ELSEVIER

Journal of Chromatography A, 870 (2000) 29–38

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Application of liquid separation techniques to the determination of the main urinary nicotine metabolites

Krystyna Tyrpień[a,*], Tomasz Wielkoszyński[a], Beata Janoszka[a], Cezary Dobosz[a], Danuta Bodzek[a], Zygmunt Stęplewski[b]

[a]*Department of Chemistry, Faculty of Medicine, Silesian Medical School, Jordana St. 19, 41-808 Zabrze, Poland*

[b]*Department of Environmental and Preventive Medicine, Faculty of Medicine, Silesian, Medical School, Jordana 19, 41-808 Zabrze, Poland*

Abstract

A rapid procedure for the analysis of the main nicotine metabolites (cotinine, *trans*-3′-hydroxycotinine) in urine has been worked out. The procedure includes isolation of nicotine and its metabolites from urine by means solid–liquid extraction technique using resin Amberlite XAD-2 and then quantitation by the use of thin-layer chromatography with densitometry (in reflection mode). GC–MS was applied to confirm the results obtained by TLC. The procedure was applied to the analysis of cotinine concentrations in urine samples taken from children living in Upper Silesia region (Poland). Among 444 investigated children we did not find cotinine almost in 60% but in 15% of this population, there were children who could have been exposed to cigarette smoke. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Nicotine; Cotinine; Hydroxycotinine

1. Introduction

Tobacco smoke consists of a heterogeneous mixture of gases, uncondensed vapours, tar and particulate phase, which contains about 4000 compounds and more than 10^{14} radicals/puff in the gas phase [1]. The biochemical evidence suggests that exposure to environmental tobacco smoke (ETS) in public places e.g., in the workplace increases oxidative stress [2] and DNA damage [3], leading to cell damage or death. Cigarette smoking increases the risk of acute coronary events [4] and other forms of atherosclerosis by influencing oxidative lipoprotein modification and by endothelium damage. Many other diseases such as malignant tumours of respiratory [5–7] and digestive tract, the bladder and renal pelvis and pancreas may be caused by cigarette smoking [8]. It has been proven that nicotine has a specific gonadotoxic effect [9]. Among several nicotine metabolites, cotinine is a specific, excellent indicator of tobacco smoke exposure [10]. The true smoking status is based on cotinine level in body fluids [11], but cut-off points and distribution of metabolites in body fluids [12] are dependent on sex, age, diet, racial and ethnic differences as well as many other factors [13,14]. The isolation of fractions, containing nicotine and its metabolites by liquid–liquid [15] and liquid–solid [16] extraction in various modification from biological material, were applied.

*Corresponding author.

0021-9673/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)01239-X

Nicotine and cotinine were detected in urine by means of ultraviolet spectrophotometry after separation on silica gel [17]. The automated colorimetric direct test with barbituric acid was compared to high-performance liquid chromatographic methods for determination of nicotine in urine [18,19]. Nicotine and up to 12 metabolites in urine of human and laboratory animals were also determined under suitable chromatographic conditions by means of pre-column derivatization with 1,3-diethyl-2-thiobarbituric acid (DETBA) followed by HPLC [20]. Moreover, several variations of the colorimetric assay and a qualitative extraction procedure were evaluated in comparison with a cotinine immunoassay [21]. An improvement of sensitivity and stability of derivatization products before HPLC analysis were obtained by O'Doherty et al. [22] using 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrums acid) as a complexing agent. In the reversed-phase HPLC, which was more often used than normal phase [17] for this purpose, several polar mobile phases have been proposed with different pH values [23].

Very sensitive liquid chromatographic [12,24] or gas chromatography–mass spectrometric techniques have been applied in the assessment of nicotine exposure [25,26].

The immunoassays which are used for cotinine measurement — because of their sensitivity for measuring non-smokers' passive exposure to ETS — are a polyclonal-antiserum-based on ^{125}I-radioimmunoassay [27] and an enzyme-linked immunoassay (ELISA) [28]. A rapid and very useful method for epidemiological studies in schoolchildren has been described by Feyreabend et al. [29] in which a very fast multipipetting and vortexing system followed by GC with nitrogen–phosphorus detection was applied.

In this study, we have worked out a rapid procedure for the isolation and determination of nicotine and its main metabolites in urine consisting of solid–liquid extraction using resin Amberlite XAD-2, and then identification and quantitation by the use of HPLC and planar chromatography with densitometry. The chosen procedure was then applied to the analysis of cotinine concentrations in urine samples taken from children living in the Upper Silesia region of Poland.

