J. Clin. Chem. Clin. Biochem.
Vol. 25, 1987, pp. 711–717

© 1987 Walter de Gruyter & Co.
Berlin · New York

Determination of Thiocyanate in Plasma and Saliva without Deproteinisation and Its Validation as a Smoking Parameter

By P. Degiampietro, E. Peheim

Chemisches Zentrallabor der Universitätskliniken, Inselspital Bern

D. Drew, H. Graf

Abt. für Parodontologie, Zahnmedizinische Kliniken, Universität Bern and

J. P. Colombo

Chemisches Zentrallabor der Universitätskliniken, Inselspital Bern

(Received February 6/June 9, 1987)

Summary: A simple photometric procedure was developed for the determination of thiocyanate (SCN^-) in plasma and saliva without deproteinisation or dialysis. Fe(III) ions form a red coloured complex with SCN^- with a maximum absorbance at 460 nm. Mercury(II) nitrate is used to run a sample blank. A manual and an automated version (COBAS BIO) of the method is described. The method is linear up to 5000 $\mu\text{mol/l}$ SCN^- . The CV of the between-run precision is 2.8–8% for the manual and 2.6–6.6% for the automated method. The SCN^- plasma concentration was 21–134 $\mu\text{mol/l}$ in nonsmokers and 44–260 $\mu\text{mol/l}$ in smokers. In mixed saliva, the concentration is much higher than in plasma: 1.57–5.5 mmol/l in smokers and 0.79–3.9 mmol/l in nonsmokers. Plasma, but not oral fluid SCN^- , is a valuable parameter for studying smoking habits in population surveys. However, its use is limited. In our experience only heavy smokers can be distinguished from nonsmokers.

Introduction

The plasma thiocyanate (SCN^-) concentration is one of several indicators for testing exposure to tobacco smoke. It has been widely used in health screening programmes for the evaluation of smoking behaviour (1–3). SCN^- is usually determined photometrically as iron(III) SCN^- in a protein-free solution. The proteins were either precipitated (4, 5) or dialysed on the autoanalyser (6, 7), but losses of thiocyanate due to coprecipitation in different deproteinisation procedures have been reported (8, 9).

Our objective was to develop a simple photometric procedure for the determination of thiocyanate in plasma and saliva without deproteinizing or dialysing the sample, and which could be easily automated.

Materials and Methods

Apparatus

Centrifugal analyser COBAS BIO (F. Hoffmann-La Roche, CH-4002 Basel, Switzerland), spectrophotometer UVIKON 810 (Kontron Instruments, CH-8010 Zürich, Switzerland).

Chemicals

Acetoacetic acid lithium salt (SIGMA A-8509); ammonium thiocyanate 0.1 mol/l (MERCK 9900); ascorbic acid (MERCK 127); Bilirubin-Control (DADE B5132); Brij 35 (MERCK 801962); chlorpromazine (Rhône Poulenc Pharma); ethylenediamine tetraacetic acid disodium salt, EDTA (MERCK 8418); iron(III) nitrate (MERCK 3883); nitric acid 1 mol/l (Merck 9966); mercury(II) nitrate (MERCK 4434); sodium chloride (MERCK 6404); sodium pyruvate (MERCK 6619); sodium salicylate (MERCK 6601).

Materials

Semi-micro glass cuvettes, HELMA No. 104-05, 10 mm (KONTRON, CH-8048 Zürich Switzerland); dilutor "micro lab m" (Hamilton Bonaduz AG, CH-7402 Bonaduz, Switzerland); dispenser Eppendorf Multipipette 4780 and pipette 3130 100 µl (Eppendorf Gerätebau, D-2000 Hamburg 65, FRG).

Reagents

Automated procedure

Reagent: Brij 50.0 g/l, iron(III) nitrate 33.3 mmol/l, nitric acid 225 mmol/l.

Start Reagent: Mercury(II) nitrate 300 mmol/l, nitric acid 750 mmol/l.

Manual procedure

Reagent A: Brij 60.0 g/l, iron(III) nitrate 40.0 mmol/l, nitric acid 270 mmol/l.

Reagent B: Mercury(II) nitrate 60 mmol/l, nitric acid 150 mmol/l.

