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Combined SPE and HPTLC as a Screening Assay of Urinary Cotinine from Male Adolescents Exposed to Environmental Tobacco Smoke**

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Abstract

Cotinine, as the main metabolite of nicotine, has been determined in urine using solid-phase extraction and the high-performance thin-layer chromatographic (SPE-HPTLC) method. The urine samples were collected from a group of 35 male adolescents which were moderate or significantly exposed to home environmental tobacco smoke (ETS). l-methyl-2-pyrrolidinone was used as the internal standard in the proposed screening procedure. The thin-layer chromatograms were evaluated densitometrically after visualization of cotinine spots with ninhydrin and cadmium acetate solution. The described SPE-HPTLC procedure indicated good selectivity, sensitivity and reproducibility, enabling reliable verification of interview collected questionnaire data in families exhibiting a diversified level of ETS. The results of cotinine measurements by the proposed method were applied for assessment of hazards from home ETS on the health status of elementary schoolboys, especially an increased risk for infectious respiratory tract diseases and exercise-induced bronchospasm.

Keywords: Cotinine; passive smoking; human urine; solid-phase extraction; HPTLC

Introduction

Trace amounts of cotinine, i.e. *(S)(-)*-l-methyl-5-(3-pyridyl)-2-pyrrolidinone (with molecular structure presented in Fig. 1), was isolated in trace amounts from *Nicotiana tabaccum* and *Nicotiana rustica* leaves. Moreover, cotininie, as probably artifact of nicotine autooxidation, was also detected in *Duboisia hopwoodii* (Solanaceae) species - a medicinal plant used by Australian aborigenes [1]. However, cotinine is the primary human metabolite of nicotine and by possesing the long elimination half-time from the body can be reliably determined in different biological fluids up to several days after a person's exposure to tobacco smoke [2]. Serum, salivary or urinary cotinine has been currently recognized as a generally specific, highly sensitive and most reliable biomarker of human smoking as well as transdermal or dietary intake of nicotine. The use of such a biomarker has been validated by reported mutual relationships between diversified biological effects of environmental tobacco smoke (ETS) and quantitated cotinine concentrations in specified biological fluids [3].

Many very sensitive and mostly entirely specific methods

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have been published for cotinine determination in urine, blood or seminal plasma, serum, saliva and extraembryonic, crevicular or cervical fluids such as simplified spectrophotometric assay with diethylthiobarbituric acid [4], capillary column gas chromatography with nitrogen-specific [5], ion-trap [6, 7] or mass spectrometry detection [8], high-performance liquid chromatography (HPLC) with UV detector [9], HPLC joined to atmospheric pressure chemical ionization tandem mass spectrometer (APCI MS-MS) [10, 11], or involving pre-column derivatization [12], as well as ^{125}I - based radioimmunoassay [13] or monoclonal antibody enzyme-linked immunosorbent assay (ELISA) [14]. However, when using these analytical procedures dietary nicotine exposure may significantly confound extremly low-level (in range of 0.3 - 2 μg/l) determination of cotinine from ETS, in particular biological fluids unable to distinguish between active and passive smoking of subjects [3].

Thin-layer chromatography (TLC) on silica gel G adsorbent layer, benzene-dioxan-ethanol-ammonia (50:40:5:5) as mobile phase and iodoplatinate reagent as visualizing agent has been applied by Bery and Grove [15] for semi-quantitative determination of cotinine along with nicotine, morphine, methadone and cocaine in urine extracts of smoking and non-smoking adults. The usefulness of the TLC procedure hyphenated with scintilation counting in pharmacokinetic studies for determination of cotinine in rat plasma concentration of cotinine or its DNA adducts in human peripherial-blood leukocytes has been reported by Adir *et al.* [16] and Phillips *et al.* [17], respectively.

In sample preparation step the solid phase extraction (SPE) distinct procedures, employing mainly cartridges with octadecylsilica [18] or large pore diatomaceous earth [19] packing as well as method supporting preliminary liquid-liquid extraction [5] was developed for reliable and quick isolation and preconcentration of cotinine from multicomponent urine probes without interference and contamination. Especially, the use of SPE procedure eliminated caffeine interference on determination and quantitation of urinary cotinine [20].

To reduce substantial bias and increase precision during chromatographic determination of urinary cotinine an appriopriate internal standard should be used, especially indicating not only structural similarity but also comparable lipophilicity and basicity as a parent analyte. Until now a variety of such internal standard substances has been proposed, including amphetamine sulfate, diphenylamine, ketamine, lidocaine, methylanabasine, 5-methylcotinine, methylprylone, N-ethylnorcotinine, norephedrine and 2-phenylmidazole. However, none of these has been approved as a universal internal standard in spite of the analytical procedure chosen, which presumably explains in part the significant differences in urinary cotinine concentrations reported in various published studies on ETS exposure [3, 10].

For adolescents aged 13 years the prevalence of cigarette smoking varies widely in different countries, ranging from 2 to 5 percent (Sweden, United States) to more than 30 percent (Australia, Uruguay). Presently, in European Community countries the prevalence of smokers among male adolescents is decreasing but it is increasing among girls; however, in developing countries male adolescent smokers still reach 40 percent [21]. Detailed studies in Poland have revealed [22] that among adolescents aged 11 to 15 years 18 per cent of boys and 8 per cent of girls reported daily smoking in 1994. In the total Polish population the prevalence of tabacco smoking was 47 percent among men and 23 percent in woman, reaching more than 30 percent in pregnant woman, in 1995. The increasing proportion of smokers begining to smoke before age 15 is also observed in Poland [22].

