ELSEVIER

Journal of Chromatography B, 746 (2000) 115–122

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

High-performance liquid chromatographic determination of cotinine in urine in isocratic mode

Franck Ceppa[a,*], Younes El Jahiri[a], Hervé Mayaudon[b], Olivier Dupuy[b], Pascal Burnat[a]

[a]*Laboratoire de Biochimie, Toxicologie et Pharmacologie cliniques, Hôpital d'Instruction des Armées Bégin, 69 avenue de Paris, 94160 Saint-Mandé, France*

[b]*Service d'Endocrinologie, Hôpital d'Instruction des Armées Bégin, 69 avenue de Paris, 94160 Saint-Mandé, France*

Received 19 November 1999; received in revised form 11 May 2000; accepted 15 May 2000

Abstract

A simple procedure for the determination of cotinine, major metabolite of nicotine in urine, is described. The assay involved a liquid–liquid extraction with dichloromethane in alkaline environment. The extract was dried at ambient temperature under a gentle stream of nitrogen. The residue was dissolved in 300 μl of mobile phase and 30 μl aliquot was injected via an automatic sampler into the liquid chromatograph and eluted with the mobile phase (10–9%, v/v methanol and acetonitrile, respectively in potassium dihydrogenphosphate buffer adjusted to pH 3.4) at a flow rate of 1 ml/min on a C_8 Symmetry cartridge column (5 μm, 150 mm×3.9 mm, Waters) at 25°C. The eluate was detected at 260 nm. Internal standard was 2-phenylimidazole. Sensitive and specific, this technique was performed to test urine of diabetic patients (smokers and non-smokers) admitted in an endocrinology service. Urinary cotinine seems to be a better marker of smoking status than thiocyanates.

Keywords: Cotinine; Nicotine; Thiocyanates

1. Introduction

Smoking is a real concern of public health, causing 10–25% of french mortality. If tobacco dependance is evaluated by questionnaires, impregnation is measured with various tobacco markers as blood carbon monoxyde, thiocyanates or total nicotine metabolites of urine. Cotinine is the major metabolite of nicotine found in the urine of smokers. This specific marker is especially used for the follow-up of withdrawal during smoking cessation program. The structure of cotinine and its place in metabolism of nicotine are presented in Fig. 1.

Different colorimetric methods have been developed to determine urinary cotinine. However, these assays are not selective and specific enough

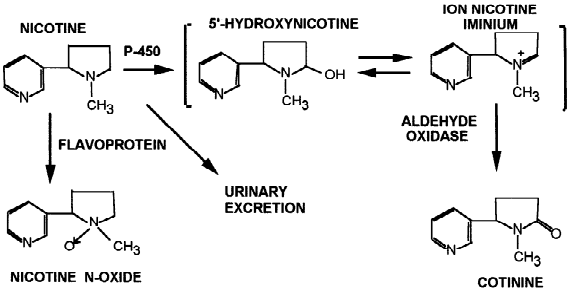

Fig. 1. Metabolism of nicotine.

*Corresponding author.

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00306-6

because many components present in urine can interfere with the reaction (erythrocytes, bilirubine, nicotinic acid, nicotinamid). Radioimmunological methods were associated with a better sensibility, but cross reactions with other nicotine's metabolites were observed [1]. If specificity was improved with ELISA and fluorescence polarization techniques, these methods remains costly. High-performance liquid chromatography (HPLC) with ultraviolet detection remains the most widely used analytical method [3,4], but these methods are sometimes time-consuming. A number of them have recoveries of about 60–70%. Also, chromatographic resolution and selectivity are often poor.

In the present work, we describe a simple, rapid, sensitive and selective HPLC method, using only 5 ml of urine, to measure urinary cotinine of diabetic patients, who are particularly susceptible to smoking damage, through macroangiopathic complications. Moreover, smoking seems to be implicated in microangiopathic complications like nephropathy. Our liquid–liquid protocol of extraction is simple and fast, with an improved extraction recovery, and didn't require clean up of sample, where multiple sample work-up procedures are needed to avoid endogenous interferences. This marker was compared to thiocyanates, another biological marker which is measured in blood by a colorimetric method.

