (Veall and Vetter, 1958, p. 225) and on the need to sample from a vessel other than that into which the dose has been injected (Sevelius, 1965, p. 76). To test this, pairs of samples were drawn simultaneously every five minutes during one run. One sample of each pair was drawn via a cannula through which the isotope had been injected and above which a venous tourniquet had been applied for two minutes before sampling. This experimental design would tend to produce additive errors in the direction of a low estimate in the arm with the tourniquet. No significant difference was detected (mean difference 0.29 per cent; \(P = 0.6\)). Syringes were therefore more reliable as well as being considerably more convenient.

The full technique.

In the light of the foregoing experiments a simple technique was evolved for the simultaneous measurement of red cell and plasma volumes. It requires a minimum of standard laboratory apparatus, involves little in the way of decontamination and can be undertaken by those not specifically equipped for radioactive tracer work. It is therefore described in detail.

All manoeuvres are performed aseptically and all equipment is sterile. All weighings are to ±0.1 mg. 2.5 ml of preservative free 3.8 per cent sodium citrate is drawn into a 10 ml plastic syringe and approximately 10 ml of blood are taken from the patient into this syringe. The mixture is discharged gently into an autoclaved 15 ml polypropylene stoppered centrifuge tube. 20 \(\mu\)c of \(^{51}\)Cr as sodium chromate in approximately 0.5 ml saline is added, and the tube capped and mixed gently before being suspended in a water bath at 37°C for 10 minutes. During this time the tube is inverted gently once or twice. The cell suspension is centrifuged for 5 minutes at 900 g and as much of the supernatant as possible is removed. Approximately 6 ml of 0.9 per cent saline are added and the tube gently mixed again. Centrifugation and resuspension are repeated twice more. Four ml of the last supernatant are retained for estimation of activity. The final suspension is made up to about 12 ml in a fresh syringe, mixed thoroughly, and about 2 ml ejected into a clean centrifuge tube; 1 ml of this is drawn up into a 1 ml plastic syringe and used to prepare the red cell standard.

Both the dose and the standard syringes are weighed with disposable needles and needle covers in position. The contents of the 1-ml syringe are ejected into a volumetric flask and made up to 100 ml with water which ensures complete haemolysis and even distribution of the isotope. The syringes are reweighed after ejection, subtraction yielding the weight received by the patient and the amount used for the standard.

A standard cartridge and disposable syringe kit as supplied by the Radiochemical Centre, Amersham, containing up to 5 \(\mu\)c of RIHSA is assembled and weighed. The contents are ejected into a 1-litre volumetric flask and the volume made up with water. Immediately after mixing, duplicate 4-ml aliquots of both standards are dispensed using disposable syringes. The aliquots of RIHSA standard are retained for all estimations involving the same batch of RIHSA, and the remainder of the contents of the flask discarded. The empty cartridge is reweighed. A second cartridge is assembled and weighed for use as the dose for the patient.
A cannula at least 16 s.w.g. is inserted into a vein and a sample of blood is drawn for estimation of pre-existing radioactivity in the patient. An infusion is attached via a three-way tap. Both tracers are injected through the rubber tubing, with the drip firmly turned off, and are then flushed in rapidly. Both dose syringes are re-weighed. Great care must be taken that no infusion fluid leaks back into either of the syringes, and that no isotope is lost at any stage between the two weighings. Both these errors would invalidate the method of determining the administered dose.

Ten minutes after injection of the tracers, 4.5 ml of blood is withdrawn from the patient into a 5-ml plastic syringe which has been wetted with concentrated heparin solution (25,000 μ/ml). The side arm of the tap is used and at least 5 ml of blood is drawn through the system before taking the sample. Three more samples are taken at 5 minute intervals. The sample syringes are thoroughly mixed and the plunger adjusted carefully to the 4 ml mark, the ejected excess from each sample being used for haematocrit estimation in quadruplicate by the micro-centrifuge method using a Hawksley centrifuge. This method eliminates trapped plasma (Strumia, Sample and Hart, 1954). The syringes are then emptied into counter tubes containing a few drops of Teepol (Shell Chemical Co.) which haemolyses the samples and ensures uniform distribution of both isotopes throughout the sample. Samples, background and standards are counted under two sets of conditions; emissions between 210 keV and 454 keV are due to \(^{51}Cr\). Emissions between 16 keV and 38 keV are largely \(^{I25}I\), but approximately 5 per cent of the \(^{31}Cr\) counts also fall within this range. Machine shielding and counting time are adjusted so that background count rate is less than 10 per cent of sample count rate and net sample counts exceed 10,000.

Calculations.

