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An Improved Method for Determination of Thiocyanate in Plasma and Urine[1])

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Summary: An improved spectrophotometric method is described for the determination of thiocyanate in plasma and urine. Thiocyanate is adsorbed on a weak anion-exchange resin with strong affinity for chaotropic ions, and eluted with perchlorate. Thiocyanate is then chlorinated by hypochlorite and quantified according to the *König* (J Prakt Chem 1904; 69:105–37) reaction by use of isonicotinic acid and 1,3-dimethyl-barbituric acid. The method affords a simple, rapid and sensitive assay for thiocyanate and has a detection limit of 0.93 μmol/l. At thiocyanate concentrations of 107.1 and 167.4 μmol/l in plasma and urine the within-day CVs were 0.69% and 1.1% respectively, and the total imprecision measured for a period of 65 days was 0.98%. Analytical recoveries were quantitative both with urine and plasma samples.

Introduction

Natural exposure of cyanide during evolution has resulted in complex mechanisms for its detoxication in man. In the major pathway, cyanide is metabolized to thiocyanate after reacting with a sulfur donor, such as thiosulfate. This reaction is catalyzed by the enzyme rhodanase (EC 2.8.1.1)[2]). Determination of thiocyanate in serum and urine is therefore of great interest in monitoring cyanide exposure from tobacco smoke (1), fire smoke (2) and from ingested cyanogenic glucosides (3). Intake of thiocyanogenic glucosides (glucosinolates) present in cabbage and vegetables of the *Brassica* family and mustard (4), and intake of thiocyanate-containing food such as milk and cheese elevate the thiocyanate concentration in body fluids.

Thiocyanate present in blood is partly bound to plasma albumin (5). When plasma concentration of thiocyanate exceed 250 μmol/l, thiocyanate is excreted into the urine as the reabsorption in the tubules becomes saturated (6, 7). The estimated plasma elimination half-life of thiocyanate is 2.7 days in healthy subjects (8).

Several methods for determination of thiocyanate have been described based on the colorimetric method developed by *König* in 1904 (9). These methods constitute multi-step reactions where thiocyanate is first halogenated to cyanogen chloride or cyanogen bromide. In the *König* method (9) the cyanogen halide then reacts with pyridine yielding glutaconic aldehyde, and this compound then reacts with a primary amine producing a dye which is quantified spectrophotometrically. After the first description of the method several modifications have appeared (10–26), but unfortunately all of them involve handling of unpleasant or toxic compounds, e. g. bromine, arsenite, benzidine, pyridine and 1-phenyl-3-methyl-5-pyrazolone.

Isonicotinic acid is unnoxious and releases no unpleasant vapour. This reagent was first used in the *König* reaction by *Ishii* et al. (21) in a modification where pyrazolone dissolved in dimethylformamide-water was used in-

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[2]) Enzyme:
Rhodanase (thiosulfate sulfurtransferase) (EC 2.8.1.1)

stead of barbituric acid. *Nagashima* (23) used sodium isonicotinate together with barbiturate and obtained a stable reagent. Earlier 1,3-dimethylbarbituric acid was used in combination with pyridine (24), and recently the combined use of isonicotinic acid and 1,3-dimethylbarbituric acid was introduced for determination of cyanide and thiocyanate in water by *Meeussen* et al. (26). This combination was also utilized for analysis of cyanogens in cassava (25). Although this is an attractive reagent combination, it has not earlier been used for analysis of thiocyanate in plasma and urine.

