J. clin. Path., 1970, **23**, 304-308

Use of Triton X-100 scintillant in a simple method for the simultaneous assay of ^{55}Fe and ^{59}Fe by liquid scintillation counting

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SYNOPSIS A method is described using Triton X-100 scintillant to simplify liquid scintillation counting of ^{55}Fe alone or in combination with ^{59}Fe. The Triton acts as an efficient solubilizing agent allowing small volumes of aqueous solutions of iron compounds in hydrochloric acid to be counted. The efficiencies achieved are comparable with those of previously described methods whilst the technique is less time-consuming and the scintillant-aqueous mixture is stable at low temperatures. Reproducible quantitative recovery of radioactivity has been obtained.

The study of iron metabolism has been greatly facilitated by the use of radioisotopic techniques. The gamma emissions of ^{59}Fe are readily measured and this nucleide has been most extensively employed. Frequently, however, double isotope techniques using ^{55}Fe simultaneously or sequentially with ^{59}Fe are desirable. The long half-life of ^{55}Fe (2·7 years) also makes this isotope more suitable for long-term studies of iron loss.

^{55}Fe decays by electron capture, emitting manganese x rays, the most penetrating of which is the K x ray. This is produced in only 25% of disintegrations and has an energy of only 0·0059 MeV. The measurement of this low energy emission in whole blood samples presents considerable difficulty. The blood sample must be digested, the iron being separated by electrodeposition (Peacock, Evans, Irvine, Good, Kip, Weiss, and Gibson, 1946; Dern and Hart, 1961) or by the precipitation of insoluble iron compounds (Katz, Zoukis, Hart, and Dern, 1964). Liquid scintillation counting provides a sensitive and reproducible method of measuring ^{55}iron activity and has permitted the simultaneous assay of ^{55}iron and the β rays of ^{59}iron. The preparation of a stable scintillation system, however, has proved difficult (Perry and Warner, 1963; Eakins and Brown, 1966; Graber, McKee, and Heyssel, 1967).

In this paper we describe the use of Triton X-100 liquid scintillant (Patterson and Green, 1965; Turner, 1968) to simplify the liquid scintillation counting of ^{55}Fe alone or in combination with ^{59}Fe. The efficiencies obtained are comparable with those of previously described methods whilst the technique is less time-consuming and the scintillant system is stable at low temperatures. The applicability of this method to the measurement of iron absorption by the double-isotope technique (Veall and Vetter, 1958) has been demonstrated by comparison of results with those obtained by the total faecal collection method.

Received for publication 8 July 1969.

Materials and Method

Chemicals used were of reagent grade. They were concentrated sulphuric acid, 70% perchloric acid, 30% ammonium hydroxide, ferric ammonium citrate solution (5 mg iron per ml) and ascorbic acid-hydrochloric acid reagent (40 mg ascorbic acid per ml N HCl prepared immediately before use).

Toluene scintillant containing 105 ml Liquifluor[1] in 2·5 l of sulphur-free toluene was mixed with Triton X-100 detergent in the proportion 2:1 (v/v) toluene/triton.

^{55}Fe was obtained as ferric ammonium citrate (specific activity 2·94 mCi per mg) from the Radiochemical Centre, Amersham, England, and ^{59}Fe as ferric citrate (specific activity 9·0 mCi per mg) from the Commonwealth X-ray and Radium Laboratories, Melbourne.

Ten ml heparinized whole blood labelled with ^{55}Fe (with or without ^{59}Fe), 1 ml ferric ammonium citrate carrier, 15 ml concentrated sulphuric acid, and 7 ml perchloric acid are mixed in a Kjeldahl flask, glass beads being added to prevent bumping. The addition of 5 mg carrier iron was shown to produce better recoveries, as found by previous workers. The mixture is then heated very slowly on a digestion rack in a fume cupboard until the solution becomes a light yellow. It is then allowed to cool when it should be colourless. To ensure complete oxidation of the blood a further 2 ml perchloric acid may be added and the solution reboiled to remove traces of acid. Once the solution is again cool, ammonium hydroxide is added very slowly, cooling the flask continuously in a water bath, until heavy brown floccules of iron hydroxide are precipitated. The contents of the flasks are quantitativeiy transferred to centrifuge tubes, rinsing twice with approximately 5 ml NH_4OH. The tubes are centrifuged at 2,000 rpm for 10 minutes, approximately one half the clear supernatant being aspirated and discarded, and the remainder, including the brown precipitate, is quantitatively transferred into a counting vial washing twice with distilled water. The suspension is then centrifuged in the vial, the supernatant removed, and the brown iron precipitate dried in an oven at 50°C. After cooling, the iron precipitate is dissolved in 0·5 ml of the freshly prepared hydrochloric-ascorbic mixture, time being allowed for complete dissolution. Ten ml of the Triton scintillant is added to each vial, mixed thoroughly, and stored at 4°C for subsequent counting.

