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HPTLC screening assay for urinary cotinine as biomarker of environmental tobacco smoke exposure among male adolescents☆

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Abstract

For selective screening determination of urinary cotinine, i.e. (*S*)-1-methyl-5-(3-pyridyl)-2-pyrrolidinone, the major metabolite of nicotine, the high-performance thin-layer chromatographic (HPTLC) method have been proposed. Prior the final HPTLC analysis the procedure required a solid-phase extraction (SPE) of cotinine from collected urine samples with 1-methyl-2-pyrrolidinone as an internal standard. Densitometrical quantitation of cotinine on the chromatograms have been performed with a 16-grayscale scanner using the specialized software implemented on a desktop microcomputer. The lower detection limit of cotinine was 6 μg/l allowing the method to be applied for the measurement a concentration of this compound in urine samples collected from 35 elementary schoolboys exposed on both moderate and/or significant ETS. The mean recovery of cotinine from urine samples was 93 %. The mean intra-day accuracy for the analysis of cotinine in range 6–750 μg/l, including four paralell measurements, was 2.9 %. The results of cotinine measurements by proposed SPE–HPTLC procedure were used in the pilot studies for assessment of hazard from home ETS on the health status of elementary schoolboys, especially an increased risk for infectious respiratory track diseases. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cotinine; Passive smoking; Solid-phase extraction; HPTLC; Human urine

1. Introduction

Cotinine i.e. (*S*)(-)-1-methyl-5-(3-pyridyl)-2-pyrrolidinone, with molecular structure presented in Fig. 1, is the primary human metabolite of nicotine and by possessing the long elimination half-time from the body can be reliably determined in hair or different biological fluids up to

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several days after a person's exposure to tobacco smoke [1]. Serum, plasma, 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 biomarker have been validated by reported mutual relationships between diversified biological effects of environmental tobacco smoke (ETS) and quantitated cotinine concentration in specified biological fluids [2]. However, recently Nakajima et al. [3] reported that homozygous whole deletion allele of the CγP2A6 gene in humans leads to deficient cotinine formation from nicotine.

Many very sensitive and mostly entirely specific methods have been published for cotinine determination in urine, blood or seminal plasma, serum, saliva and extraembryonic, cervicular 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 ultraviolet detection [9], HPLC hyphenated with atmospheric pressure chemical ionization tandem mass spectrometry (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].

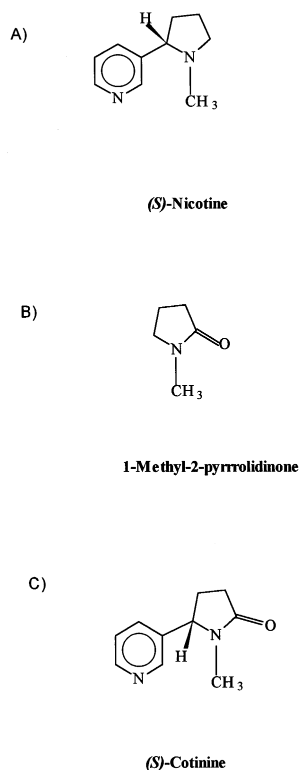

Fig. 1. Neutral forms of compounds studied: (a) nicotine, (b) 1-methyl-2-pyrrolidinone (internal standard), (c) cotinine.

Thin-layer chromatography (TLC) employing 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 Berry 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 TLC procedure hyphenated with scintilation counting in pharmacokinetic studies for determination of 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.

For adolescents aged 13 years prevalence of cigarette smoking varies widely in different countries, ranging from 2 to 5% (Sweden, USA) to more than 30% (Australia, Uruguay) [18]. Presently, in European Community countries prevalence of smokers among male adolescents is decreasing but it is increasing among girls, however, in developing countries male adolescent smokers still reach 40% [18]. Detailed studies in Poland revealed [19] that among adolescents, aged 11–15 years, 18% of boys and 8% of girls reported daily smoking in 1994. In total Polish population the prevalence of tabacco smoking was 47% among men and 23% in woman, reaching more than 30% in pregnant woman, in 1995. The increasing proportion of smokers beginning to smoke before age 15 is also observed presently in Poland [19].

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 develop-

ment of premature coronary heart disease [20], increased lower respiratory tract infections [21], middle ear effusions [22], decreased lung function [23], lower rates of lung growth [24] and more frequent exacerbation of asthma [25]. The associations between level of ETS exposure to adolescents, measured as urinary cotinine excrection, and dwelling conditions [26] as well as enabling, predisposing and reinforcing factors has been recently described [27]. Furthermore, significant correlations were observed between the concentration of cotinine and benzene metabolites in urine indicating extended risk of leukemia and lymphomas in humans exposed to ETS [28].