2. Experimental

2.1. Patients

All patients ($n=436$) were admitted in an endocrinology unit for the annual visit about their diabetes. They were asked about their smoking status and macroangiopathic complications were evaluated. Cotinine was measured in urine as well as thiocyanates in serum.

2.2. Analytical standards

Cotinine and 2 phenylimidazole used as internal standard were obtained from Sigma (Saint Quentin Fallavier, France).

2.2.1. Reagents

All reagents used were of HPLC grade and included dichloromethane, acetonitrile and methanol (Carlo Erba, Milan, Italy). Potassium hydroxyde (5 M), potassium dihydrogenphosphate (5.15 g/l), citric acid (7.92 g/l), sulfuric acid (95–98%) and water for chromatography were of analytical grade (Merck, Chelles, France). Sodium octane sulfonate, hydrated salt (99%, 0.372 g/l) came from Acros (Geel, Belgique). Triethylamine (99%, 45 mmol/l) was purchased by Sigma.

2.2.2. Apparatus and chromatographic conditions

The chromatographic system consisted of a Waters 600 reciprocating piston pump, a Waters 717 intelligent sample processor (WISP TM) and a 996 programmable photodiode-array detector (Waters, Milford, MA, USA).

Analysis was performed on a C_8 reversed-phase cartridge column (Symmetry cartridge, 5 μm, 150 mm×3.9 mm I.D., Waters) linked to a C_8 precolumn (5 μm, 30 mm×3.9 mm I.D., Waters). The separation was carried out at stable temperature (25°C) in an oven. The isocratic mobile phase used was a mixture of aqueous phase, methanol and acetonitrile in the respective proportions 81:10:9 (v/v/v).

The aqueous phase contained 0.372 g of sodium octane sulfonate, 5.15 g of potassium dihydrogenphosphate, 7.92 g of citric acid with 5 ml of triethylamine before completing to 800 ml with distilled water. The pH was adjusted to 3.4 with citric acid. This solution was degassed by vacuum and sonicated before use, and the flow rate was 1 ml/min.

2.2.3. Preparation of standard solutions and calibration samples

Stock solutions of cotinine and 2-phenylimidazole were prepared by dissolving accurately weighed quantities in methanol to give a concentration of 1.0 and 2.0 mg/ml, respectively, and were stored at −80°C.

These stock solutions were used to spike the drug-free urine (obtained from a urine bank) to cover various calibration ranges: 0–2400 μg/l. This urine bank had been previously tested to verify it was free of cotinine.

Standards were extracted together with 20 unknown samples and run as a batch processed by the automatic sampler, which was normally operated overnight. Five standards were placed at the beginning and at the end of the batch of unknowns. This was used to check the linearity and the reproducibility of the chromatographic system.

2.3. Extraction method

Several techniques of extraction have been described in literature. Ji et al. developed recently a GC–MS method for both detection of trans-3-hydroxycotinine and cotinine in urine after transdermal administration of nicotine [2]. This original method requires hydrolysis of conjugated metabolites by β-glucuronidase followed with an liquid–liquid extraction. Using this mode of detection, this method remains the most specific between all GC techniques. Pacifici et al. [3] have compared a simple HPLC method after solid–liquid extraction (using dichloromethane and methanolic hydrochloride acid) to a coupling of HPLC and mass spectrometry (MS), using a μBondapack® C_{18} column. Both techniques gave similar results, with CVs below 5%.

Cotinine was still detected in serum after 8 h of tobacco withdrawal and two or three cigarettes smoked. Optimization of technique was problematic. Zuccaro et al. [4] proposed a determination by a gradient mode which requires two columns, the first one (Supelcosil LC-18DB 3 μm) was used to shorten retention time of basic components. However the limit of linearity is only 500 μg/l. Among all the methods using a gradient, HPLC coupled to MS developed by Bernert et al. [4] remains the most sensitive technique (0.05 μg/l), but requires extreme precautions concerning smoking status of technicians and cleanness of glassware. A review of chromatographic methods for determination of cotinine in different biological fluids including those mentioned above is presented in Table 1.