2. Experimental

2.1. Chemicals

Analytical grade of solvents, for spectroscopy or for HPLC [dichloromethane (DCM), acetonitrile; Baker, Fluka] were used as eluents and for extraction and separation procedures.

Standards: *S*-(−)-nicotine (1-methyl-2-(3-pirydyl)pyrrolidine), (−)-cotinine (1-methyl-5-[3-pyridyl]-2-pyrrolidinone) and sodium 1-octanesulfonate were obtained from Sigma-Aldrich, and *trans*-3′-hydroxycotinine from Toronto Research Chemicals (Ontario, Canada). Non-polar resin Amberlite XAD-2 was purchased from Supelco and natrium hydroxide from local commercial sources (POCh-Gliwice, Poland).

2.2. Urine sampling

The parents of the sampled child collected a 24-h urine, starting on Sunday morning and ending on Monday morning. Urine was collected in plastic containers, previously delivered and washed with distilled water. During sampling the container was kept at +4°C. The containers were collected each Monday afternoon. The amount of urine in each container was measured in the laboratory, then each sample of urine was split into two separate plastic tubes. The samples were frozen at −80°C until analysis.

Creatinine measurements were carried out in the urine sample by the modification of the Jaffe method [30], the detection limit was 0.3 mg/ml of urine.

2.3. Isolation of nicotine and its metabolites fraction from urine

Urine sample was adjusted with 2 mol/l natrium hydroxide (in proportion about 20:1, v/v) to pH>10. After that, the analytes were extracted by solid–liquid extraction followed by the centrifugation and evaporation. For this purpose urine samples (10–20 ml) were shaken with 2–3 g of Amberlite XAD-2, which was prepared according to published procedure [16] for 5 min and centrifuged at 2000 *g* for 10 min. Then, the water layer was discarded.

The fraction containing nicotine and its main metabolites was isolated from XAD-2 resin by shaking twice with 5 ml of acetone–dichloromethane (1:3, v/v). After centrifugation, the organic phase was transferred to a fresh tube to evaporate, first in waterbath (at 40°C) and then to dryness under a nitrogen stream. The residue was dissolved in acetonitrile and analysed by HPLC and TLC–densitometry as well as by GC–MS.

2.4. HPLC

A Shimadzu (Kyoto, Japan) LC 9A liquid chromatograph equipped with a UV detector and a Rheodyne injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop was used. An analytical Supelcosil LC-PAH C_{18} column (250×4.6 mm, Supelco, Bellefonte, PA, USA) connected with guard column LC-18 (5 cm, Supelco) was used.

The flow-rate of mobile phase consisting of acetonitrile–buffer (88:12, v/v) at pH 4 adjusted by triethylamine was 0.5 ml/min.

2.5. TLC

TLC separations were performed on 4×8 cm chromatographic plates coated with silica gel bound with C_{18}(Machery–Nagel, Düren, Germany). Standards (0.5 μg/μl in acetonitrile) and dichloromethane sample solutions were applied (1.5 μl from 20 μl) on the plates by means of a Nanomat applicator (Camag, Muttenz, Switzerland). Chromatograms were developed to a distance of 7.5 cm in a horizontal chamber (DSII-Chromdes, Lublin, Poland) [18] using acetonitrile–water (88:12, v/v) with addition of sodium 1-octanesulfonate (50 mg/100 ml) as mobile phase.

Visualisation was first carried out under UV illumination at λ=254 nm. Next the spots were quantified by scanning in reflectance mode (UV absorbance at λ=260 nm, zig-zag scanning mode) using a CS9301 PC scanner (Shimadzu). The calibration range was from 50 to 200 ng/μl of each standard.

2.6. GC–MS

GC–MS was used to confirm the results obtained by TLC. A mass spectrometer (QP 2000-Shimadzu) connected with a gas chromatograph (GC-14) was used. The standards and analytes were chromatographed by 1-μl splitless injection onto a 25 m×0.2 mm (film thickness 0.25 μm) fused-silica capillary column HP Ultra 1. Conditions for nicotine, cotinine and hydroxycotinine analysis were as follows: electron impact (EI) 70 eV; helium flow-rate 1 cm^3/min; temperatures: injector 250°C, interface 270°C, ion source 200°C; GC temperature programme; 80°C (2 min) heating 6°C/min to 190°C (held for 1 min), 4°C/min to 230°C, 6°C/min to 270°C (held for 5 min).