Recently, the US Enviromental Protection Agency (EPA) classified secondhand tobaccco smoke as a Group A carcinogen [23]. The possible acute and persistent adverse health effects of short- or long-term ETS exposure to human preadolescent and adolescent children include such evidences as elevated risk for development of premature coronary heart disease [24], increased lower respiratory tract infections [25], middle ear effusions [26], decreased lung function [27], lower rates of lung growth [28] and more frequent exacerbation of asthma [29]. The associations between level of ETS exposure to adolescents, measured as urinary cotinine excrection, and dwelling conditions [30] as well as enabling, predisposing and reinforcing factors has been recently described [31]. Furthermore, significant correlations between the concentration of cotinine and benzene metabolites in urine noted indicating extended risk of leukemia and lymphomas in humans exposed to ETS [32].

In view of growing interest in the negative health consequences of chronic or single ETS exposure to adolescents we present in this report the usefulness of the HPTLC method for sreening determination of cotinine in urine samples as a supporting tool into guidelines for planning, implementing and evaluating comprehensive national health promotion programs related to the reduction or elimination of ETS.

Materials and Methods

Subject Data

Subjects for the study consisted of a random sample of 35 Caucasian, healthy, non-smoking schoolboys, with mean age 14 years and 7 months (range from 14 years to 15 years 3 months), all urban citizens, who were diagnosed with different levels of environmental tobacco smoke (ETS) exposure over the past one-year period confirmed by self-reported smoking status in a family interview. In addition to obtaining data on lifetime histories of tobacco use both for subjects and their family members, information was elicited about such exposure characteristics as sociodemographic factors, general somatic development indices (body height, body mass, head and chest circular), nutritional status (calculation of body mass index - BMI), measurements of thickness of body fat foldings on scapula, abdomen and arm muscles), medication use and recreational physical activity. A complete medical history was taken from each schoolboy including the birth health status, past and current childhood diseases (especially infectious respiratory tract diseases). Information was collected by a structured interview using a specialized questionnaire administered by trained personnel. Preliminary statistical analyses were conducted using U-test for comparison of mean values and structural descriptors extracted from questionnaire data set. Results of these calculations were accepted as statistically significant at significance level $p < 0.05$.

Collection of Urine Samples

Half hour morning urine samples (5 ml as minimal volume) in fasting subjects were collected in pasteurized glass urine collection containers, once per week within the whole month, especially after weekends, in time close as much as possible to the particular subjects morning wake-up and before leaving his home for school. All samples were stored at -20°C after the addition of 50 µl of 6 *M* hydrochloric acid to preserve microbial growth.

Reagents and Standard Solutions

Standards of *(S)(-)*-cotinine, *(S)(-)*-nicotine and 1-methyl-2-pyrrolidinone (abbreviated as 1-Me-Pyr) were supplied from Sigma-Aldrich (St.Louis, MO, USA). A standard solution of cotinine and nicotine were prepared by dissolving 10 mg each in 10 ml of 0.012 *M* aqueous solution of hydrochlorid acid and stored at 4°C until use. Internal standard solution was prepared by dissolving 400 µl of glass distilled, anhydrous l-methyl-2-pyrrolidinone (d = 1.033 g/ml) in 4 ml of 1% (v/v) methanolic solution of concentrated hydrochlorid acid (d = 1.410 g/ml) and rinsing up to volume of 10 ml with 0.2 *M* aqueous solution of hydrochlorid acid.

A methanolic solution (10 %, v/v) of *p*-toluenesulfonic acid monohydrate (Sigma-Aldrich, St.Louis, MO, USA) was prepared. HPLC grade methanol (J.T.Baker, Phillipsburg, NJ, USA) was used for preparation of standard solution and in all performed laboratory experiments.

Ninhydrin (Merck, Darmstadt, Germany), cadmium acetate monohydrate (Sigma - Aldrich, St.Louis, MO, USA) and glacial acetic acid (POCh, Gliwice, Poland) were applied in HPTLC experiments to prepare the visualizing reagent.

SPE Procedure

Solid-phase extraction disposable polypropylene columns of Bakerbond-spe series (maximal probe volume 6 ml) prepacked with 500 mg of high capacity rever-sed-phase octadecylsilica (40 µm, 60 A°, 475 m^2/g, surface pH = 6.8) (J.T.Baker, Phillipsburg, NJ, USA, product no. 7020-06) were used along with an all-glass vacuum Baker-12 SPE processing system (Witko-Eurocolor, Lodz, Poland) equipped to hold and perform simultaneous analysis with twelve SPE columns. An IP 20 portable vacuum pump (Veb Reglerwerk, Dresden, Germany) was applied during the SPE procedure. All SPE experiments were made at controlled room temperature (21 +/- 0.5°C).

In SPE of urinary cotinine the method proposed by Jacob III *et. al.* [6] was used with some modifications. Amount of 100 µl of l-methyl-2-pyrrolidinone internal standard solution was added to each collected 5 ml urine sample in a 10 x 1.5 cm glass tube. Next, 1 ml of 2 *M* sodium hydroxide aqueous solution was added to each tube followed by vortex-mixing for 1 min at 50 r.p.m. on the benchtop laboratory shaker Elpan 357 (Unipan, Warsaw, Poland).

In preliminary step (i) of SPE of urinary cotinine the octadecylsilica SPE columns placed in the manifold cover of Baker-12 SPE processing system were pre-conditioned by washing them successively (turn vacuum off) using 6 ml aliquots of methanol and deionized water, respectively, as well as discarding collected eluates. After that particular urine samples, containing previously added internal standard, were aspired on the top of the wetted packing bed of the SPE columns.

