Estimation of Calcium, Magnesium, and Phosphorus in Diet and Stool

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A procedure that is simple and convenient for general use for the estimation of calcium, magnesium, and phosphorus in diet and stool is described. It includes a \( \text{HNO}_3-\text{HClO}_4 \) digestion in a micro-Kjeldahl flask. Calcium subsequently may be estimated by atomic absorption spectrophotometry or by the Clark-Collip procedure, magnesium by atomic absorption spectrophotometry, and phosphorus by the method of Fiske and SubbaRow. Chromium, used as a fecal collection marker, does not interfere in the measurement of calcium and magnesium, whereas the measurement of phosphorus requires a preliminary ketone extraction of the chromium. The precision and accuracy of the procedure have been evaluated and found to be quite satisfactory.

The investigation of a number of diseases involving disturbances in calcium metabolism requires not only the estimation of serum and urine calcium (Ca), but also balance studies in which determinations of dietary and fecal calcium must be made. The general technic, duration of collections, dietary precautions, and interpretation of results have been adequately considered by several authors (1–6). The value of such balance studies is considerably enhanced by the simultaneous measurements of phosphorus (P) and magnesium (Mg).

Most references in the literature, which deal with the initial preparation of diet and stool and their subsequent analyses for metals, lack adequate practical details. We considered it worthwhile to present a method of estimation of Ca, Mg and P in diet and stool that is simple, yet accurate, and which provides sufficient detail to enable it to be used conveniently by technical staff.

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A wet digestion method, originally devised by the authors for the estimation of dietary sodium (Na) and potassium (K), has been extended to the analysis of Ca, Mg, and P in both diet and stool. The method possesses the added advantages of requiring only micro-Kjeldahl equipment and a minimum of space. It is applicable to the analysis of chromium (Cr), if chromium sesquioxide is used as a fecal collection marker, and meets the required analytic precision and accuracy.

**Procedures**

In the following procedures, only CP quality reagents are used, and all solutions are prepared in double-distilled water.

**Diet Analysis**

**Preparation of Diet Homogenate**

The solid (approximately 1250 g) and fluid (approximately 1000 ml) portion of the patient's 24-hr diet are supplied by the dietitian in separate, 1-gal, wide-mouthed, polypropylene bottles fitted with polypropylene screw closures. Before analysis, this diet is converted into a homogenate of suitable consistency. The solid, followed by the liquid portion of the diet, is transferred to a Waring Blender, Model CB4. Any residual diet left in the bottles is removed by adding a small quantity of water, shaking, and adding the residue to the blender. The blender is run at low speed (14,000 rpm) for 3 min, medium speed (17,000 rpm) for a further 3 min, and finally, high speed (19,000 rpm) for 5 min.

**Digestion**

1. The whole of the freshly made homogenate is immediately poured into a preweighed, 4-liter beaker. A small amount of water is added to the blender, shaken to disperse any adhering homogenate, and transferred to the beaker. A rubber kitchen spatula with a long wooden handle has been found useful in removing the last traces of homogenate. The beaker and contents are reweighed, using a 5-kg balance (Ohaus Scale Corp.). The increase in weight of the beaker is the weight of the homogenized day's diet (in milligrams).

2. A clean, dry 100-ml Pyrex Kjeldahl flask is inserted into a 250-ml beaker and placed on the platform of an 800-g balance, Model K7 (Mettler Balance Co.). The combined weight is tared.

3. The homogenate is well stirred with a glass rod. Approximately 25 ml of the homogenate is transferred immediately to the Kjeldahl flask, using a wide-tipped 25-ml transfer pipet. The Kjeldahl flask plus
the homogenate is then reweighed and the weight of the aliquot of homogenate noted (Z g).

4. Six pieces of antibumping granules (BDH) are added and a 35-mm diameter glass filter funnel inserted into the neck of the flask.

5. A 5-ml volume of conc. HNO₃ is added from a buret and the flask placed in an electrically heated, temperature-controlled digestion rack (Laboratory Construction Co.) set to maintain a low temperature (rheostat Position 2). The flask is rotated frequently; it is removed periodically from the rack and its contents well mixed by rotation. After heating for 1–2 min, a brisk evolution of gas tends to cause the homogenate to rise up the neck of the flask. At this stage, the flask is removed from the rack, and the contents of the flask are mixed by gentle rotation. This assists the bubbles of gas to escape. The flask is then returned to the digestion rack, and the careful heating is continued. This sequence is repeated until the formation of gas bubbles has ceased (5–10 min), and the digestion then continued at a moderate temperature (rheostat Position 4).