3. Results and discussion

Very simple techniques, both the isolation and determination of cotinine and nicotine and its metabolites using solid–liquid extraction followed by TLC–densitometry were carried out for a short period of time and at minimum solvent consumption

Table 1
Comparison of HPLC and TLC techniques used to urinary cotinine determination

	HPLC	TLC
Detection limit	4.7 ng/ml	13.5 ng/spot
Min. cotinine concentration in 10 ml urine sample	9.4 ng/ml	1.35 ng/ml
Min. cotinine concentration in 20 ml urine sample	4.7 ng/ml	0.65 ng/ml
Calibration curve	$y = 16971x + 3222.2$	$y = 2.1584x + 15.784$
(regression coefficient)	(r=1.00)	(r=0.997)
Amount of sample separations/working day	30	70
Average solvent consumption/sample	15 ml	0.2 ml
RSD (%)	4.2	6.3

in comparison to HPLC technique (Table 1). In this way several samples can be analysed in parallel. On 1 day, we were able to isolate a fraction containing nicotine and its metabolites from 70 urine samples, and on the next day it was possible to separate and determine the main metabolites in these samples using TLC–densitometry, but we were only able to determine the cotinine level in 30 samples by means of HPLC, under the same chromatographic conditions. Although the detection limit for cotinine is smaller in HPLC than in TLC (Table 1), because of using the same urine sample volume we had to determine smaller urinary cotinine concentrations using TLC–densitometry, since the 20-μl loop in the HPLC equipment requires a reliable sample volume, but in TLC we can apply the whole extract onto chromatographic plates. The separation of the main metabolites gives better results in TLC than HPLC under the described conditions, but the applied chromatographic system allows to determine cotinine concentration in urine using each of these techniques (Figs. 1 and 2). Therefore we decided to use TLC–densitometry for the determination of cotinine concentration in the children's urine samples.

Using the described procedure, an 80% recovery of cotinine was found, but the RSD of cotinine determination in smokers urine was 9.2%. A 2-ml volume of urine is, in our work, the least volume necessary to analyse cotinine in the case of passive smokers. In the case of active smokers, cotinine determination requires less urine (unpublished data).

A cotinine content below the detection limit of TLC–densitometry was found in 234 (52.7%) of all the tested (444) urine samples. We tried to analyse chosen urine samples comparing HPLC and TLC results. The cotinine concentrations determined by these techniques were a little different, although in the same order (Table 2). An example of HPLC chromatograms are shown in Fig. 1. In addition, for some of these samples GS–MS was used as a reference method, for which detection limit is lower (5 ng GC–MS; 13.5 ng TLC–densitometry) [31]. GC–MS was also applied to compare the results of analysis, particularly in those samples in which a high cotinine concentrations were determined (>79 ng/ml using TLC technique). The comparison of the results obtained by both methods is presented in Table 3. These results are in agreement with high cotinine concentrations. But GC–MS (which can determine lower contents of cotinine) can be useful

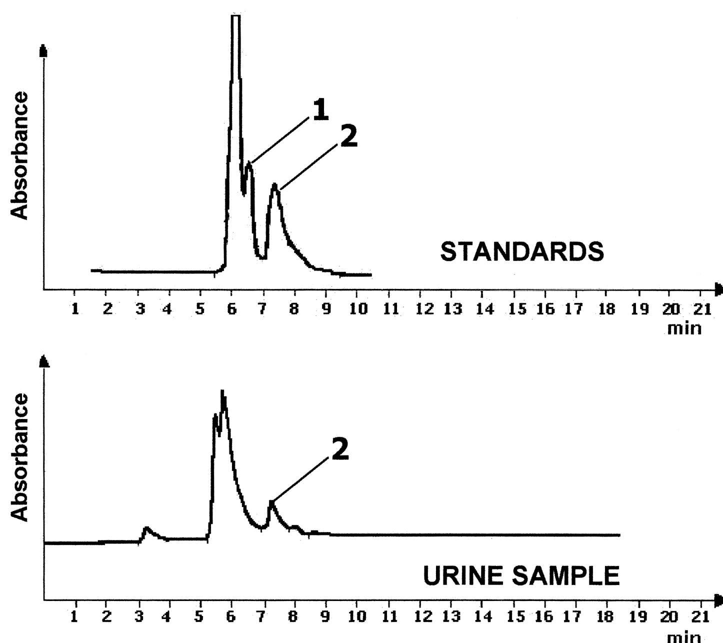

Fig. 1. HPLC chromatograms standards of nicotine metabolites and urine sample. 1 = Hydroxycotinine; 2 = cotinine.