Specimen

Blood from 324 persons was placed in heparinized tubes and centrifuged at 1200 g.

Whole mixed saliva was collected with the Salivette system (SARSTEDT, D-5223 Nümbrecht, FRG). After centrifugation at 400 g the clear saliva was used. Parotid saliva was collected using a canula and a 5 ml syringe. Pure lemon juice on the tongue was used as a stimulant. Average collection time was 15 min for 1 ml of saliva.

Procedures

The method was designed for an automated (COBAS BIO) and a manual (UVIKON 810) procedure. The absorbances were read at 460 nm (tab. 1). The calibration was performed with ammonium thiocyanate 250 µmol/l in sodium chloride 103 mmol/l.

Statistics

The Wilcoxon and the z-test were used for statistics.

Results

Spectral characteristics

Thiocyanate forms a red coloured complex with Fe(III). The absorbance is maximal at 460 nm (fig. 1). Chloride also forms a coloured complex with Fe(III).

Interference from chloride is always encountered, since it is present in all body fluids. The interference of FeCl^{2+} , however, is negligible at wavelengths above 460 nm (fig. 2). Therefore the absorbance of

Tab. 1. Procedures of thiocyanate determination.

Automated determination of thiocyanate (COBAS BIO)

1	Units	µmol/l
2	Calculation factor	2820
3	Standard 1 conc	0
4	Standard 2 conc	0
5	Standard 3 conc	0
6	Limit	5000
7	Temperature (°C)	25.0
8	Type of analysis	6
9	Wavelength (nm)	460
10	Sample volume	25
11	Diluent volume (µl)	50
12	Reagent volume (µl)	150
13	Incubation time (s)	600
14	Start reagent volume (µl)	05
15	Time of first reading (s)	1.0
16	Time interval (s)	10
17	Number of readings	04
18	Blanking mode	1
19	Printout mode	1

Manual determination of thiocyanate (UVIKON 810)

100 µl	sample and	
300 µl	distilled water are pipetted into semi-micro glass cuvette and mixed.	
500 µl	Reagent A is added and mixed. After 10 min, read absorbance A_1 at 460 nm.	
100 µl	Reagent B is added and mixed. Read absorbance A_2 within 30 seconds.	
	$c = 2820 (0.9 A_1 - A_2)$	(µmol/l)

the iron thiocyanate complex was measured at 460 nm (spectrophotometers) or at 492 nm (mercury line photometers).

Hg(II) forms colourless complexes with thiocyanate as well as with chloride. A sample blank can be performed by reading the absorbance before and after the addition of mercury(II) nitrate.

Composition of reagents

In our laboratory Brij in hydrochloric acid 200 mmol/l is used in the bilirubin method to prevent protein precipitation (10). Therefore Brij 30 g/l was also introduced in the thiocyanate method. The reagent concentrations were optimized at 460 nm on a COBAS BIO at 25 °C. Nitric acid and iron(III) nitrate were varied between 25–400 mmol/l and 5–80 mmol/l respectively. Heparin plasma spiked with sodium thiocyanate 250 µmol/l was used. No proteins

Fig. 1. Absorbance spectrum of iron(III) thiocyanate
500 μ l Reagent A, 300 μ l distilled water and 100 μ l ammonium thiocyanate (μ mol/l):
0 (—), 125 (---), 250 (-.-)

Fig. 2. Absorbance spectrum of iron(III) chloride
500 μ l Reagent A, 300 μ l distilled water and 100 μ l sodium chloride (mmol/l):
0 (—), 51.5 (---), 103 (-.-)

Fig. 3. Absorbance at different iron(III) nitrate and nitric acid concentrations
Brij 30 g/l, nitric acid 25–400 mmol/l, sodium chloride 10 mmol/l, ammonium thiocyanate 25 μ mol/l, iron(III) nitrate (mmol/l):
5 (Δ), 10 ($\square$), 20 (*), 40 ($\blacktriangle$), 80 ($\blacksquare$)

Fig. 4. Absorbance at different mercury(II) nitrate concentrations
Brij 30 g/l, iron(III) nitrate 20 mmol/l, nitric acid 100 mmol/l, sodium chloride 12 mmol/l, ammonium thiocyanate 25 μ mol/l, mercury(II) nitrate 0–6.0 mmol/l

precipitate with nitric acid below 200 mmol/l and iron(III) nitrate below 40 mmol/l if the samples have been diluted with distilled water before adding the reagent (fig. 3).

