ANALIZA ŞI STANDARDIZAREA MEDICAMENTELOR

CONTRIBUŢII LA CERCETAREA ŞI VALIDAREA METODEI DE DETERMINARE A NICOTINEI ŞI COTININEI CU AJUTORUL GAZ-CROMATOGRAFIEI CUPLATE CU MASS-SPECTROSCOPIA

Doina Şpaiuc[1,3*], Adrian Şpac[2], Luminița Agoroaei[1], Rodica Peredelcu[4] , Elena Butnaru[1]

University of Medicine and Pharmacy "Grigore T. Popa", Faculty of Pharmacy, 16th University Street, 700115, Iași, Romania

[1] Department of Toxicology

[2] Department of Physico-Chemistry

[3]Emergency Military Hospital, Iași

[4]University of Medicine and Pharmacy "Nicolae Testemițanu", Chișinău, Republica Moldova

Rezumat

Nicotina şi cotinina sunt principalii markeri biotoxicologici ce se determină în intoxicaţiile acute şi cronice (tabagismul) la persoanele care fumează sau recurg la tratament substitutiv cu diferite produse farmaceutice pe bază de nicotină (chewing-gum, plasture, spray nazal, inhalator bucal, tablete sublinguale). Obiectivul acestui studiu a fost dezvoltarea şi validarea unei metode GC-MS de determinare a nicotinei şi cotininei din ser (metoda cu standard intern). Determinările au fost efectuate pe un cromatograf de gaze tip Agilent Technologies 7890A echipat cu coloană cromatografică DB5MS, 30 m x 0,25 mm, 0,25 µm. Detecţia s-a realizat prin spectrofotometrie de masă în modul SIM (m/z pentru nicotină: 84 pic de bază, 133 şi 162 pic molecular şi respectiv cotinină: 98 pic de bază, 118 şi 176 pic molecular cu temperatura sursei MSD de 230 °C şi cea a cvadrupolului MSD de 150°C). Metoda validată a fost liniară pe domeniul 1-500 ng/mL, selectivă, exactă şi precisă.

Cuvinte cheie: GC-MS, validare, nicotină, cotinină.

Abstract

Contributions to the development and the validation of the method to determine the nicotine and the cotinine by gas chromatography coupled with mass spectrometry

The nicotine and the cotinine are the main bio-toxicological markers that are determined in acute and chronic intoxication (tabagism) in people who smoke or use different substitution treatments based on nicotine (chewing-gum, patch, nasal spray, oral inhaler, sublingual tablet). The objective of this study was to develop and validate a GC-MS method for nicotine and cotinine determination in serum (a method with an internal standard). Measurements were performed on a gas chromatograph Agilent Technologies 7890 equipped with a DB 5 MS chromatographic column (30 m x 0.25 mm, 0.25 µm film thickness). Detection was performed by mass spectrometry in SIM mode (m / z for nicotine: 84 basic peak, 133 and 162 molecular peak and m / z for cotinine: 98 basic peak, 118 and 176 molecular peak; the temperature of the MSD source was 230°C and that of the MSD qvadrupol was 150 ° C). The validated method was linear in the range 1-500 ng / mL, selective, accurate and precise.

Keywords: GC-MS, validation, nicotine, cotinine

Introduction

The nicotine is found either in tobacco products used as drugs or in medicines used in withdrawal/smoking cessation

In Romania, according to the data of a GATS study (Global Adult Tobacco Survey), coordinated by the Ministry of Health, the adult smoking prevalence in 2011 was of 26.7%. The nicotine enters the body by breathing, through the oral mucosa and through the skin.

The nicotine is absorbed very quickly through the lung, reaching to the central nervous system within 15 seconds after inhaling the smoke. Absorption through the buccal mucosa is relatively slow. The half-life of nicotine is two hours. A person, who smokes about 20 cigarettes per day, absorbs 58 mg of nicotine [1].