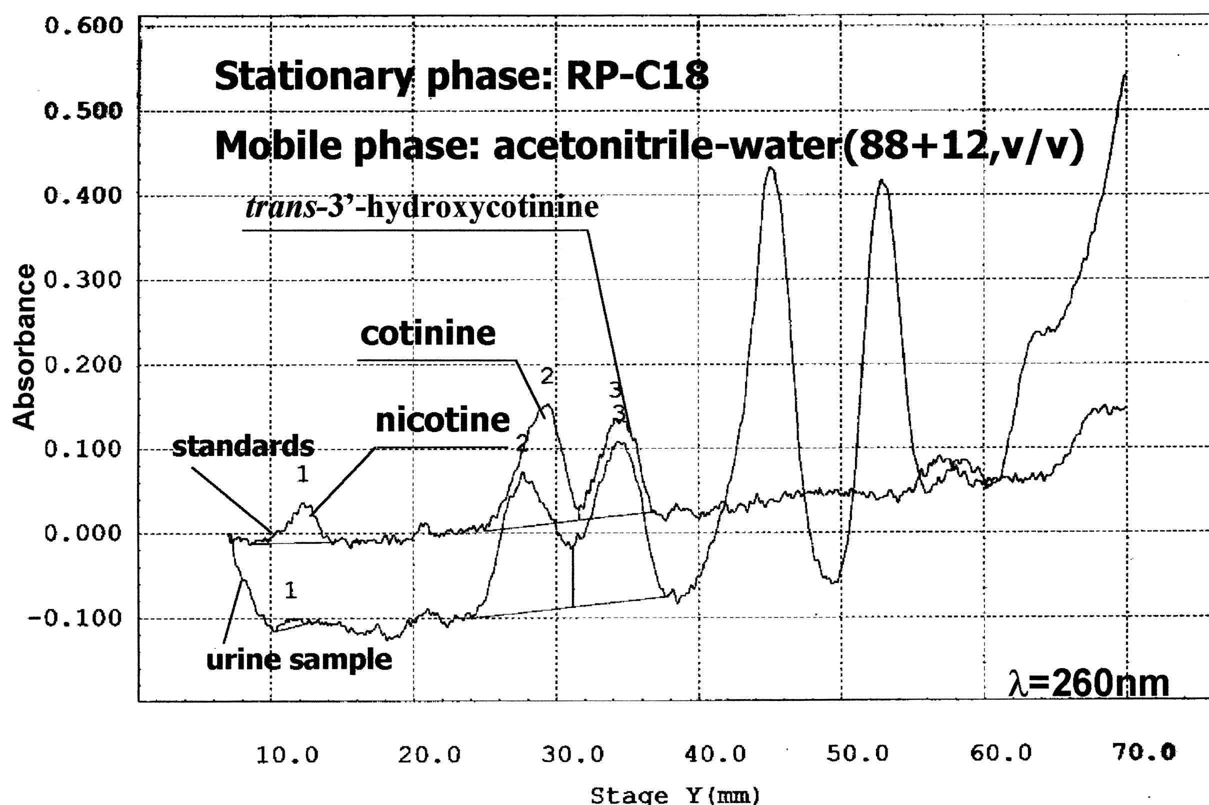

Fig. 2. TLC chromatogram of fraction containing nicotine (1); cotinine (2) and *trans*-3′-hydroxycotinine isolated from a child's urine sample.

as a complementary method where cotinine was not found using TLC–densitometry.

In one case, among 40 investigated samples, cotinine was absent, but the presence of *trans*-3′-hydroxycotinine as the main metabolite was confirmed, using GC–MS. But in several cases, besides nicotine, cotinine or *trans*-3′-hydroxycotinine were found.

Table 2
Comparison of cotinine concentrations determined by HPLC and TLC in chosen children's urine

Sample	HPLC ($ng \cdot ml^{-1}$)	TLC ($ng \cdot ml^{-1}$)
U38	212.2	140.4
U391	57.3	163.8
U157	147.6	120.6
U100	97.8	47.2
U406	51.7	104.7

An example of a GC–MS chromatogram of a child's urine sample with marked presence of nicotine and its main metabolites is presented in Fig. 3. A TLC chromatogram of the sample in which the same compounds were identified is presented in Fig. 2, as well as TLC chromatograms of the selected samples, in which only cotinine and only *trans*-3′-hydroxycotinine were found, are shown in Fig. 4a and b, respectively.

