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Determination of nicotine metabolites in urine by LC-MS/MS: trends of smoke and smokeless products exposure in ice hockey

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Abstract

Since nicotine is both a stimulant and a relaxant, consumption of smokeless tobacco or nicotine-containing medication may benefit sport practice without usual smoking harms. Thus, serious interest has been rising about the prevalence of snus in winter sports.

A method involving liquid-liquid extraction followed by liquid chromatography-tandem mass spectrometry for the detection and quantification of nicotine and its metabolites cotinine, *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide in urine was developed. Then, nicotine exposure during the 2009 Ice Hockey World Champs was assessed by analyzing 72 samples. Nicotine and/or metabolites were detected in every urine sample, with exposure within the last three days for eight specimens out of ten and consumption prior and/or during the games for half of the hockey players. Assuming detrimental respiratory effects of smoking, extensive smokeless nicotine consumption may be hypothesized.

Introduction

Various consumption patterns of nicotine exist, from tobacco smoke to smokeless tobacco (snus, snuff,…) and replacement therapies (patch, gum,…), corresponding to different intakes and metabolism pathways. Nicotine is metabolized in numerous compounds, such as cotinine, *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide (Figure 1)[1].

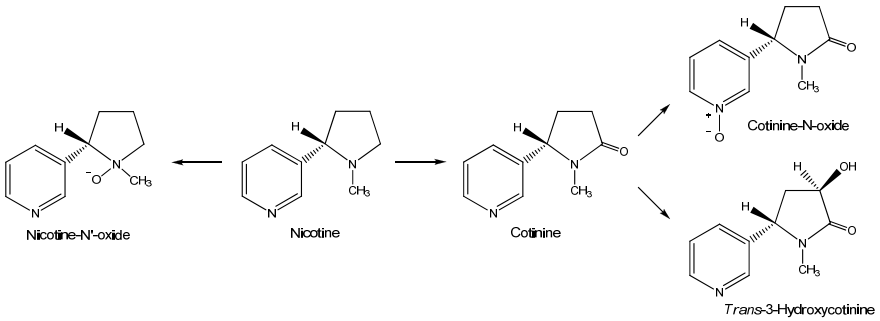

Figure 1. Simplified metabolism of nicotine.

Nicotine may enhance performances due to stimulant and relaxant properties, while snus has

been reported as a growing trend in winter sports[2-6]. However, nicotine did not appear in the 2009 World Anti-Doping Agency (WADA) Prohibited List and Monitoring Program[7]. Thus, a liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the simultaneous determination and quantification of nicotine and metabolites in urine was developed and further applied to urine samples collected during the 2009 Ice Hockey World Championships to measure the prevalence of nicotine exposure.

Materials and Methods

Nicotine (NIC) and cotinine (COT) from Sigma-Aldrich (Buchs, Switzerland), *trans*-3-hydroxycotinine (3-HC), nicotine-*N'*-oxide (NNO) and cotinine-*N*-oxide (CNO) from TRC (Toronto, Canada), *d4*-nicotine (*d4*-NIC), *d3*-cotinine (*d3*-COT) and *d3*-*trans*-3-hydroxycotinine (*d3*-3-HC) from LGC Promochem (Molsheim, France).

1 mL urine spiked with 10 µl of 10 µg/ml internal standard (I.S.) solution (*d4*-NIC, *d3*-COT and *d3*-3-HC), diluted with 1 mL phosphate buffer (0.2 M, pH 7.0) prior to vortex mixing. LLE performed with 2.5 mL chloroform: propan-2-ol (95:5, v/v) for 10 min and centrifugation for 5 min at 2500 rpm. Organic layer evaporated to dryness and reconstituted in 1 mL ACN: ammonium formate (10mM, pH 3.0) (98:2, v/v). Separation carried out on a LC-MS/MS system using a Phenomenex Luna® HILIC column (150 mm × 3.0 mm, 5 µm). Mobile phase consisted of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with 0.3 mL/min flow. Initial mobile phase condition was 98% A for 3 min, decreased to 35%, held from 10-13 min and increased to 98% from 13.1-16 min. Analyses performed using a linear ion trap mass spectrometer LTQ-MS (ThermoFinnigan, San Jose, CA, USA) with an atmospheric pressure ionization (API) interface in positive ESI mode.

Experiments conducted following the guidelines on bioanalytical method validation from the US Food and Drug Administration (FDA).

Results and Discussion

HILIC mode provided a complete separation of all analytes, with excellent peak shape and repeatability of retention times (RSD ≤ 4.1%) (Figure 2). Direct infusion of standard solutions allowed optimization of MS parameters and identification of SRM transitions (NIC 163 → **132**, 120, 106; COT 177 → **146**, 98, 80; 3-HC 193 → **134**, 118, 80; NNO 179 → **132**, 130, 117 and CNO 193 → 134, **96**, 98).

