

Use of cuprous thiocyanate as a short-term continuous marker for faeces

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The main technical difficulty in the use of insoluble markers for faeces such as chromium sesquioxide (Whitby and Lang, 1960) or barium sulphate (Dick, 1967) is in the solubilization of these very inert compounds. Digestions with perchloric acid or chelations with EDTA are time-consuming procedures. Accordingly a search has been made for a compound which although insoluble under physiological conditions, could be decomposed by relatively mild chemical treatment.

Cuprous thiocyanate (CuSCN) is a compound which has solubility characteristics in water rather similar to those of barium sulphate. In neutral or mildly alkaline conditions the solubility is about 0.5 mg% (BaSO_4 0.25 mg%) and in 0.1 N HCl about 3 mg% (BaSO_4 1 mg%). Cuprous thiocyanate is, however, readily decomposed by 50% nitric acid at room temperature, or by 10% nitric acid at the boiling point. Thus to estimate CuSCN in faeces it is only necessary to add nitric acid, boil and filter, in order to obtain a solution for analysis of copper. This may be estimated by conventional chemical methods, or more simply, by atomic absorption spectroscopy.

MATERIALS AND METHODS

PREPARATION OF CuSCN Since at the beginning of this study no pure sample of CuSCN was available commercially, it was necessary to prepare such a compound. In doing so we have used the well tried gravimetric method of estimation of copper as CuSCN, reasoning that this would give a pure preparation (Vogel, 1961).

Copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ AR), 200 g, is dissolved in about 3 litres of water with a few millilitres of concentrated HCl and brought to the boiling point. About 50 ml of saturated sulphurous acid (prepared from $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ AR and HCl) is added and mixed and then 10% KSCN AR is added slowly with stirring. Further portions of sulphurous acid are added from time to time to maintain reducing conditions. The KSCN is added until CuSCN is no longer precipitated. The precipitate should be white and the supernatant colourless and smelling of sulphur dioxide. The CuSCN is then filtered (Green's Hydruro 904 $\frac{1}{2}$ is suitable) and washed well with distilled water until the filtrate is free from

SO_4 (BaCl_2 test). The pure CuSCN¹ is then dried in an oven at 120°C, powdered in a mortar, and weighed out into gelatin capsules.

ORAL ADMINISTRATION OF CuSCN AND COLLECTIONS OF SAMPLES For reasons which will be discussed under Toxicology, three different levels of oral CuSCN were used: 1 g, 500 mg, and 250 mg per day. The lower the oral dose, the greater will become the interference from dietary copper. On normal diets faecal copper is of the order of 1 to 2 mg per day; thus with an oral dose of 250 mg CuSCN the likely error from dietary copper will be up to about 1.5%. This would not represent a serious error in fat estimations.

All daily doses were weighed out (to less than 1% error) and packed in three gelatin capsules, no. 2 for the 1 g dose and no. 4 capsules for the smaller doses. One capsule was then given with each of the three main daily meals.

Our general method for faecal fat collections is to start the patient on CuSCN, either 250 or 500 mg per day, and at the same time give a single carmine marker. Two days after the carmine marker is passed one or more faecal specimens are obtained and delivered to the laboratory for estimation of fat and copper, the oral CuSCN then being terminated. In this procedure the patient is usually on oral CuSCN for less than one week.

ESTIMATION OF COPPER IN FAECES The simplicity of this method is due to the ease with which the copper compounds present are decomposed by dilute nitric acid at the boiling point. Filtration and washing then yield a solution suitable for analysis of copper, the results of which are expressed here in terms of CuSCN.