The nicotine binds to the plasma proteins at a rate of 2-5%. The metabolism occurs mainly in the liver (80-90%) but it can be metabolized in the lungs and kidneys [2]. The nicotine is metabolized by CYP2A6 to cotinine, the main toxic metabolite. 20 metabolites of nicotine have been identified, which are less active than nicotine. The half-life of cotinine is 15-20 hours. The nicotine and its metabolites are eliminated via urine, saliva, sweat, lungs and breast milk. The nicotine elimination is conditionated by the urinary pH: at alkaline pH it is eliminated about 10% and at acidic pH, the nicotine is eliminated unchanged at a rate of

3-4%. The nicotine binds to the nicotinic receptors widely distributed in the brain, and stimulates neurons that are part of pleasure and reward [3].

Nicotine causes psychic addiction, which is 6-8 times higher than alcohol addiction. 95-100% of smokers are addicted and this is the most common of all known addiction [4].

In this paper we propose the development and validation of a method for quantitative determination of nicotine and cotinine in serum (internal standard method) by GC-MS.

The method validation was performed according to the ICH guidelines [5] (International Council of Harmonization) as well as methodologies in the literature [6-13].

Materials and methods

- Nicotine - C^3 99%, density 1.009 g/mL, Merck Germany
- Cotinine - 98% Sigma - Aldrich - Germany
- Methanol - Sigma - Aldrich - Germany
- Dichloroethane - Promochim
- Human serum from smokers.

Standards

- Standard of nicotine in methanol: 500 m g / mL
- Standard of cotinine in methanol: 500 m g / mL

Sample preparation

0.1 mL methanolic solution of nicotine + 0.1 mL methanolic solution of cotinine + 4.8 mL serum . The concentration of nicotine and cotinine is 10 mg / mL.

To 1 mL of serum it was added 0.5 mL of 5% ammonium sulphate solution and 1 mL of dichloroethane: methanol (1: 1).

Energic stirring for five minutes at 1000 vibrations / min, it was added 0.5 mL dichloroethane and it was stirred again for five minutes. The mixture was centrifuged at 4000 rotations / min for 10 minutes. The organic phase was separated, evaporated to dryness and it was repeated with 0.5 mL mixture of dichloroethane / methanol (1: 1).

Equipment and chromatographic conditions

We used a gas chromatograph 7890 Agilent Technologies Agilent Technologies 7683B autoinjector equipped with mass spectrometer detector and Agilent Technologies 5975 inert MSD;

Analyses were performed on a chromatographic column DB 5 MS (30 m x 0.25 mm, 0.25 µm film thickness) with mobile phase consisting of helium with a flow rate of 1 mL / min; the injector's temperature was 250 ° C, the MSD quadrupole's temperature was 150 ° C. The temperature gradient in the column compartment was initially 100 ° C, followed by a ramp of temperature - 10 ° C / min up to 250 ° C and immediately, a second ramp - 20 ° C / min up to 280 ° C and finally the temperature was kept constant for 3.5 minutes; the analysis lasts 20 minutes and the last approximately 8 minutes are required to remove any volatile components extracted simultaneously with nicotine and cotinine in serum, as in the conditions described above. The volume of solution injected was 0.5 µL, at a split ratio of 1/5. Detection was performed by mass spectrometry in SIM mode (single ion monitoring) (m / z for nicotine: 84 - basic peak, 133 and 162 - molecular peak; m / z for cotinine: 98 - basic peak, 118 and 176 molecular peak) . Interpretation of results: Agilent Technologies ChemStation software.

The validation parameters: specificity, linearity, precision, accuracy and limits of detection were studied.

Results and discussions

Method validation for nicotine and cotinine

The identification of nicotine and cotinine in serum was based on comparison of retention times from the sample's chromatogram with those obtained in the chromatogram of the reference substance.

To identify the separate components, it can be used (for MS detection in SCAN mode) the comparison of the mass spectra for corresponding peaks of the sample, with the mass spectra from the spectral libraries (Wiley, NIST).

Figures 1 and 2 show the mass spectrum of nicotine and cotinine from the Wiley mass spectra library [14].

