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Short communication

The enzyme-linked immunosorbent assay (ELISA) method for nicotine metabolites determination in biological fluids

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ABSTRACT

The aim of this study was to present the usefulness of enzyme-linked immunosorbent assay (ELISA) in nicotine metabolites determination in urine and serum samples from active smokers and compare it with the reference to thin layer chromatography (TLC) with densitometry.

The specific anti-cotinine antibodies were obtained from rabbit sera after sequential immunization with 4′-carboxycotinine–hemocyanine conjugate. The immunoaffinity chromatography technique with the use of self-prepared cotinine-aninohexyl-sepharose bed enabled the isolation of the specific anti-nicotine metabolites antibodies from the antiserum. Affinity of isolated antibodies to cotinine was passively immobilised on ELISA plates and competition between nicotine metabolites in samples and tracer (horseradish peroxidase–cotinine conjugate) was applied. After the washing stage the enzymatic activity of solid-phase-bound peroxidase was determined. For calibration cotinine perchlorate solutions in appropriate matrix were used. Determination ranges for serum and urine samples were from 3 to 1500 and from 3 to 5000 ng/mL, respectively. Precision within-run and between-run was below 8.7 and 11.3%; mean recovery of cotinine was 100.59% from serum and 88.56% from urine samples.

The ELISA method, used in determination of the main nicotine metabolites showed high accuracy and sensitivity. However, this method was less specific than the reference technique (TLC). The high correlation coefficients, $r > 0.9$, between the results of determined nicotine metabolites in urine by means of ELISA and TLC with densitometry confirmed the possibility of the application of ELISA method to practical monitoring of tobacco smoke exposure in large population groups.

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1. Introduction

There are many biomarkers applied to the evaluation of tobacco smoke exposure.

The determination of main nicotine metabolites, cotinine and *trans*-3′ hydroxycotinine, is considered the most specific one [1–3].

The analytical techniques most often used for the nicotine and its metabolites determination are chromatographic (GC, HPLC, TLC) and immunochemical methods. The chromatography methods enable the determination of particular metabolites but they require time-consuming biological sample preparation [4,5–8].

Whereas immunochemical methods allow faster and less laborious evaluation of the nicotine exposure based on the sum of nicotine metabolites presented in investigated samples.

The estimation of exposure using immunochemical methods is analysed by cross-reactivity of nicotine metabolites with applied antibodies specific for this method [9]. So far methods using monoclonal as well as polyclonal antibodies and isotopic (RIA), enzymatic (ELISA) or fluorescent (FPIA) tracers have been developed [4]. In most cases these methods are based on the competition of the antigen (nicotine derivative) present in the sample/standard with the labelled antigen for the constant number of binding sites on the specific antibody.

The aim of the study was the elaboration of the immunoenzymatic determination of nicotine metabolites in body fluids with the application of rabbit polyclonal antibodies against cotinine and the evaluation of sensitivity, specificity and precision of the applied method.

2. Experimental

2.1. Obtaining the specific anti-cotinine antibodies

Specific anti-nicotine metabolites antibodies were raised in New Zealand albino rabbits (6-months old males with the initial body weight 3000 ± 500 g). Immunization was carried out with the use of 4′-carboxycotinine (Aldrich, USA), Limulus polyphemus hemocyanin (Sigma, USA) conjugate as described in Langone et al. [4]. At 7–10 days after each immunization blood samples were collected from the ear vein and antibodies' concentration was measured.

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2.2. The evaluation of anti-cotinine antibodies concentration

Antibodies to cotinine were measured by enzyme-linked immunosorbent assay (ELISA) method using 4′-carboxycotinine - ε-poly-L-lysine (conjugation described in Ref. [4]) as antigen and secondly, peroxidase conjugated anti-rabbit immunoglobulin antibodies. ELISA plates were coated with antigen (5 mg/L, 12 h). Next the plates were washed in phosphate buffered saline with 0.05% Tween 20 (PBST). For blocking of uncoated sites and dilution of sera and second antibody TBSTG buffer (0.1 M TRIS/HCl, 0.15 M NaCl, 0.1% gelatin, 0.1% Tween 20, 0.02% Tiomerosal, 0.2% Proclin 300, pH 7.4) was used. Diluted rabbit antisera were incubated for 1 h and after washing in PBST (four times), diluted the second antibody was dispensed (1 h, room temperature). After the washing stage the chromogen solution (*o*-phenylenediamine dihydrochloride 1 mg/mL in 0.1 mol/L phosphate–citrate buffer pH 4.8 with 0.015% hydrogen peroxide) was pipetted. After termination of reaction by 2 mol/L sulphuric acid solution the absorbance at 492 nm (reference wavelength 630 nm) was read using a PowerVawe XS plate reader (BioTek, USA). For publication purposes the antibody concentration was expressed in mg/mL of antiserum and calibration was prepared with the use of immunochromatography purified antibody as described below.