A faecal homogenate of the sample is made as in the faecal fat method of van de Kamer, Huinink, and Weyers (1949). From this homogenate samples from 20 to 40 g are weighed out into a 250 ml conical flask. Ten ml concentrated HNO_3 is added, a filter funnel is placed in the neck of the flask, the contents are brought to the boiling point and allowed to simmer for 10 minutes. Some frothing occurs near the boiling point but this is seldom serious. The sides of the flask (and funnel if necessary) may be washed down with water and this is usually sufficient to prevent further frothing. The contents of the flask are then filtered through Whatman no. 54 paper

¹Recently Messrs Koch-Light Laboratories Ltd have agreed to produce CuSCN to a puriss standard. The price will depend upon the demand for this compound.

into a 250 ml standard flask; the filter paper is well washed with water and the volume made up to the mark. Filtration with this grade of paper is generally very rapid; a small amount of fine material may pass through the filter but this should be of no consequence to subsequent analysis. This solution may be used directly for analysis or may be diluted to an appropriate concentration.

CHEMICAL METHOD

Many complexometric methods are available for the estimation of copper. The only difficulty in this case is the yellow colour of the extract which might interfere with the photometry. It was found that at the dilution employed here interference was negligible using an alkaline extraction with amyl alcohol-ether mixture of the complex with sodium diethyldithiocarbamate. The method is essentially that described by Varley (1967) for the estimation of serum copper.

REAGENTS

- 1 *Ammonia*, SG 0-88
- 2 *Sodium pyrophosphate*, 6% solution in water
- 3 *Sodium diethyldithiocarbamate*, 200 mg% in water
- 4 *Amyl alcohol-ether mixture*, 1 to 1
- 5 *Powdered anhydrous sodium sulphate AR*
- 6 *Stock standard CuSCN*, 1 mg per ml Weigh out 500 mg CuSCN, dissolve in 10 ml concentrated HNO₃ and make up to 500 ml.
- 7 *Working standards*, 10 to 50 µg per ml These are prepared by diluting 1 to 5 ml of the stock standard to 100 ml.

Glass-distilled water is used throughout.

TECHNIQUE One ml of a suitable dilution (usually 1 in 10) of the acid extract is pipetted into a 20 ml glass-stoppered tube. To this is added 5 drops of ammonia and about 5 ml of glass-distilled water. The contents are mixed and 1 ml of 6% sodium pyrophosphate and 1 ml sodium diethyldithiocarbamate are then added and mixed. Five ml of the amyl alcohol-ether mixture is then added, the tube is stoppered and well shaken for at least one minute. The organic layer is removed to a photometer cuvette, a little powdered anhydrous sodium sulphate added and mixed in order to dry, and the colour read at 440 mµ against a suitable standard and blank treated in the same manner as the test. The calibration curve should be linear over the range quoted.

ATOMIC ABSORPTION METHOD

The introduction of atomic absorption spectroscopy has resulted in the simplification of many analytical methods for metals. A dilution of the acid extract is suitable for analysis by this method and the concentration of copper present is such that no difficulty should be experienced in obtaining good sensitivity. Accordingly it is not proposed to go into technical details of the method which are similar to those already described for copper (see for example 'Technical Data Sheets', Hilger and Watts Ltd¹), but to mention specific points.

Nitric acid has been found to have a slight effect on

¹The apparatus used in this work was supplied by Messrs Hilger and Watts Ltd and contained the H1150 atomic absorption unit.

the estimation. In order to minimize this the standards should have approximately the same concentration of nitric acid as the dilution of the acid extract. Dilute standards may be made up from a stock standard (1 mg/ml) prepared by dissolving CuSCN in nitric acid, an additional volume of HNO₃ being then added to each dilute standard in order to bring it to approximately the same concentration of HNO₃ as in the acid extracts for analysis.

Interference from other substances likely to be present in faeces has been investigated, particularly the effects of Ca, P, Mg, Na, and K. Neither in normal nor pathological conditions should there be any substantial degree of interference from these substances. Added evidence for lack of interference is given by the good correlation between results obtained by the chemical method and the atomic absorption method (Table I). However, due to the technical simplicity, atomic absorption is the method of choice, and this method was used in most of the work quoted in this paper.