It is more difficult to achieve narrow, symmetrical densitometric peaks with cotinine and *trans*-3′-hydroxycotinine than with nicotine, due to the higher polarity and higher R_f values of these nicotine metabolites. The addition of natrium octane sulfonate to the mobile phase in TLC analyses improves the sharpness of the peaks. Scanning techniques are very

Table 3
TLC and GC–MS results of nicotine and its metabolites identification in urine samples[a]

Sample no.	Nicotine		Cotinine		*trans*-3′-Hydroxycotinine	
	TLC	GC–MS	TLC	GC–MS	TLC	GC–MS
14	−	−	−	+	+	−
16	−	−	+	+	+	+
38	−	−	+	+	+	+
39	+	+	+	+	−	−
55	−	−	−	(+)	−	−
74	−	−	+	+	−	−
95	−	−	+	+	+	+
96	−	+	+	+	−	−
128	−	−	−	−	−	−
133	+	−	(+)	+	−	−
140	−	−	−	−	−	−
151	−	−	+	+	(+)	−
172	(+)	−	(+)	+	(+)	−
174[a]	−	−	+	−	(+)	−
175	−	−	−	−	(+)	−
184	−	−	+	+	−	−
231	+	−	+	+	+	+
238	−	−	+	+	−	−
239	+	−	−	+	−	−
249	−	−	−	−	−	−
263	−	−	+	+	−	−
290	−	−	+	+	(+)	−
304	−	−	−	+	+	+
337	(+)	+	+	+	+	+
343	+	+	+	+	(+)	−
351	+	+	−	+	(+)	−
352	+	+	+	+	(+)	−
360	−	−	−	−	−	−
362	−	−	+	+	+	−
367[a]	−	−	−	−	+	+
369	−	−	−	−	−	−
385	−	−	+	+	−	−
386	−	−	+	+	−	−
409	−	−	+	+	+	+
414	−	−	+	+	+	+
416	−	−	+	+	+	+
425	−	−	+	+	−	−
433	+	+	+	+	+	+
442	−	−	+	+	−	−
444	−	+	−	−	−	−

[a] −, Below detection limit; +, detected; (+), weakly detected.

important in this case, because zig-zag scanning improves signal intensity coming from cotinine, at reflectance mode of measurement. The comparison of zig-zag and linear scanning modes for standard nicotine and its metabolites are shown in Fig. 5.

The presented multisteps analytical procedure for the isolation and determination of the main metabolites of nicotine in such an easily accessible biological material as urine, allowed assessment of cotinine levels, as one of the main nicotine metabolites in urine in children from Upper Silesia Region in a quick and simple way.

It is known that 22.5 ng of cotinine/ml urine qualified a person as a passive smoker according to

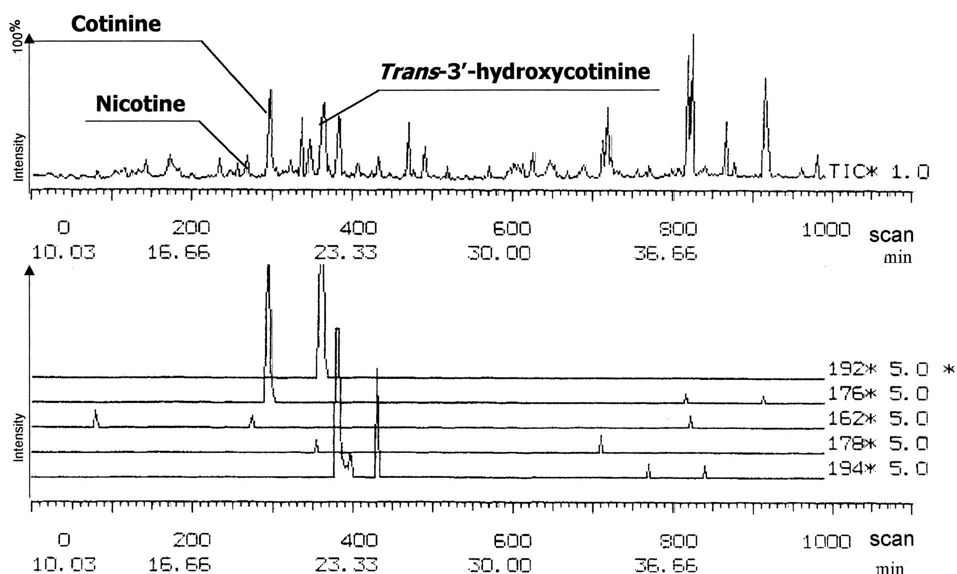

Fig. 3. GC–MS chromatogram of fraction containing nicotine (1), cotinine (2) and *trans*-3′-hydroxycotinine isolated from child urine sample.