In sample application step (ii) of SPE for the next 5 minutes the urine samples were enabled to flow freely through the packing bed of SPE columns. After this time a vacuum 3 mm Hg was applied to the Baker-12 SPE processing system until all volume of urine samples flowed through the SPE columns. Fractions collected in this fashion were discarded.

In the next washing step (iii) of SPE the columns were eluted with 1 ml of deionized water for 2 minutes, applying vacuum 3 mm Hg. The vacuum was turned off and columns were air dried for 10 min. These collected eluates were also discarded.

Finally, in selective elution step (iv) of SPE the columns were washed two times with 0.5 ml of methanol for 2 minutes using vacuum 2 mm Hg. To these collected eluates, containing urinary cotinine, 100 µl of 10% methanolic solution of/j-toluenesulfonic acid were added using an adjustable pipettor. From this final SPE extracts the volume of 1 µl was taken with Hamilton 701 microsyringe to spot on the sample application position of HPTLC plate. Between daily analyses the urinary cotinine SPE extracts were stored in a laboratory refrigerator at 4°C.

HPTLC Analysis

HPTLC experiments were performed on 10x10 cm, glass-backed, ready-for-use HPTLC plates precoated with wettable (partly silanized) bonded polymeric octadecylsiloxane silica (0.25 mm, mean d_p = 9 µm) supplied from Macherey-Nagel (Diiren, Germany, product no. 811 075).

The HPTLC plates were used as received. Mixtures of methanol with deionized water in the proportions 80:20 (v/v) was applied as mobile phase. Before use the appropriate mixture of solvents was left to stand for lh at room temperature (21 +/- 0.5°C).

Samples (1 µl) of internal standard solute and final SPE extracts were spotted individually on the sample application position (10 mm from the lower edge of HPTLC plate) with a type 701 Hamilton (Reno, CA, USA) 10 µl microsyringe. The plates were developed vertically in normal all-glass chambers (20 x 20 x 10 cm, Glassverke, Ilmenau, Germany), without prior saturation, on migration distance (Z_m) 8.5 cm.

Developed zones of cotinine and l-methyl-2-pirrolidinone were visualized by spraying the HPTLC plates with freshly prepared derivatizing solution proposed by Devenyi [33] containing 0.2 g of ninhydrin, 0.5 g of cadmium acetate monohydrate dissolved in 2 ml of glacial acetic acid and adjusted to 100 ml with methanol. Subsequently the HPTLC plates were heated at 80°C for 60 min.

Densitometry

The chromatograms on the HPTLC plates were densitometrically evaluated with the 16 grayscale (4-bit) scan-

ner ScanJet 3p (Hewlett-Packard, Warsaw, Poland) connected to an IBM-compatible Pentium MMX 166 MHz desktop microcomputer and working under Quantiscan v.2.0 (Biosoft, Cambridge, England, UK) specialized software [34] enabling integration of peak area referred to individual chromatographic zone. The chromatograms were scanned along to the direction of mobile phase development. Displayed densitometric plots were analyzed after automatic medium smooting to provide rejection of small peaks and noise caused by gradients in thickness of layer or particle size distribution in stationary phase deposited on HPTLC plate. Peak location and integration algorithm of Quantiscan software was used with typical settings as follows: smooth cycles = 3, smooth width = 4, rejection width = 1, rejection height = 1, slope = 1, area = 3.

Calculations

Cotinine concentration C_k (μg/L) in analyzed urine samples was calculated using the following formula:

$$C_k = [C_s \bullet V_{spe} \bullet 10 (LA_k - LA_s)] / [V_{ex} \bullet V_{ur}] \quad (1)$$

where:

C_s -(μ g) is mass of internal standard (l-methyl-2-pyrrolidinone) added to urine sample before applying SPE procedure,

V_{spe} - (liters) is total volume of final SPE extract of cotinine fraction,

LA_k - is logarithm of integrated area of densitometric peak refered to cotinine zone on the chromatogram of final SPE extract,

LA_s - is logarithm of integrated area of densitometric peak refered to internal standard zone on the chromatogram of final SPE extract,

V_{ex} - (liters) is total volume of final SPE extract deposited on the sample application position of HPTLC plate,

V_{ur} - (liters) is total volume of urine sample extracted in SPE procedure.

The retention parameters of solutes on HPTLC plates were calculated from quadrupole measurements of experimental values of retardation factor R_f and Bate-Smith and Westall [35] formula for logarithmic retardation factor R_M:

$$R_f = Z_s/Z_m = \quad (2)$$

$$R_M = \log k' = \log [(1 / R_f) - 1] \quad (3)$$

where:

Z_s - is migration distance (mm) of the centre of developed solute zone from the sample application position on the HPTLC plate,

Zm - is the migration distance (mm) of the mobile phase front from the sample application position to mobile phase front, and

k' - is capacity factor of solute.

The average plate height values , H_a *(μm),* number of average plate height equivalents, N' resolution, R_s, and separation factor, α, was calculated directly from the chromatograms using equations proposed by Poole and Fernando [36]:

$$H_a = \sigma^2/[(Z_f - Z_o) - R_f] \quad (4)$$

$$N' = 0.80 \bullet Z_f / H_a \quad (5)$$

$$R_s = 0.25 \bullet N'^{0.5} \bullet \ln [(1 + k_2) / (1 + k_1)] \quad (6)$$

$$\alpha = R_{f2} / R_{f1} \quad (7)$$

where:

σ^2 - is observed variance (mm) of developed solute zone,

Z_f - is distance (mm) of the mobile phase front position above the mobile phase entry position,

Z_o - is distance (mm) of the mobile phase entry position to the sample application position, and

R_{f2} and R_{f1} - are retardation factors of more and less ($R_{f2} > R_{f1}$) retained neighboring zones of solutes, respectively.

