Technical methods

Desferrioxamine chelatable body iron

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Further experience with the differential ferrioxamine test (Fielding, 1965) for the measurement of body-iron chelation has led to modifications which include (1) an improved standard for the estimation of ferrioxamine, (2) simplified preparation of the test dose, (3) restriction of fluid during the test and (4) simplified methods of calculation.

The principle of the test remains the same: an intravenous injection is given consisting of two components, the chelating agent desferrioxamine, and the iron chelate ferrioxamine, labelled with approximately $2 \mu\text{C } ^{59}\text{Fe}$. The urinary excretion of injected ferrioxamine is measured by isotope assay. Total ferrioxamine excretion is assayed chemically: this is derived both from injected ferrioxamine and from the action of desferrioxamine on body iron. The difference (Fd) between total ferrioxamine excretion (Ft) and ferrioxamine excreted from the test dose (Fex) represents the excretion derived from chelation *in vivo*. Total chelation *in vivo* (Fv) is then calculated by simple proportion from the isotope excretion data.

The simultaneous injection of chelator and isotope-labelled metal chelate in the measurement of chelation *in vivo* produces a system which may be of wider interest in the study of chelation, not only of iron but of other metals. The characteristics of the system are:

1 The observed value depends on the ratio of urinary chelate excreted from two independent sources (Fd/Fex): therefore, the timing of urine specimen collection is not critical since the ratio reaches a constant value in a relatively short time; thus, essentially the same result is obtained from a six-hour or seven-hour urine.

2 The results are independent of renal function, since both terms of the ratio are equally affected by variations in renal function. Thus, it is possible to study chelation *in vivo* in the presence of reduced renal function, provided sufficient excretion takes place to make chemical assay possible.

3 The results are not greatly affected by minor losses of urine during collection, since both terms of the ratio tend to be equally affected by such losses.

4 As shown below, the result is unaffected by variation in the volume of test dose within easily achieved limits.

THE TEST DOSE

The preparation of the test dose has been simplified by Ciba Laboratories Ltd. who have prepared vials con-

taining a mixture of 500 mg. desferrioxamine methane-sulphonate and 50 mg. ferrioxamine hydrochloride. The contents of one vial is dissolved in exactly 6.0 ml. sterile distilled water containing approximately $2 \mu\text{C } ^{59}\text{Fe}$ as ferric chloride. (Sterile distilled water and ^{59}Fe may be added in two separate volumes providing the total volume is exactly 6.0 ml.) Chelation of the labelled iron is completed within one minute. The standard intravenous dose is 1 ml. of this solution per 10 kg. body weight and is adjusted to body weight to the nearest 0.5 ml.; for example, 5.5 ml. is given to a 54 kg. subject. A little more than the volume required for the dose is prepared to provide a standard for the ^{59}Fe assay and for the chemical estimation of ferrioxamine in the test dose. It will be found convenient for both purposes to prepare a 1 in 100 dilution of the test dose by diluting a 0.25 ml. aliquot to 25 ml. in a volumetric flask.

PREPARATION OF THE PATIENT

On the morning of the test, fluids should be restricted to 6 fl. oz. before the test and only a further 6 fl. oz. permitted during the test period, in order to keep the six-hour urinary volume to less than 400 ml.

URINE SPECIMEN

All urine passed in the six hours after the test injection is collected, the last urine being voided at six hours. The containers should be iron free: a convenient iron-free container of 2-litre capacity, known as a Vico-Uriket¹, has been found useful for this purpose. It is fitted with a carrying handle and disposable polythene liners for both containers and lid.

ESTIMATION OF FERRIOXAMINE

A laboratory preparation is used as a standard for the estimation of ferrioxamine instead of the commercial preparation.

PREPARATION OF STANDARD FERRIOXAMINE SOLUTION

Dissolve 550.7 mg. ferric chloride, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (AR), in ion-free water and make up to exactly 100 ml. This contains 113.75 mg. iron per 100 ml. Dissolve 500 mg. desferrioxamine methanesulphonate (1 vial, Desferal Ciba) in 4.5 ml. ion-free water. Add an accurately measured 1 ml. ferric chloride solution and dilute to 50 ml. in a volumetric flask. This standard solution now contains 25 mg. per 100 ml. ferrioxamine base. Calibration of the standard and the estimation of urine specimens are as previously described (Fielding and Brunström, 1964) except that results are referred to ferrioxamine base not to hydrochloride.