Jarvis et al. [32], but 79 ng of cotinine/ml urine, is the cut-off value which has been set by Spierto et al. [33]. The range of cotinine concentration in our investigated samples was 0–214.6 ng/ml (1.236 μmol/l) of urine. The distribution of the investigated child population dependent on cotinine level in urine

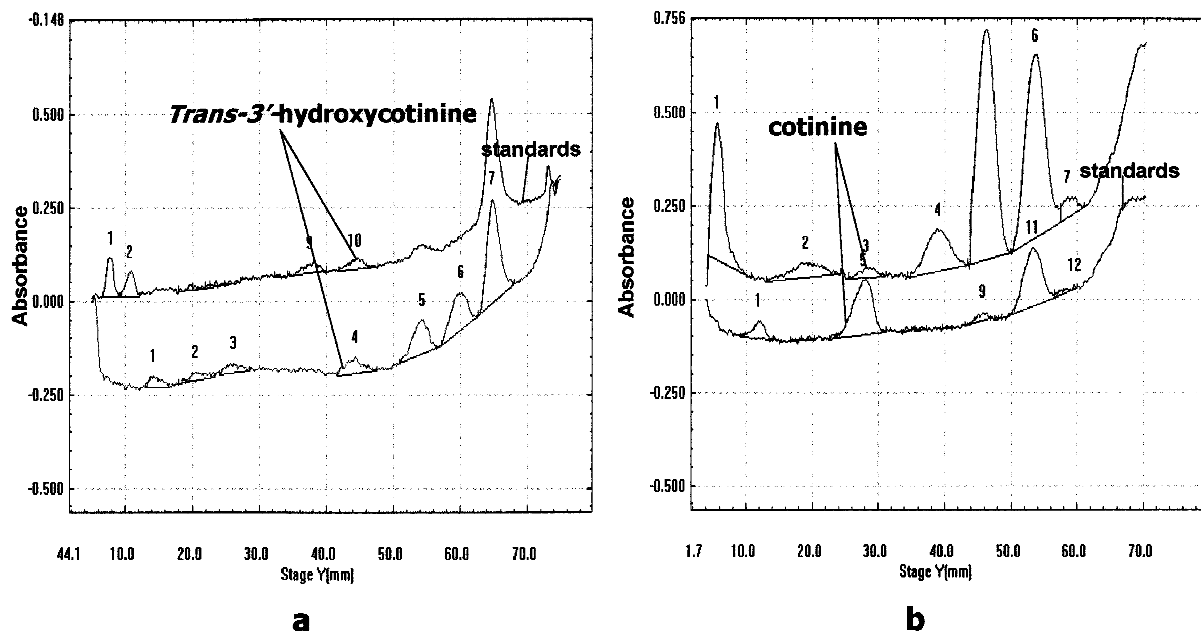

Fig. 4. TLC chromatograms of the samples in which (a) only *trans*-3′-hydroxycotinine and (b) only cotinine were identified.