The optimal mercury(II) nitrate concentration was determined with a maximal chloride concentration of 120 mmol/l in the sample. Due to its large excess chloride is bound first, although the chloride complex has a smaller complex formation constant than the thiocyanate complex (fig. 4).

The optimized final concentrations were: Brij 30 g/l, iron(III) nitrate 20 mmol/l, mercury(II) nitrate 6.0 mmol/l, nitric acid 150 mmol/l, sample volume fraction 0.1. The molar lineic absorbance of the iron(III) thiocyanate is 355 m^2/mol at 460 nm and 291 m^2/mol at 492 nm.

Linearity

The linearity was tested with ammonium thiocyanate 0–5000 μ mol/l in sodium chloride 103 mmol/l. A linear relationship between absorbance and thiocyanate concentration was found up to 5000 μ mol/l ($y = a + bx$, $a = 0.0085$, $b = 0.004$, $r = 0.999$, $s_{y-x} = 0.0066$).

Interferences

A pool of heparin plasma samples was used and spiked with acetoacetate, ascorbic acid, bilirubin, chlorpromazine, EDTA, haemoglobin, pyruvate and salicylate. The thiocyanate was determined at 460 and 492 nm on the COBAS BIO (tab. 2). Ascorbic acid, chlorpromazine, sodium pyruvate, sodium salicylate, EDTA and bilirubin do not interfere. Ace-

Tab. 2. Interferences.

Substance		$\lambda = 460 \text{ nm}$		$\lambda = 492 \text{ nm}$	
		Found thiocyanate ($\mu\text{mol/l}$)	Interference (%)	Found thiocyanate ($\mu\text{mol/l}$)	Interference (%)
No addition (reference)		85	—	80	—
Acetoacetic acid (mmol/l)	2.0	99	+ 16.5	100	+ 25.0
	4.0	112	+ 31.8	117	+ 46.3
	6.0	125	+ 47.1	131	+ 63.8
Ascorbic acid (mmol/l)	2.0	87	+ 2.4	83	+ 3.8
	4.0	87	+ 2.4	83	+ 3.8
	6.0	86	+ 1.2	82	+ 2.5
Bilirubin ($\mu\text{mol/l}$)	17	85	0.0	78	— 2.5
	34	84	— 1.2	81	+ 1.3
	51	84	0.0	81	+ 1.3
Chlorpromazine (mg/l)	0.3	85	0.0	77	— 3.7
	0.6	85	0.0	79	— 1.2
	0.9	85	0.0	80	0.0
EDTA (mmol/l)	5.0	85	0.0	81	+ 1.3
	10.0	84	— 1.2	82	+ 2.5
	15.0	83	— 2.4	80	0.0
Haemoglobin (g/l)	0.9	96	+ 12.9	84	+ 5.0
	1.8	108	+ 27.1	88	+ 10.0
	2.7	120	+ 41.2	90	+ 12.5
Sodium pyruvate (mmol/l)	2.0	86	+ 1.2	76	— 1.2
	4.0	89	+ 3.5	79	0.0
	6.0	90	+ 5.9	77	+ 2.8
Sodium salicylate (mmol/l)	2.0	82	— 3.5	76	— 5.0
	4.0	81	— 4.7	79	— 1.2
	6.0	82	— 3.5	77	— 3.7

toacetic acid (1.0 mmol/l) causes an overestimation of the thiocyanate concentration by 7 $\mu\text{mol/l}$ (460 nm) or 15 $\mu\text{mol/l}$ (492 nm), and haemoglobin (1.0 g/l) by 9 $\mu\text{mol/l}$ (460 nm) or 5 $\mu\text{mol/l}$ (492 nm).

Precision

The precision was examined at three concentration levels, using heparin plasma samples spiked with ammonium thiocyanate. For the between-run study, portions of frozen plasma were thawed each day, centrifuged and the thiocyanate determined in the supernatant.