¹Vicsons Ltd., Pinner Road, Harrow, Middlesex.

CALIBRATION OF STANDARD FERROXAMINE SOLUTION

Reagents

Standard ferrioxamine solution 25mg./100ml.
Desferrioxamine methanesulphonate 0.5g/100ml.
Citric acid $\text{H}_3\text{C}_6\text{H}_5\text{O}_7\cdot\text{H}_2\text{O}$ (AR) 10.5g/100ml.
Disodium hydrogen phosphate $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$
 19.8g./100ml
Disodium ethylenediamine tetracetic acid
 (Na_2EDTA) powder

Distribute standard ferrioxamine solution into nine numbered iron-free test tubes as follows: 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 0.5, 5.0 ml.

Make up each tube to 5 ml. with ion-free water to give the following concentrations of ferrioxamine base: 0, 2.5, 5, 10, 15, 20, 25, 2.5, 25 mg. per 100 ml. To each tube add 0.15 ml. desferrioxamine (0.5%) followed by 0.4 ml. citric acid solution, and mix. To tubes 8 and 9 add about 1.5 g. crystalline $\text{Na}_2\text{E.D.T.A.}$, mix and stand for 10 minutes, by which time decolorization should be complete. To all tubes add 1.5 ml. disodium hydrogen phosphate solution: mix and lightly centrifuge tubes 8 and 9 until supernatants are clear. Measure the extinctions in a suitable spectrophotometer at 430 $\text{m}\mu$, setting tube 8 to zero. Tube 9 should give zero reading; if not, an inadequate amount of $\text{Na}_2\text{E.D.T.A.}$ has been added and decolorization of ferrioxamine is incomplete. Plot the extinctions of the first seven tubes arithmetically against the concentrations of ferrioxamine base in the 5ml. volumes tested, to obtain a straight line through the origin. A concentration of 15 mg. per 100 ml. in a 1 cm. cell gave an extinction of 0.48 at 430 $\text{m}\mu$. The slope using the same apparatus is stable and it is unnecessary to recalibrate at each test.

ESTIMATION OF FERROXAMINE IN URINE

Measure into each of two iron-free test tubes 5 ml. of test urine: add 0.15 ml. desferrioxamine, followed by 0.4 ml. citric acid, and mix. To the second tube add 1.5 g. $\text{Na}_2\text{E.D.T.A.}$ and stand for 10 minutes. Now add 1.5 ml. $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$ reagent to both tubes and mix well. Centrifuge both specimens at 3,000 r.p.m. for 10 minutes or until the supernatants are water clear. Set the spectrophotometer to zero with tube 2 and measure the extinction of tube 1 at 430 $\text{m}\mu$. The concentration of ferrioxamine base in the test urine is read directly from the standard calibration curve.

THE DIFFERENTIAL FERROXAMINE TEST

Four observations are made: 1 The ^{59}Fe activity of the test dose, c.p.m./ml.; 2, the ^{59}Fe activity of the six-hour urine, c.p.m./ml.; 3 the ferrioxamine concentration of the six-hour urine, mg./100 ml.; and, 4, the ferrioxamine concentration of the test dose, mg./100 ml.

CALCULATION OF RESULTS

If V_s = volume of test dose (ml.)

V_u = volume of 6-hour urine (ml.)

C_s = ^{59}Fe activity of test dose (c.p.m./ml.) (this is $\times 100$ of the activity of the diluted test dose prepared as a standard)

C_u = ^{59}Fe activity of six-hour urine (c.p.m./ml.)

$[F]$ = ferrioxamine concentration in test dose (mg./100 ml.)

$[Ft]$ = ferrioxamine concentration in six hour urine (mg./100 ml.)

Then F_v , *in-vivo* chelation,

$$= \frac{C_s}{C_u} [Ft] - [F] \text{ (}\mu\text{g./kg.) as ferrioxamine}$$

F_{ex} %, proportion of intravenous ferrioxamine excreted in six hours

$$= \frac{100 C_u V_u}{C_s V_s} (\%)$$

F_d , six-hour excretion of ferrioxamine formed by *in-vivo* chelation,

$$= \frac{F_v C_u V_u}{C_s V_s} \text{ (}\mu\text{g./kg.)}$$

The results expressed as ferrioxamine ($\mu\text{g./kg.}$) may be converted to iron ($\mu\text{g./kg.}$) by the factor 0.09.