The aim of the present investigation was therefore to optimize the *König* reaction for analysis of thiocyanate in plasma and urine with isonicotinic acid and 1,3-dimethylbarbituric acid as substitutes for pyridine and barbituric acid. The reaction mechanism is shown in figure

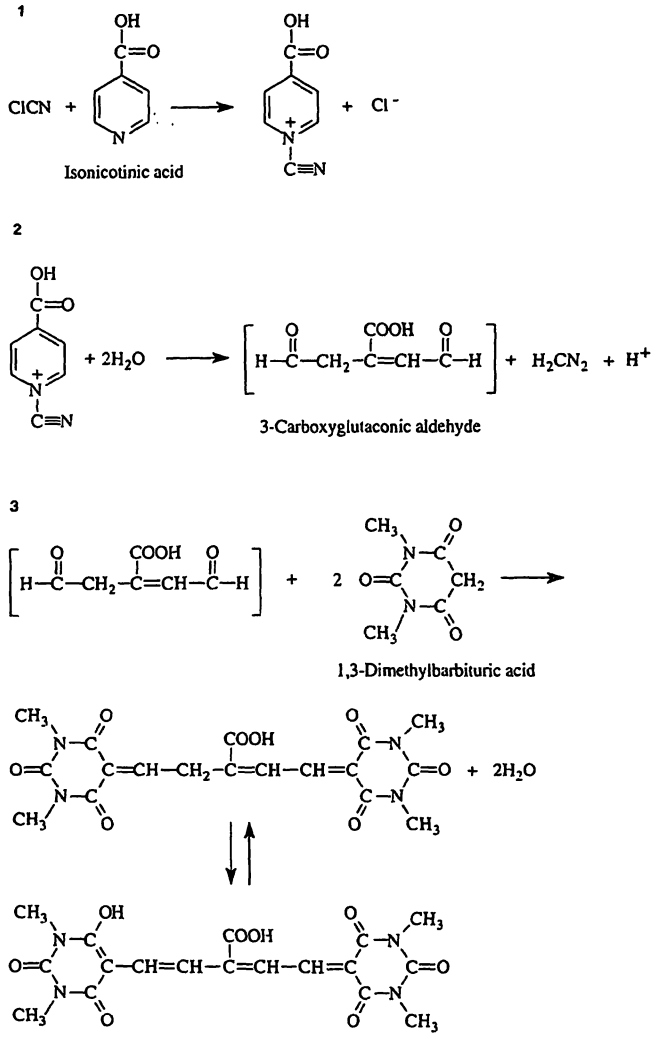

Fig. 1 Reaction scheme for determination of thiocyanate and cyanide
After cyanogen chloride has been formed from the reaction between thiocyanate and hypochlorite it reacts with isonicotinic acid to produce 3-carboxyglutaconic aldehyde (intermediate). Condensation of the aldehyde with two molecules of 1,3-dimethylbarbituric acid finally gives the dye.

1. The clean-up and chlorinating of the purified sample with sodium hypochlorite is an updated modification of our earlier method.

Materials and Methods

Materials

Ion exchanger

Amberlyst A-21, 20–50 mesh was purchased from Sigma Chemical Co. (St. Louis, MO). The resin was dried in an oven at ≤ 100 °C for 24 hours and thereafter ground in a laboratory mill (Cemotec 1090 Sample Mill, Tecator AB, Höganäs, Sweden). A particle size of 0.1–0.3 mm was obtained by sieving the dry resin. Then the resin was slurried into 2 volumes of de-ionized water and allowed to sediment for 5–10 minutes. The supernatant containing fine particles was decanted to ensure an optimal column flow rate, and the procedure was repeated 3–4 times. The resin was transferred to a suitable column, washed with 3 volumes of hydrochloric acid, 1 mol/l, followed by de-ionized water to neutral pH (pH-indicator paper). Then the resin was washed with 10 volumes of sodium hydroxide, 1 mol/l, followed by de-ionized water to neutral pH. The resin could be stored for at least one year at + 4 °C. For preparing 1-ml columns of the resin, 0.7 (i.d.) × 2.5 cm, we used the "Econo-Columns", 0.7 (i.d.), × 4.0 cm, from Bio-Rad Laboratories. Alternatively, the cheaper Poly-Prep Columns, 0.8 × 4.0 cm (Bio-Rad Labs.) could be used.

Reagents

All reagents were prepared from analytical grade chemicals (unless otherwise specified) and were dissolved in de-ionized water.