2.3. Isolation of specific anti-cotinine antibodies from antisera

The isolation of the anti-cotinine antibodies from antisera was carried out by immunoaffinity chromatography on carboxycotinine-aminohexyl-sepharose support. The synthesis of the stationary phase included the activation of Sepharose 4B with bromocyan [10] and 1,6-diaminohexane coupling. The resulting AH-Sepharose was covalently bonded with 4′-carboxycotinine (using EDCA). Binding of the antibodies from antisera obtained after 5 and subsequent immunizations was done by a constant mixing of 5 mL antiserum, 10 mL packed support and 30 mL PBST buffer for over 4 h. After sequentially washing with PBST and PBS (absorbance at 280 nm in effluent below 0.02) absorbed antibodies were eluted with 0.5 mol/L acetic acid and after neutralization with solid TRIS base dialyzed to 0.1 mol/L $NaHCO_3$ (three times). After addition of 0.1% sodium azide, antibody solution was stored in a refrigerator (or after addition of 50% glycerol, in a freezer). The IgG concentration was determined at 280 nm (E1% = 12.5). Antibody solution was stable for at least 2 years at 4 °C.

2.4. Synthesis of the tracer—horseradish peroxidase–cotinine conjugate (HRP–cotinine)

Cotinine, which was applied as a tracer in the ELISA method, was labelled with horseradish peroxidase (Fluka). The coupling was performed with the use of the Erlanger method where cotinine carboxy-derivative was applied as haptene and isobutyl chloroformate as a coupling agent [11]. Activation was performed by dissolving 10 μmol cotinine–4′-carboxylic acid and 12,6 μl *N*-ethylmorpholine in 275 μl dimethylformamide, cooling in ice bath and adding 13 μl isobutyl chloroformate. After 5 min 15, 37.5 and 75 μl portions of activated mixture were added to solutions of 10 mg HRP in 0.5 mL water with 2 μl *N*-ethylmorpholine. 4′-Carboxycotinine to enzyme ratio in coupling stage were 2:1; 5:1 and 10:1, respectively for three conjugate preparations. The conjugates, after the cleaning step on the Sephadex G-50 column, were mixed with an equal volume of glycerol, then 1% bovine serum albumin, 0.5% cytochrome *c* and 0.3% Proclin were added. The obtained solutions were kept in the freezer (−20 °C).

Optimization of conjugate dilution for ELISA method was done by analyzing their binding properties to anti-cotinine antibody-coated ELISA plates. The conjugates were diluted in TBSTG buffer from 100 to 10,000 times (tracer concentrations 50–5000 ng/mL) and incubated on plates at night. The next steps (washing, HRP activity assay, absorbance measurements) were done as described for the ELISA method. For construction of ELISA method conjugate dilution that gave about 70% of maximal signal was selected.

2.5. Optimization of antibody coating on ELISA plates and conjungate concentration

ELISA microtiter plates were coated with immunoaffinity chromatography isolated antibodies (0.5–10 mg specific IgG/L 50 mmol/L carbonate buffer (pH 9.6). The plates were kept in the TBSTG in a fridge until used in assays (14 days at maximum). The optimum concentration of coating antibody and conjugate dilution were chosen by checkerboard titration.

The preliminary studies showed that the highest sensitivity is achieved with the use of conjugate obtained by coupling 4′-carboxycotinine and peroxidase in molar ratio 5:1, with the working dilution 1:2000 (which corresponds to 250 ng of labeled HRP/mL incubation solution) and 5 mg/L coating antibody concentration.

2.6. ELISA assays

For performing assay in urine 10 μl cotinine standard or urine sample and 90 μl HRP–cotinine diluted 1:1800 in TBSTG buffer were placed in the antibody-coated microtiter wells. All assays were performed in duplicates in a fridge overnight (14 h minimum) after which the wells were emptied and washed four times with PBST and were incubated with 100 μl substrate solutions (TMB Substrate, Sigma, USA). The plates were incubated in the dark for 15–20 min, the reaction was stopped with diluted H_2SO_4 and the absorbance was measured at 450 nm (reference wavelength 630 nm) as described above. Assays in serum samples were essentially performed as described for urine; however, 25 μl standard serum sample and 75 μl HRP–cotinine diluted 1:1500 in TBSTG buffer (the final tracer dilution was 1:2000). Increased volumes of samples allowed for the determination of lower nicotine metabolite concentrations in a serum.

