Validation of urinary thiocyanate as a biomarker of tobacco smoking

M. Buratti, D. Xaiz, G. Caravelli and A. Colombi

Thiocyanate ion (SCN) is the major detoxication product of cyanide, which is converted to SCN by a thiosulphate sulphurtransferase, mainly in hepatic mitochondria. Low-level cyanide exposure for man is caused by factors such as dietary intake of cyanogenic glucosides, tobacco smoking, drug administration and occupational exposure to organic nitriles. Urinary SCN concentration was determined through a commercial kit for the analysis of cyanide in water. Spot urine samples were collected at 7:30 h and 12:30 h, from 99 healthy male white-collar office workers (non-smokers *n*=72, smokers *n*=27). Comparison of SCN excretion values did not show any difference between the morning and midday samples. The SCN median value of non-smokers was 24 μmol l^{-1} (range 9–24 μmol l^{-1}) and was statistically different from that of smokers (SCN = 92 μmol l^{-1}, range 33–275 μmol l^{-1}) ($p<0.001$). In smokers median levels of SCN increased with the intensity of tobacco smoking and SCN individual excretion rose in relation to the number of cigarettes smoked daily ($r=0.78$, $p<0.0001$, $n=16$). In the whole group of investigated subjects SCN excretion was correlated with urinary cotinine, a well know biomarker of tobacco consumption ($r=0.86$, $p<0.001$, $n=99$). When the assay was applied as a smoking habit screening test, with a cut-off value of SCN=38 μmol l^{-1} adopted to discriminate between non-smokers and smokers, the following smoker track-down predictive values resulted: 93% negative for non-smokers and 96% positive for smokers.

Keywords: *thiocyanate, urine, tobacco smoke biomarker, predictivity.*

Introduction

Thiocyanate ion (SCN) is the major detoxication product of cyanide, which is converted to SCN by a thiosulphate sulphurtransferase (EC 2.8.1.1), mainly in hepatic mitochondria (Himwich and Saunders 1948). Low-level cyanide exposure for man is caused by factors such as dietary intake of cyanogenic glucosides, tobacco smoking, drug administration and occupational exposure to organic nitriles (IPCS 1993). SCN is also an important metabolite of sulphur-containing compounds, such as glucosinolates contained in some vegetables, especially those of the brassica family (Bourdoux 1995). As a consequence SCN is usually present in biological fluids (serum, saliva and urine) in concentrations of some μmol l^{-1} (Nogang *et al.* 1993) The measurement of SCN in biological fluids has been suggested for some time as a biomarker of tobacco consumption Gardener *et al.* 1984, Pojer *et al.* 1984, Junge 1985, Jarvis *et al.* 1987, Robertson *et al.* 1987). While the determination of SCN in serum and saliva has found wide application, urinary SCN determination, in spite of the ease of biological sample collection, has received little attention. This was probably due to serum analytical methods not being sufficiently accurate when applied to urine specimens (Maehly and Swensson 1970, Lundqvist *et al.* 1979, Pre' and Vassy 1991, Michigami *et al.* 1992, Ensafi and Tajebakhsh-E-Ardanasy 1995). The aim of this work was to confirm the validity of urinary SCN excretion as a tool for the evaluation of cigarette smoking. SCN excretion, determined by a simple colorimetric method based on König synthesis of pyridine dyes, was studied in a group of subjects of varying cigarette consumption. The results of this study were then used to evaluate the predictivity of the test in discriminating between smokers and non-smokers.

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EXPERIMENTAL

Chemicals

Potassium thiocyanate (analytical grade) was obtained from C. Erba (Milan, Italy), the Spectroquant 14800 kit for analysis of cyanide in water was from Merck (Darmstadt, Germany), SPE octadecyl Sep-Pak C18 cartridges, with 360-mg filling, were from Waters (Milan, Italy).