Activities were counted in a Nuclear-Chicago Unilux TM II liquid scintillation system. Counting channels were first selected to allow balance point counting of ^{55}Fe and ^{59}Fe separately. When counting these isotopes simultaneously, efficiency was necessarily reduced, the channel width for ^{59}Fe being reduced to exclude ^{55}Fe (less than 0·02% efficiency of balance point counting for ^{55}Fe), while the contribution of ^{59}Fe to the ^{55}Fe was decreased to 18% of the counts in the ^{59}Fe channel by reducing the upper level discriminator of the ^{55}Fe balance point channel.

True ^{55}Fe counts = recorded ^{55}Fe counts-18% of recorded ^{59}Fe counts, and true ^{59}Fe counts = recorded ^{59}Fe counts.

[1]Pilot Chemicals Inc, Watertown, USA

MEASUREMENT OF IRON ABSORPTION

A dose or approximately 5 μCi ^{59}Fe-ferric citrate was given in a standard breakfast which contained 5 mg of food iron and approximately 20 mg of ascorbic acid. Approximately 25 μCi ^{55}Fe-ferric citrate was incubated with normal plasma and injected intravenously about one hour after the breakfast. Standards were kept of both the ^{59}Fe-ferric citrate ingested and the ^{55}Fe-labelled plasma injected. Twelve to 14 days after administration of the isotopes, 20-30 ml of blood was removed for measuring ^{55}Fe and ^{59}Fe activity. Samples were prepared in duplicate by the technique described above. Aliquots of 0·5 ml each of the ^{55}Fe and ^{59}Fe standards were mixed with 10 ml of non-radioactive heparinized blood and again prepared for counting in duplicate. Absorption of orally administered ^{59}Fe was then calculated from the formula:

$$\% \text{ Absorption of } {}^{59}Fe = \frac{\text{Fraction of } {}^{59}Fe \text{ dose in blood sample}}{\text{Fraction of } {}^{55}Fe \text{ dose in blood sample}} \times 100$$

$$= \frac{{}^{59}Fe \text{ activity in blood}}{{}^{59}Fe \text{ activity administered orally}} \times \frac{{}^{55}Fe \text{ activity administered intravenously}}{{}^{55}Fe \text{ activity in blood}} \times 100$$

In all patients accurate faecal collections were made for 14 days after ingestion of the ^{59}Fe solution and the absorption of iron was calculated by the faecal recovery method.

Results

REPRODUCIBILITY AND RECOVERY OF ACTIVITY

This was evaluated by counting aliquots of whole blood, previously tagged *in vivo* with ^{59}Fe, in a sodium iodide well counter, then digesting and transferring them as above and recounting. Recovery in six such aliquots was 96·4 ± 1·3 SD%.

SELECTION OF ACID SOLVENT

Type

Hydrochloric acid was selected as the most suitable acid solvent since sulphuric acid did not decolorize the iron completely and orthophosphoric acid caused considerable quenching when used in sufficient concentration to dissolve the iron precipitate completely. Complete decolorization of the ferric iron with fresh ascorbic acid was necessary to avoid quenching. The amount of ascorbic acid added had little effect