6. Another 5-ml volume of conc. HNO₃ is added and the digestion is continued until the contents begin to thicken and bump. The final 5-ml volume of conc. HNO₃ is then added and the boiling continued until the fluid is small in volume, pale brown in color, and free from solid.

7. The flask is then allowed to cool for 2–3 min and 5 ml HClO₄ added from a buret. The digestion is resumed at a suitable temperature. Eventually the mixture begins to carbonize. The flask is removed, and the gas is allowed to escape as before by gently rotating the flask in a circular manner. Digestion is continued until the color of the solution has changed from a brown, through a green, to a final colorless solution.

8. As the digestion proceeds, the temperature is raised progressively. White fumes (HClO₄) begin to issue from the flask, and the volume of the colorless digest becomes quite small. Eventually, the clear concentrate becomes turbid and the digest begins to crystallize. At this stage, the Kjeldahl flask is rotated continually while on the digestion rack until almost all the liquid appears to have been driven off. The flask is then allowed to cool.

9. The filter funnel is washed with 20 ml of hot double-distilled water, so directed that the washings rinse the inside of the neck of the flask. The contents of the flask are dissolved by holding the flask with a metal clamp as a handle and heating over a naked bunsen flame until the solution begins to boil; the boiling is continued for a further 5 min (to hydrolyze any meta- or pyrophosphate to orthophosphate). Water

*The addition of HClO₄ to each flask is staggered by 10 min.
is added if required. The contents are then cooled and quantitatively transferred to a 100-ml volumetric flask. This sequence of washing and transferring is repeated, using two further 10-ml quantities of warm water. After cooling, the contents of the flask are made up to the mark with water, stoppered, and well shaken. A slight turbidity is apparent. The solution is then centrifuged in 50-ml conical centrifuge tubes for 10 min at 3000 rpm, and the clear supernatant stored in a 100-ml plastic bottle.

10. Stages 1–9 (above) are repeated with the remaining portions of the homogenate to be analyzed.

11. A reagent blank is run, using 25 ml double-distilled water in lieu of the homogenate, and the digestion with HNO₃ and HClO₄ proceeds exactly as with the homogenate.

12. The solutions are then analyzed for Ca, Mg, and P by the appropriate methods.

**Stool Analysis**

**Absence of Chromium**

**Preparation of Fecal Homogenate**

The stools passed during a prescribed number of days, preferably six (and collected directly into ½-gal plastic containers fitted with lids), are transferred to the Waring Blender with the aid of hot double-distilled water and a rubber kitchen spatula fitted with a long wooden handle. The total volume should be approximately 3½ liters (for a 6-day collection). A 1-ml volume of octanol is added, and the mixture is homogenized according to the instructions issued for the diet.

**Digestion**

The procedure used in the digestion of the fecal homogenate (size and number of homogenate aliquots analyzed, final volume of the digest supernatants, etc) is exactly as described previously for the diet digestion.

A wide-mouth pipet attached to a Propipet is used for transferring the homogenate. Visible carbonization may or may not occur during the HClO₄ digestion, depending upon the concentration of the organic matter in the homogenate.

**Presence of Chromium**

When the stool contains chromium (ingested by the patient as a collection marker), the preparation of the fecal homogenate and its subsequent digestion is identical to that described above. Particular care, however, is taken to ensure that the homogenate is adequately restirred with a glass rod just prior to the pipetting of each 25-ml
aliquot. The digestant assumes the typical red dichromate color; when made up to 100 ml, this becomes a yellow solution. The solution is centrifuged, and the clear supernatant analyzed as described below.

Analytic Technics

Chromium

The stool chromium is estimated (7) at 440 nm using a Bausch and Lomb Spectronic 20 fitted with a "wide range" kit and ½ in. cuvets. The calibration curve is linear up to 20 mg Cr₂O₃ per 100 ml. Two standards, 10 and 20 mg Cr₂O₃ per 100 ml, are used for the preparation of the curve.

Calcium

A modification of the atomic absorption method of Trudeau and Freier (8) involving the principle of bracketing was used. The diet and fecal digestants were diluted 1:50 with 0.5% (w/v) lanthanum ion. These were then estimated at 4227 Å in a Perkin-Elmer atomic absorption spectrophotometer Model 303 fitted with a Boling burner.