Instrumentation

Colorimetric analyses were done by means of a 552 UV/visible spectrophotometer (Perkin Elmer, Monza, Italy) with 10-mm optical path glass cuvettes, set at 585 nm, 1 nm slit width. For solid-phase extraction (SPE) a 24-port vacuum manifold was used (Alltech, Milan, Italy).

Study group

A group of 99 healthy male white-collar office workers was studied. Information regarding occupation, tobacco consumption, work-related passive tobacco smoke exposure (ETS) and confounding factors (age, vegetarian diet, alcohol consumption, drug intake, etc.) were recorded through an anamnestic questionnaire. Subjects were classified according to tobacco smoke exposure and smoking habit: (a) non-smokers (*n*=72)—48 not exposed to ETS in the working environment (NS, *n*=48) and 24 passively exposed (PS, *n*=24), (b) smokers (*n*=27)—mean number of cigarettes smoked per day, 17 (range 2–40, with an average of five cigarettes being smoked during the monitored time interval); moderate smokers (LS, smoking no more than 15 cigarettes per day, *n*=13) and heavy smokers (HS, smoking more than 15 cigarettes per day, *n*=14). Self-reported tobacco smoking status was verified by means of urinary cotinine determination.

Urine sample collection and storage

Spot urine samples were collected from each subject twice a day at 7:30 h (second urination, beginning of workshift) and at 12:30 h (after a 5 h shift, before lunch interval); shortly afterwards 0.5 ml aliquots were separated and stored at –18°C until analysis.

Standard preparation

A stock standard solution of SCN (100 mmol l^{-1}) was prepared by dissolving 970 mg of potassium thiocyanate with 100 ml of deionized water; working standards for calibration were prepared daily by diluting stock solution with water to obtain concentrations of 5, 10 and 40 μmol l^{-1})

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Sample processing

A 0.5 ml aliquot of urine was diluted to 5 ml with deionized water and the resulting solution was loaded onto an SPE cartridge preactivated with 3 ml of methanol and 4 ml of water. The sample solution was aspirated by negative pressure through the cartridge and the eluate was collected. Then 0.5 ml of eluated or working standard solution was further diluted to 5 ml with water and, according to supplier instructions, Spectroquant reagents (Chloramine T, buffers, pyridine, dimethylbarbituric acid) were added, by use of the kit green measuring spoon. After mixing and waiting for 5 min, sample colour was read using water as a blank. The SCN concentration in the sample was calculated based on the standard calibration curve.

Other methods

Urinary creatinine was determined photometrically through a Cobas Fara analyser and urinary cotinine was determined through a modified HPLC method (Pichini *et al.* 1991).

Non-parametric tests, assuming non-normal distribution of data, were used for statistical elaborations.

Results

Analytical quality

The colorimetric analysis adopted here is based on König synthesis of pyridine-derived dyes. Briefly, SCN reacts with chlorine and forms cyanogen chloride, CNCl. The CN^+ ion reacts with pyridine to produce an intermediate which hydrolyses to a conjugated dialdehyde, glutaconic aldehyde. The coupling of the dialdehyde with 1, 3-dimethyl barbituric acid results in a violet polymethine dye.

The standard calibration curve ($y=1.32x$) was linear up to an SCN concentration equivalent to 1000 μmol l^{-1} in the water or urine sample and its slope appeared not to be affected by the nature of the matrix used to prepare diluted SCN working solutions. The limit of detection, calculated as three times the blank standard deviation, was 2 μmol l^{-1}. The within-day precision of the method, evaluated by repeated analysis of SCN low- and high-level urine samples (SCN=21 μmol l^{-1} and 87 μmol l^{-1}, $n=10$) gave relative standard deviations (RSD) of 5.2% and 2.4% respectively. Between-day precision, determined by repeated analysis of the same urine over a 3 month period, gave an average SCN concentration of 105 μmol l^{-1}, RSD=5.7%, $n=12$. The recovery rate of SCN (100 μmol l^{-1} added to urine samples containing 13 μmol l^{-1} of thiocyanate) was higher than 95%.