Our protocol of extraction is simple and fast, and does not require any particular precaution. Urines of the morning were collected in plastic tube, then kept frozen at −80°C till the day of analysis. Thawned urines were centrifuged in order to avoid eventual impurities.

A total of 5 ml of urine (standard, control or patient) and 100 μl of internal standard (2 phenyl imidazole, 100 μg/ml) were added to 20 ml screw capped (teflon joint) glass tubes.

After the addition of 1 ml of 5 N potassium hydroxyde and 5 ml of dichloromethane, tubes were sealed hermetically, shaken 15 min and centrifuged 15 min at 2500 rpm. The supernatants were discarded by aspiration and 3 ml of the inferior organic phase were collected in hemolysis tubes.

After evaporation to dryness under gentle stream of nitrogen at ambiant temperature, residues could either be dissolved in 300 μl of mobile phase or stored sealed at +4°C and sheltered from light during 24 h. An aliquot (30 μl) was injected into the HPLC system via the automatic sampler. A pool of urine was obtained from healthy volunteer smokers and constituted a control. This pool was tested (treated as a specimen) many times to obtain a mean value, and was included in each sample set.

2.4. Quantitation

A calibration curve (eight points between 0 and 2400 μg/l) was done for each sample set. The calibration graphs were repeated five times for measurement of reproducibility.

2.5. Recovery

Various amounts of cotinine were dissolved in 5 ml human blank urine in order to obtain solutions of 2000, 500, 200 and 125 μg/l, respectively. These samples were extracted as mentioned above. The extraction residues obtained were dissolved in 300 μl of mobile phase containing 5 μg of internal standard and chromatographed. A second set of samples was prepared simultaneously by extraction of 5 ml aliquots of human blank urine. Cotinine and internal standards were then added to the extraction residue at the concentration noted above.

The analytical recovery was calculated by comparing the peak-area ratios of cotinine/internal standard in extracted samples, to the ratios obtained from the samples to which, studied compound and internal standard had been added after extraction.

Table 1
Chromatographic methods for determination of cotinine in different biological fluids

	Extraction solvant	Column	Mobile phase	Flow (ml/min)	Internal standard	Specimen	Limit of detection (μg/l)	Ref.
CG–MS	dichloromethane *n*-butyl acetate	DB-5 MS (15 m×0.32 mm)	helium	30	cotinine-D_3	urine 1 ml	20	[2]
HPLC (isocratic mode) UV 254 nm	dichloromethane methanolic Hcl	Supelchem LC8 DB 5 μm	water acetonitrile triethylamine sodium heptane sulfonate potassium hydrogen-phosphate citric acid	1.6	*N*-ethyl norcotinine	plasma 5 ml	5	[3]
HPLC (gradient mode) UV 254 nm	dichloromethane isopropyle alcohol	Supelcosil LC8 DB 5 μm and Suplex pKb 100 5 μm	A: water–acetonitrile B: water–acetonitrile C: water KH_2PO_4 D: aceto-nitrile KH_2PO_4	1.5 0.3	*N*-ethyl norcotinine	serum 1.5 ml	5	[4]
HPLC (gradient mode) mass spectrometry	methylene chloride on poly-propylene column	C_{18} 3 μm	methanol–ammonium acetate	1	cotinine-D_3	serum 1 ml	0.05	[5]

2.6. Evaluation of the technique

Linearity of the technique was appreciated by successive dilutions of high concentration cotinine sample. Limit of detection and quantitation were determined, as well as precision and specificity of the method.

2.7. Expression of results

We chose to give our results without taking into account of creatinine concentration of samples, since many conflicting discussions still take place about this concern [6]. Usuals concentrations were chosen with reference to international literature [7]: <20 μg/l for non-smokers; between 20 and 50 μg/l for passive or occasionnal smokers and >50 μg/l for regular smokers. For thiocyanates, usuals concentrations are: <70 μmol/l for non-smokers and ≥ 70 μmol/l for smokers.