The calibration of the method was performed using the crystaline cotinine perchlorate. Solutions prepared in phosphate-buffered saline (PBS; 20–5000 ng cotinine/mL) were used for the calibration of urine assays and solutions prepared in 5% human serum albumin solution in PBS (10–1500 ng cotinine/mL) were used for the calibration of serum assays.

2.7. Assessment of analytical precision, sensitivity and accuracy of method

The assessment of analytical precision was based on within-assay and between-assay coefficients of variation obtained in assays of control urine samples with various nicotine metabolites concentratios as well as control serum sample. Assessment of analytical sensitivity was defined as a metabolite concentration that leads to a decrease in maximal tracer binding by 10% (value B/Bo = 90%, ED 90). Analytical accuracy was controlled by the estimation of the cotinine recovery from biological material loaded with the known amounts of the standard. In order to estimate the effect of the biological sample dilution on the assay results, urine samples from people who smoked approximately 20 cigarettes/day were diluted serially with PBS and such prepared material was used to assess the nicotine metabolites concentrations (linearity test).

2.8. Assessment of antibody cross-reactivity

The cross-reactivity of used antibody was assessed based on the read-out values (ED20, ED50 and ED80) from the calibration

curves for a particular compound (S-nicotine *N*-oxide, S-cotinine *N*-oxide, R,S-nornicotine, R,S-anabasine, *trans*-3′-hydroxycotinine and cotinine α-and β-*N*-glucuronides. The cross-reactivity was calculated as a ratio between ED50 of a given metabolite and ED50 of cotinine and specified in percent (it was possible only for *trans*-3′-hydroxycotinine and S-nicotine *N*-oxide). In the case of other compounds cross-reactivity was measured by performing assay with standard substance solution (concentration range 5–10,000 ng/mL) and calculated as the ratio of measured cotinine equivalents concentration to real standard concentration.

2.9. Comparison of ELISA assay with chromatographic method

The nicotine metabolite concentrations were determined in 30 urine samples from patients exposed to tobacco smoke and 20 urine samples from non-smokers. The immunoenzymatic technique (devoid of glucuronide hydrolysis) and thin-layer chromatography with densitometry, which allowed cotinine and *trans*-3′-hydroxycotinine determination according to previously published procedure [5] were applied. The results obtained were submitted to statistical analyses.

3. Results

The production of anti-cotinine antibody in response to repetitive immunizations with 4′-carboxycotinine–hemocyanin conjugates from *Limulus polyphemus* was equal in all three immunised animals. The concentration of the specific antibodies in native serum ranged between 4 and 8 mg/mL after 4–5 doses of immunogen and was stable after subsequent immunisations. The efficiency of antibodies isolation with the use of the imunoaffinity method was (approximately 80–90%) and enabled, in a relatively simple manner, obtaining specific anti-cotinine antibodies, presumably removing simultaneously part of the cross-reactive anti-cotinine antibodies with low affinity to cotinine. The assay range for cotinine in urine was from 3.5 to 5000 ng/mL, but in serum samples – from 3 to 1500 ng/mL. Within-assay and inter-assay precision of nicotine metabolites measurements performed by the use of proposed ELISA method were from 4.4 to 8.7% and from 6.1 to 11.3%, respectively. Within-assay and inter-assay precision for measurements in serum samples was 4.2% and 6.7%, respectively (mean cotinine concentration 102 ng/mL). The cross-reactivities (in percent) determined for S-cotinine, S-nicotine *N*-oxide, *trans*-3′-hydroxycotinine determined by the method used for urine samples as well as the ED90, ED80, ED50 and ED20 values (in ng/mL) are shown in Table 1. For S-nicotine, R,S-nornicotine, R,S-anabasine, S-cotinine *N*-oxide, α- and β-cotinine *N*-glucuronides cross-reactivities were extremelly low and accounted for 0.04%; 0.04%; 0.02%; 0.37%; 0.06% and 0.06%, respectively. The determination limit for the method calculated as an ED90 value for cotinine was 3.5 ng/mL for determinations in urine samples and 2.9 ng/mL for determinations in serum samples (mean values from 10 independent calibration curves). Mean cotinine recoveries for the serum were 100.59 ± 2.75%, whereas for the urine, 88.56 ± 11.38%. The results of the dilution test for an exemplary urine samples showed high linearity of assay. Original sample from active smoker (nicotine metabolites, 3285 ng/mL) serially diluted with PBS gave results with high correlation to the calculated metabolites concentration: [calculated conc.] = (0.9883 [measured conc.] + 34.4). Correlation coefficient was 0.995. The comparison of nicotine metabolites determinations using ELISA assays with those of cotinine and *trans*-3′-hydroxycotinine using thin layer chromatography (TLC) [5] for urine samples of active smokers is shown in Fig. 1. The correlation coefficients (*r*) were as follows: 0.92 (nicotine metabolites (ELISA) vs. cotinine (TLC), 0.89 (nicotine metabolites (ELISA) vs. *trans*-3′-hydroxycotinine (TLC) and 0.91 (nicotine metabolites (ELISA) vs. the sum of cotinine and *trans*-3′-hydroxycotinine (TLC).