The analytical quality of the procedure appeared to be greatly affected by the SPE purification step. In fact, calibration curves obtained from seven different unpurified urines (spiked with four SCN concentrations in the range 20–400 μmol l^{-1}) showed variable slope values in the range from 0.57 to 2.14. In contrast, when SPE purification was adopted, the calibration curve slopes became very consistent and were independent of matrix influence.

Study group results

Urinary SCN values observed in the studied subjects before and after a 5 h workshift, with subjects grouped according to their tobacco smoking habit, are reported in Table 1. In smokers or non-smokers neither the time at which SCN median excretion values were obtained (morning and lunch) nor the comparison of individual paired data revealed any significant statistical differences. On the other hand the comparison between median values of non-smokers versus smokers showed highly significant differences ($p<0.01$): the median concentration of SCN in non-smoker urine samples, collected at 12:30 h, was 24 μmol l^{-1} ($n=72$), while in smokers it was 92 μmol l^{-1} ($n=27$). When the studied subjects were grouped according to self-reported tobacco smoke exposure status (Table 2), urinary SCN concentrations were observed to increase with growing exposure levels and statistically significant differences were apparent between NS and LS median values ($p<0.01$) and between LS and HS ($p<0.01$); no statistically significant difference was observed between NS and PS groups. Table 2 shows the urinary cotinine values observed in the different tobacco smoke exposure groups: NS and PS values never exceeded the analytical detection limit of 0.28 μmol l^{-1}, while LS and HS groups showed median values of 3.97 and 6.72 μmol l^{-1} respectively. Self-reported tobacco

	Urinary SCN concentration							
	Sampling time 7:30 h				Sampling time 12:30 h			
	Non-smokers		Smokers[a]		Non-smokers		Smokers[b]	
	μmol l^{-1}	μmol $mmol^{-1}$ cr	μmol l^{-1}	μmol $mmol^{-1}$ cr	μmol l^{-1}	μmol $mmol^{-1}$ cr	μmol l^{-1}	μmol $mmol^{-1}$ cr
Average	23	1.50	106	6.02	25	2.04	105	8.00
Standard deviation	8	1.07	77	4.08	8	1.15	61	4.41
Median	22	1.13	99	5.59	24	1.82	92	6.83
Geometric mean	22	1.26	82	4.60	24	1.76	90	6.80
Min. value	10	0.35	20	1.08	9	0.27	33	1.60
Max. value	52	7.34	320	14.97	42	5.46	275	17.38
n	72	70	25	23	72	70	27	26

Table 1. Thiocyanate (SCN) concentration in spot urine samples of the studied group (samples collected at 7:30 h and 12:30 h). Values are expressed in μmol l–[1] and μmol $mmol^{-1}$ creatinine (μmol $mmol^{-1}$ cr).

[a] Mean number of cigarettes smoked daily = 17.

[b] Mean number of cigarettes smoked during the monitored time interval = 5 gig./5 h.

Exposure level	NS SCN (μmol l^{-1})	NS COT (μmol l^{-1})	PS SCN (μmol l^{-1})	PS COT (μmol l^{-1})	LS SCN (μmol l^{-1})	LS COT (μmol l^{-1})	HS SCN (μmol l^{-1})	HS COT (μmol l^{-1})
Mean	24	<0.28%[a]	26	<0.28%[a]	76	4.06	132	8.68
Standard deviation	8		8		49	1.99	60	4.87
Median	23		27		70	3.97	115	6.27
Geometric mean	23		25		66	3.60	120	7.27
Min. value	9		14		33	1.43	40	1.19
Max. value	42		45		223	8.59	275	18.39
n	48		24		13	13	14	14

Table 2. Urinary concentrations of thiocyanate (SCN, μmol l^{-1}) and cotinine (COT, μmol l^{-1}), determined in samples collected at 12:30 h, in subjects classified according to tobacco smoke exposure level. *Key* : NS, Non-smokers not exposed to environmental tobacco smoke (ETS); PS, non-smokers passively exposed to ETS; LS, smokers smoking no more than 15 cigarettes per day; HS, smokers smoking more than 15 cigarettes per day.