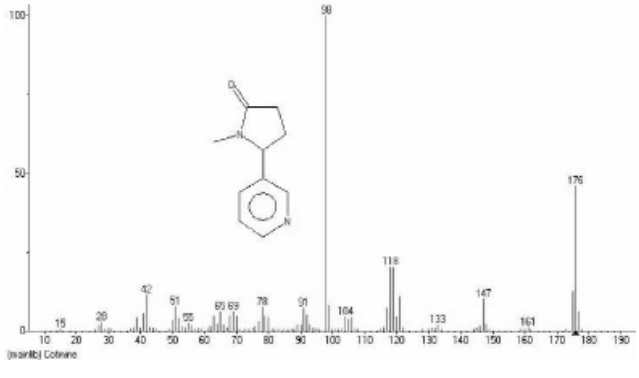

Fig. 1. *The mass spectrum of nicotine*

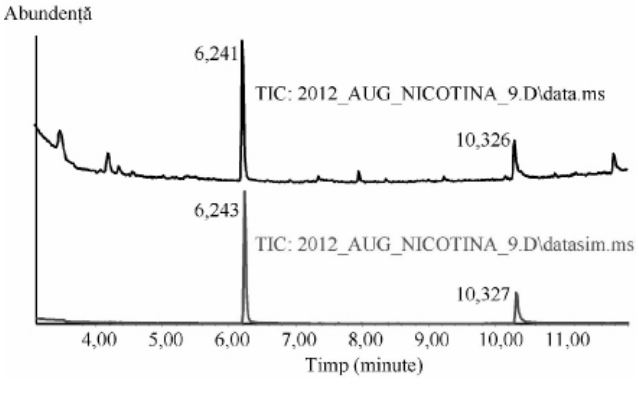

Fig. 2. *The mass spectrum of cotinine*

Analyzing the two mass spectra it can be noticed that these present main peaks at values m / z 42, 84 (basic peak), 133, 162 (molecular peak) to nicotine and respectively 98 (basic peak), 118, 147 and 176 (molecular peak) to cotinine. These values were confirmed by the literature [13-18].

An important criterion in developing a method of analysis by gas chromatography with capillary column is the judicious choice of the split ratio. The tests were conducted using various split ratios from 1/100 to complete analysis of this volume (SPLITLES). Finally, we chose as the best value of split ratio, a report of 1/5. Figure 3 presents an example of a chromatogram of a serum sample containing nicotine and cotinine. To increase the safety of identification, the chromatogram was performed with detection in both ways (SCAN and SIM).

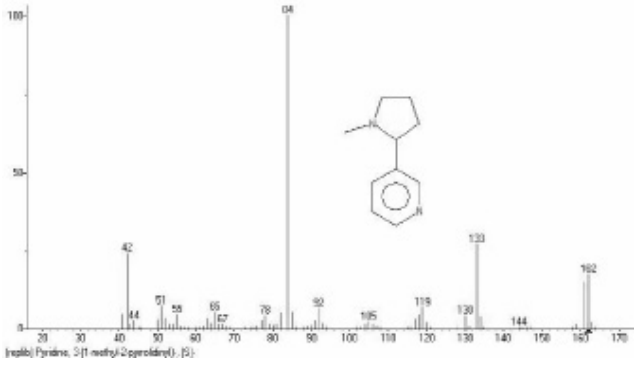

Fig. 3. *The chromatogram of a serum sample containing nicotine and cotinine*

From this chromatogram we can notice a peak at the retention time of 6.241 minutes and one at 10.326 minutes in SCAN mode; a peak at the retention time of 6.243 minutes and respectively, one at 10.327 minutes, in SIM mode. For these peaks the mass spectra shown in Figure 4 and Figure 5 were recorded.