4. Discussion

Chromatography (GC, HPLC, and TLC) as well as immunochemical methods are the most common analytical techniques used for nicotine metabolites determination.

In the presented study and those previously described by other authors the method of rabbit antibodies formation against cotinine was used [4]. Administration of 4–5 doses of immunogene led to obtaining immune serum containing the specific antibodies concentration of approximately 4–6 mg/mL. The application of the immunoaffinity chromatography technique with the use of self-prepared cotinine-aninohexyl-Sepharose bed enabled fast and simple isolation of specific antibodies against nicotine metabolites from the immune serum.

It is possible, that in the same manner, the isolation of antibodies with high affinity to cotinine was achieved with simultaneous elimination of crossreacting antibodies with other nicotine metabolites. The efficiency of antibody isolation process was relatively high and enabled obtaining 5 mg of specific antibodies against cotinine from 1 mL of immune serum.

Cotinine is most often determined as nicotine metabolites in biological samples. Apart from cotinine [4,6–8,14–27] and nicotine [12,13] different nicotine metabolites were determined [31]. Many tracers were used in immunochemical methods [4,6–8,16,19,20,22–24,27]. There are also methods that do not require labelled nicotine or its metabolites [15,21].

Most immunoenzymatic methods are the solid phase techniques (ELISA techniques) where immobilised on solid phase nicotine derivative and soluble in water, high-molecular weight carrier conjugates are applied (e.g. ε-polylysine or proteins than ones used as carrier protein in the immunization of animals) [4,6,18]. The simultaneous addition of sample/standard and antibody leads to the bond inhibition of antibody with haptene to the solid phase that is proportional to the added amount of nicotine metabolite. Finally, formed immunocomplexes are determined with the use of the second, coupled with the enzyme or biotin antibody directed against animal immunoglobulines (second antibody against first antibody).

The method sensitivity depends considerably on the affinity of the applied antibody and is often significantly higher for monoclonal antibodies. The advantage of this version of the ELISA test is the possibility of applying the native immune serum (polyclonal) to the analysis without its fractionation and isolation of specific anti-

Table 1
ED20, ED50, ED80 and ED90 values (in ng/mL) for cotinine, *trans*-3′-hydroxycotinine and S-nicotine *N*-oxide. Cross-reactivity was calculated as ratio of ED50 for investigated compound to ED50 for cotinine (in percent). Measurements were done with use of urine version of method. For cotinine mean and S.D. from 10 independent calibration curves were presented.

Investigated substance	ED 90 (ng/mL)	ED 80 (ng/mL)	ED 50 (ng/mL)	ED 20 (ng/mL)	% Cross-reactivity
Cotinine	3.50 ± 1.04	9.98 ± 2.36	97.1 ± 10.1	1 534 ± 127.3	100%
Trans-3′-OH-cotinine	4.31	37.6	520.6	40 385	18.6%
Nicotine *N*-oxide	74.0	210.2	1 465	12 780	5.1%