Calcium may be estimated satisfactorily also by the Clark-Collip method (9). In the presence of chromium, however, the slight modification indicated below is required.

To an aliquot of the stool supernatant (2–5 ml), in a 15-ml conical centrifuge tube, is added 1 ml of 4% (w/v) ammonium oxalate. The appropriate number of drops of 2% (v/v) ammonium hydroxide are added, the contents well mixed, and 1 drop applied at the end of a glass rod to a piece of "Hydrion" test paper (pH 3.0–5.5). Generally, 5 drops of NH₄OH suffice to achieve the desired pH of 4.5. The estimation is then completed according to the original procedure (9).

Magnesium

The diet and fecal digestants were diluted 1:50 with water and read at 2852 Å in a Perkin-Elmer atomic absorption spectrophotometer Model 303 fitted with a D.C.R.1, or estimated manually, using the principle of bracketing.

Phosphorus

In the absence of chromium, phosphorus is measured by a modification (10) of the method of Fiske and SubbaRow at 610 nm in a Bausch and Lomb Spectronic 20 using ½ in. cuvets.

The method of estimating fecal phosphorus in the presence of chromium is based on the suggested extraction of dichromate by methyl isobutyl ketone (11). A 3-ml volume of the digest supernatant is transferred to a 20-ml stoppered culture tube; 0.1 ml of conc. HCl
is added, followed by 6 ml methyl isobutyl ketone. The contents of the stoppered tube are mixed on a Fisher "Roto-Rack" for 10-15 min and then centrifuged for 15 min. The yellow ketone layer is removed by aspiration. A further 6 ml of the ketone is added, and the mixing and centrifuging are repeated as before. The ketone layer is finally removed, leaving a colorless aqueous layer for phosphorus analysis.

**Calculation**

\[
C = \text{milligrams of desired constituent per 100 ml (Ca, Mg, P, or Cr) in the digest supernatant analyzed;}
\]

\[
Z = \text{weight of aliquot of homogenate analyzed (g)};
\]

\[
M = \text{weight of entire homogenate (g)}.
\]

\[
\text{mg constituent in aliquot analyzed} = \frac{C}{100} \times 100 = C
\]

\[
\text{mg constituent in diet or stool} = \frac{C \times M}{Z}
\]

**Comments**

The digestions should be carried out in a fume hood specially designed for safe use with HClO$_4$ (19). This will comply with fire regulations. The technician is advised to wear a pair of comfortable and strong safety goggles. The use of heat-insulated cotton gloves enables the hot Kjeldahl flasks to be handled with comfort.

If the patient does not eat all his meals, the dietitian should transfer the unconsumed solid portions of the meal to one container and the liquid portion to a second container. These two containers should be large enough to take all the returns from the patient during a given analytic period. The dietitian should supply the laboratory with an estimated calcium content of the total returns. The returns are then homogenized with sufficient distilled water to give the desired calcium concentration, digested, and analyzed.

The occasional use of a deodorizer spray (eg, Ozum) will render the preparation and analysis of the fecal homogenate acceptable. A face mask may be preferred. Disposable plastic gloves should be used.

The "fluid portion of the patient's 24-hr diet" (see Preparation of Diet Homogenate) refers to all fluids taken by the patient in the 24 hr, except drinking water (which preferably should be distilled).

**Experimental Results**

**Diet or Chromium-Free Stool**

The possibility was investigated that any excess of HClO$_4$ used in the stool and diet digestion might interfere with the formation of the phosphomolybdate blue color in the estimation of phosphorus by oxi-
dizing the reducing agent. The data showed that HClO₄ up to the maximum amount used in the digestion of an aliquot of homogenate is without effect on the estimation of phosphorus.

The analytic precisions of the determinations of Ca, Mg, and P in diet and stool were obtained by the estimation of their concentrations in different weighed aliquots (N = 6) of the homogenates. The analyses were then expressed as the amount of Ca, Mg, and P (milligrams per day) present in the diet or stool.

The mean daily input (or output), in milligrams per day, and the range obtained were (1) diet: Ca 773 (769–776), Mg 215 (213–218), and P 1183 (1172–1195); (2) stool: Ca 682 (669–689), Mg 153 (150–154), and P 412 (407–418). Thus it is clear that the precisions of the three determinations are satisfactory for both diet and stool.