The within-run precision ($n = 10$) was checked in three separate runs (tabs. 3, 4). The standard deviation varies in the manual procedure from 2.3 to 6.9 $\mu\text{mol/l}$ ($= 0.0008$ to 0.0024 absorbance) and in the automated procedure from 0.57 to 1.70 $\mu\text{mol/l}$ ($= 0.0002$ to 0.0006 absorbance). The coefficient of variation in the manual procedure appears high, especially at the low concentration range. The photometer has a resolution of ± 0.001 absorbance, so

that the differences between the cuvette absorbances lead to a background deviation comparable to that of the standard deviation.

The between-run precision ($n = 10$) was examined over 10 days (tabs. 3, 4). The coefficient of variation is 2.8–8.0% for the manual and 2.6–6.6% for the automated procedure. In the analysis of Monitrol II and Precinorm U, we found thiocyanate concentrations below 20 $\mu\text{mol/l}$. They cannot be used as quality control material. Pooled patient serum or thiocyanate solutions are preferable.

Recovery

Four different plasma samples (900 μl) were each spiked with 100 μl ammonium thiocyanate 1000 $\mu\text{mol/l}$. The concentration found was 2–3% lower than the added one (tab. 5).

Plasma of nonsmokers and smokers

Thiocyanate was determined in the plasma of 207 nonsmokers and 117 smokers on the COBAS BIO.

Tab. 3. Precision with automated procedure.

		Thiocyanate (μmol/l)			between-run (n = 10 days)
		within-run (n = 10)			
		run 1	run 2	run 3	
Plasma A	$\bar{x}$	62.1	61.1	63.0	46.0
	SD	0.74	0.88	0.87	3.06
	CV (%)	1.19	1.43	1.37	6.64
Plasma B	$\bar{x}$	120.8	119.8	121.9	101.0
	SD	0.63	0.92	0.57	2.58
	CV (%)	0.52	0.77	0.47	2.56
Plasma C	$\bar{x}$	243.1	244.1	247.7	210.4
	SD	0.88	0.57	1.70	5.42
	CV (%)	0.36	0.23	0.69	2.58

Tab. 4. Precision with manual procedure.

		Thiocyanate (μmol/l)			between-run (n = 10 days)
		within-run (n = 10)			
		run 1	run 2	run 3	
Plasma A	$\bar{x}$	53.0	52.2	53.2	44.9
	SD	5.62	2.30	5.18	3.60
	CV (%)	10.60	4.41	9.74	8.03
Plasma B	$\bar{x}$	114.1	111.4	112.9	100.2
	SD	4.89	4.25	3.76	4.05
	CV (%)	4.28	3.81	3.33	4.04
Plasma C	$\bar{x}$	227.7	225.0	238.8	208.0
	SD	3.39	6.86	3.80	5.89
	CV (%)	1.49	3.05	1.59	2.83

Tab. 5. Recovery.

Sample	Thiocyanate ($\mu\text{mol/l}$)		Recovery (%)
	Reference	+ 100 $\mu\text{mol/l}$	
Plasma 1	51	148	97
Plasma 2	53	151	98
Plasma 3	116	214	98
Plasma 4	199	296	97

Tab. 6. Thiocyanate in plasma of nonsmokers and smokers.

Subject	Nonsmokers percentiles				Smokers percentiles			
	Thiocyanate ($\mu\text{mol/l}$)				Thiocyanate ($\mu\text{mol/l}$)			
	n	2.5	50	97.5	n	2.5	50	97.5
Females	49	24	60	121	34	34	141	252
Males	158	21	59	134	83	45	124	264
Total	207	21	59	134	117	44	127	260

Thiocyanate concentrations of 21–134 $\mu\text{mol/l}$ (median 59) for nonsmokers and 44–260 $\mu\text{mol/l}$ (median 127) for smokers were found (tab. 6). The results of the smokers overlapped widely those of the nonsmokers. In the nonsmokers thiocyanate concentrations were identical in females and males. Smoking females had a higher median value than males but their 95% range was very similar. The mean plasma thiocyanate concentration increased significantly according to the daily tobacco consumption (fig. 5). Heavy smokers (> 20 g/d) had a very small overlap with nonsmokers.