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

2. Experimental

2.1. Subjects data

This studies were performed with approvement of local ethical commision for medical research. Subjects for the study consisted of 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 level of ETS exposure over the past 1 year period confirmed by self-reported smoking status in family interview. In addition to obtaining data on a lifetime histories of tobacco use for both subjects and their family members, informations 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, the past and actual childhood diseases, especially an infectious respiratory track diseases. Study informations were collected by structured interview with specialized questionnaire administered by trained personel. 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 meaningful at significance level $P < 0.05$.

2.2. Collection of urine samples

The urine samples (5 ml as minimal volume) in fasting subjects were collected in a previously delivered, pasteurized glass urine collection containers. The respective urine samples were collected in each subject home after week-end time at every Monday morning, within the month period ($n = 4$). The fresh urine samples were taken in time between subject wake-up and before his home leaving to the school practice. The collected urine samples were moved in the same morning as quick as possible from the subject home to the laboratory. All samples were stored until analysis at -20°C after addition of 50 μl of 6 M hydrochloric acid to preserve microbial growth.

2.3. 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). Standard solution of cotinine and nicotine were prepared by dissolving 10 mg each of them in 10 ml of 0.012 M aqueous solution of hydrochloric acid and stored at 4°C until use. Internal standard solution was prepared by dissolving of 400 μl of glass distilled, anhydrous 1-methyl-2-pyrrolidinone ($d = 1.033$ g/ml) in 4 ml of 1% (v/v) methanolic solution of concentrated hydrochloric acid ($d = 1.410$ g/ml) and rinsing up to volume of 10 ml with 0.2 M aqueous solution of hydrochloric acid.

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

2,2′-Dihydroxyindan-1,3-dione (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 visualizing reagent.

2.4. SPE procedure

Solid-phase extraction (SPE) disposable polypropylene columns of Bakerbond-spe series (maximal probe volume 6 ml) prepacked with 500 mg of high capacity reversed-phase octadecylsilica (40 μm, 60 Å, 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. The portable vacuum pump IP 20 (Veb Reglerwerk, Dresden, Germany) was applied during developed SPE procedure. All SPE experiments were made at controlled room (21 ± 0.5°C) temperature.

In SPE of urinary cotinine the method proposed by Jacob III et al. [6] were used with some modifications. The 100 μl of 1-Me-Pyr internal standard solution was added to the each collected 5 ml urine samples in 10 × 1.5 cm glass tubes. Next, the 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 bench top 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 have been 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 sample, containing previously added internal standard, were aspired on the top of wetted packing bed of SPE column. In sample application step (ii) of SPE for the next 5 min the urine samples were enabled to flow freely through the packing bed of SPE columns. After this time a vacuum 3 mmHg was applied to the Baker-12 SPE processing system until the 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 min applying vacuum 3 mmHg. The vacuum was turned off and columns were air dryied for 10 min. The collected eluates were also discarded. Finally, in selective elution step (iv) of SPE the columns were washed with two successive 0.5 ml aliquots of methanol for 2 min using vacuum 2 mmHg. To these collected eluates, containing urinary cotinine, 100 μl of 10% methanolic solution of *p*-toluenesulfonic acid were added using adjustable pipettor. From this final SPE extracts the 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 laboratory refrigerator at 4°C.

2.5. HPTLC analysis

HPTLC experiments were performed on 10 × 10 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 (Düren, 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 were left to stand for 1 h at room (21 ± 0.5°C) temperature.

The volume of 1 μl of each final urinary cotinine SPE extracts were spotted individually on the sample application position of HPTLC plate (10 mm from the lower edge) with a type 701 Hamilton (Reno, CA, USA) 10 μl microsyringe. Similary, on the same HPTLC plate, the 1 μl of

standard solutions of cotinine and 1-methyl-2-pirrolidinone (see Section 2.3 of experimental part) were spotted on the particular starting points to support identification of both substances after a plate development. The plates were developed vertically in normal all-glass chambers (20 × 20 × 10 cm, Glassverke, Ilmenau, Germany), without prior saturation, on the migration distance (Z_m) 8.5 cm.