Natural background is subtracted from the standards, and patient background is subtracted from the samples. The standard count is the result of diluting a known weight of isotope in a known volume. The sample counts result from the dilution of another known weight of the same activity in an unknown volume in the patient. This dilution volume is thus calculated by the formula:

\[
\text{Dilution volume (litres) = } \frac{\text{Net standard counts} \times \text{weight injected into patient} \times \text{volume of standard in litres}}{\text{Net sample counts} \times \text{weight used to prepare standard}}
\]

In one channel all the counts are derived from \(^{51}Cr\) and the net counts from standard and samples on this channel are used in the above formula. The red cell mass is calculated by multiplying the \(^{51}Cr\) dilution volume by the haematocrit of the sample. In the case of the \(^{125}I\) dilution volume, however, all net counts must be corrected to allow for the small proportion of \(^{51}Cr\) counts recorded in the second channel. This proportion is determined by dividing the net counts recorded in the \(^{125}I\) channel by the net counts recorded in the \(^{51}Cr\) channel during counting of the \(^{51}Cr\) standard. With the conditions described above this correction factor is around 0.05 (5 per cent); and this proportion of the net counts of each sample in the \(^{51}Cr\) channel is subtracted from the net counts in the \(^{125}I\) channel before the \(^{125}I\) dilution volume is calculated. The plasma volume is derived by multiplying the \(^{125}I\) dilution volume by the proportion of plasma in the sample.

Accuracy and reproducibility.

The foregoing technique was employed in 7 volunteer subjects. The study was performed twice on each subject at an interval of two hours. Samples were assayed on an automatic gamma counter (Nuclear Enterprises Ltd., Gammamatic Mark II) for 200 seconds. Background and standards were counted before, halfway through, and at the end of each batch of samples, and the complete sequence was counted twice to minimize the influence of small changes in counting conditions; serious systematic changes would also be easily detected by this routine. Red cell and plasma volumes were calculated for each of the 10 to 25 minute samples in each run (table II). In two of the runs only three samples were obtained so that in all, 54 estimations of red cell and plasma volumes were made. Total blood volumes and whole body haematocrits were calculated using the mean values of red cell volume and plasma volume in each run. The whole body/venous haematocrit ratio was calculated for each run. The red cell volume two hours after the first tracer injection was calculated from the sample taken for estima-
### Table II

Results of duplicate red cell and plasma volume estimations in 7 subjects.

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Run No.</th>
<th>Sample time (min)</th>
<th>Plasma volumes</th>
<th>Red cell volumes</th>
<th>Total blood volume (ml)</th>
<th>Venous haematocrit</th>
<th>Whole body haematocrit</th>
<th>F&lt;sub&gt;cell*&lt;/sub&gt; ratio</th>
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<td></td>
<td>10</td>
<td>15</td>
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<td>15</td>
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</table>

* Whole body/venous haematocrit ratio.
tion of patient background during the second run. Results are presented in table II.

Accuracy.
The values based on the individual samples of each run can be expressed as a percentage of the mean for that run. This normalization enables the data from all the studies to be combined. By excluding variations due to difference between individual patients and between duplicate runs, the coefficient of variation will estimate the variability due to errors in counting and weighing, sample and standard preparation and dispensing, and haematocrit estimation. The coefficient of variation was 2.0 per cent for both red cell and plasma volumes.

Reproducibility.
Mean results for the two runs on each subject were compared using Student’s t test for paired samples. Reproducibility was also good, there being no significant difference between the means of the two runs on each subject. (Mean differences: red cell volume, +1.7 per cent, P = 0.65; plasma volume, +3.8 per cent, P = 0.06). The measured increase in plasma volumes between estimations was accompanied by a mean fall of 0.7 per cent in the venous haematocrit. Such a fall in haematocrit would account for nearly 60 ml (1.8 per cent) of the increase in plasma volume and probably reflects a contribution from the volume of saline infused to maintain patency of the cannulae between estimations. Independent of this, one should expect an overestimation of the plasma volume on the second run: during the second run, RIHSA from the first injection is still being lost from the circulation and this is not accounted for by using a single background sample throughout the run. By comparing the activity in the background sample at the start of the second run with the value at the end of the first run, the rate of fall in activity can be calculated. On log/linear plotting the fall was found to be 11.9 per cent, and this could lead one to expect a further fall of 2.0 per cent during the second run. This corresponds exactly to the remaining mean difference found between the two runs.

The whole body/venous haematocrit ratio was relatively constant from subject to subject, and did not differ significantly between the two runs (1st run, mean: 0.913, SD 0.029; 2nd run, mean: 0.916, SD 0.039; mean difference 0.003, P = 0.89).

The red cell volume two hours after the first tracer injection did not differ significantly from the means of either the 1st or 2nd runs (mean differences: 1st run, +1.9 per cent, P = 0.17; 2nd run, −0.2 per cent, P = 0.96).