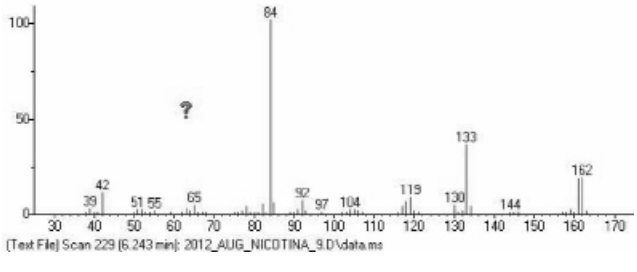

Fig. 4. *The mass spectrum of the peak with retention time 6.243*

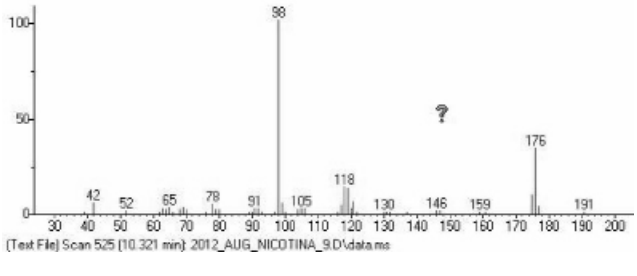

Fig 5. *The mass spectrum of the peak with retention time 10.321*

A comparison of the presence of the recorded spectral lines as opposed to the mass spectra of nicotine and cotinine was made (Figure 6 and Figure 7).

The identification of nicotine and cotinine was made according to the retention time and mass spectrum in SCAN mode; for the subsequent determinations performed in SIM mode, we took in consideration only the retention time (with a deviation of ± 5%), that means there were accepted values of the retention time in the range 5.931 to 6.555 minutes for nicotine and 9.805 to 10.837 minutes for cotinine. It must be noted that the retention times experimentally obtained, do not vary by more than 1%.

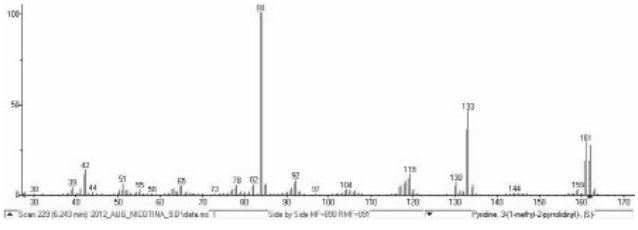

Fig. 6. *Comparison of the mass spectrum of the compound from the sample with the retention time of 6.243, with the ones from the database (nicotine - Wiley mass spectral library)*

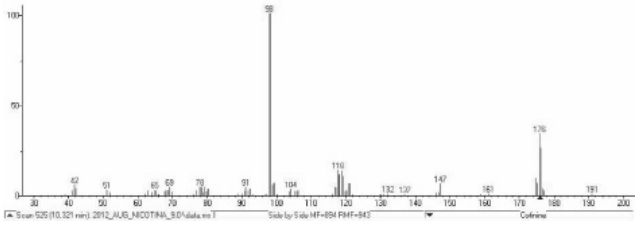

Fig. 7. *Comparison of the mass spectrum of the compound from the sample with the retention time of 10.321, with the ones from the database (cotinine - Wiley mass spectral library)*

To determine the specificity of the method for the simultaneous determination of nicotine and cotinine in serum, a blank sample was processed in the mentioned conditions. The sample thus prepared was analyzed by GC-MS in the specified operating conditions. From the analysis of the obtained chromatogram it can't be noticed the apparition of any chromatographic peak at the values of the retention times corresponding to the nicotine (6.24 minutes) and cotinine (10.32 minutes) (Figure 8).

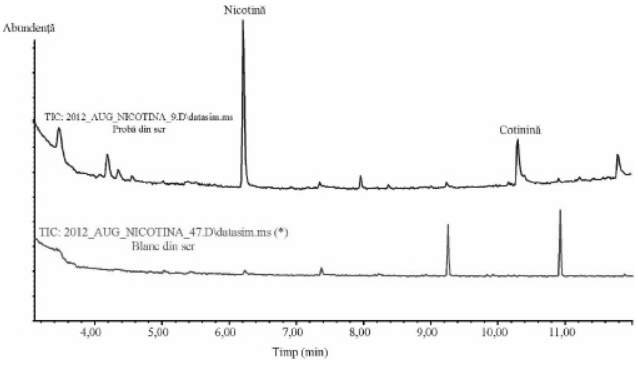

Fig. 8. *Chromatograms for a blank sample containing nicotine and cotinine*

To determine linearity we studied three sets of standard solutions (nicotine and cotinine) in the range 1-500 ng / L for nicotine and 1-500 ng / L for cotinine.

Following the analyzes, we determined: the coefficient of correlation (r), the coefficient of regression (r^2), the intercept with the ordinate, the curve and standard error of the regression curve (Table I).