2.8. Statistical analysis

Statistical analysis are based on calculation of coefficient of correlation, on Kruskal–Wallis unilateral variance analysis (equivalent to khi2 for non-parametric tests), and on Mann–Whitney test. Treshold of significance was set for a P below 0.05.

ROC curves (Receiver Operating Characteristic) were built up for comparison of theses two markers based on study of smokers and patients who have never smoke.

Theses curves are made from points representing the couple sensibility-specificity at a given treshold concentration [8]. The closer the curve is close to the superior left corner of the graphic, the more the concentration tested is both sensitive and specific.

ROC curves were constructed by COMPAR® software.

3. Results and discussion

3.1. Performance of the HPLC system

Cotinine's retention factor was $k'=0.47$. The run time was set at 16 min in order to elute endogenous substances before next run. The analytical peaks of cotinine, caffeine (if present) and 2-phenylimidazole were well resolved, ($R=6.54$, Fig. 2). With this type of method, no clean-up of sample was necessary (amplitude of baseline is low). This technique is characterized by a fast and easy mode of extraction and a good selectivity ($\alpha=2.73$). Symmetry of cotinine's peak is acceptable (0.71). The good recovery explains a sensitivity comparable to other HPLC techniques mentioned above. The specificity is better owing to photodiode-array detector. None interference with cafeine was observed and only six interfering molecules were found during 436 assays.

3.2. Recovery

Area ratios between signals for cotinine to internal standard at different points of calibration are presented in Table 2. Calculation of extraction ratio takes into account factor 5/3 (extraction with 5 ml of dichloromethane and recovering of 3 ml of extract only). The mean extraction ratio of cotinine is 92.1%.