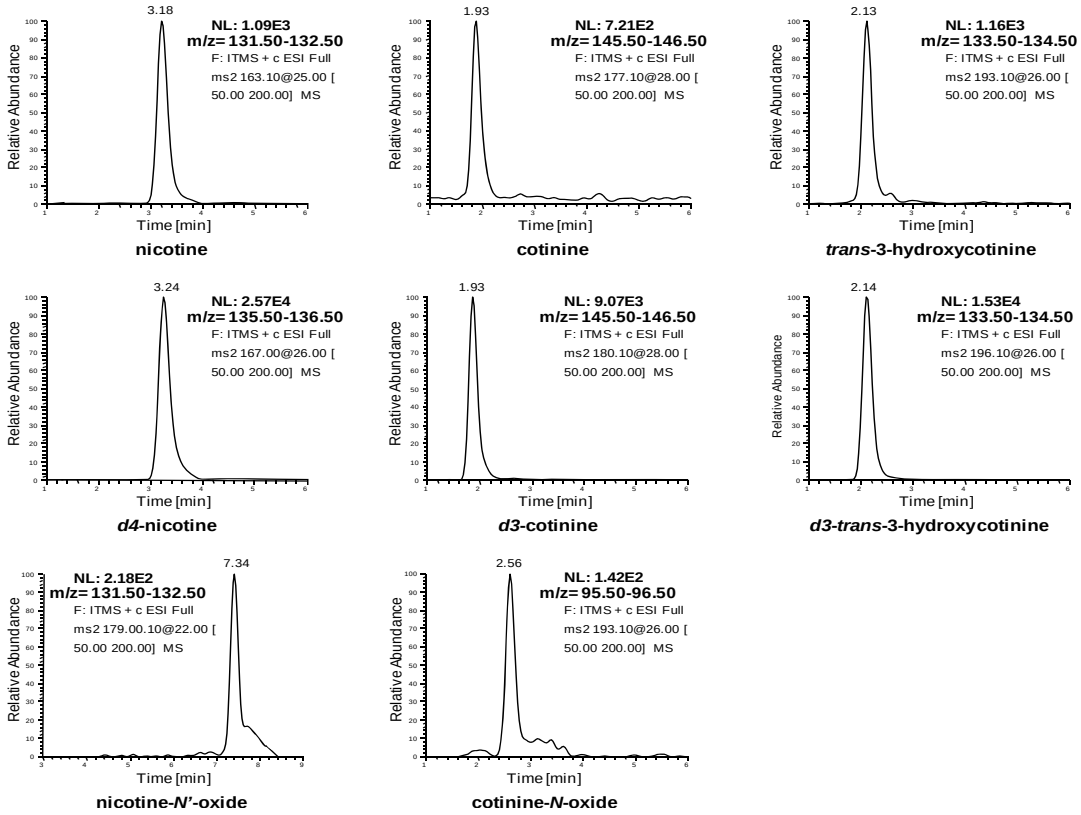

Figure 2. Urine sample with nicotine and metabolites at 10 ng/mL (LLOQ) and IS at 100 ng/mL.

Extraction efficiency ranged from 70.4-100.4% depending on the analyte and concentration, with evidence of good repeatability (RSD < 15%). Different curves fitting were evaluated after achieving three validation series (calibration standards and validator standards (QCs) at k = 6 and k = 4 concentration levels, respectively). A profile of confidence interval in the dosage range for each compound (10-10'000 ng/mL for NIC, COT, 3-HC and 10-5'000 ng/mL for NNO and CNO) was built combining accuracy and intermediate fidelity variance. Unweighted linear least-squares regression proved best and was chosen for quantification purpose. Due to the linear response, calibration standards and QCs were reduced to two and three concentration levels, respectively, with satisfactory repeatability (≤ 9.4%) and intermediate precision (≤ 9.9%) (Figure 3)[8].

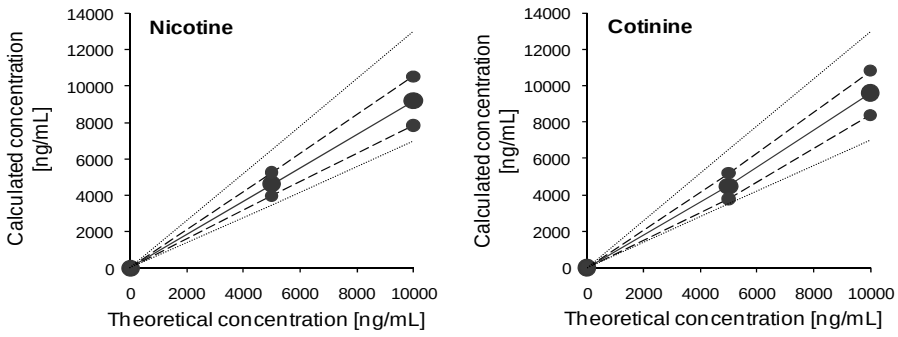

Figure 3 Accuracy profiles (— trueness, – – – accuracy as confidence interval, **....** acceptance limits (± 30 %)).