Comparison with prediction formulae.
Predicted blood volumes for the subjects were calculated from their heights and weights according to the formulae derived by Nadler, Hidalgo and Bloch (1962), and the formula derived by Allen and associates (1956) (table III). All these methods of prediction are derived from studies using independent plasma and red cell tracers or single tracer with correction for whole body/venous haematocrit ratio. The measured data correlated well with all these predictions but the best appeared to be the prediction from the computer corrected surface area formula of Nadler, Hidalgo and Bloch (col. (2) table III) which gave a mean difference of −134 ml ± 308 ml. This compares favourably with the results of Nadler,
Hidalgo and Bloch (1962) whose results showed a standard error of estimate of 392 ml for males and 413 ml for females.

**DISCUSSION**

This technique can give extremely accurate and repeatable simultaneous measurements of red cell and plasma volume with a minimum of special apparatus. The reproducible accuracy of the method is comparable with that of Wood and Levitt (1965) whose technique and calculation are more complex. It is not possible to make a comparison with the work of Button, Gibson and Walter (1965) or Albert and associates (1965) who have used the same isotopes because they did not publish duplicate studies.

The technique enables observations to be made of the whole body/venous haematocrit ratio, the $F_{cc}$ ratio of Reeve and associates (1953). Before the results can be compared with those of other workers, however, some consideration must be given to the method of calculation of the two volumes from the data. In the case of the red cell volume in subjects with a normal circulation no difficulties arise: red cells do not normally leave the circulation and mixing of tagged cells is complete in ten minutes (Albert, 1963). In fact, in this study samples which were drawn 5 minutes after injection showed no significant difference when compared with the mean of the 10–25 minute samples (mean difference 0.4 per cent, $P=0.72$). RIHSA, however, continuously leaves the circulation after mixing and many workers derive plasma volume at the time of tracer injection by logarithmic plotting of activity against a linear plot of time and extrapolation zero time (Gregersen and Rawson, 1959). Others have derived an average loss rate over a series of studies and applied a correction factor to their mean sample results (Chaplin, Mollison and Vetter, 1953). This assumes the same loss rate in all individuals and is probably valid if the circulatory state of the subjects is the same.

Both these methods assume instantaneous mixing and loss of tracer starting at the moment of injection. This simplification can only be an approximation: it would appear from the work of Chien and others (1964) that RIHSA is not detected in the lymph for at least 20 minutes after intravenous injection; Gitlin (1957), investigating the concentration of protein in interstitial fluid, assumed that there was insignificant loss of RIHSA from the plasma to the interstitial fluid during the first 15 minutes after intravenous injection. For these reasons it seems preferable to assume that loss of tracer before 25 minutes is negligible and that mixing is complete at 10 minutes. Thus the mean of the samples drawn between 10 and 25 minutes has been used to estimate the plasma volume. Graphical procedures are probably only indicated when much longer intervals are used or when loss rates are thought to be abnormally high. This view is shared by Remington and Baker (1961) and Button, Gibson and Walter (1965). The latter workers studied 71 patients, calculating the mean of three samples (10, 15 and 20 minutes) and found a mean $F_{cc}$ ratio of 0.902, SD 0.033; this is very similar to results presented here: mean 0.914, SD 0.033.

Calculation of Wood and Levitt's (1965) data shows a significantly greater variability in the $F_{cc}$ ratio in their subjects (variance ratio 7.11, $P<0.01$). Since their subjects were stated to be normal it would appear that the increased variability must reflect greater variability in their technique.

The most common reason for using a dual tracer technique is to elucidate or at least take account of variation in the $F_{cc}$ ratio; in these circumstances the use of an average correction factor for plasma tracer loss with extrapolation to zero time would seem to be particularly unsatisfactory, since this method of calculation itself may mask any change. The reasons usually cited for variations in the $F_{cc}$ ratio depend on the state of dilatation of all or part of the peripheral circulation (Albert, 1963), vasoconstriction tending to lower the ratio. However, vasoconstriction would also be expected to lessen the rate of RIHSA loss from the circulation and the use of an average correction factor would then overestimate the plasma volume. This would give an incorrectly high value for the $F_{cc}$ ratio, thus partially obscuring any actual change.

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REFERENCES


UNE SIMPLE METHODE DE DETERMINATION SIMULTANEE DU VOLUME PLASMATIQUE ET ERYTHROCYTAIRE

SOMMAIRE

Divers aspects de la méthodologie et de l'interprétation des déterminations à double traceur du volume sanguin ont été examinés. Une simple technique utilisant des érythrocytes autologues chromatés au 51Cr et 125I RIHSA est présentée en détail. L'exactitude et la reproductibilité ont été évaluées au cours d'essais doubles chez des volontaires. Les volumes mesurés ont été comparés avec les volumes dérivés de formules de prédiction standard.

EINE EINFACHE METHODE ZUR GLEICHZEITIGEN BESTIMMUNG DES PLASMA- UND ERYTHROZYTENVOLUMENS

ZUSAMMENFASSUNG