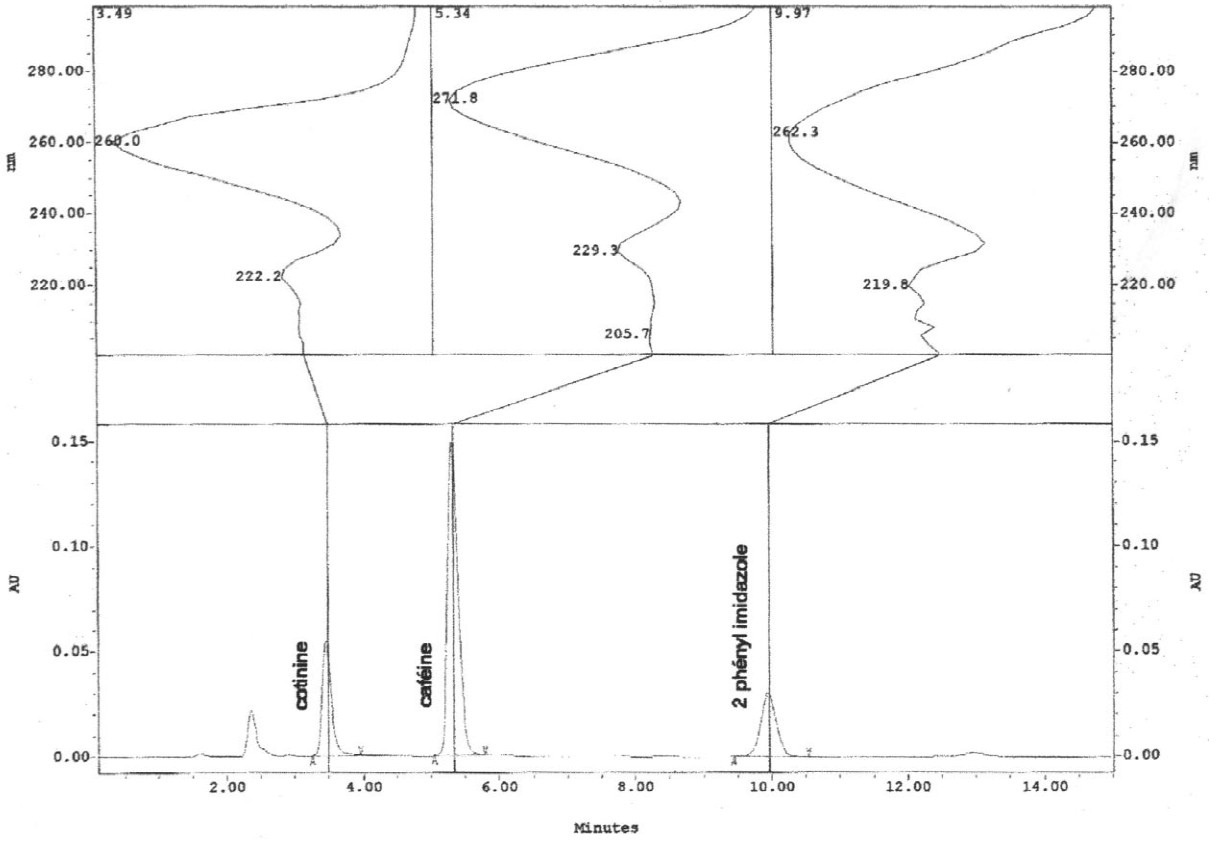

Fig. 2. Chromatogram of the analysis of a patient urine sample (concentration=104 μg/l) after addition of internal standard and extraction; spectra corresponding to each peak are represented above.

Table 2
Recovery of cotinine in human urine ($n=10$)

Calibrators (μg/l)	2000	500	200	125
Area ratios before extraction (CV)	3.26 (3.1%)	0.75 (3.8%)	0.30 (3.8%)	0.41 (4.1%)
Area ratios after extraction (CV)	1.74 (4.3%)	0.42 (4.4%)	0.16 (4.8%)	0.24 (4.7%)
% of extraction	88.9	93.3	88.8	97.5

3.3. Limits of detection and quantitation

The limit of detection of the assay was 5 μg/l. A signal-to-noise ratio of approximately 3:1 was observed and concentration was calculated with ten samples. The limit of quantitation of the assay was evaluated as the concentration equal to ten-times the value of the signal-to-noise ratio.

In our study, it was 17 μg/l, which is better than most of the previous published works, for this type of technique [7]. We decided to choose 20 μg/l as our treshold concentration.

With this cut-off, the non-tobacco sources of cotinine (tomatoes, potatoes, eggplant, green pepper or green tea) do not give a positive result [9].

3.4. Linearity

Calibration curves were obtained by weighed least-squares linear regression analysis (weight factor $1/y^2$) of the peak-area ratio of cotinine/internal standard versus the concentration of cotinine. Equation of the regression curve was $y=0.0009x+0.0003$ (correlation coefficient $r^2=0.99$). Technique was linear up to 10 000 μg/l, but as precaution, we diluted all specimens for which concentration was over the concentration of last calibration point. For Lequang et al. [10], limit of linearity was 40 mg/l.

3.5. Precision

The precision (expressed by the coefficient of variation of replicate analyses) of the method was evaluated at three levels of concentration (46, 468 and 1723 μg/l). The intra-assay reproducibility was determined by analysing ten successive extractions of each urine on the same day. Coefficients of variation were 1.08, 1.68 and 2.14% at theses tested concentrations, respectively. The inter-assay reproducibility was obtained by analysing each urine on various days along a 15-day period, using a separate calibration each day. At these concentrations, coefficients of variation were 3.52, 3.71 and 4.23%, respectively. Stability of urinary cotinine is noteworthy: Riboli et al. [11] could distinguish smokers and non-smokers after 10 years of conservation.

3.6. Specificity

Using the technique discribed above, non-interference due to cafeine, to other bases or eventual drugs has been observed. Theobromine, which is present in urine of tea drinkers, is not extracted by this method.

3.7. Clinical study

Prevalence of smoking among our population sample is 14%, a data which is usually found in other studies [12]. The mean age of women who smoke is lower than for men, as previously described [13]. As presented in Table 3, mean concentrations of cotinine or thiocyanates are in good relation to smoking status ($P<10^{-6}$). However no significant difference was observed between non-smokers and former smokers, because of relatively short half-life of theses two markers (data not shown).

Observation of Table 4 seems to confirm great specificity of cotinine, with only 4% of false positive [14].