Table I. Statistical calculation of the regression curve for nicotine and cotinine

	Nicotine	Cotinine
coefficient of correlation (r)	0,99996	0,9999
coefficient of regression (r2)	0,99992	0,9999
standard error of the regression curve (SE)	2623,723	1294,535
intercept	185788,3	46638,4
curve	2153,6	3821,8

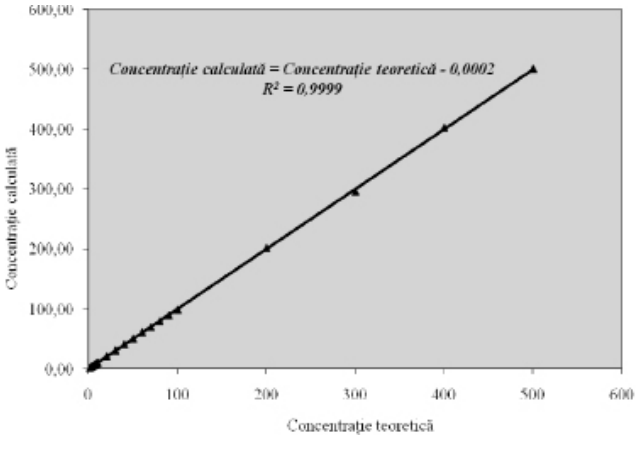

Fig. 9. *Calibration curve for nicotine*

The calibration curves (Figures 9 and 10) revealed the relation of direct proportionality between the area of the analytical signal and the sample's concentration in the studied range, demonstrating the linearity of the method.

The limits of detection (LD) were calculated:

- Nicotine - 1.12 ng / mL and
- Cotinine - 4.02 ng / mL and, the limits of quantification (LQ):
- Nicotine - 12.18 ng / mL and
- Cotinine - 3.39 ng / mL.

Using the estimation of these limits based on the coefficient of deviation and the regression curve, the working range was determined: 12.18 to 500 ng / mL for nicotine and 3.39 to 500 ng / mL for cotinine.

To demonstrate the method's precision, the repeatability of the analysis was performed for three independent solutions with different concentrations, in the range of 10-50 ng / mL. The relative standard deviation (RDS) was 1.6804% for nicotine, and 1.0351% for cotinine.

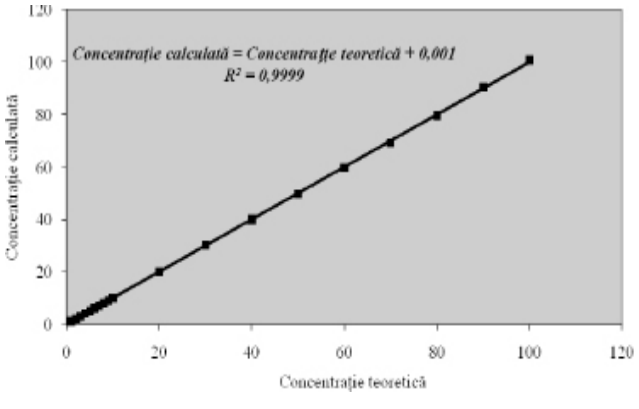

Fig. 10. *Calibration curve for cotinine*

Table II. Accuracy of the method of determination of nicotine and cotinine by GC-MS

Crt. nr.	Theoretical concentration (ng / mL)	Nicotine			Cotinine		
		Peak area	Calculated concentration (ng / mL)	Recovery %	Peak area	Calculated concentration (ng / mL)	Recovery %
1	10	206875	9.79	97.9	84436	9.89	98.9
2		207664	10.16	101.6	85274	10.11	101.1
3		208107	10.36	103.6	84043	9.79	97.9
4	30	250839	30.21	100.7	162819	30.40	101.3
5		251757	30.63	102.1	161653	30.09	100.3
6		250388	30.00	100.0	161737	30.12	100.4
7	50	293399	49.97	99.9	237447	49.93	99.9
8		293715	50.11	100.2	236341	49.64	99.3
9		293651	50.08	100.2	237637	49.98	100,0
		Statistical data	Average	100.7 %	Statistical data	Average	99.9 %
			Minimum	97.9 %		Minimum	97.9 %
			Maximum	103.6 %		Maximum	101.3 %

To establish the accuracy of the method of determination of nicotine and cotinine, we chose to work with a minimum of 9 determinations covering the specific areas of concentrations. The experimental values are shown in Table II.