Potassium thiocyanate and sodium perchlorate monohydrate were obtained from E. Merck (Darmstadt, Germany). Isonicotinic acid (purum) and 1,3-dimethylbarbituric acid (puriss) were obtained from Fluka Chemie AG, (Buchs, Switzerland). Sodium hypochlorite, 0.5 mol/l dissolved in NaOH 0.1 mol/l (reagent no. 23039) was obtained from BDH Chemicals (Poole, England) and was diluted 10-fold to a final concentration of 50 mmol/l. This reagent is stable for at least one month at + 4 °C.

Potassium thiocyanate working standards, 20, 50, 100, 200, and 300 µmol/l, were prepared daily from a 100.0 mmol/l stock standard solution. The colour reagent was prepared by dissolving 0.925 g of NaOH in 50 ml water. Then 1.75 g 1,3-dimethylbarbituric acid and 1.43 g isonicotinic acid were added to the alkaline solution. This reagent is stable for at least 4 months at + 4 °C. As a blank we used de-ionized water.

Apparatus

During the optimization of the method we used a Shimadzu Model UV-2101 PC UV-VIS Scanning spectrophotometer (Shimadzu Corporation, Analytical Instruments, Kyoto, Japan). Otherwise, we used a Zeiss PM 2K spectrophotometer.

Procedure

A 0.5-ml aliquot of blank, working standards, plasma or urine was diluted with 5.0 ml of NaOH, 1.0 mol/l, and applied to a 2.5 × 0.7 cm column of Amberlyst A-21. The column was washed three times with 5-ml portions of water, and then thiocyanate was eluted by 8.0 ml of $NaClO_4$, 1 mol/l. To a 4.0-ml aliquot of the eluate we added 0.2 ml of acetic acid, 0.35 mol/l, and mixed on a Vortex mixer. The chlorinating reaction was then performed by adding 0.1 ml of sodium hypochlorite, 50 mmol/l, and the sample was again mixed. Then within 2 min, 0.6 ml of the colour reagent was added.

After 10 min the absorbance was read at 607 nm and the amount of thiocyanate was calculated from a calibration graph.

Plasma and urine specimens

Venous blood samples from 15 healthy non-smokers and 5 smokers were drawn into 5 ml Vacutainer tubes containing sodium heparin (Becton Dickinson, Rutherford, NJ). Plasma was separated by centrifugation at 2500 *g* for 10 minutes and stored at −20 °C until analysis. Untimed urinary samples were obtained from 15 healthy non-smokers and 5 smokers. Thiocyanate in plasma and urine samples are stable for at least six months when stored at −20 °C (18).

Results

Clean-up of plasma and urine

In the earlier method (18) urine and plasma samples were diluted with sodium hydroxide, 0.1 mol/l, for dissociation of thiocyanate from albumin (28). With this procedure we occasionally found recoveries of added thiocyanate as low as 83%. With a stronger alkaline solution, 1.0 mol/l, we now obtained quantitative recoveries both with urine and plasma samples.

Chlorination

Sodium hypochlorite in water solution is in a dynamic equilibrium with chlorine as follows:

$$ClO^- + Cl^- + H_2O \leftrightarrow Cl_2 + 2\ OH^-$$

The chlorine then reacts with thiocyanate:

$$4\ Cl_2 + SCN^- + 4\ H_2O \rightarrow CNCl + 8\ H^+ + 7\ Cl^- + SO_4^{2-}$$

It is advantageous if both the chlorinating reaction, the formation of glutaconic aldehyde derivative, and the colour development can be performed at the same pH and in sequence.

The chlorination of thiocyanate was therefore investigated together with isonicotinic acid and 1,3-dimethylbarbituric acid as colour reagents. The pH optimum of the reaction was broad (fig. 2), and the addition of 0.2 ml of 0.35 mol/l acetic acid to a 4.0-ml effluent gave a pH of 4.0. At this pH the reaction between thiocyanate and hypochlorite was practically instantaneous and the absorbance was constant within the tested range of 10–120 seconds.