[a] All samples had cotinine <0.28 μmol l^{-1}, analytical limit of detection.

smoking status fully agreed with urinary cotinine excretion. A highly significant relationship between urinary SCN and cotinine has been found in the whole group of subjects studied; this relationship is described by the equation $y=27.4+12.1x$ ($r=0.86$, $n=99$, $p<0.01$) (Figure 1). In smoker group (LS+HS), a statistically significant relationship between SCN concentration and self-reported daily smoked cigarette number was evident ($y=e^{(3.64+0.05x)}$, $r=0.78$, $p<0.01$, n-16) (Figure 2).The confounding factors studied had no influence on SCN excretion.

The statistical results did not change when data elaboration was done with SCN excretion expressed in μmol mmol^{-1} creatinine instead of μmol l^{-1}.

Predictivity of the test

SCN cumulative frequency distributions of smokers and non-smokers are reported in Figure 3. They appear to be fully distinct, with an optimum discrimination value between non-smokers and smokers set at 38 μmol l^{-1}. Indeed, 69 out of 72 non-smokers have SCN values lower than and 25 out of 27 smokers have SCN values higher than 38 μmol l^{-1}. According to this cut-off value the predictivity of urinary SCN as a screening test for tobacco consumption has been calculated: the SCN positive predictive value (VP$^+$, percent of true smokers among all those who were positive to SCN test) is 89%, and the negative value (VP$^-$, percent of true non-smokers among all those who were negative to the SCN test) is 97%, with a sensitivity, Se, of 93% (Se, percent of

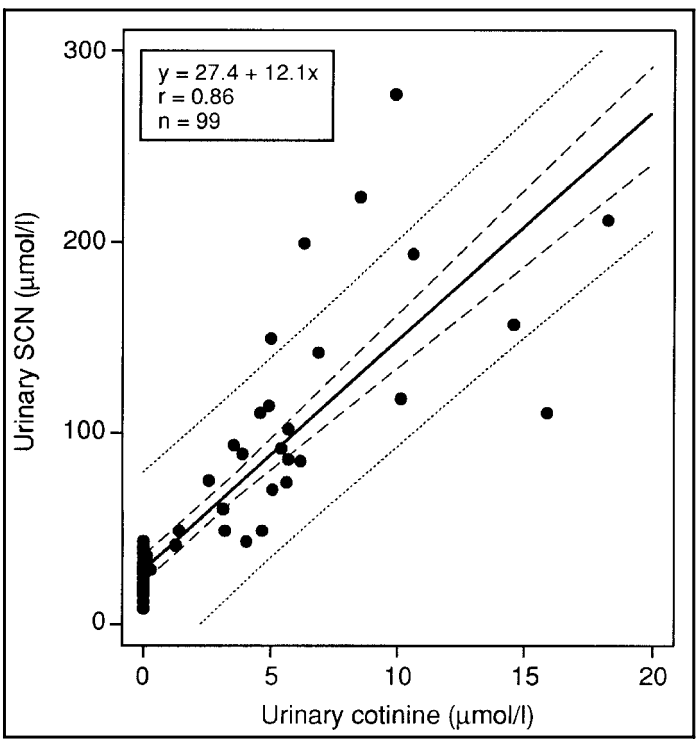

Figure 1. Relationship between urinary SCN (y, μmol l^{-1}) and urinary cotinine concentration (x, μmol l^{-1}): $y=27.4+12.1x$, $r=0.86$, $p<0.01$, $n=99$.