The solvation parameters, *i.e.* R_2, an excess of molar refraction, π^H the dipolarity/polarizability, $\Sigma\alpha^H$ the overall hydrogen-bond acidity, $\Sigma\beta^H$ the effective hydrogen-bond basicity, and V_x the McGovan characteristic volume were calculated using the algorithm and fragmental constants reported by Abraham [37].

All statistical treatment of experimental data and calculations of structural parameters of solutes were performed, respectively, with Statistics 4.3 (StatSoft, Inc., Tulsa, OK, USA) or ACD/Labs (Advanced Chemical Development Inc., Toronto, ONT, Canada) and HyperChem 4.5 (Hypercube Inc., Waterloo, ONT, Canada) software implemented on an IBM-compatible Pentium MMX 166 MHz desktop microcomputer.

Results and Discussion

Subject Questionnaire Data

Inspection of data collected in the interview questionnaires revealed that 59% of 35 investigated schoolboys were exposed to different forms of passive smoking in their house life. The mean time of common stay of smoking parents with their male adolescent children at home was 12 hours per day (range of 6-18 hours) and 45 hours per weekend (range 20 to 70 hours). In such active smoking families mostly one of the parents (51% of fathers and 35.75% of mothers) were exclusively active smokers. In 25% of smoking families both parents were active smokers. Elementary or secondary general educational background were indicated among 81% of fathers in such smoking families. In non-smoking families 45% of fathers indicated analogous educational background. These data exhibits on statistically significant positive correlation between increasing general educational background of men and their reduced active smoking.

Among women the persistence of continous active smoking was observed in range from 10 to 28 years (mean 16 years). In the group of active smoking mothers women indicating elementary and secondary general educational background prevailed (74%). The 26% of active smoking mothers possessed higher education. Similar results were found in the group of non-smoking mothers, indicating that

degree of general education did not influence the smoking status of women.

It was revealed that 25% of interviewed women smoked 'while pregnant. However, infants born to such active smoking women indicated acceptable mean Apgar points equaling 8.7. Only a single infant was born with low Apgar (4 points). The mean value of inborn body mass of the mentioned group of infants were 3469 grams (range 2450 to 4500 grams). Most of such infants were born in the proper time after mean 38.8 weeks of pregnancy (range 35 to 41 weeks). Only two infants were born before expiration of 37 weeks of pregnancy. Breast feeding was used for 56.8% of infants born to active smoking women. The mean period of breast feeding was 3 months and 1 day (range from 1 week to 8 months).

There was observed any statistically significant difference ($p > 0.05$) between ETS exposed and non-exposed groups of investigated schoolboys as considering mean values of their somatical development indices, *i.e.* mean body height (168.5 cm and 171.7 cm, respectively), mean body mass (54.5 kg and 55.7 kg) and head circumference (55.3 cm and 55.5 cm). An analogous relation was observed between indices characterizing nutritional status of both groups of schoolboys, *e.g.* mean BMI values 19.2 and 18.9, respectively.

Surprisingly, and contrary to published reference data [21, 24-31, 38], no statistically significant difference ($p > 0.05$) was revealed in a general health status between ETS exposed and non-exposed groups of schoolboys. In the first 5-year period of their life the fraction of subjects seeking any kind of infectious respiratory tract diseases was 40% and 31.5% in, respectively, passive smoking and non-passive smoking group of investigated schoolboys. Similary, in both groups comparable numbers of subjects were included in the health care of specialized dispersaries, *i.e.* 40% for exposed and 26% for non-exposed schoolboys on the home passive smoking. However, in the last mentioned fraction of ETS-exposed subjects, a prevalence of lower respiratory tract infections (near 75%) during the year preceding the current study was noticed. No cases of asthma were detected in the whole set of subjects studied. However, in 8% of ETS-exposed and 15.8% ETS non-exposed male adolescent subjects indicated preliminary symptoms of dust disease, indicating that passive smoking was one of the multiple risk factors increasing evidences of such disease. All investigated subjects lived in residences with technically well-equipped sanitary arrangements. Both groups of subjects, *i.e.* ETS-exposed and ETS non-exposed, lived in comparable housekeeping conditions as indicated by mean flat area (54.27 m^2 and 51.44 m^2, respectively) and crowding index (0.74 and 0.64, respectively). However, a statistically significant difference ($p < 0.05$) was detected in a fraction of schoolboys possessing a separate room in their family home, *i.e.* 84% of ETS exposed and 53% of ETS non-exposed subjects. This means that active smoking parents tried to reduce exposure of their male adolescent children to passive smoking by enabling them to use the separate room in the residence.