Amount of colour reagent

When we varied the volume of colour reagent in the assay from 0.1 to 1.0 ml the absorbance increased up to the addition of 0.9 ml reagent (fig. 3), but only a slight increase in absorbance was observed after addition of 0.5 ml reagent. Maximum absorbance was obtained within 10 min after the addition of the colour reagent (fig. 4). Then the colour slowly decreased with time.

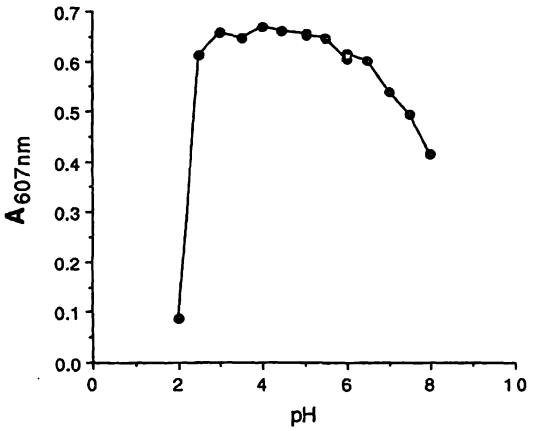

Fig. 2 Effect of pH on the formation of the dye
Tubes containing 4 ml sodium perchlorate, 1.0 mol/l, 0.125 ml acetic acid/acetate buffer, 1.0 mol/l (pH 2–5), citrate/phosphate buffer, 1.0 mol/l pH 5–6), or phosphate buffer, 1.0 mol/l (pH 6–8) and 0.25 ml of potassium thiocyanate, 100 µmol/l, were added, pH was confirmed on a pH-meter. Colour development was performed as described.

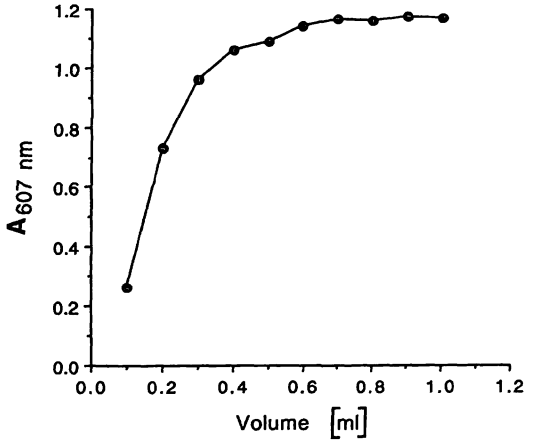

Fig. 3 Effect of amount of colour reagent on dye development
Procedure as described in methods with exception of added water to a constant volume of 1.0 ml for the colour reagent. 50 nmol thiocyanate was added to assay.

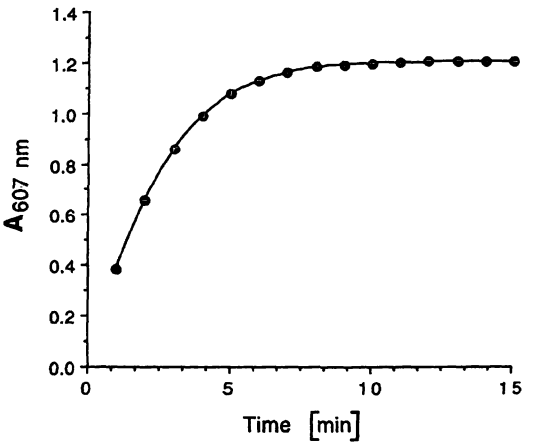

Fig. 4 Effect of time on colour development

The absorption maximum of the dye formed in sodium perchlorate was at 607 nm.

Standard curve

The standard curve was linear up to 500 µmol/l (fig. 5). However, a smaller amount of the sample should be taken to the assay if high thiocyanate concentrations are expected.