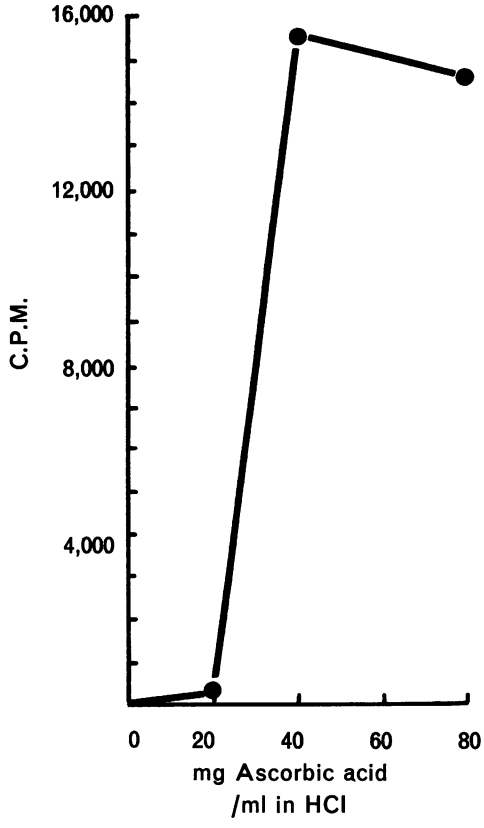

Fig. 1 *Relationship between count rate and ascorbic concentration.*

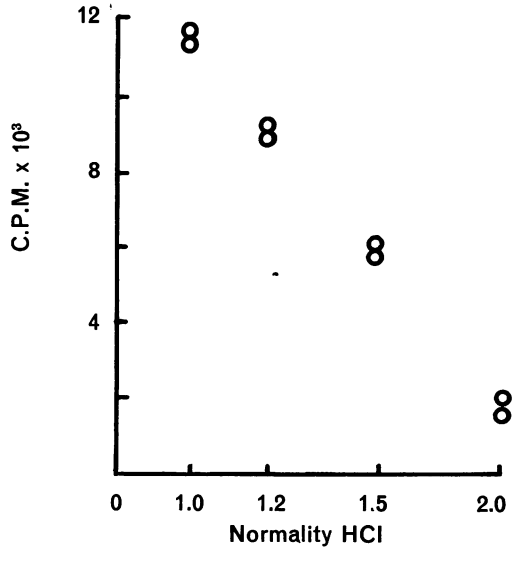

Fig. 2 *Effect of hydrochloric acid concentration on counting rate.*

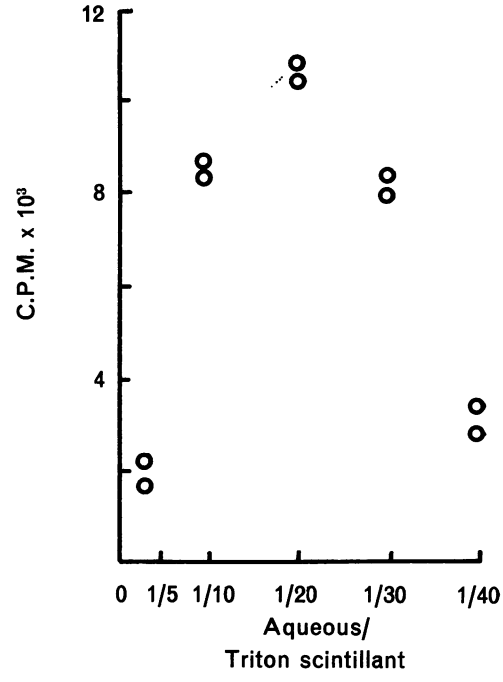

Fig. 3 *Effect of the aqueous/Triton scintillant ratio on counting rate.*

on the counting rate or cross counting ratio above concentrations of 40 mg/ml of the mixture (Fig. 1).

Normality of hydrochloric acid

Figure 2 shows the effect of hydrochloric acid concentration on the counting rate. It can be seen that the greatest efficiency was obtained using a normality of 1·0 HCl although it took rather longer to dissolve the ferric iron with this concentration and the precipitate did not dissolve satisfactorily with weaker concentrations of HCl. Reproducibility was good with both 1·0 and 1·2 N HCl.

AQUEOUS/TRITON SCINTILLANT RATIO

The optimum ratio for the toluene-scintillant/triton mixture was found to be 2:1 v/v. It produced a clear solution with maximum counting rates. Greatest efficiency was obtained with an aqueous to Triton-scintillant ratio of 1 in 20 (Fig. 3). Although the ratio is not very critical, counting rate falls rapidly if the aqueous phase is markedly increased, eg, 1 in 5, when an emulsion is formed, or if excess detergent is present, when a milky solution results. A 10 ml counting mixture, ie, 0·5 ml aqueous solution plus 9·5 ml Triton scintillant, was found to give greater efficiency than large volumes.

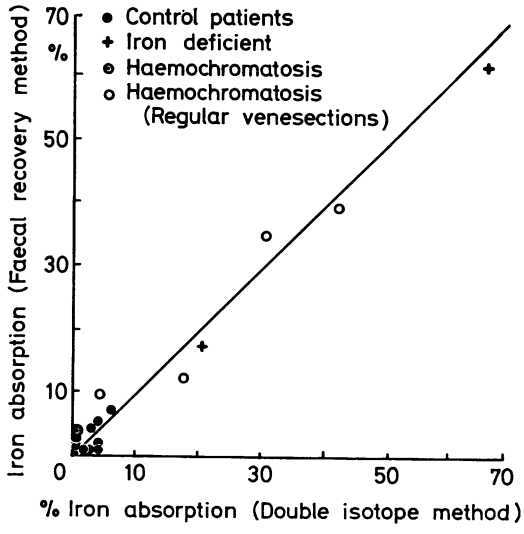

Fig. 4 *Correlation between iron absorption as measured by faecal recovery method and by double isotope method (r = 0·98).*

EFFICIENCY OF COUNTING

For each isotope, a known amount was added to six samples of whole blood *after* digestion and precipitation. The mean counting efficiency at balance point obtained for ^{55}Fe was 9·1 ± SD 1·2%. In terms of the K *x* ray emissions, efficiency was therefore 36%. Similarly the counting efficiency for ^{59}Fe was 70·5 ± SD 1·9%. When counting both isotopes simultaneously (see above) efficiencies were reduced to 3·2% and 40·2% for ^{55}Fe and ^{59}Fe respectively.