Saliva of nonsmokers and smokers

Smokers showed significantly higher values of thiocyanate than nonsmokers in whole mixed saliva (1.57–5.5, median 2.92 mmol/l versus 0.79–3.91, median 1.67 mmol/l; $p < 0.05$). In stimulated parotid saliva we found concentrations from 1.27 to 2.40 mmol/l (tab. 7). They were higher than in whole mixed saliva of the same individual (tab. 7). All the saliva values were higher than those in plasma. Rinsing the oral cavity two times lowered the value from 1.58 to 1.08 mmol/l (median, $n = 10$, $p < 0.005$). This is probably due to a washout effect.

Fig. 5. Thiocyanate in plasma ($\bar{x} \pm 2s$) in relation to the tobacco consumption (g/d). Nonsmokers versus smokers 10, 11–20, > 20 g/d, (0 to > 20, $p < 0.001$); 1 g of tobacco roughly compares to one cigarette (11).

Tab. 7. Thiocyanate in 10 whole saliva and parotid saliva samples of the same individuals.

Subject	Thiocyanate (mmol/l)	
	whole saliva	parotid saliva
1	1.59	2.34
2	1.28	1.51
3	1.48	2.38
4	1.87	2.40
5	1.40	2.01
6	0.72	1.27
7	1.75	2.30
8	1.73	1.85
9	1.71	2.36
10	1.57	2.03
Minimum	0.72	1.27
Median	1.58	2.17
Maximum	1.87	2.40

Discussion

The method allows the determination of SCN^- without protein precipitation. A sample blank is prepared by adding mercury nitrate. The performance of the method described is good. Interference by the substances tested is minimal. Acetoacetate and haemoglobin interfere, leading to falsely elevated concentrations, of SCN^- . However, even the lowest concentration of acetoacetate used for the interference studies is rarely encountered in patients. Haemolysis of the sample must be avoided. The automated method is rapid and useful for large population surveys.

The plasma level of SCN^- is influenced by the diet, drugs, elimination rate and mainly smoking habits.

Certain vegetables of the genus *Brassica*, e.g. cabbage, turnips, different types of kales and rape, contain appreciable amounts of SCN^- (up to 300 mg/kg fresh material) (12). 3-Indolylglucothiocyanates are split by thioglucosidase (EC 3.2.3.1) into glucose and isothiocyanates. Isothiocyanate is converted to SCN^- by thiocyanate isomerase (EC 5.99.1.1) (13). Normal amounts of SCN^- -containing foods do, however, not significantly influence SCN^- plasma levels (1).

Sodium nitroprusside (sodium nitrosylpentacyanoferrate(II)), often used as a hypotensive agent, contributes to SCN^- plasma levels (14). It is first degraded to HCN and then to SCN^- by thiosulphate sulphurtransferase (rhodanase) (EC 2.8.1.1) in the liver, kidney and the gastrointestinal tract.

Another factor influencing the sensitivity of plasma SCN^- is the elimination rate. Recently *Junge* determined the biological half life of SCN^- to be 6 days (15). This is half the previously cited time of 10 to 14 days (1), based on the findings of *Pettigrew & Fell* (16).

The main application of SCN^- determination in plasma is the evaluation of smoking exposure. The SCN^- measured in the plasma of smokers originates from HCN inhaled from tobacco smoke. Our data, as well as those of others, demonstrate that plasma SCN^- discriminates the groups of heavy smokers but its sensitivity is too low for detection of light smoking (1). This was observed in an intervention study for cardiovascular risk factors in Switzerland (11).