Recovery, imprecision, and detection limit

The absolute recovery of the method was tested by comparing the absorbance obtained from a standard solution of thiocyanate, 200 µmol/l, passed through the column with that obtained after direct addition of thiocyanate to sodium perchlorate. In a triplicate experiment the absolute recoveries were 101.9, 99.6 and 98.9% respectively.

Analytical recovery of thiocyanate, 50 µmol/l, added in duplicate experiments to plasma samples with thiocyanate concentrations of 35.0 and 71.5 µmol/l respectively, were 96, 99% and 97, 97% respectively. Thiocyanate, 50.0 µmol/l, added in duplicate to urine samples containing 49.0 and 96.3 µmol/l respectively of thiocyanate gave recoveries of 97, 98% and 102, 104% respectively. The within-day imprecision of the method was evaluated by analysis of ten aliquots of a plasma sample and of 10 aliquots of a urine sample. The results obtained (mean and SD) with plasma was 107.1 ± 1.17 µmol/l (CV 1.1%) and with urine 167.4 ± 1.15 µmol/l (CV 0.69). The long-term imprecision of the method was determined by analysis of aliquots of a urine sample stored at −20 °C on 10 different days for a period of 65 days. The total mean was 117.9 µmol/l, SD 1.16 µmol/l, and CV 0.98%. The detection limit was 0.93 µmol/l when defined as three times the SD value added to the mean of ten blank determinations.

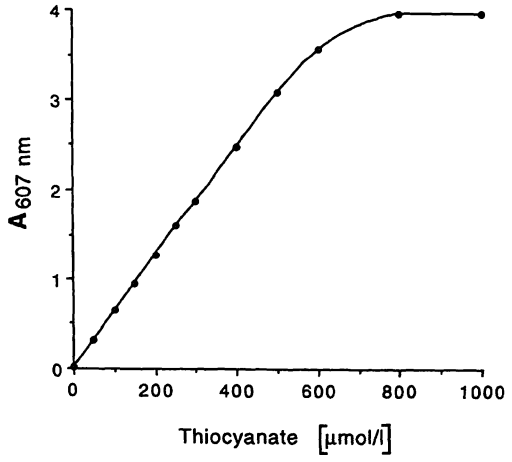

Fig. 5 Standard curve
To the alkaline application solution 25−500 nmol potassium thiocyanate was added. Regression line from the added standard concentrations 0−500 µmol/l was $y = 0.030 + 0.006x$ ($r^2 = 1.00$).

Colour reagent comparison

To further validate the new colour reagent we analyzed 20 plasma and 20 urine samples from healthy non-smokers and smokers and compared the obtained values with the pyridine based reagent (18). The results obtained were in satisfactory agreement (fig. 6).

Interference studies

Compounds normally present in body fluids were tested for possible interference at concentrations encountered in urine. The following compounds neither gave any absorbance when added alone to the samples, nor affected the absorbance with thiocyanate, 100 µmol/l, by more than ± 5%: sodium chloride, ammonium chloride, creatinine, urea, glycine and cystine. Some commonly prescribed drugs such as salicylic acid, 20 mmol/l, ascorbic acid, 10 mmol/l, and thiamine, 1 mmol/l, also did not interfere. Isoniazid did not interfere when added to a concentration of 1 g/l to a urinary sample.

Sodium nitroprusside had a strong negative interference. However, this interference could be eliminated by washing the ion-exchange column twice during the clean-up of the sample with 5 ml of ammonium chloride, 4 mol/l, followed by 5 ml of water. This procedure should include the calibrators since the absorbance decreased about 20%.

Cloxacillin, 2 g/l, did not interfere when added to a urine sample. However, a positive interference was obtained when benzylpenicillin, 2 g/l, was added both to water blank and to five different urine samples. An apparent "thiocyanate" concentration of 25 µmol/l was observed in water samples and an additional "thiocyanate" concentration ranging from 13 to 24 µmol/l was found in the urine samples. For the urine samples the absorbance of benzylpenicillin was on molar basis only 0.25−0.44% of that given by thiocyanate but administration of high doses of the interfering antibiotics may significantly affect the absorbance. Cephalothin interfered when added to water and urine samples on a molar basis half of that by benzylpenicillin. This interference could be eliminated in the same way as described for sodium nitroprusside.