STABILITY OF THE SAMPLES

Both ^{55}Fe and ^{59}Fe counts remained stable for five to seven days when kept refrigerated. Thereafter, there was a gradual fall in counts which was parallel in both samples and mixed standards as well as digests of pure ^{55}Fe. Occasional samples showed a white opalescence at room temperature but this cleared completely on cooling.

IRON ABSORPTION

Figure 4 shows the results of iron absorption obtained by the double isotope method compared with the faecal collection method in 10 control subjects, two patients with iron deficiency, and five with idiopathic haemochromatosis, three of whom were undergoing regular venesection therapy. There was good correlation between two methods (r = 0·98, t < 0·001).

Discussion

The results of this study confirm that ^{55}Fe can be counted simply and with satisfactory efficiency by solubilizing aqueous solutions using Triton X-100 in the scintillant system.

Early methods of assay of ^{55}Fe in whole blood involved digestion and separation of the iron by electroplating, the activity being determined by the use of Geiger-Muller tubes (Peacock *et al*, 1946), by gas-flow counters (Hallberg and Brise, 1960), or by a beryllium-window counter (Pitcher, Williams, Parsonson, and Williams, 1965). Dern and Hart (1961) employed electrodeposition of iron followed by redissolving the iron for liquid scintillation counting. Later, Katz *et al* (1964) avoided the need for electroplating by using a stable emulsion of an aqueous solution of iron in a thixotrophic gel. A similar method was described by Perry and Warner (1963) who used a liquid scintillation system containing equal parts of toluene and ethanol. However, this scintillant does not mix with the aqueous solution of iron below 10°C. Hence samples must be maintained above this temperature for counting and this increases the background count rate due to thermal emissions from the photomultiplier tube. Eakins and Brown (1966) claimed greater efficiency by converting the iron into an insoluble white ferriphosphate complex and holding the precipitate in suspension in the scintillator by silica gel. This method and similar techniques described by others (Graber *et al*, 1967) were still complex.

Patterson and Greene (1965) investigated a number of emulsifiers for the measurement of low energy beta emitters in aqueous solution by liquid scintillation counting and found that, of those tested, only Triton X-100 had acceptable counting properties. It did not quench light emitted from scintillators and had a low concentration of phosphorescent contaminants. The solubilization with toluene solutions of 2, 5/diphenyloxazole (PPO) and 1, 4 bis-2-5 phenyloxazolyl-benzene (POPOP) was found to allow counting of large volumes of aqueous solutions with high efficiency. Compounds normally insoluble in organic solvents may be taken up in acid, alkali, or buffer and incorporated into Triton-scintillant, the detergent Triton X-100 acting as a very efficient solubilizing agent (Turner, 1968). The clear solutions formed were found to be stable for a long time. The use of Triton-scintillant in this study allowed considerable simplification of the method of sample preparation without sacrifice of counting efficiencies. As samples were stable at low temperatures, background counting rate was reduced to a minimum.

Although the digestion procedure used in the present method involves prolonged heating of sulphuric acid at its boiling point, we were not troubled by coloured digests or precipitates

although it was found necessary to use heparinized blood, as blood collected in acid-citrate-dextrose solution often produced a white precipitate in the digests. The use of perchloric acid for digestion and decolorization requires caution in view of its explosive potential. During the procedure samples must never be allowed to evaporate to complete dryness. Perchloric acid, however, has been found to be a most suitable decolorizer, especially when moderately large volumes of blood must be counted.

The application of the method to the double isotope technique gave results which compared favourably with those obtained by the faecal collection method for subjects with a wide range of iron absorption. With careful technique, excellent recovery of radioactivity after digestion and precipitation has been obtained. This is not essential where both sample and standard contain a mixture of two isotopes, as in the method employed here to calculate iron absorption, since the percentage recovery of each isotope will be identical. When samples contain ^{55}Fe alone, however, quantitative and reproducible recovery is mandatory.

We wish to acknowledge the skilled technical help of Miss Elizabeth Wilson, BSc (Qld), in this work.

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