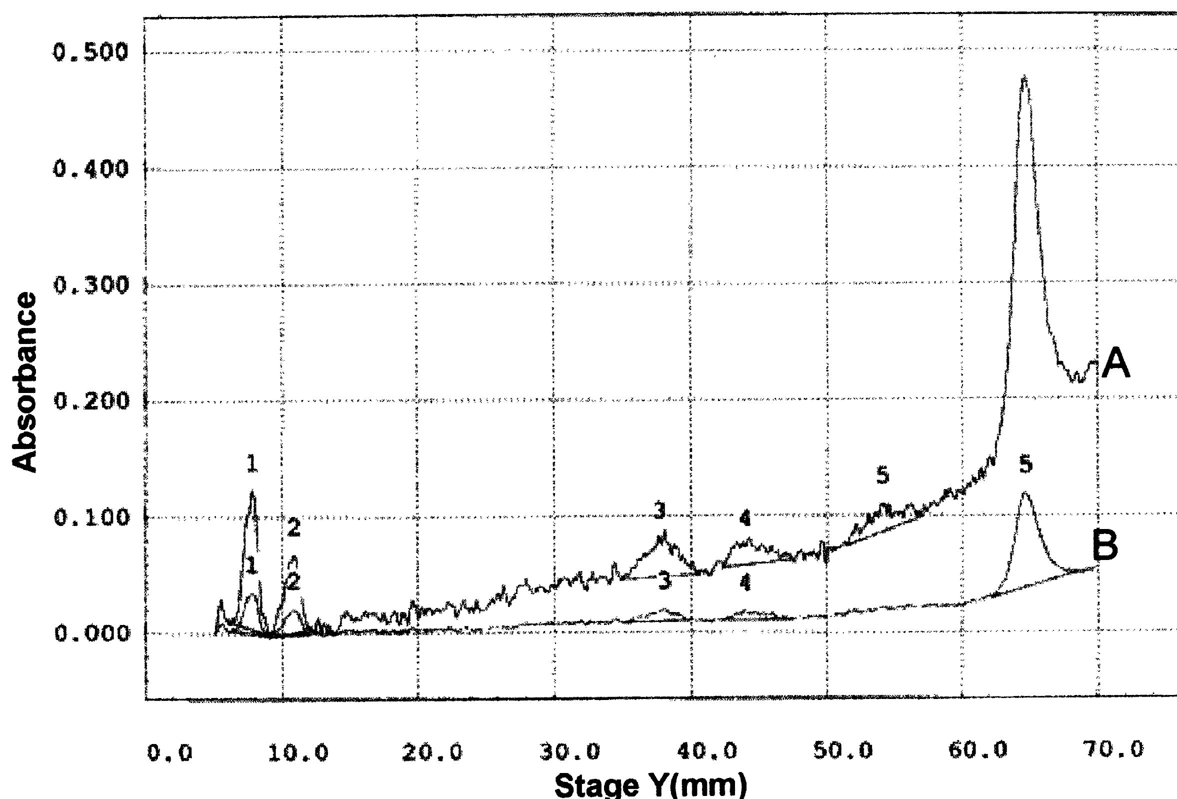

Fig. 5. Comparison of zig-zag (A) and linear (B) scanning modes for standard nicotine-2 and its metabolites: 1=1′-nicotineoxide; 2=cotinine; 4=*trans*-3′-hydroxycotinine.

is shown in Table 4. Among 444 investigated children, we did not find cotinine in almost 60%, but in 15% of this population there were children who could have been exposed to cigarette smoke or it is possible that some of them could have been occasional smokers.

Table 4
Distribution of the cotinine levels in urine of the investigated Silesian child population (n=444)

Cotinine level	Percentage (%)
(ng/mg creatinine)	
Not detected	53
0–12	21
>12[a]	26
(ng/ml urine)	
Not detected	57.6
0–22[b]	27.1
22–79	11.0
>79[c]	4.3

[a] Cut-off point according to Preston et al. [28].
[b] Cut-off point according to Jarvis et al. [32].
[c] Cut-off point according to Spierto et al. [33].

On the other hand, on the basis of investigation of Puerto Rican children, the cut-off was set about 12 ng cotinine/mg creatinine [28]. Creatinine production and excretion by the body is fairly constant for healthy individuals, so an urinary creatinine measurement can be used as an estimate of urinary dilution or concentration [34]. It has been suggested that expressing urinary cotinine as a ratio urinary creatinine concentration may be better than the use of urinary cotinine concentration alone [35]. According to these results the distribution between passive and non-smokers for the population investigated by us is presented in Table 4. The results of the study carried out by Jędrychowski et al. strongly support the thesis that children who were exposed to ETS in their home environment were more susceptible to

acute respiratory tract illnesses than unexposed children [36].

4. Conclusions

It is alarming that so many children are exposed to passive smoking at home or/and in public places. Among these there are not only healthy children, but children with various diseases. Moreover, they live in the most polluted region of Poland, where many toxic substances can be the cause of numerous illnesses. Therefore, there is a necessity for cheap and simple procedures to determine these compounds and their metabolites in body fluids, particularly, if they are non-invasive methods. What is more, the application of planar chromatography to analyse urine samples may be useful not only for assessment of tobacco smoke exposure but also in the metabolic investigations of other toxic substances, which occur in regions as polluted as Upper Silesia.

The application of HPLC under the same chromatographic conditions requires much more solvent consumption and bigger sample volume. It is not possible to carry out the separations simultaneously many fold with standard substances with different detection parameters as it is possible in TLC.

The obtained results will be verified by questionnares for finding the exact cut-off point for distribution of investigated population between passive or non-exposed children.

Acknowledgements

The studies were partially sponsored by the Polish State Committee for Scientific Research (Project No 6 PO4G00716) financed in 1999–2001.