Cotinine and thiocyanates are well correlated to numbers of smoked cigarettes per day ($r=0.58$ and $r=0.62$, respectively, Fig. 3) with regard to number of patients tested. People for which urinary cotinine is high with a negative answer to the questionnaire are supposed to be untruthful. Those for which cotinine is low when they declare to be smokers are

Table 3
General characteristics of the 436 diabetic patients studied

	Number (women)	Age		Age of diabetes (years)		Thiocyanates (μmol/l)	Cotinine (μg/l)
		Men	Women	Insulino-dependent	Non-insulino-dependent		
Non-smokers	189 (101)	55.4±15	62.7±15	12±11	11±10	44.4±16.7	23.0±25.6
Smokers (mean number of packets–years=25.5±18)	62 (11)	54.0±17	48.8±13	15±11	12±11	109.9±47.7	627.4±652.5

Table 4
Breakdown of patients by smoking status and cotinine determined in urine

	Smokers	%	Non-smokers	%	Total
Cotinine >20	44	10	19	4	63
Cotinine ≤20	18	4	355	81	373
Total	62		374		436

supposed to have quit since the admission in the diabetology unit.

In the contrary theses markers are not related to numbers of packets–years (data not shown) thus cannot assess cumulative smoking. As a matter of fact, theses parameters have to be considered as instantaneous markers and as tool to follow withdrawal, especially when gum or transdermal patch are used [15].

Cotinine and thiocyanates are well correlated to each over, either for the whole population sample, or for patients for which cotinine is positive (r=0.78, Fig. 4).

Optimal treshold concentrations calculated by COMPAR® software for urinary cotinine and thiocyanates are 28 μg/l and 64 μmol/l, respectively. Treshold concentrations giving 100% of specificity (positive predictive value) for urinary cotinine and thiocyanates are 330 μg/l and 107 μmol/l, respectively. It is important to note that superposition of ROC curves show a constant superiority of the

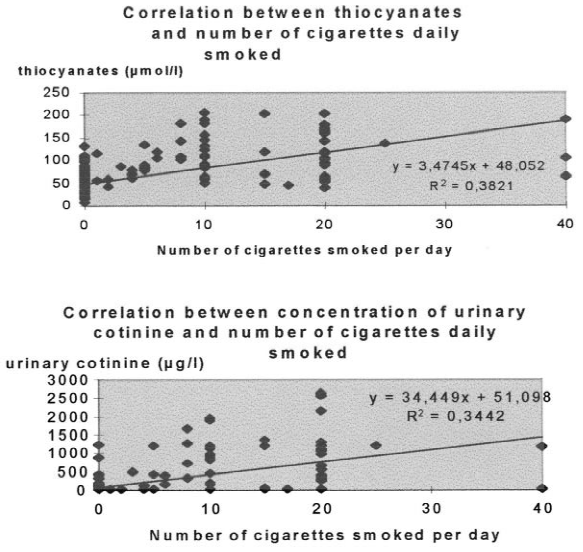

Fig. 3. Quantitation of smoking by thiocyanates (top) and urinary cotinine (bottom), n=436.

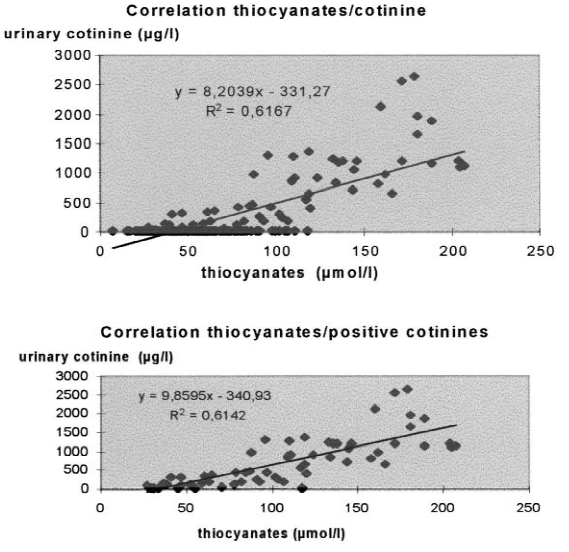

Fig. 4. Correlation between cotinine and thiocyanates for the whole population sample (top) and for patients for which cotinine is positive (bottom, n=63).