RESULTS

RECOVERIES OF CuSCN FROM FAECES When on four separate occasions CuSCN in the usual quantity was added to stools before homogenization, and the described methods followed, the recoveries of CuSCN were found as in Table I.

TABLE I
PERCENTAGE RECOVERIES OF CuSCN ADDED TO
STOOLS

<i>Atomic Absorption</i>	<i>Chemical</i>
100	102
103	100.5
100	99.5
102	99.5
Means 101	100

The total copper recovery in faeces (calculated as CuSCN) over 79 four-day balance periods on 14 human subjects on 1 g CuSCN per day from the first carmine marker to the last was 99.7% of the CuSCN administered. The minimum number of four-day periods was three and the maximum nine. The minimum recovery in any individual was 97.0% and the maximum 102.8%. A histogram of the recoveries and a comparison with those using BaSO₄ (Dick, 1967) and Cr₂O₃ (Rose, 1964) are shown in Figure 1.

TOXICITY The first consideration with a compound of this type is of course the possibility of toxicity from absorption of either copper or thiocyanate, and these will be dealt with separately.

Experiments on the human adult began with the

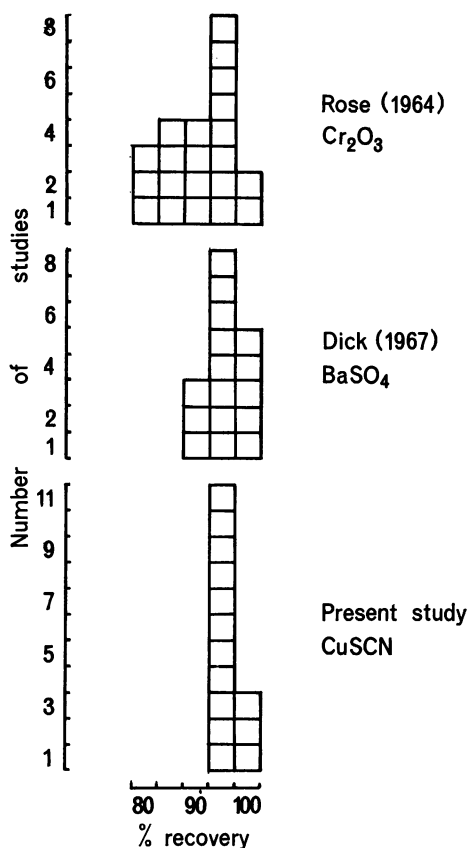


FIG. 1. Histograms of overall recoveries.

author taking small oral doses of the compound; first 30 mg, then five days later 300 mg, and eventually 1 g. Blood copper levels (Varley, 1967) were estimated before and one, three, five, eight, and 24 hours after each dose. Copper oxidase activity (Ravin, 1961) was also estimated on all the latter specimens. Twenty-four-hour urines were collected and analysed for copper (Varley, 1967). In no case was there any significant change in copper or copper oxidase from the control samples, and no clinical symptoms were experienced. In the case of the 1 g dose an attempt was made to recover the copper from the subsequent stools and this is shown in Table II. It might be argued that 20 mg CuSCN remains to be accounted for, but under the experimental conditions involved in analysing separate stools, a recovery of 98% is very reasonable.

The author then undertook a trial of this compound as a continuous marker under metabolic conditions, taking 1 g per day split into four doses. After a run-up period of four days, five four-day

TABLE II
CuSCN RECOVERIES (INDIVIDUAL STOOLS) FROM
1 g CuSCN GIVEN ON FIRST DAY

Stools	Recovery of CuSCN (mg)
1st day	—
2nd day	559
3rd day	169
4th day	132
5th day	93
5th day (2nd stool)	27
6th day	—
Total recovery	980

stool collections were made with carmine markers between all four-day periods. The recovery of copper (expressed as CuSCN) between the first and last carmine marker was 19.87 g or 99.4% of the intake. No change occurred in the blood copper, copper oxidase, or urine copper over this period.