References

[1] L.A. Mooney, R.M. Santella, L. Covey, A.M. Jeffrey, W. Bigbee, M.C. Randall, T.B. Cooper, R. Ottman, W.-Y. Tsai, L. Wazneh, A.H. Glassman, T.-L. Young, F.P. Perera, Cancer Epidemiol., Biomarker Prev. 4 (1995) 627.
[2] D.J. Howard, R.B. Ota, L.A. Briggs, M. Hampton, Ch.A. Pritsos, Cancer Epidemiol., Biomarker Prev. 7 (1998) 981.
[3] G. van Poppel, H. Verhagen, P. van 'tVeer, P.J. van Bladeren, Cancer Epidemiol., Biomarker Prev. 2 (1993) 441.
[4] N.L. Benowitz, G.A. Fitzgerald, M. Wilson, Q.I. Zhang, J. Am. Coll. Cardiol. 22 (1993) 1159.
[5] X. Xu, L. Wang, Arch. Environ. Health 53 (1998) 44.
[6] W. Rokicki, M. Rokicki, H. Wyl, Pol. Przegl. Chir. 67 (1995) 101, in Polish.
[7] K. Kashiwabara, H. Nakamura, T. Kiguchi, H. Yagyu, T. Yokoi, T. Matsuoka, Clin. Chim. Acta 276 (1998) 179.
[8] C. Malaveille, P. Vineis, J. Esteve, H. Ohshima, G. Brun, A. Hautefeuile, P. Galet, G. Ronco, B. Terracini, H. Bartsch, Carcinogenesis 10 (1989) 577.
[9] N.K. Kavitharaj, P.L. Vijayammal, Pharmacology 58 (1999) 2–7.
[10] P.M. Suter, A. Spitzbarth, K. Gautschi, L. Erdmenger, D.J. Vonderschmitt, W. Vetter, Praxis 84 (1995) 821.
[11] E. Jauniaux, B. Gulbis, G. Acharya, P. Thiry, C. Rodeck, Obstet. Gynecol. 93 (1999) 25–29.
[12] G.D. Byrd, R.A. Davis, W.S. Caldwell, J.H. Robinson, J.D. de Bethizy, Psychopharmacology 139 (1998) 291–299.
[13] I.M. Sasson, D.T. Coleman, E.J. LaVoie, D. Hoffmann, E.L. Wynder, Mutation Res. 158 (1985) 149–157.
[14] A.J. Wells, P.B. English, S.F. Posner, L.E. Wagenknecht, E.J. Perez-Stable, Am. J. Public Health 88 (1998) 1503–1509.
[15] P.P. Rop, F. Grimaldi, C. Oddoze, A. Viala, J. Chromatogr. 612 (1993) 302.
[16] E. Yamasaki, B.N. Ames, Proc. Natl. Acad. Sci. USA 74 (1977) 3555–3559.
[17] I.D. Watson, J. Chromatogr. 143 (1977) 203–206.
[18] S.A. Kolonen, E.V.J. Puhakainen, Clin. Chim. Acta 196 (1991) 159–166.
[19] J. Moore, M. Greenwood, N. Sinclair, J. Pharmaceut. Biochem. Anal. 8 (1990) 1051–1054.
[20] K. Rustemeier, D. Demetriou, G. Schepers, P. Voncker, J. Chromatogr. 613 (1993) 95–103.
[21] R.F. Smith, H.M. Mather, G.A. Ellard, Clin. Chem. 42 (1998) 275–280.
[22] S. O'Doherty, M. Cooke, D.J. Roberts, J. High Resolut. Chromatogr. 13 (1990) 74–77.
[23] N.T.L. Thuan, M.L. Miqures, D. Roche, G. Roussel, G. Mahuzier, J. Chrestien, O.G. Ekindjan, Clin. Chem. 35 (1987) 1456.
[24] K.T. McManus, J.D. debethizy, D.A. Garteiz, G.A. Kyerematen, E.S. Vesell, J. Chromatogr. Sci. 28 (1990) 510.
[25] H. James, Y. Tizabi, R. Taylor, J. Chromatogr. B 708 (1998) 87.
[26] P. Voncken, G. Schepers, K-H. Schäfer, J. Chromatogr. 479 (1989) 410–418.
[27] G.J. Knight, G.E. Palomaki, D.H. Lea, J.E. Haddow, Clin. Chem. 35 (1989) 1036–1039.
[28] A.M. Preston, L.J. Ramos, C. Calderon, H. Sahai, Prevent. Med. 26 (1997) 1.
[29] C. Feyerabend, M.A.H. Russel, J. Pharm. Pharmacol. 42 (1990) 450–452.
[30] N.W. Tietz (Ed.), Textbook of Clinical Chemistry, W.B. Sanders, Philadelphia, 1986.

[31] K. Tyrpień, C. Dobosz, T. Wielkoszyński, B. Janoszka, D. Bodzek, Proceedings of the 10th International Symposium on Instrumental Planar Chromatography, Visegrad, Hungary, 1998, p. 254.
[32] M.J. Jarvis, H. Tunstall-Pedoe, C. Feyerabend, C. Vesey, Y. Saloojee, Am J. Public Health 77 (1987) 1435.
[33] F.W. Spierto, W.H. Hannon, J.S. Kendrick, J.T. Bernert, J. Pirkle, P. Gargiullo, J. Smoking Relat. Dis. 5 (1994) 65.
[34] S.G. Thompson, R.D. Barlow, N.J. Wald, H. Van Vunakis, Clin. Chim. Acta 187 (1990) 289–296.
[35] D.W. Sepkowic, N.J. Haley, Am. J. Pub. Health 75 (1985) 663–665.
[36] W. Jędrychowski, E. Flak, Przegl. Epid. 50 (1996) 457–465.