INTERPRETATION OF RESULTS

F_v is ferrioxamine formed from body iron by the standard intravenous dose of Desferal. F_d is the amount of such ferrioxamine, excreted in the urine in six hours, and is therefore a measure of the efficacy of Desferal in removing body iron in each case.

DERIVATION OF THE FORMULA FOR IN-VIVO CHELATION

Using the symbols as above,

$$F, \text{ ferrioxamine in test dose} = [F] \frac{V_s}{100} \text{ mg.} \quad (1)$$

F_{ex} , ferrioxamine in 6-hour urine

$$\text{derived from test dose,} = F \frac{C_u V_u}{C_s V_s} \text{ mg.} \quad (2)$$

$$= [F] \frac{C_u V_u}{100 C_s} \quad (1) \text{ and } (2)$$

$$Ft, \text{ total ferrioxamine in six-hour urine} = [Ft] \frac{V_u}{100} \text{ mg.} \quad (3)$$

F_d , ferrioxamine in 6-hour urine

$$\text{derived from } in\text{-}vivo \text{ chelation,} = Ft - F_{ex} \text{ mg.} \quad (4)$$

Since F mg., intravenous ferrioxamine, gives F_{ex} mg. in six-hour urine

and F_v mg., *in vivo* chelated ferrioxamine, gives F_d (mg.) in six-hour urine,

therefore

$$F_v = \frac{F \cdot F_d}{F_{ex}} \text{ mg.} \quad (5)$$

$$= \frac{F(Ft - F_{ex})}{F_{ex}} \text{ mg.} \quad (4) \text{ and } (5)$$

$$= [F] \frac{V_s \left([Ft] \frac{V_u}{100} - [F] \frac{C_u V_u}{100 C_s} \right)}{[F] \frac{C_u V_u}{100 C_s}} \text{ mg.} \quad (1), (2) \quad (3), (5)$$

$$= \frac{C_s V_s}{100 C_u} \left([Ft] - [F] \frac{C_u}{C_s} \right) \text{ mg.}$$

$$= 10 V_s \left([Ft] \frac{C_s}{C_u} - [F] \right) \mu\text{g.}$$

Since $V_s = 1$ ml. per 10 kg. body weight,
and $10 V_s =$ body-weight in kilograms
therefore

$$F_v = [Ft] \frac{C_s}{C_u} - [F] \mu\text{g./kg.}$$

INHERENT COMPENSATION FOR MINOR VARIATION IN VOLUME OF TEST DOSE

As stated above, the test dose is adjusted to the nearest 0.5 ml., *i.e.*, only subjects with body weight an exact multiple of 5kg. will receive the exact dose on which the formulation depends, 1 ml. per 10 kg. body weight. However, the system is self-compensating for this variation:

Let the dose equivalent of 1 ml./10 kg. be D ml., and the actual dose given be D' ml.

Then, D' will contain $\frac{D'}{D}$ of the standard dose of desferrioxamine; the amount of *in-vivo* chelation will also be changed by this fraction, assuming that chelation is a linear function of the dose of desferrioxamine. In fact, chelation is a log-log function of the dose of desferrioxamine and for a small change of D , may be taken as linear. The amount of ferrioxamine given in the test dose will also change by $\frac{D'}{D}$, although the concentration

in the test dose remains the same.
Thus, when the dose volume is D ,

$$F_v = [Ft] \frac{C_s}{C_u} - [F] \mu\text{g./kg.}$$

then, when the dose volume is D'

$$[Ft] = [F_{ex} + F_d], \text{ becomes } \left[\frac{D'}{D} F_{ex} + \frac{D'}{D} F_d \right]$$

C_s remains the same

C_u becomes $\frac{D'}{D} C_u$

$[F]$ remains the same
therefore,

$$\begin{aligned} \text{when the dose volume is } D', \\ \text{observed } F_v &= \left[\frac{D'}{D} F_{ex} + \frac{D'}{D} F_d \right] \frac{C_s D}{C_u D'} - [F] \\ &= [F_{ex} + F_d] \frac{C_s}{C_u} - [F] \\ &= [Ft] \frac{C_s}{C_u} - [F] \end{aligned}$$

which is the same value when dose volume is D .

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REFERENCES

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