The recoveries of Ca, Mg, and P added to diet and stool were then determined, using the methods described. The ranges of the amount of Ca, Mg, and P added were (1) diet: Ca 240–841 mg, Mg 12–120 mg, P 241–2754 mg; and (2) stool: Ca 803–6570 mg, Mg 189–821 mg, P 201–3217 mg.

The mean percent recovery and range were (1) diet: Ca 99.7 (97.1–101) N = 8, Mg 101 (97.0–106) N = 10, P 99.5 (95.0–105) N = 10; and (2) stool: Ca 101 (96.2–105) N = 12, Mg 101 (95.0–104) N = 11, P 99.8 (97.1–103) N = 12.

These data would suggest that the recovery for all three elements, both in diet and stool, is also satisfactory.

**Chromium-Containing Stool**

**Chromium**

The purity of the acid-washed chromium sesquioxide used (11) was determined. The mean purity of six weighed portions was 100%, with a range of 100–101%.

In order to determine the recovery of Cr₂O₃ added to stool, 4.5 g Cr₂O₃ were added to a 3-day stool collection which had been partly homogenized with 1800 ml H₂O, and the homogenization then completed. Six weighed aliquots of the homogenate were digested, made up to volume, and analyzed for Cr. The mean recovery was 101%, with a range of 99.9–102%.

The precision of the Cr determinations was obtained by the analysis of a 3-day stool collection from a patient receiving 1.5 g Cr₂O₃ per day. The mean value of nine aliquots analyzed was 3607 mg, with a range of 3554–3651 mg Cr₂O₃ per 3-day stool. This indicates that only 80% of the chromium ingested in the 3-day period was passed in the stool.
Such data confirm the need of a continuous fecal collection marker in balance studies. It is clear that the precision of the chromium estimates is satisfactory.

**Phosphorus**

The amount of HCl, if any, required for ketone extraction of phosphorus in presence of chromium, was determined. It was found that HCl was required, and that 0.1 ml conc. HCl per 3-ml aliquot of digest supernatant is sufficient for an accurate P analysis as described above (*Analytic Techniques*).

A number of experiments were carried out to determine the ratio of total ketone volume to digestant volume required for complete dichromate extraction. It was found that two ketone extractions of 6 ml each are needed to completely free 3 ml of digest supernatant of the yellow dichromate.

Experiments in which standard aqueous P solutions, with and without added ketone, were analyzed, showed that ketone extraction per se had no effect on the absorbance of the P solutions.

An examination was made of the error in stool P analysis due to chromium at a concentration likely to be found in patients ingesting 1.5 g Cr₂O₃ per day, and of the effect of the ketone extraction of the chromium on the phosphorus content of the stool. The solutions examined were (A) digestants from a stool homogenate containing no Cr, (B) solution in (A) to which dichromate was added to produce a concentration of 60 mg Cr₂O₃ per 100 ml and (C) solutions in (B) subjected to ketone extraction. These data are shown in Table 1. It is clear that the presence of chromium enhances the apparent phosphorus content of feces and that the ketone extraction of the chromium cause no loss of fecal phosphorus.

The recovery of P added to aliquots of a fecal digestant containing

<p>| <strong>Table 1. Effect of Chromium and Its Subsequent Ketone Extraction on the Phosphorus Content of Stool</strong> |
|-------------------------------------------------|---------------------------------|--------------------------|</p>
<table>
<thead>
<tr>
<th>Aliquot No.</th>
<th>Stool</th>
<th>Stool + Cr</th>
<th>Stool + Cr extracted with ketone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.338</td>
<td>0.349</td>
<td>0.338</td>
</tr>
<tr>
<td>2</td>
<td>0.338</td>
<td>0.352</td>
<td>0.339</td>
</tr>
<tr>
<td>3</td>
<td>0.338</td>
<td>0.350</td>
<td>0.340</td>
</tr>
<tr>
<td>4</td>
<td>0.333</td>
<td>0.353</td>
<td>0.333</td>
</tr>
<tr>
<td>5</td>
<td>0.340</td>
<td>0.358</td>
<td>0.339</td>
</tr>
</tbody>
</table>

* Concentration of aliquots diluted 1:50.
† Aliquots 1-5 are portions of the same homogenate, having approximately the same weights.
Ca, Mg, P in Diet and Stool

Cr was determined. The added P was sufficient to increase the concentration by 20–100 mg/100 ml. These solutions were then extracted with ketone, as described previously, and analyzed for phosphorus. The mean recovery of five duplicate aliquots analyzed was 101%, with a range of 98.8–102%.