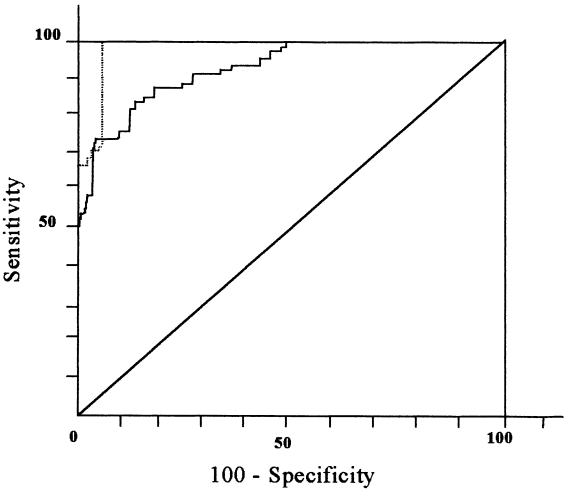

Fig. 5. ROC (Receiver Operating Characteristic) curves of urinary cotinine (···) and thiocyanates (—).

marker «cotinine» on the marker «thiocyanates» (Fig. 5).

Sample studied ($n=151$, number of couples of points after exclusion of doubloons by COMPAR®) is great enough to consider urinary cotinine (determined by high-performance liquid chromatography) as a more performant biological marker than thiocyanates in the assessment of smoking status.

References

[1] S.R. Jones, S. Amatayakul, Clin. Chem. 31 (1985) 1076.
[2] A.J. Ji, G.M. Lawson, R. Anderson, L.C. Dale, I.T. Croghan, R.D. Hurt, Clin. Chem. 45 (1999) 85.
[3] R. Pacifici, S. Pichini, I. Altieri, M. Rosa, A. Bacosi, A. Caronna, J. Chromatogr. 612 (1993) 209.
[4] P. Zuccaro, I. Altieri, M. Rosa, A.R. Passa, S. Pichini, G. Ricciarello, R. Pacifici, J. Chromatogr. 621 (1993) 257.
[5] J.T. Bernert, W.E. Turner, J.L. Pirkle, C.S. Sosnoff, J.R. Akins, M.K. Waldrep, Q. Ann, T.R. Covey, W.E. Whithfield, E.W. Gunter, B.B. Miller, D.G. Patterson, L.L. Needham, W.H. Hannon, E.J. Sampson, Clin. Chem. 43 (1997) 2281.
[6] S.G. Thompson, R.D. Barlow, N.J. Wald, H. Van Vunakis, Clin. Chem. Acta 187 (1990) 289.
[7] J.E. Haddow, G. J Knight, G.E. Palomaki, L.M. Neveux, B.A. Chilmonczyck, Clin. Chem. 40 (1994) 562.
[8] P. Hannequin, J. C Liehn, M.J. Delisle, G. Deltour, J. Valeyre, Eur. J. Nucl. Med. 13 (1987) 203.
[9] E.F. Domino, Clin. Pharmacol. Ther. 57 (1995) 479.
[10] N.T. Lequang, M.L. Migueres, D. Roche, G. Roussel, G. Mahuzier, J. Chretien, O.G. Ekindjian, Clin. Chem. 35 (1989) 1456.
[11] E. Riboli, N.J. Haley, F. De Waard, R. Saracci, Int. J. Epidem. 24 (1995) 354.
[12] G.V. Gill, M. Rolfe, I.A. Macfarlane, K.R. Huddle, Diabetic Medicine 13 (1996) 996.
[13] G. Lagrue, S. Cormier, Press. Med. 27 (1998) 1966.
[14] L. Meyer, B. Bauduceau, H. Mayaudon, T. Le Guyadec, H. Combe, D. Gautier, Med. Armées. 21 (1993) 537.
[15] G. Lagrue, C. Phillipon, N. Lafnoune, S. Cormier, F. Lebargy, Sem. Hôp. Paris 70 (1994) 387.