The data presented in Table II show that the average retrieval in the study regarding the accuracy of the method of determination of nicotine and cotinine has a value of 100.7% (97.9%- 103.6%) for nicotine and 99.97% (97 9%-101.3%) for cotinine, in the range 10-50 ng / mL. The chromatographic methods accept a range of ± 10%; meaning that the method is accurate.

Conclusions

The method of determination by GC-MS for nicotine and cotinine (after the optimization of the working parameters) was validated according to the standards developed by ICH Q2B. The results obtained indicate that in the concentration range of 1-500 ng / mL, the method is linear, precise and accurate. The limits of detection and quantitation of nicotine and cotinine in the conditions of extraction from serum (internal standard method) were established. The average retrieval was 100.7% for nicotine and 99.9% for cotinine, in the range of concentrations of 10-50 ng / mL.

References

1. Hukkanen J., Jacob P. 3rd., Benowitz N.L. Metabolism and disposition kinetics of nicotine. *Pharmacol. Rev.*; 57(1):79-115 (2005).
2. Butnaru E., Proca M. Toxicologie vol.II. Editura Timpul. Iaşi. (2001).
3. Riley A., Curvall M. Determination of nicotine and its metabolites in biological fluids: in vivo studies. . In: *Analitical determination of nicotine and related compounds and their methabolites.* Gorrod J.W., Jacob P., editors. Elsevier Science B.V., Amsterdam, Netherlands; 583-644 (1999).
4. Dani J.A., De Biasi M. Cellular mechanisms of nicotine addiction. *Pharmacol. Biochem. Behav.*;70:439-446 (2001).
5. * * * – ICH Topic Q2B: Validation of Analytical Procedures (CPMP / ICH / 281 / 95) – The European Agency for the Evaluation of Medicinal Products.
6. * * * – Chromatography and Spectroscopy Supplies, Reference Guide, Agilent Technologies, (2009).
7. Robert L. , Grob PhD. , Eugen F. , Barry PhD. – Modern Practice of Gas Cromatography 4 th Edition, (2004).
8. Roman L., Săndulescu R, Bojiţă M., Muntean D.L. – Validarea metodelor analitice, Ed. Medicală, Bucureşti, (2007).
9. Oprean R., Rozet E., Dewé W., Boulanger B., Hubert Ph. – Ghid de validare a procedurilor analitice cantitative, Ed. Medicală Universitară „Iuliu Haţieganu", Cluj Napoca, (2007).
10. Eugen F. , Barry PhD., Robert L., Grob PhD. – Columns for Gas Cromatography : Performance and Selection, Hardcover, (2007).
11. * * * – Chromatography and Spectroscopy Supplies, Reference Guide, Agilent Technologies, (2009).
12. Robert L., Grob PhD., Eugen F., Barry PhD. – Modern Practice of Gas Cromatography 4 th Edition, iunie (2004).
13. * * * – NIST Chemistry WebBook (http://webbook.nist.gov.chemistry).
14. * * * – Wiley Mass Spectral Library, Agilent Chemstation.
15. Jin-gu Kim, Ueon-Sang Shin, Ho-Sang Shin - Rapid Monitoring Method of Active and Passive Smoker with Saliva Cotinine by Gas Chromatography-Mass Spectrometry, Bull. Korean Chem. Soc., 23,10:1496-1498 (2002).
16. Karaconji I. B., Skender L., Visnja K. Determination of Nicotine and Cotinine in Urine by Headspace Solid Phase Microextraction Gas Chromatography with Mass Spectrometric Detection. Acta Chimica Slovenica;, Vol. 54 Issue 1,74 (2007).
17. Culea M. Exposure Assessment of Nicotine and Cotinine by GC-MS. Indoor Air.;14(2):83-6 (2004).
18. Shakleya D.M., Huestis M.A. Simultaneous and Sensitive Measurement of Nicotine, Cotinine, *trans*-3'-Hydroxycotinine and Norcotinine in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry J Chromatogr. B. Analyt. Techno.l Biomed Life Sci. 1; 877(29): 3537–3542, (2009).