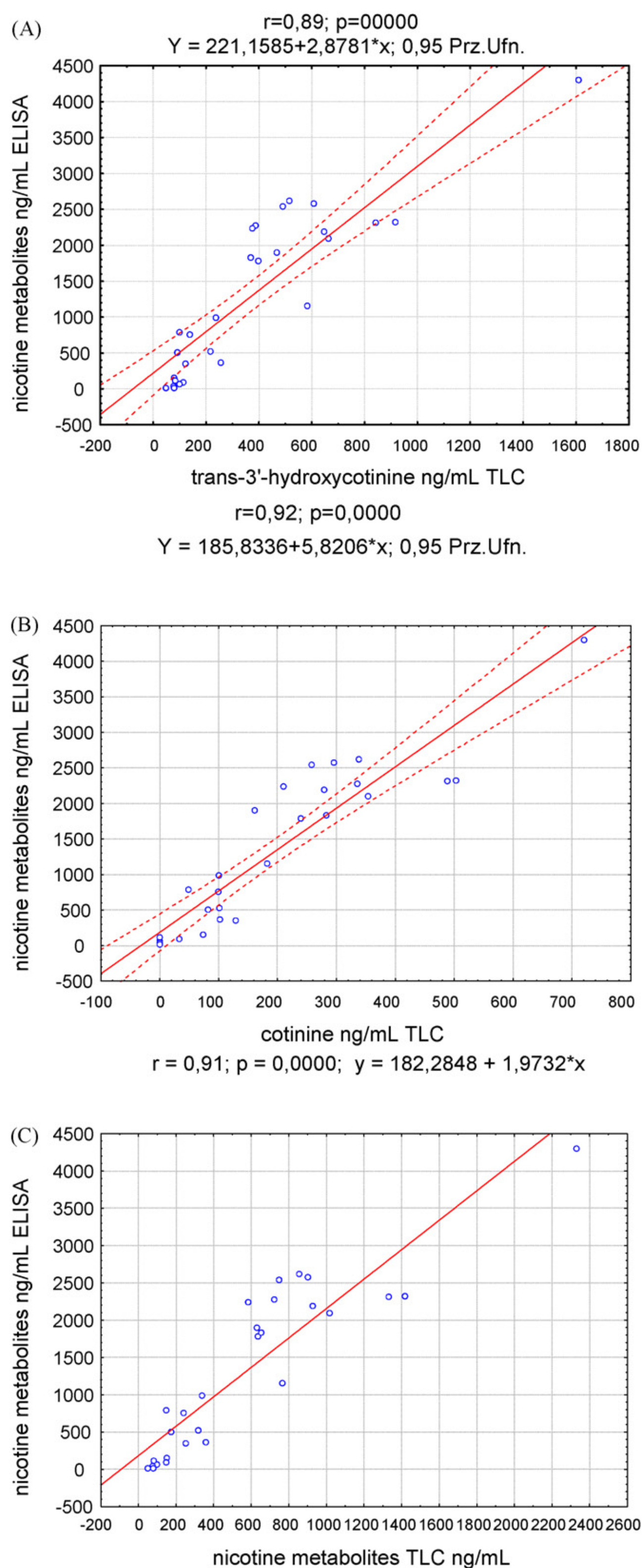

Fig. 1. Comparison of nicotine metabolites concentrations in urine samples of active smokers determined by ELISA and TLC with densitometry; (A) nicotine metabolites (ELISA) vs. *trans*-3′-hydroxycotinine (TLC); (B) nicotine metabolites (ELISA) vs. cotinine (TLC); (C) nicotine metabolites (ELISA) vs. sum of cotinine and *trans*-3′-hydroxycotinine (TLC).

bodies and the negligible influence of possibly presented marker enzymes inhibitors in biological material.

In the presented study a novel technical solution of an elaborated method was utilized.

This new approach related to the immobilization of the antibody in solid phase where the competitiveness took place between the analyte (nicotine metabolite in the analyzed sample or standard) and cotinine derivative coupled with signaling molecule (horseradish peroxidase). When the stationary condition was achieved (the incubation time 12–14 h) the elimination of reagents unbound with solid phase took place and the substrate for peroxidase was added. The intensity of the obtained coloured products (absorbance) was inversely proportional to the concentration of nicotine metabolites.

As the tracer 4′-carboxycotinine conjugated to horseradish peroxidase (HRP–cotinine) was used. The Erlanger method was applied for the formation of this tracer. The previous experiments of coupling haptene (4′-carboxycotinine) to HRP with the use of carbodiimide (EDC) led to the formation of conjugates with a low haptene:enzyme ratio which caused significant reduction in the sensitivity of the ELISA method (data not shown). It was proved that the optimal ratio of haptene to HRP was 5:1 when the Erlanger coupling method was applied (method sensitivity with the use of conjugate obtained at 2:1 and 10:1 haptene/HRP ratios have lower analytical sensitivity, results not shown). The stability of concentrated tracer solution (kept at a temperature of −20 °C) was at least 5 years.