Cyanide did not separate from thiocyanate in the chromatographic step, and it reacts quantitatively in the *König* reaction. The interference from cyanide, however, could be eliminated by washing the column twice with 5 ml of hydrochloric acid, 1 mol/l, but the cyanide con-

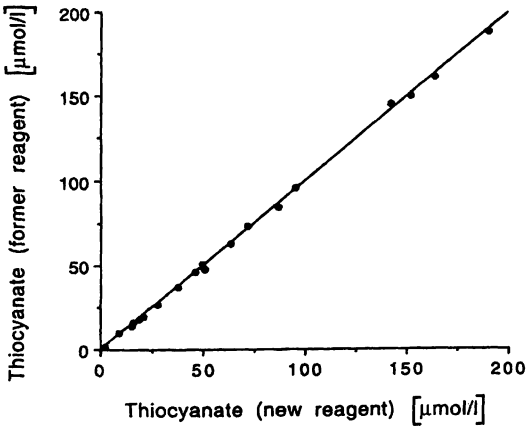

Fig. 6a Correlation between old and new reagent for determination of thiocyanate in serum
Regression line; $y = 3.792 + 0.917x$ ($r^2 = 0.992$).

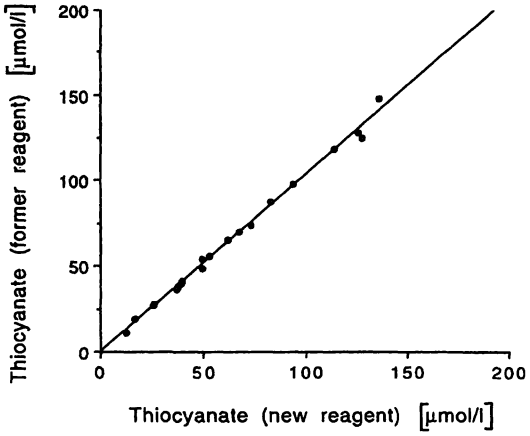

Fig. 6b Correlation between old and new reagent for determination of thiocyanate in urine
Regression line; $y = 0.0911 + 1.037x$ ($r^2 = 0.995$).

centration in plasma and urine is normally < 0.1 µmol/l and this interference can therefore be neglected.

We recommend the additional column washings when the above mentioned interferences are expected in the samples.

Discussion

Several methods have been described for analysis of thiocyanate by use of *König* reactions (9–26). However only a few of them (14, 18, 19) have been developed for quantitative determinations of thiocyanate in plasma and urine. In the method of *Boxer & Richard* (14) the samples were deproteinized and thiocyanate was oxidized to hydrogen cyanide by acid permanganate. By a stream of nitrogen the hydrogen cyanide was then transferred to a collection tube containing sodium hydroxide and determined by reacting with pyridine and 1-phenyl-3-methyl-5-pyrazolone. An advantage of this procedure is the elimination of interfering compounds during aeration, but the procedure is cumbersome and requires special glass equipment, which makes the method applicable only to small series of samples.

In the methods developed earlier in our laboratory (18, 19) the sample was first purified by use of the affinity of selected ion exchangers for thiocyanate. This affinity is probably due to a chaotropic effect rather than an anion-exchange mechanism, and the thiocyanate could be eluted by the chaotropic perchlorate ion even at high pH when the resins are uncharged. As discussed in the second communication (19) the Lewatite MP 7080 resin first used in the clean-up (18) had to be replaced because the manufacturers modified the resin resulting in a reduced affinity for thiocyanate, and it could not be used in our clean-up procedure. Several other ion exchange resins were therefore tested for their ability to selectively bind thiocyanate, and we found that some weakly basic anion-exchange resins with a polystyrene matrix had strong affinity to thiocyanate (19). Amberlyst A-21 (Röhm & Haas Co., Philadelphia, USA) had a tertiary amine as functional group on a styrene-divinylbenzene copolymer matrix and was as effective as the original Lewatit MP 7080 resin in adsorbing thiocyanate (19). Unfortunately, Amberlyst A-21 is only available in particle size 0.05–0.1 mm (too small) and 0.3–1.0 mm (too large), and the larger particles therefore have to be ground in a laboratory mill to the size of 0.1–0.3 mm to obtain an optimal flow. Upon request the service of grinding and sieving can be offered from our laboratory. In separate experiments we also used the unground resin and reduced the flow by the use of a stop-cock. Although this procedure is a little trickier it gave the same results as grinding the resin.