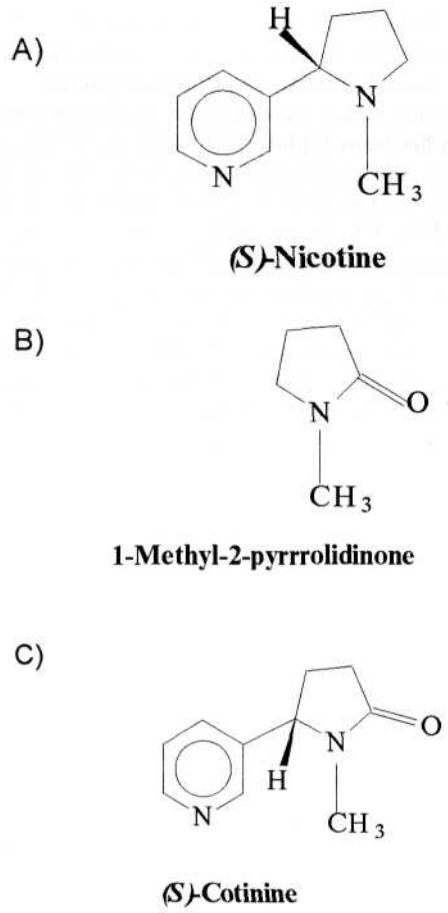
SPE and Chromatography

In human liver nicotine is metabolized to cotinine with an average efficency of 70-80 per cent, but only 10-15 per cent of itself is extracted with urine in an unchanged form [3]. Both compounds can be regarded as moderate basic analytes, respectively, on the inspection of their pK_{a1} values, presented in Table 1, typical for pyridinium ion (see Fig. 1) of their moyeties. However, lack of the carbonyl group in the pyrrolidine ring of nicotine molecules cause increased basicity of tertiary ammonium ion formed in this moiety, which shifts second dissociation constant pK_{a2} to high values

Table 1. Calculated structural parameters of solutes.

No	Parameter	Unit	Nicotine	1-Me-Pyr[a]	Cotinine
1.	Formula	–	$C_{10}H_{14}N_2$	C_5H_9NO	$C_{10}H_{12}N_2O$
2.	Molecular weight	a.m.u.	162.23	99.13	176.21
3.	Molar refractivity	ml	49.25	26.85	49.36
4.	Molar volume	ml	157.1	96.2	153.6
5.	Parachor	ml	394.2	232.1	399.4
6.	Refraction index	–	1.539	1.469	1.555
7.	Surface tension	dyne/ml	39.6	33.7	45.6
8.	Density	g/ml	1.032	1.029	1.146
9.	Polarizability	ml	$19.52 \cdot 10^{-24}$	$10.64 \cdot 10^{-24}$	$19.57 \cdot 10^{-24}$
10.	*logP*	–	0.72	0.84	1.01
11.	pK_{a1}	–	4.23 (6.16[b])	–0.41	4.72
12.	pK_{a2}	–	9.13 (10.96[b])	–	–2.67
13.	Hydration energy	kcl/mol	–1.15	+1.14	–2.07
14.	Approximate surface area	A^2	273.99	263.43	290.63
15.	McGovan volume, $V_x/100$	ml/mol	1.3710	0.8200	1.3867
16.	Excess molar refraction, $R_2/10$	ml/mol	0.938	0.487	1.049
17.	Dipolarity/polarizability, π^H	–	1.63	1.65	2.49
18.	Hydrogen-bond acidity, $\Sigma\alpha^H$	–	0.0	0.0	0.0
19.	Hydrogen-bond basicity, $\Sigma\beta^0$	–	0.83	1.35	0.85

[a] l-Methyl-2-pyrrolidinone (internal standard).
[b] Experimental values in 15°C according to *Merck Index*, 20, 6611, 1996.



Figl. Neutral forms of compounds studied: (a) nicotine, (b) l-methyl-2-pyrrolidinone (internal standard), (c) cotinine.

(9.13 as calculated or 10.96 experimental), characteristic for strong organic bases. In contrast, hydrogen-bond basicity of cotinine and nicotine is comparable as indicated by the aproximate values of $\Sigma\beta^H$ parameter. Cotinine is more hydrophobic in nature than nicotine, but hydrophobicity of l-methyl-2-pyrrolidinone (1-Me-Pyr), proposed as internal standard in developed here SPE and HPTLC procedure, is in the middle range of respective *logP* values of both former compounds. In the pyrrolidine ring of cotinine and 1-Me-Pyr molecules the influence of strong positive mesomeric effect *+M* from tertiary amine group significantly enhancing basicity (nucleophilicity) of oxygen atoms in the carbonyl substituent. However, negative values *of* pK_{a1} and pK_{a2} for 1-Me-Pyr and cotinine, respectively, indicate that such molecules can be regarded as very week Bronsted-Lowry bases. All three analytes considered indicate probably only residual hydrogen-bond acidity as expressed by calculated $\Sigma\alpha^H$ parameters.

The structural parameters mentioned above predominantly influence observed retention of all considered solutes in developed HPTLC systems employing silanized, polymeric octadecylsiloxane silica gel layer and binary aqueous-methanol (20:80, v/v) as mobile phase. As proven by Sykora *et al.* [39], a high methanol concentration in the aqueous mobile phase significantly increased ionization of moderate and strongly basic pyridine-like compounds to cationic forms, enabling their ion-exchange specific interactions with acidic sites existing on the octadecylsiloxane silica gel layer in the form of underivatized residual silanol groups. Such retention behaviour has been recently reported by Bazylak and Aboul-Enein [40, 41] for set of bifunctional, highly polar, basic compounds with diversified hydrophobicity in HPTLC systems employing monomeric as well as polymeric octadecylsiloxane silica stationary phase and aqueous-methanol binary mobile phase. Indeed, according to enlarged ionization providing ion-exchange interactions, observed chromatographic retention of more basic nicotine is higher compared to cotinine (see respective *Rf* and *k'* values in Table 2 and Fig. 2A). However, on the low capacity factor *k'* value of cotinine one can assume that its retention is governed mostly by hydrophobic interactions which prevailing the impact of potential ion-exchange silanophilic interactions. Such result indicate also on rather satisfactorily deactivation of residual silanols on applied chemically modified silica surface enabling sufficient overall efficiency of HPTLC system as revealed by calculated values of H_a, *N'*and R_s parameters summarized in Table 2. Additionaly, some part of pyridinum ions of cotinine moieties have been engaged probably in process of stable ion-pair formation with deprotonated molecules of p-toluenesufonic acid (pK_a = 4.82), added to final SPE extract before HPTLC analysis (see Materials and Methods), which also improved chromatographic retention of this analyte, diminishing undesirable ion-exchange interactions with free silanols.