The above elaborated methods enabled the nicotine metabolites (mainly cotinine) determination in urine samples in the concentration range from about 3.5 to 5000 ng/mL (range ED90–ED10). The limit of determination for serum samples was 3–1500 ng/mL and that was in agreement with other data concerning the level of nicotine metabolites occurring in this kind of biological material. In the case of individuals smoking large amounts of cigarettes (>20–30 cigarettes per day) it could be necessary to dilute urine samples (event 20–30 times). The equal detection limits for assay version in urine and serum are probably due to matrix effect (high protein concentration in serum) and “blocking” of active sites in antibodies. Within-run and between-run coefficients below 12% proved the high analytical precision of assay results and the stability of the presented method. The applied antibody (obtained after isolation with the use of immunoaffinity chromatography) was characterised by relatively low cross-reactivity with *trans*-3′-hydroxycotinine (approximately 19%) and S-nicotine *N*-oxide (approximately 5.1%). The rest of the analysed nicotine derivatives (R,S-anabasine, R,S-nornicotine, S-nicotine, S-cotinine Noxide, S-cotinine α- and β-*N*-glucuronides) did not show significant reactivity with the antibody (cross-reactivity below 0.1%). These compounds (except for cotinine β-*N*-glucuronide) do not occur at high levels in biological material, so this fact does not have any clinical significance. The problem can be encountered with the necessity of releasing the cotinine and *trans*-3′-hydroxycotinine from its conjugates with glucuronic acid as some quantity of these compounds is excreted in this form (applying basic or enzymatic hydrolysis). However, in most chromatographic and immunochemical methods nicotine metabolites are determined only in their free (non-conjugated) form as analyzed in the presented study [28–30].

Since some people can metabolize nicotine solely to *trans*-3′-hydroxycotinine, the results for these individuals could be underestimated by approximately 80% (cross-reactivity for *trans*-3′-hydroxycotinine, about 19%). However, the comparison of the ELISA and TLC method showed a relatively good correlation between the excretion of *trans*-3′-hydroxycotinine and that of the excretion of nicotine metabolites determined by TLC (Fig. 1).

The comparison of the presented method to the chromatography technique (TLC with densitometry) showed a high compatibility of obtained data. Urine samples that showed the concentration

of nicotine metabolites below 20 ng/mL ($n = 20$) gave the negative results in TLC analysis (absence of cotinine as well as *trans*-3′-hydroxycotinine). The data obtained from urine samples taken from active smokers ($n = 30$) showed high correlation with the data obtained in TLC related to cotinine and *trans*-3′-hydroxycotinine excretion (Fig. 1). The Pearson correlation coefficient (r) ranged from 0.89 to 0.92 depending on the correlated compound (however, mostly for cotinine) which proved the high coincidence of the results.

The sensitivity of the TLC method was 13.5 ng of cotinine per spot (which corresponded with approximately 27 ng/mL of urine sample when the extraction was carried out using 10 mL of urine sample). The results of 20 urine samples of individuals that declared lack of active and passive smoking exposure from TLC and ELISA techniques did not show the presence of nicotine metabolites. Thus, the calculated diagnostic sensitivity and diagnostic specificity of ELISA method reached 100% proving that this test can be used as screening tool in population studies.

Many authors suggest to measure 3′-*trans*-hydroxycotinine as more beneficial method, because this nicotine metabolite was shown to be predominant especially in urine samples [32]. But in Fig. 1C due to cross-reactivity of obtained antibodies, probably not only against these two main nicotine metabolites, it is observed that higher nicotine metabolites concentration were determined by ELISA in comparison to TLC results. The best chromatographic technique to determine all nicotine metabolites presented in the investigated samples is liquid chromatography coupled with mass spectrometry, the technique that gives various possibilities, but is not sufficiently popular in many laboratories, yet (due to resructed access to apparatus, lack of professional staff) [33].

5. Conclusion

The presented elaborated immunoenzymatic method for nicotine metabolites determination is characterised by its simplicity from a technical point of view as it does not require complex and expensive apparatus (the ELISA reader) as well as isotopic markers.

Taking into consideration these advantages this method can be applied to practical monitoring of tobacco smoke exposure levels in large population groups. However, its specificity is not sufficient in every case.

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