The ion exchanger Amberlyst A-21 has a high affinity for the thiocyanate ion and efficient elution was obtained with 3.0 ml of sodium perchlorate, 1 mol/l, from a column containing 1.0 ml of Amberlyst A-21, demonstrating a more effective elution compared to Lewatite MP 7080. This made it possible to reduce the sample volume from 0.5 ml to 0.25 ml and the elution volume from 8.0 to 4.0 ml. We analysed two different plasma and urine samples with these reduced volumes and compared the result with the conventional procedure. The results obtained agreed excellently. Thus with the present ion exchanger we obtain results in accordance with that obtained with the formerly used ion exchanger. Since the earlier method (18) also gave excellent correlation with an oxidation method (14) we conclude that also the present method would give similar results.

Analytical methods should be evaluated by estimation of their precision and accuracy as well as by the costs

and practicability of the method. As shown in results the imprecision of this method is quite satisfactory, and the recoveries were quantitative. Although satisfactory recovery is a requisite for an accurate method this is not enough for evaluation of accuracy. We therefore also investigated possible interference from other compounds. Several endogenous and exogenous compounds were tested and positive interferences were found with benzylpenicillin and cephalothin. These interferences are easily eliminated as described.

The present method is useful for detection of low endogenous concentrations of thiocyanate (detection limit 0.93 μmol/l) as well as the extremely high levels (up to 4.1 mmol/l) seen in some urine samples from populations in Africa depending on intake of cassava (3, 29). Cassava (manioc, *Manihot utilissima*) is a daily staple food for 400 million people (20) and contains the cyanogenic glucoside linamarin, which, if not properly processed before consumption, may cause cyanide intoxication. The normal human thiocyanate level (18) in serum is 42.5 μmol/l (SD 17.1 μmol/l, n = 20) and the urinary excretion of thiocyanate is 43.0 μmol/24 h (SD 22.1 μmol/24 h, n = 20). In an earlier paper we found the mean ± SD serum concentration to be 48.7 ± 30.6 μmol/l in non-smoking subjects (n = 212), and smoking 1–9 cigarettes per day increased the concentration to 120 ± 56.7 μmol/l. With higher cigarette consumption (more than 20 cigarettes per day) the mean concentrations increased to 150 ± 29.3 μmol/l (30). Measurement of thiocyanate levels after inhalation of fire smoke indicates exposure to hydrogen cyanide production from combustion of nitrogen containing materials such as plastics. The hypotensive drug sodium nitroprusside contains cyanide (44% of weight) which is metabolised to thiocyanate. Intake of thiocyanate-containing food such as milk, and intake of thiocyanogenic glucosides (cabbage, mustard) or cyanogenic glucosides (kernels, linseed, bamboo spurts) elevate the thiocyanate concentration in plasma and urine (31).

The present method affords several advantages over earlier methods (14, 18, 19) for thiocyanate determination in biological materials such as plasma and urine, and we have also found the method suitable in the determination of saliva thiocyanate. As compared with our earlier method (18, 19) the important improvement with the present method is the replacement of the odorous pyridine with isonicotinic acid. This may be a particular advantage in the tropics in the monitoring of cyanide exposure from cassava. Facilities such as fume chambers may be missing in these developing countries, but the elimination of pyridine is also of great interest in well-equipped laboratories. The solid state of isonicotinic acid also facilitates transport and handling.

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