The lower limit of quantification (LLOQ) for all analytes was 10 ng/mL and the limit of

detection (LOD) was ~500 pg/mL. Selectivity tests demonstrated no interfering endogenous or exogenous molecules within selected scan windows. Matrix effect was found insignificant despite very slight analyte and concentration dependant ion enhancement or suppression.

During the 2009 IIHF Worlds, urine samples were collected after every game (n = 72). In agreement with the 2009 International Standards for Laboratories (ISL), a storage period of three months and complete removal of identification means were ensured prior to use for research purpose[9]. NIC, COT, 3-HC, NNO and CNO were detected in 87%, 91%, 94%, 97% and 97% of samples, respectively (Figure 4).

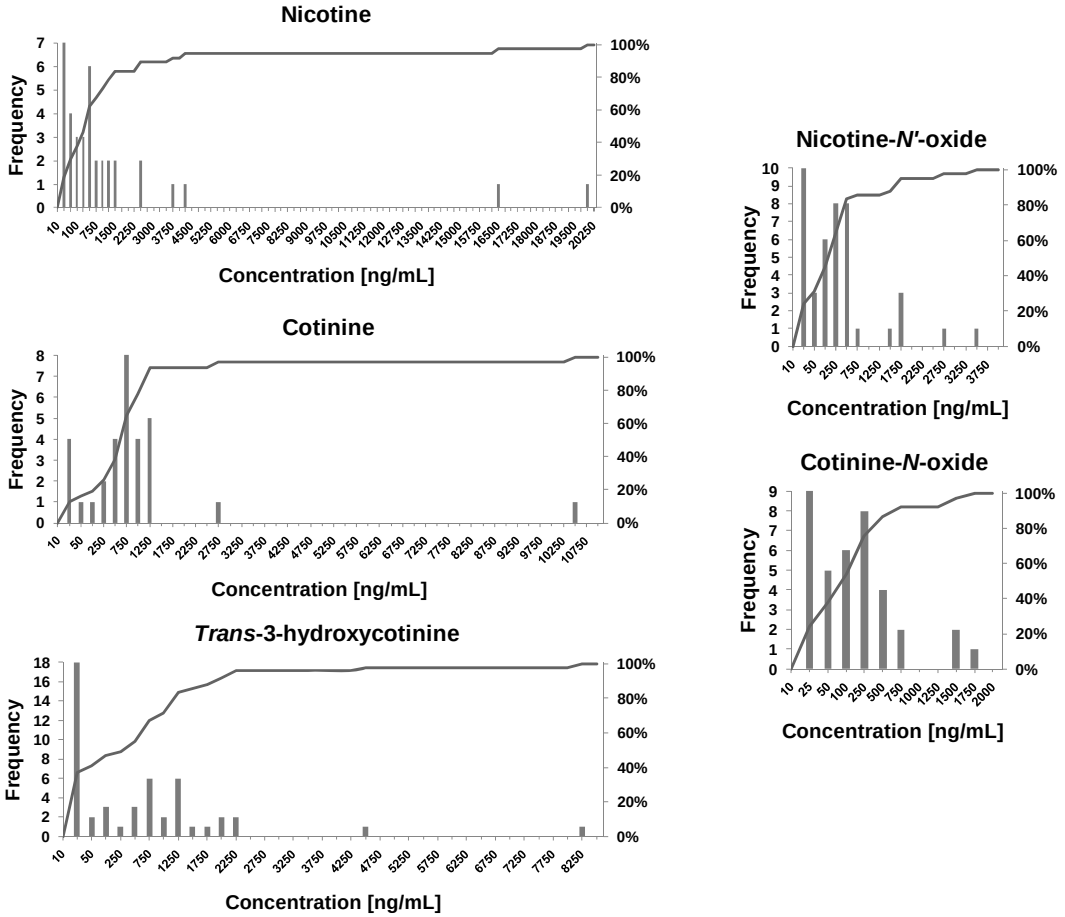

Figure 4 Concentrations distribution for nicotine and metabolites. The solid line indicates the cumulative percentage.

At least one of the five different metabolites was present in every sample. These findings suggest that every athlete was exposed to nicotine, either environmentally or from active consumption, during the competition period. Above LLOQ levels were measured in 51%, 43%, 68%, 58% and 51% of samples, respectively. One of the five different metabolites was detected at such concentrations in 82% of samples. These results also indicate exposure within the last three days for approximately eight ice hockey players out of ten[10]. Prevalence of nicotine consumption close to or/and during the games was evaluated by relying on conservative concentration limits for active consumption (50 ng/mL for NIC, COT and 3-HC

and 25 ng/mL for NNO and CNO). Accordingly, active nicotine use was highlighted in 36% - 44% of samples, depending on the analyte. Noteworthy, at least one of the metabolites was present at such levels in 53% of urine samples. Thus, according to the quantitative measurements performed and the detrimental respiratory effects due to smoking, sound evidence on smokeless nicotine use may be hypothesized[11, 12]. However, means of distinction between forms of nicotine consumption need to be further investigated.

Acknowledgements

This project was entirely supported by a grant from ADS. The authors gratefully acknowledge the IIHF for making this study possible.

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