Developed zones of cotinine and 1-methyl-2-pirrolidinone were visualized by spraying the HPTLC plates with freshly prepared derivatizing solution proposed by Dévényi [29] containing 0.2 g of 2,2'-dihydroxyindan-1,3-dione, 0.5 g of cadmium acetate monohydrate dissolved in 2 ml of glacial acetic acid and filled up to 100 ml with methanol. Subsequently the HPTLC plates were heated at 80°C for 60 min.

2.6. Densitometry

The chromatograms on the HPTLC plates were densitometrically evaluated with the 16 grayscale (4-bit) scanner ScanJet 3p (Hewlett-Packard, Warsaw, Poland) connected to IBM compatible Pentium MMX 166 MHz desktop microcomputer and working under Quantiscan v.2.0 (Biosoft, Cambridge, England, UK) specialized software [30] 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 analysed after automatic medium smoothing to provides 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.

2.7. Calculations

Cotinine concentration C_k (μg/l) in analysed urine samples was calculated using following formula:

$$C_k = [C_s * V_{\mathrm{spe}} * 10^{(\mathrm{LA}_k - \mathrm{LA}_s)}]/[V_{\mathrm{ex}} * V_{\mathrm{ur}}] \qquad (1)$$

where C_s (μg) is mass of internal standard (1-methyl-2-pyrrolidinone) added to urine sample before applying of SPE procedure, V_{spe} (l) is total volume of final SPE extract of cotinine fraction, LA_k is logarithm of integrated area of densitometric peak referred to cotinine zone on the chromatogram of final SPE extract, LA_s is logarithm of integrated area of densitometric peak referred to internal standard zone on the chromatogram of final SPE extract, V_{ex} (l) is total volume of final SPE extract deposited on the sample application position of HPTLC plate, V_{ur} (l) is total volume of urine sample extracted in SPE procedure.

The retention parameters of solutes as its retardation factors R_f on HPTLC plates were calculated from quadrupole measurements of experimental values:

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

where Z_s is migration distance (mm) of the centre of developed solute zone from the sample application position on the HPTLC plate, Z_m is the migration distance (mm) of the mobile phase front from the sample application position to mobile phase front.

All statistical treatment of experimental data were performed with Statistica 4.3 (StatSoft, Inc., Tulsa, OK, USA) software implemented on an IBM-compatible Pentium MMX 166 MHz desktop microcomputer.

3. Results and discussion

3.1. Subjects questionnaire data

Inspection of data collected in the interview questionnaires revealed that 59% of 35 investigated schoolboys were exposed on the 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 h per day (range of 6–18 h) and 45 h per week-end (range 20–70 h). In such active smoking families mostly one of the parents (51% of fathers and 35.75% of mothers) were exclusively active

smoker. In 25% of smoking families the both parents were active smokers.

In the first five-year period of their life the fraction of subjects seeking on the any kind of infectious respiratory track diseases was 40 and 31.5% in, respectively, passive smoking and non-passive smoking group of investigated schoolboys. Similary, in both groups comparable amounts of subjects were included in the health care of specialized dispensaries, 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, the prevalence of lower respiratory track infections (near 75%) during the year preceding of reported here studies were noticed. None 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 probably only one of multiple risk factors increasing evidences of such disease.

3.2. SPE and chromatography

Solid phase extraction (SPE) as giving higher recovery rates and better sensitivity of detection has been found as useful and efficient preconcentration and purification method of diversified biological samples before final analysis with HPTLC or HPLC procedure [31,32]. In human liver nicotine is metabolized to cotinine with an average efficiency of 70–80 %, but only 10–15 % of itself is excreted with urine in an unchanged form [2]. For reliable and quick isolation and preconcentration of cotinine from multicomponent urine probes — without interference and contamination — the SPE distinct procedures, employing mainly cartridges with octadecylsilica [33] or large pore diatomaceous earth [34] packing, as well as method supporting preliminary liquid–liquid extraction [5] were developed. Especially, the use of SPE procedure eliminated caffeine interference on reproducible determination and quantitation of urinary cotinine [35].

To reduce substantial bias and imprecision 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 parent analyte. Until now a variety of such internal standard substances has been proposed, including deuterocotinine [11], amphetamine sulfate [10], pheniramine maleate [36], diphenylamine [10], desmethylimipramine [37], ketamine [8], quinoline [37], lidocaine [38], methylanabasine [10], 5-methylcotinine [10], *N*-ethylnorcotinine [5,9], methylprylone [10], norephedrine [39] and 2-phenylmidazole [5,40]. However, none of them was approved as universal internal standard in spite of analytical procedure chosen, which presumably explain in part the significant differences in urinary cotinine concentrations reported in various published studies on ETS exposure [3,10]. Inappriopriate choice of internal standard for chromatographic assay of urinary cotinine can lead to the unreliable analytical data as some of mentioned substances exhibits a limited availability, a reduced chemical stability during storage of urinary cotinine extracts, an unsufficient selectivity of chromatographic separation related to cotinine peak or lack of reproducibility in extraction efficiency for a broad range of cotinine concentration in urine of passive and active tobacco smokers [10,38].