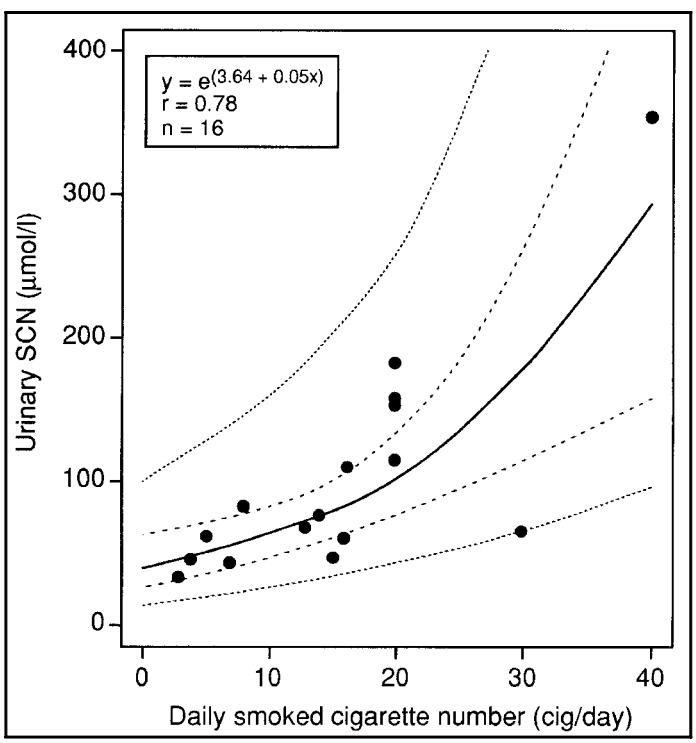

Figure 2. Relationship between urinary SCN concentration (y, μmol l^{-1}) and number of cigarettes smoked daily (x, cig./day): $y=e^{(3.64+0.05x)}$, $r=0.78$, $n=16$.

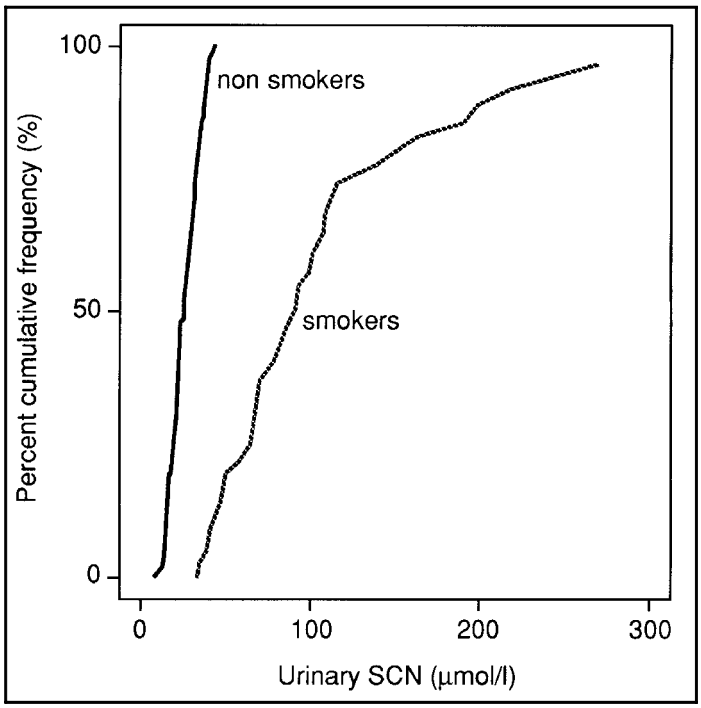

Figure 3. Cumulative percent (%) frequency distribution of urinary SCN concentrations (µmol l^{-1}) in 72 non-smokers (—) and 27 smokers (– – –).

smokers correctly detected among all the smokers) and a specificity, Sp, of 96% (Sp, percent of non-smokers correctly detected among all non-smokers).