The SCN^- concentrations in whole saliva were in the same range and higher in smokers than in nonsmokers, as reported by others (1, 17, 18). In stimulated parotid saliva the concentrations found (1.27–2.40 mmol/l) are higher than those reported by *Pruitt et al.* of 0.76 ± 0.55 (SD) (19). The SCN^- concentrations in parotid and whole saliva are about 30 times higher than in plasma. Thus SCN^- must accumulate in the parotid gland and be secreted through a process which is still unexplained. This is also the case for hypocyanate (OSCN^-), a degradation product of SCN^- (19), which functions as an antimicrobial agent (20). Extremely high flow rates (up to 3 ml/min) could lower the SCN^- concentration by dilution. However, it is improbable that it could account solely for these large concentration differences. The levels of SCN^- in saliva are also influenced by diet and smoking habits and show a larger scatter than the plasma values. In addition, the production of hypocyanate in the oral cavity by the antimicrobial peroxidase system requires SCN^- , thereby contributing to the interindividual variations (19, 20). It must also be emphasized that rinsing the oral cavity changes SCN^- concentrations. Whole salivary SCN^- (oral fluid) is therefore certainly inferior to plasma with respect to its sensitivity for the evaluation of smoking habits (1, 3, 17). In our opinion, it should not be used.

Plasma SCN^- is an accepted parameter for the validation of smoking habits. However, based on our findings it seems to be more useful for the evaluation of the cessation of smoking in intervention studies (11), rather than for the detection of smokers in general population surveys.

References

1. Bliss, R. E. & O'Connell, K. A. (1984) *Health Psychology* 3, 563–581.
2. Gardner, M. J., McCarthy, T. L. & Jusko, W. J. (1984) *J. Toxicol. Environ. Health* 14, 393–406.
3. Puddey, I. B., Vandongen, R., Beilin, L. J. & Ukich, A. W. (1984) *Aust. NZ J. Med.* 14, 408–414.
4. Bowler, R. G. (1944) *Biochem. J.* 38, 385–388.
5. Augsten, M. & Depersdorff, J. (1982) *Z. Med. Labor.-Diagn.* 23, 226–228.
6. Butts, W. C., Kuehnemann, M. & Widdowson, G. M. (1974) *Clin. Chem.* 20, 1344–1348.
7. Vesey, C. J. & Kirk, C. J. C. (1985) *Clin. Chem.* 31, 270–274.
8. Stoa, K. F. (1957) *Studies on thiocyanate in serum*. University of Bergen, ARBOK 1957, Medisinsk rekke Nr. 2, A. S. John Griegs, Baktrykkeri, Bergen, Norway.
9. Thürkow, B., Jess, G. & Weuffen, W. (1982) *Pharmazie* 37, 264–269.
10. Colombo, J. P., Peheim, E., Kyburz, S. & Hoffmann, J. P. (1974) *Clin. Chim. Acta* 51, 217–219.
11. Nater, B., Gutzwiller, F., Abelin, Th., Degiampietro, P., Junod, B. & groupe d'étude des PNR 1A: Evolution de la consommation de tabac dans 2 villes d'intervention et 2 villes témoins suisses. (1985) *Rev. Epidém. et Santé Publ.* 33, 80–89.
12. Weuffen, W., Franzke, C. & Thürkow, B. (1984) *Nahrung* 28, 341–355.
13. Wagner, H. (1982) *Pharmazeutische Biologie, Drogen und ihre Inhaltsstoffe* 2. Aufl., Gustav Fischer Verlag, Stuttgart, pp. 88–91.
14. Vesey, C. J. & Cole, P. V. (1985) *Br. J. Anaesthesiol.* 57, 148–155.
15. Junge, B. (1985) *Brit. Med. J.* 291, 22.
16. Pettigrew, A. R. & Fell, G. S. (1972) *Clin. Chem.* 18, 996–1000.
17. Biglan, A., Gallison, Ch., Ary, D. & Thompson, R. (1985) *Addictive Behaviors* 10, 137–144.
18. Lamberts, B. L., Pruitt, K. M., Pederson, E. D. & Golding, M. P. (1984) *Caries Res.* 18, 488–494.
19. Pruitt, K. M., Mansson-Rahemtulla, M. & Tenovuo, J. (1983) *Arch. Oral Biol.* 28, 517–525.
20. Tenovuo, J., Pruitt, K. M. & Thomas, E. L. (1982) *J. Dent. Res.* 61, 982–985.

Prof. J. P. Colombo, M. D.
Dept. of Clinical Chemistry
Inselspital, University of Bern
CH-3010 Bern