Table 2. Mean values of chromatographic retention parameters of solutes (*n* = 4) as calculated according to eqs. (2) - (7) (see Materials and Methods for details).

No	Parameter	Unit	Nicotine	1-Me-Pyr[a]	Cotinine
1.	Colour of the spot [b]	–	dark blue	dark blue	dark violet
2.	R_f	–	0.12[c]	0.44[d]	0.92[e]
3.	R_M	–	0.85	0.10	–1.05
4.	k'	–	7.10	1.27	0.09
5.	H_a	μm	354	83	38
6.	N'	–	215	919	1975
7.	R_s	–	–	27.1	23.1
8.	α	–	–	3.6	2.1

[a] l-Methyl-2-pyrrolidinone (internal standard).
[b] after vizualization with ninhidrine - cadmium acetate reagent (see Experimental).
[c] Calculated for zone containing equivalent of 10 ng of nicotine with σ^2 = 3.7 mm, Z_s = 10.5 mm
[d] Calculated for zone containing equivalent of 10 ng of 1-Me-Pyr with σ^2 = 3.1 mm, Z_s = 37.5 mm.
[e] Calculated for zone containing equivalent of 10 ng of cotinine with σ^2 = 3.0 mm, Z_s = 78.0 mm.
The values of Z_m = 85 mm, Z_f = 95 mm, Z_o = 10 mm were taken for all calculations of retention parameters.

A relative increase of hydrogen-bond acidity of hydrocarbonaceous stationary phases in the binary aqueous mobile phase containing high concentrations of methanol has been reported by Abraham and Roses [42] and Poole *et al.* [43]. So, observed high retention of 1-Me-Pyr in an applied HPTLC system can probably be explained by its high value

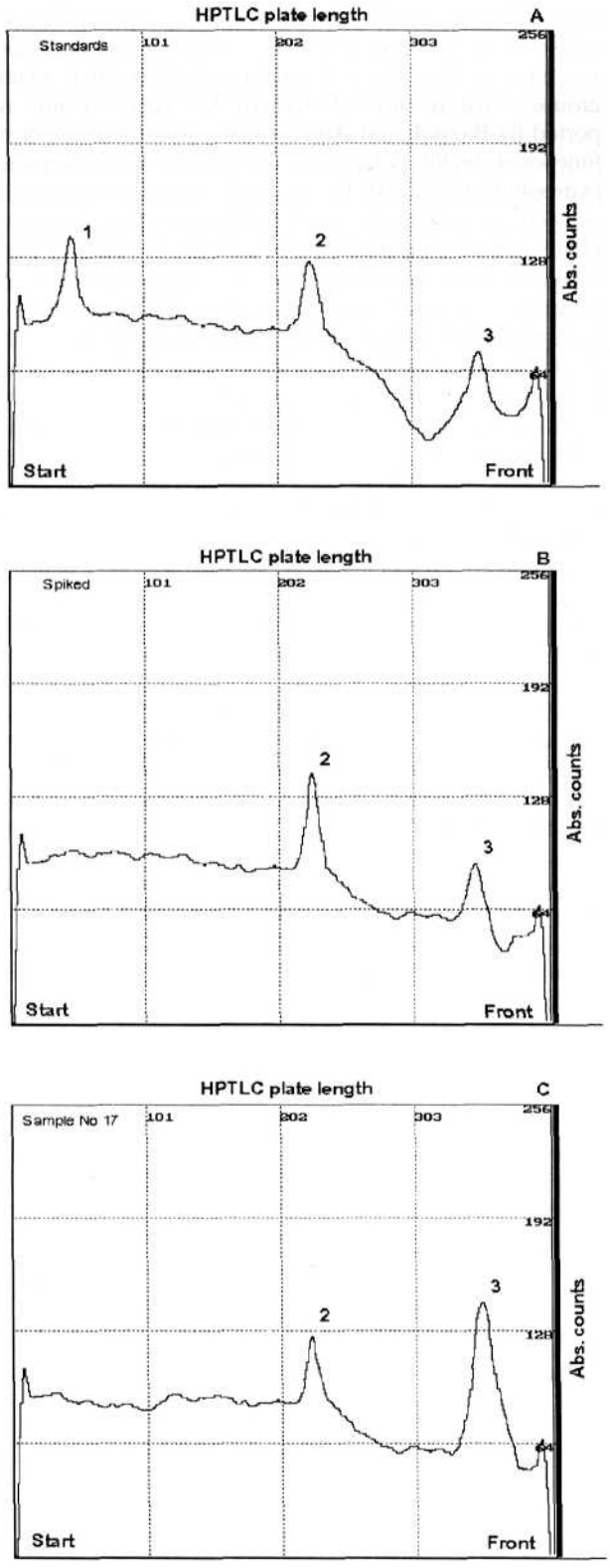


Fig. 2. Densitograms recordered in proposed HPTLC procedure for separations (A) mixture of standard compounds (ca. 10 µg/ 1), (B) SPE extract of spiked blank urine with internal standard l-methyl-2-pyrrolidinone (20 µg/l) and cotinine (20 µg/l) (C) SPE extract of urine sample collected from significantly ETS exposed subject No. 17 (see Table 5). Peaks: (1) nicotine, (2) l-methyl-2-pyrrolidinone (internal standard), (3) cotinine. For detailed description of measurement conditions see *Materials and Methods.*

of hydrogen-bond basicity $\Sigma\beta^H$ (compare Table 1) leading to enhanced hydrogen bonding, in part due to electrostatic interactions, with octadecylsilica stationary phase.