In SPE system proposed here, when the equimolar mixture of aqueous-methanol was used as eluent, the basic pyridine compouds as tobacco alkaloids are mostly in the unprotonated forms [41]. So, one can assume that retention process of cotinine and 1-Me-Pyr, as internal standard proposed here, on the SPE octadecylsilica column is moderated exclusively according to hydrophobicity of such solutes (logarithm of partition coefficient in *n*-octanol–water system log P equal 1.01 and 0.84, respectively). 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.

In proposed here SPE procedure the methanolic solution of *p*-toluenesulfonic acid was added to the final cotinine urine SPE extracts to convert cotinine to the non-volatile *p*-toluenesulfonic acid cotinine salt. This salt is formed between anionic form of easy deprotonated *p*-toluenesulfonic acid and basic pyridinium ion of cotinine moiety [42].

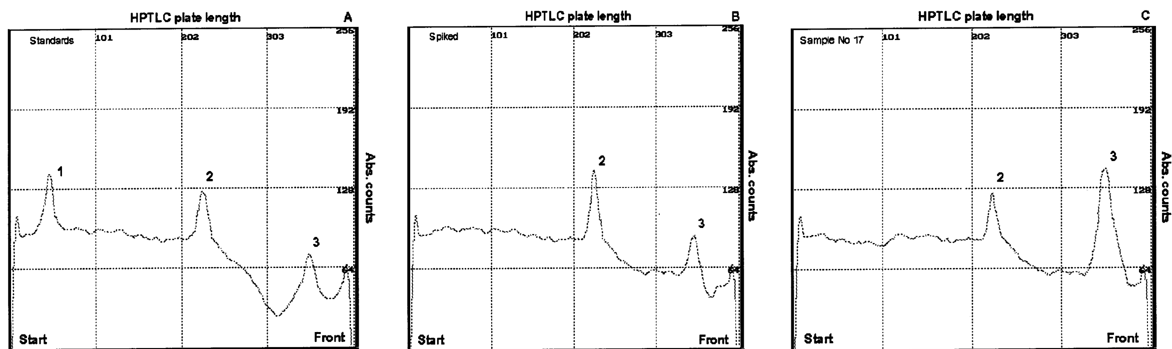

Fig. 2. Densitogram recordered in proposed HPTLC procedure for separations (A) mixture of standard compounds (ca. 10 μg/l), (B) SPE extract of spiked blank urine with internal standard 1-methyl-2-pyrrolidinone (20 μg/l) and cotinine (20 μg/l), (C) SPE extract of urine sample collected from significantly ETS exposed subject No. 17 (see Table 3). Peaks: (1) nicotine, (2) 1-methyl-2-pyrrolidinone (internal standard), (3) cotinine. For detailed description of measurement conditions see Sections 2.5 and 2.6.

Moreover, other structurally related tobacco alkaloids as norcotinine, nicotine, nornicotine, anatabine, neonicotine [2,6], accidentally co-extracted in trace amounts from urine samples with cotinine fraction during of SPE process, can be converted to analogous non-volatile salts. Additionally, spotting of mentioned *p*-toluenesulfonic acid alkaloide salts on the HPTLC plate can enhance the final chromatographic separation of nicotine and cotinine zones and improving sensitivity and specifity of the proposed method. Compare to cotinine increased stability of *p*-toluenesulfonic acid nicotine salt caused stronger retardation of this solute on deposition point and longer retention of nicotine zone. Reaction of *p*-toluenesulfonyl acid with norcotinine, nornicotine, anatabine and neonicotine, as possessing secondary amine group in pyrrolidine or piperidine ring of their moieties, yields in situ the *bis*-derivative, i.e. salt and tosylate [43,44]. In such way, in proposed here HPTLC conditions, co-elution of nornicotine, anatabine and neonicotine from the starting point on the chromatographic plate with cotinine zone can be prevented enabling further unambigously identification and sensitive detection of cotinine.