The compound was then used as a continuous marker for faecal fat estimations over short periods of four or five days on patients on the metabolic ward. Plasma copper estimations done from time to time showed no changes and no symptoms attributable to copper toxicity were experienced. Later CuSCN was used as a continuous marker of 1 g per day in 14 human subjects on metabolic balance. In the 79 four-day periods involved, 99.7% of the administered copper was recovered between the first and last carmine markers. The compound has now also been used in over 100 patients for estimating short-term fat outputs without encountering any difficulty due to copper toxicity. Our experience, and especially the results of recovery experiments, leads us to believe that copper is not absorbed from this compound in human subjects on a normal diet.

When, however, tests were made for thiocyanate it became apparent that this radical is invariably present in plasma and urine in human subjects on oral CuSCN, and that the plasma thiocyanate level varies considerably in different individuals on the same dose. Plasma thiocyanate was estimated by the method of Bowler (1944) modified for the Auto-Analyzer (unpublished). Figure 2 shows an average response to 1 g CuSCN per day and also the highest levels we have seen over 10 days on this dose. The toxicology of this radical has been extensively studied in the past when thiocyanate was widely used in the treatment of hypertension and it is evident that levels of this magnitude would be unacceptable in patients. It is generally considered that the upper limit of safety is about 12 to 15 mg% of thiocyanate (Beckman, 1958; Sollmann, 1957).

With lower oral doses of CuSCN plasma levels of thiocyanate are also reduced. Thus with 0.5 g

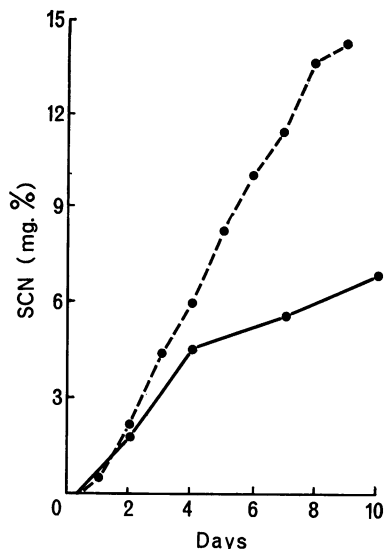


FIG. 2. Plasma SCN levels in human subjects on 1 g CuSCN od. --- 'extreme' response. — 'average' response.

per day average plasma values are about 4 mg% after seven days, while Fig. 3 shows the plasma levels in 20 individuals taking 250 mg per day for seven days. The mean plasma value here after seven days was 2.6 mg%. Over longer-term administration of a constant dose of CuSCN plasma levels of thiocyanate tend to stabilize. In general a dose of 1 g per day leads to values of about 10 to 20 mg%, 0.5 g per day to levels of about 5 to 10 mg%, and 0.25 g to levels of 2 to 5 mg%.

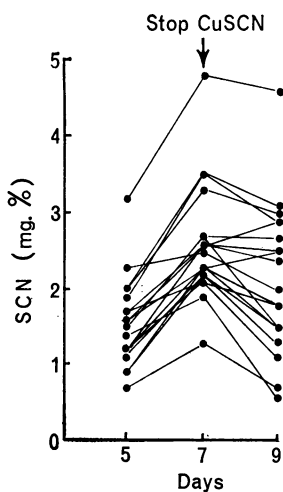
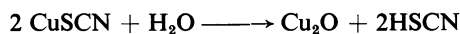


FIG. 3. Plasma SCN levels in 20 human subjects on 250 mg CuSCN od. for seven days.

The passage of CuSCN through the gut thus results in a quantitative excretion of the copper in the faeces, but with a variable absorption of the thiocyanate radical. This is an unexpected finding in terms of gut pH as CuSCN is unaffected by the physiological range, *ie*, from 0.1 N HCl to pH 9: an active physiological process must be involved.