Discussion

The awareness of a person's smoking habit is essential in different medical activities such as epidemiological investigation of exposure to chemicals and in clinical practices where patients may present smoking-related diseases. However, given that smokers tend almost systematically to under-rate their tobacco consumption, and that smoking and inhaling habits are difficult to evaluate, an objective validation of current consumption rate through quantitative measurement of tobacco products in body fluids is mandatory. While measurement of SCN in plasma and saliva was suggested long ago as a biomarker of tobacco consumption, urinary SCN values have received little attention.

The effective performance of the proposed procedure in evaluating the smoking habit of individuals is based primarily on its improved analytical specificity. Urine contains many pyridine-like compounds able to undergo a König reaction, as is the case, for instance, of nicotine metabolites (O'Doherty and Cooke 1990) and possibly of vitamin B6 metabolites, such as 4-pyridoxic acid and pyridoxamine. These organic compounds are retained in the C18 cartridge during the SPE purification step, and thus they can no longer interfere with subsequent determination of SCN which, on the other hand, is unretained in the cartridge. In fact when the SPE step was omitted and SCN determination was done on simply diluted urine samples, the inter-subject variability of the measure increased and the discriminating power of the assay was greatly reduced. The adequate performance of the proposed assay is supported by the positive correlation between urinary SCN concentration and daily cigarette consumption. An exponential model was preferred to a linear model to describe this association because it reflects the curvilinear relationship observed between self-reported cigarette consumption and serum SCN concentration. This concentration apparently levels out to approximately 175 µmol l^{-1}, corresponding to about 20 cigarettes per day (Woodward *et al.* 1991, Bourdoux 1995). The relationship observed here is significantly stronger than that reported between plasma SCN and cigarette number in previous studies (Gardner *et al.* 1984, Pojer *et al.* 1984). Moreover, the validity of urinary SCN in the categorization of subjects according to their tobacco smoking habit gave more consistent results than other studies (only 70% of smokers were identified by use of the same biomarker, Pre' and Vassy 1991), and comparable results to those obtained in much more elaborate nicotine or cotinine tests (Jarvis *et al.* 1987). Because non-smoker and smoker groups did not differ in relation to occupation, dietary habits, and other confounding factors, the increased validity of the test can only be attributed to analytical improvements. The lack of differences between morning and midday individual excretion values observed in this study confirms that urinary levels of SCN are good indicators of chronic consumption of tobacco because they are not sensitive to daily variation in tobacco smoking. SCN-delayed excretion is due to the slow transformation of cyanide and possibly nitriles, as well as the final elimination of the ion from urine (Dalhamn *et al.* 1968). For instance, acetonitrile was recently found in exhaled air from non-smokers (6 ppb) and smokers (69 ppb) and its concentration was observed to drop only slowly when smoking ceased ($t_{\frac{1}{2}}$=48h), corresponding to the slow transformation rate of acetonitrile to cyanide (Jordan *et al.* 1995).

Quantifying exposure to tobacco products is useful for epidemiological studies concerned with the efficacy of smoking cessation methods. With its relatively long mean biological half-life (SCN $t_{\frac{1}{2}}$= 6 days) (Junge 1985), by far exceeding that of cotinine ($t_{\frac{1}{2}}$= 30 h), SCN gives improved long term estimates of tobacco consumption. Other biomarkers of tobacco smoking, such as carbon monoxide in exhaled air, nicotine in plasma ($t_{\frac{1}{2}}$= 30 min), and carboxyhaemoglobin in whole blood ($t_{\frac{1}{2}}$= 4 h), are time dependent indicators of recent smoke inhalation. They are sensitive to the number of puffs per cigarette, length of the butt and inhaling habit to the point that values drop rapidly depending on the time elapsed since the last cigarette was smoked (Robertson *et al.* 1987). Lastly, SCN determination in urine does not require trained medical staff for sample collection, and it is more acceptable to people than collecting other biological fluids, such as blood or saliva.

Urinary SCN determination could thus represent a useful tool in the assessment of long-term individual tobacco smoke consumption because it is easy to perform on a large scale in laboratories and it has an acceptable specificity

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