3.3. Validation study

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

Accuracy and precision of the assay were determined on the four separate occasions by analysis of spiked blank urine samples taken from eligible non-smoking subject. 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 1) were considered satisfactory as in typical range observed for thin-layer chromatographic determinations [17,31]. Furthermore, the slightly increased mean inter-day (day-to-day) accuracy ($<3.2\%$) and precision ($<7.3\%$) demonstrated the good reproducibility of proposed procedure (see Table 2). The lower accuracy of the urine fortified with 6 μg/l of cotinine, compared to those containing of 50 and 150 μg/l (Tables 1 and 2), is probably due to the linear scanning mode which cause a low signal-to-noise ratio in the densitometric analysis of developed HPTLC chromatograms using

office-desk flat scanner coupled with personal microcomputer (see Experimental 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 the all manual laboratory operations applied in this assay the reproducibility and robustness of proposed method allow increased sample throughput, i.e. maximum of 12 urine samples can be SPE processed and analysed on the chromatographic plate in under 60 min. The observed throughput of this procedure can be improved by introducing automated multisample SPE extraction block [45] combined with specialized TLC-plates application device. Additionally, such instrumental modification can lead to further reduction of cost of consumables involved in the proposed here assay of cotinine in urine.

3.4. Human study

According to the extended epidemiological data recently summarized by Benowitz [2] the dietary nicotine intake from different vegetables and tea consumption leads to cotinine concentration in human urine not exceed a range 0.6–1.5 μg/l. Typical value of cotinine in the urine of a person incidentally exposed to ETS was found in range 1.5–6 μg/l [2]. 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 [2,10].

Table 1
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)) × 100.
[b] Precision = (S.D. × 1000)/mean determined.

Table 2
Inter-day accuracy and precision for the analysis of cotinine in urine

Concentration (μg/l)		S.D.	Accuracy (%)	Precision (%)	*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	763.1	53.4	1.8	7.0	4
		Mean:	3.2	7.3	

Table 3
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	Two 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.	Non-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.	Non-exposed	No smokers in home	24.4 ± 1.4
35.	Non-exposed	No smokers in home	47.4 ± 2.5

[a] Calculated acc. to Eq. (1) from Experimental. 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.

In compliance with the self-reported questionnaire data the study cohort of considered 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 self-reported ETS exposure level of subjects and mean measured concentrations of cotinine in the collected urine samples are summarized in Table 3. These data reports only to the achieved experimental results which exceed the limit of detection (6 μg/l) of performed cotinine determination with proposed SPE–HPTLC procedure. The lowest mean concentration of cotinine as 16.9 μg/l were determined in urine samples collected from subject No. 20. This means that none of collected set of urine samples contained cotinine in range from 6 to 16.9 μg/l. Such result suggest that urine collection time implemented in our pilot studies was too short (see Experimental) and should be extended on the all day.

In group of home ETS exposed 19 schoolboys the recall bias of 42% was exhibited by exact SPE–HPTLC quantitation of cotinine in their urine 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 USA.

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 3 the high cotinine concentrations determined for the ETS exposed male adolescent subjects No. 1, 2, 13, 14, 17 enable to classifying them as the 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 separate children room, the reduced flat area, the home overcrowding and low general education background of both parents. In described sub-group of subjects the highly increased rate of lower respiratory tract infections were also

observed in time of the one year period before of studies reported here. This conclusions supports recent suggestions of Dell 'Orco et al. [46] formulated in view of results of their studies on children and adolescents passive smoking.

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

In set of 16 male adolescent subjects which were not-exposed to the home ETS the recall bias was 18%. Three subjects in this group, i.e. numbered as 20, 33, 35 in Table 3, indicated the moderate level of ETS exposure as was shown by determined cotinine mean concentrations 16.9, 24.7 and 47.4 μg/l, respectively. This phenomenon can be probably explained by the incidental contact of mentioned subjects with any kind of not-family-related active smokers. These results suggest that such type of incidental lifestyle opportunities should be included in the interview questionnaire as the one of exclusion criteria used in the future design of epidemiological study on the passive smoking hazard in a male adolescents.

4. Conclusions

Accurate and precise analytical method for determination of cotinine in human urine has been developed. The achieved selectivity and sensitivity of this method enable relative low-cost, quick and valuable screening analysis of the moderate and significant level of home ETS exposure to male adolescent 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 adolescent children the proper time and period of collection of their urine samples should be carefully selected. Compare to the previously published data [18,20–27,40,46–48] the results reported here exhibits that some groups of male adolescents in Poland may have 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 to the routine application of developed SPE–HPTC method for the more objective verification of frequently doubtful truthfulness of intreview collected 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 adolescent groups especially from the elementary and secondary schools.

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