SPE as giving higher recovery rates and better sensitivity of detection has been found to be useful and efficient preconcentration and purification method of diversified biological samples before final analysis with HPTLC or HPLC procedures [44, 45]. In the SPE system proposed here, when the equimolar mixture of aqueous-methanol was used as eluent, the basic pyridine compounds are mostly in unprotonated forms [46], so it can be assumed that the retention process of cotinine and 1-Me-Pyr on the SPE octadecylsilica column is moderated exclusively according to their hydrophobicity. Observed high recovery *(ca.* 95%) of both solutes eluting in the same volume fraction from SPE column can be explained also in part by this effect.

Validation Study

Typical densitogram is shown for urine samples spiked with cotinine (20 µg/l) and internal standard 1-Me-Pyr (Fig. 2B). Cotinine and internal standard were eluted with reproducible *Rf* values of 0.92 and 0.44, respectively, as compare to the chromatogram of standards (see Fig. 2A and Table 2). Reflectance detection of chromatographic zones with an applied office-desk flat scanner satisfactorily combines selectivity with sufficient sensitivity, giving the lower limit of cotinine quantification of 6 µg/l.

Accuracy and precision of the assay were determined on four separate occasions by analysis of spiked blank urine samples taken from eligible non-smoking subjects. The linearity of the proposed method was confirmed in the range of 6-750 µg/l using a 5 ml sample of urine. The correlation coefficient of regression lines was 0.9930.

The mean intra-day accuracy (< 2.9%) and precision (< 6.2) for this assay (see Table 3) were considered satisfactory as in typical range observed for thin-layer chromatographic determinations [17, 36, 40, 41, 44]. Furthermore, the slightly increased mean inter-day (day-to-day) accuracy (< 3.2 %) and precision (< 7.3 %) demonstrated the good reproducibility of proposed procedures (see Table 4). The lower accuracy of urine fortified with 6 µg/l of cotinine, compared to those containing of 50 and 150 µg/l (Tables 3 and 4), is probably due to the linear scanning mode, which causes a low signal-to-noise ratio in the densitomctric analysis of developed HPTLC chromatograms using an office-desk flat scaner coupled with personal microcomputer (see Materials and Methods for details).

SPE extraction mean recoveries was 93% for cotinine and 95% for internal standard from the urine matrix were found, independent of the concentration in the range from 6 to 750 µg/l.

Nevertheless, all manual laboratory operations applied in this assay the reproducibility and robustness of proposed method allow increased sample throughput, *i.e.* a maximum of 12 urine samples can be SPE processed and analyzed on the chromatographic plate in under 60 min. The observed throughput of this procedure can be improved by introducing an automated multisample SPE extraction block combined with specialized TLC-plates application device. Additionally, such instrumental modification can lead to further

Table 3. Intra-day accuracy and precision for the analysis of cotinine in urine.

Concentration (µg/l)		S.D.	Accuracy[a] (%)	Precision[b] (%)	*n*
Prepared	Mean determined				
6	5.4	0.8	–9.9	12.1	4
20	19.4	1.2	–3.0	6.3	4
50	51.1	2.9	2.2	5.6	4
150	149.3	7.5	–0.5	5.0	4
250	253.1	11.4	1.2	4.5	4
500	512.1	21.5	2.4	4.2	4
750	760.3	46.4	1.4	6.1	4
		Mean:	2.9	6.2	

[a] Accuracy = [(mean determined value - prepared value) / (prepared value)] x 100.

[b] Precision = (S.D. x 100) / mean determined.

Table 4. Inter-day accuracy and precision for the analysis of cotinine in urine.

Concentration (µg/l)		S.D.	Accuracy[a] (%)	Precision[b] (%)	*n*
Prepared	Mean determined				
6	5.4	0.9	–9.9	13.0	4
20	19.3	1.6	–3.3	8.1	4
50	51.2	3.1	2.5	6.1	4
150	148.6	8.6	–0.9	5.8	4
250	253.5	13.4	1.5	5.3	4
500	512.7	30.2	2.5	5.9	4
750	736.1	53.4	1.8	7.0	4
		Mean:	3.2	7.3	4

reduction of cost of consumables involved in the assay of cotinine in urine proposed here.

Human Study

According to the extended epidemiological data recently summarized by Benowitz [3] the dietary nicotine intake from different vegetables and tea consumptions leads to cotinine concentrations in human urine not exceeding 0.6-1.5 µg/l. Typical values of cotinine in the urine of a person incidentally exposed to ETS was found in range 1.5-6 µg/l [3]. The cotinine range 6-50 µg/l and 50-85 µg/l in the human urine can be used as the cut-off values for moderate and significant ETS exposure, respectively [3, 10].