Calcium and Magnesium

The influence of Cr on the determination of fecal Ca and Mg was studied. Dichromate, in amounts required to produce a concentration of 60 mg of Cr₂O₃ per 100 ml, was added to digests from several different weighed portions of a stool homogenate. These were diluted and measured by the atomic absorption spectrophotometer. The data showed that chromium, at a concentration likely to be found in the stools of patients receiving 1.5 g Cr₂O₃ per day, does not interfere with either the Ca or Mg determined by atomic absorption spectrophotometry.

The effect of chromium on the Clark-Collip determination of fecal Ca was examined by the analysis of (A) digestants of 11 different weighed aliquots from two stool homogenates, (B) the digestants in (A) to which 60 mg Cr₂O₃ (as dichromate) per 100 ml was added, and (C) the solutions in (B) after subjection to ketone extraction. In the presence of Cr, it was found to be impossible to adjust satisfactorily the pH of the solutions with the internal methyl red indicator used because of the masking yellow color of the dichromate. If, however, the internal indicator were omitted, whether Cr was present or not, the desired pH of 4.5 could be attained using Hydron pH test paper. The slight modification of the standard Clark-Collip method found necessary is described in the section Analytic Technics.

The data show that chromium causes an apparent mean reduction in the Ca content of the stool supernatant of 1.4% (range: −0.5 to 3.2), whereas if the chromium is first removed by ketone extraction, the mean reduction in apparent Ca content is 2.6% (range: 0.0 to 5.7).

Thus, it is clear that the slight modification in the standard Clark-Collip procedure described above renders it adequate for the direct estimation of stool Ca in the presence of chromium. Furthermore, there is no advantage to prior removal of chromium.

Discussion

The literature (12–16) contains many references to both the dry and wet ash procedures. Some were developed specially for tissue, others for diet and/or stool analyses. The dry ash procedures differ mainly in the type of crucible used (porcelain, Vyeor, or platinum) and the
temperature of the furnace. The wet ash procedures differ principally in the method of homogenization, the oxyacid used (H₂SO₄; HNO₃ and H₂O₂; HNO₃ and HClO₄) and their amounts, and the type of glassware in which the digestion takes place. The elements most frequently measured include Na, K, Ca, Mg, and P. Equally variable are the analytic methods used to estimate the Ca and Mg.

The use of a wet, rather than a dry, ash method for the determination of Ca, Mg, and P in diet and stool possesses a number of inherent advantages. These can be considered as (1) technical—the absence of the need for a muffle furnace and expensive platinum crucibles, (2) chemical—smaller losses of Na, K, and Cl (17) by volatilization and phosphorus (18) by loss and conversion of ortho- to meta- and pyrophosphates. The use of HNO₃ and HClO₄, rather than H₂SO₄, permits the method to be used in those digestants which precede the analyses of Na, K, Ca, Mg, and P (and any element forming an insoluble sulfate but soluble perchlorate). Moreover, the use of HNO₃-HClO₄ renders the method particularly appropriate where chromium sesquioxide is used as a fecal collection marker (4), since the Cr₂O₃ cannot be oxidized to dichromate by dry ashing. In this case, minor modifications of the standard technics for estimating Ca and P enable the interference of Cr in their measurements to be removed.

The advantages claimed for the method described in this paper are that: (1) it constitutes an analytic system generally suitable for the determination of those inorganic cations which form soluble perchlorates, such as Na, K, Ca, and Mg, as well as P, (2) it utilizes the minimum space requirements and convenience of a micro-Kjeldahl technic, and (3) satisfied the simplicity, precision, and accuracy required of a method suitable for the clinical laboratory.

The minor disadvantages are that, unlike dry ashing, which can be completed unattended overnight in the furnace, wet ashing requires a number of hours of the technician’s attention. Perchloric acid, though recommended to be handled with care (19), is not dangerous when its use is preceded by a preliminary HNO₃ digestion, such as in this method.

In practice, this method has been found to be convenient and esthetically acceptable when used with disposable plastic gloves and the occasional use of an aerosol.

References