At much higher pH values, *eg*, with 0.1 N NaOH, CuSCN is hydrolysed to cuprous oxide (Cu₂O)



The presence of a reddish compound in the faeces of human subjects on oral CuSCN supports the hypothesis that some reaction of this type occurs in the gut. We have so far been unable to prove this by analysis of the reddish compound, as the task of separating it in a pure state from faeces is formidable in the presence of food residues and of unchanged CuSCN (which is visible as a white powder).

Experiments suggest that protein, and by implication amino acids, are concerned in the splitting of CuSCN in the gut. While glycine itself, and also a mixture of 18 common amino acids, chelates copper from CuSCN very slowly and to a limited extent *in vitro*, the introduction of a low protein diet to a human volunteer on CuSCN results in an immediate fall in the plasma thiocyanate level (Fig. 4). Human duodenal juice does not by itself split CuSCN *in vitro*. Sterilization of the gut by neomycin produces a small fall in plasma thiocyanate, suggesting that the gut flora may be involved to some extent; however, this fall might be a non-specific mal-absorption affect due to the neomycin. It would thus seem that CuSCN is partly converted to Cu₂O in the gut by some active process involving protein or amino acids.

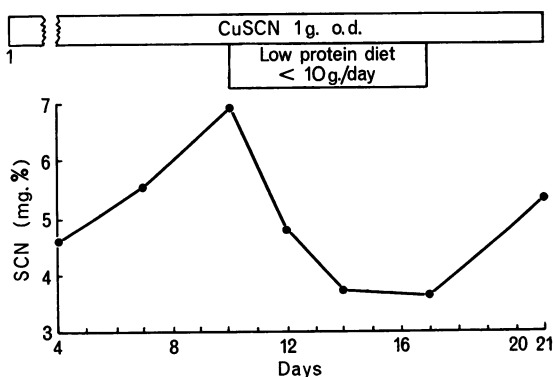


FIG. 4. Effect of a low protein diet (<10 g/day) on plasma SCN in a human subject on 1 g CuSCN od.

DISCUSSION

This compound provides a new approach to the problems of faecal marking. CuSCN starts its transit as a compound insoluble in stomach acid, but is presumably partially changed lower in the gut to a compound (Cu₂O) insoluble under neutral or alkaline conditions. (It should be mentioned that Cu₂O itself would be unsuitable as a marker as it is appreciably soluble in acid.)

The simplicity of estimating the compound in faeces is especially useful in such short-term studies as fat outputs. It has become evident that three- or five-day faecal collections without a continuous marker can be quite erroneous. Penfold (1967) states that 'there is reason to believe that of 1,085 faecal fat determinations which we have carried out during the past three years, the collections may have been incomplete or unrepresentative in as many as 30% of the cases'. Hitherto most laboratories have been deterred from using conventional markers such as Cr₂O₃ or BaSO₄ due to the considerable labour involved. With CuSCN the laboratory labour is much reduced; indeed it is simpler to estimate faecal copper (in this quantity) than faecal fat. In addition it is possible to estimate faecal fat accurately on an outpatient basis with only a relatively small sample of stool sent to the laboratory.

The absorption of thiocyanate from the compound is a distinct disadvantage. However, the toxicology of thiocyanate has been thoroughly studied in the past and we consider that with doses not greater than 500 mg per day over a limited time, there is very little possibility of a toxic reaction. This will be discussed in greater detail in a subsequent paper

dealing with the clinical evaluation of the method. It should perhaps be emphasized that although for experimental reasons we have employed doses of 1 g per day for extended periods (up to 38 days) without serious toxic reactions, we do not recommend this practice without control by estimating plasma thiocyanate.

This work was supervised by Professor C. E. Dent. I am also deeply grateful to Dr Lyal Watson for help and encouragement during all stages of the project. Thanks are also due to all the volunteers who took the compound, especially the dietitian, Miss M. Bethel, and to the nursing staff of the Metabolic Unit.

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