In compliance with the self-reported questionnaire data, study subjects consisted of 19 exposed and 16 non-exposed to home ETS schoolboys. In Fig. 2C the representative densitogram of SPE extract of cotinine from urine sample collected from significantly ETS-exposed subject No. 17 is presented. The mean measured concentrations of cotinine in the collected urine samples are summarized in Table 5. These data report only to the achieved experimental results which exceed the limit of detection (6 µg/l) of performed cotinine determination with a proposed SPE-HPTLC procedure. The self-reported exposure level of subjects was also indicated in Table 5. In the group of home ETS exposed 19 schoolboys a recall bias of 42% was exhibited by exact SPE-HPTLC quantitation of cotinine in their uirine samples. For the 11 schoolboys from this group of subjects their interview-reported home ETS exposition was strictly confirmed with applied chromatographic analyses. The recall bias determined here was comparable with the range of 30-70% as reported previously by Sampson *et al.* [10] for the large cohort studies mantained in three states of the USA. However, the recall bias observed in our studies can also be explained in part by the variability of cotinine clearence by each investigated subject. Cotinine, as biomarker of nicotine absorption from ETS, indicates a half-life averaging 17 hours [3]. This means that at least 48 to 77 hours are needed to reasonably collect all the cotinine after the incidence of ETS exposure by a particular subject [3]. In pilot studies presented here such average times of urine collection do not exceed 12 hours. Thus, in some instances this time was not adequate for the complete and reliable metabolic transformation of the all inhaled nicotine to cotinine by various male adolescent subjects. In the future, extended studies on the passive smoking hazard of male adolescents this fact requires precise synchronization of urine collection moment with the variability of cotinine clearence indicated by individual subjects.

Sampson *et al.* [10] has suggested a cotinine cut-off at 85 µg/l for urine to assign passive (or ETS-exposed humans) and active smokers. As it is seen from Table 5 the high cotinine concentrations determined for the ETS-exposed male adolescent subjects No. 1, 2,13,14,17 enable classifying them as persons which probably initiated active smoking. This assignment in all five instances match perfectly with the interview-reported extremly high home ETS exposure caused by both strongly active smoking parents consuming more than 40 cigarettes per day, the lack of a separate chidren's room, reduced flat area, home overcrowding and low general education background of both parents. In the described sub-group of subjects highly increased rate of lower respiratory tract infections were also observed in one-year-period of studies reported here. This conclusion, supporting recent suggestions and conclusions of Dell'Orco *et al.* [47], has been formulated in view of their studies on children and adolescents passive smoking.

The average concentration of urine cotinine in a whole group of the ETS exposed male adolescents subjects was determined as 71 µg/l. This result supports classification of the group of mentioned subjects as depending on the significant level of home ETS exposure. This result also suggests more care with detailed verification of questionnaire collected data which relate to the smoking habits in families of investigated schoolboys.

In the set of 16 male adolescent subjects which were not-exposed to home ETS the recall bias was 18%. Three subjects in this group, *i.e.* numbered as 20, 33, 35 in Table 5, indicated moderate level of ETS exposure as was shown by determined cotinine mean concentrations, 16, 24 and 47 µg/l, respectively. This phenomenon can probably be explained by the incidental contact of mentioned subjects with any kind of non-family-related active smokers and suggest that such type of lifestyle opportunities should be included in the interview questionnaire as one of the exclusion cri-

Table 5. Cotinine concentrations from urine samples (n = 4) of home ETS exposed and non-exposed male adolescents as determined by developed SPE-HPTLC procedure.

Subject No.	Self-reported status from interviews data	Exposure level from interviews data	Cotinine[a] (mean +/– SD) (µg/l)
1.	Exposed	Both parents smoked[b]	233.0 ± 9.9
2.	Exposed	2 or more smokers in home[b]	402.0 ± 14.1
11.	Exposed	Mother smoked[c]	31.1 ± 1.7
12.	Exposed	Mother smoked[c]	35.2 ± 1.9
13.	Exposed	Both parents smoked[b]	96.5 ± 4.7
14.	Exposed	Both parents smoked[b]	146.0 ± 6.5
15.	Exposed	Father smoked[d]	56.7 ± 2.9
17.	Exposed	Both parents smoked[b]	260.0 ± 10.8
19.	Exposed	Mother smoked[c]	34.3 ± 1.9
20.	Not-exposed	No smokers in home	16.9 ± 1.1
29.	Exposed	Mother smoked[c]	24.7 ± 1.4
30.	Exposed	Mother smoked[c]	18.8 ± 1.2
33.	Not-exposed	No smokers in home	24.4 ± 1.4
35.	Not-exposed	No smokers in home	47.4 ± 2.5

[a] Calculated ace. to eq. (1) in *Materials and Methods.*
The limit of detection of the proposed SPE-HPTLC method is 6 µg/l
[b] more than 40 cigarettes per day
[c] range of 5-10 cigarettes per day
[d] range of 20-40 cigarettes per day

teria used in the future design of epidemiological study on the passive smoking hazard in male adolescents.

Conclusions and Suggestions

An accurate and precise analytical method for determining of cotinine in human urine has been developed. The achieved selectivity and sensitivity of this method allows low-cost, quick and valuable screening analysis of moderate and significant levels of home ETS exposure to male adolescents children, as well as the active smoking status of adults. This procedure can be useful for independent verification and/or confirmation of questionnaire data gained during interviews of smokers. However, in view of sensitivity, the proposed method is not suitable for adequate detection of humans dietary and/or incidental home ETS exposure of humans.

The results of presented studies indicates that for reliable assessment of home ETS exposure of male adolescents children the proper time and period of collection of their urine samples should be carefully selected. Compare to previously published data [21, 24-31, 38, 47-49] the results reported here show that some groups of male adolescents in Poland may have an elevated risk on the not acceptable significant (or even high) level of home ETS exposure.

The results of presented pilot studies indicate on the rather strong recall bias of self-reported smoking habits which should enhance the routine application of developed SPE-HPTC method for the more objective verification of questionable questionnaire data in smokers' families. The results of presented pilot studies could be useful for the reliable evaluation of tobacco smoke pollution prevention and control among diversified adolescents groups, especially from elementary and secondary schools.

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