

An anti-doping perspective on nicotine detection in the peri-exercise period in a cohort of trained male cyclists



Toby Mündel

School of Sport, Exercise and Nutrition, Massey University, Palmerston North, New Zealand

ARTICLE INFO

Keywords:

Nicotine
Doping
Sport
Athlete
WADA
HPLC

ABSTRACT

The purpose of the present study was to investigate the detection of nicotine in athletes: before vs. after 90 min exercise, and in serum vs. urine. Serum [cotinine] was not different before vs. after exercise (31 ± 14 vs. 29 ± 14 ng mL⁻¹, $P = 0.43$), although urine [cotinine] measured greater than serum (148 ± 142 vs. 31 ± 14 ng mL⁻¹, $P < 0.01$). Urinary [nicotine] measured greater than [cotinine] (790 ± 1176 vs. 148 ± 128 ng mL⁻¹, $P = 0.05$), which significantly correlated ($r = 0.57$, $P = 0.04$). These results indicate that urine [cotinine] concentration is the optimal biomarker to detect nicotine use in athletes.

1. Introduction

Cross-sectional, self-report data and anti-doping urine analyses indicate a prevalence of nicotine use at ~25–50% amongst elite and professional athletes (Mündel, 2017); although the analysis of biological samples is not able to distinguish between sources, it is likely that a majority of use stems from smokeless tobacco with several sports (e.g. baseball, ice-hockey, football codes) reporting high rates of use (Mündel, 2017). The use of nicotine (i.e. nicotine replacement therapies) or nicotine-containing substances (i.e. smokeless tobacco) is not presently prohibited by the World Anti-Doping Agency (WADA), and a plethora of nicotine-containing products are available over the counter. WADA did, however, place nicotine on its Monitoring Program (World Anti-Doping Agency, 2012) to determine whether it satisfied the criteria of i) enhancing sport performance, ii) being a potential health threat to athletes, and iii) altering the spirit of sport that could see it upgraded to the List of Prohibited Substances.

When considering the anti-doping stance of detection, nicotine's tendency to fluctuate in blood and half-life of only ~1–2 h do not make it an attractive biomarker for nicotine intake (Benowitz, Hukkanen, & Jacob, 2009). Cotinine, its major metabolite with a longer retention time (half-life of ~16 h) and with less daily fluctuation is preferred, particularly in urine samples (Dhar, 2004). There is considerable inter-individual variability in nicotine metabolism, and due to the high degree of hepatic extraction, any physiological event that changes liver blood flow (e.g. exercise) will likely affect the rate of nicotine clearance (Benowitz et al., 2009).

No previous study has investigated the detection of this WADA-monitored substance during the peri-exercise period, whereby the timing of samples taken (e.g. pre-vs. post-exercise) and medium of biomarker (e.g. serum vs. urine) are compared. This was the purpose of the current study.

2. Material and methods

2.1. Participants

Fourteen male cyclists (mean \pm standard deviation age: 37 ± 12 years, body mass: 76 ± 9 kg) volunteered to participate in this study. All participants were competing at a club-to-national level on a regular basis and maintained a weekly training volume of more than 200 km. According to (De Pauw et al., 2013), these participants were classified as performance levels 3/4, or a trained/well-trained participant group due to their peak aerobic power (348 ± 22 W and 4.6 ± 0.6 W kg⁻¹) and peak rate of O₂ consumption ($\dot{V}O_{2\text{peak}}$, 4.9 ± 0.6 L min⁻¹ and 66 ± 10 mL kg⁻¹.min⁻¹). All participants were non-smokers and did not habitually use any form of nicotine administration.

2.2. Experimental overview

All participants attended the laboratory on five occasions: 1) preliminary submaximal and maximal tests, 2) experimental familiarisation,

Abbreviations: HPLC, high-performance liquid chromatography; $\dot{V}O_2$, rate of O₂ consumption; $\dot{V}O_{2\text{peak}}$, peak rate of O₂ consumption; WADA, World Anti-Doping Agency.

E-mail address: t.mundel@massey.ac.nz.

<https://doi.org/10.1016/j.crphys.2020.04.001>

Received 5 April 2020; Received in revised form 23 April 2020; Accepted 23 April 2020

2665-9441/© 2020 The Author. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

and 3–4) experimental trials of nicotine (NIC) or placebo (PLA) administration. The two experimental trials were completed in a randomised, crossover, double blind design. All visits were separated by seven days, conducted at the same time of day (± 1 h), following >24 h of dietary and exercise control, with participants also having refrained from alcohol and caffeine during this period. All exercise was on an electromagnetically braked cycle ergometer (Lode Excalibur, The Netherlands) with participant-specific set up for the seat, handlebars and pedals, which was maintained for each trial within a participant. All testing was conducted in a standard laboratory environment (18–22°C) with a fan-generated airflow of 19 km h⁻¹ facing participants.

2.3. Preliminary testing and familiarisation

Following body mass (Jandever, Taiwan) and height (Seca, Germany) measurements, participants completed a submaximal (consecutive 5-min stages at 100, 150, 200, and 250 W) and maximal test (40 W min⁻¹ until volitional fatigue) as described previously (Mündel, Houltham, Barnes, & Stannard, 2019). Expired gases were collected continuously (VacuMed Vista Turbofit, USA) for the determination of ventilation and O₂ uptake ($\dot{V}O_2$). Following this, a linear relationship between the mean rate of $\dot{V}O_2$ during the last 2 min of each submaximal stage and power output was determined and used to calculate a power output which would elicit 55 and 75% of $\dot{V}O_{2peak}$ for each participant for the remaining trials.

The familiarisation trial was undertaken to ensure participants were accustomed to the experimental procedures and to minimise learning effects. This trial replicated entirely the experimental trial outlined below.

2.4. Dietary and exercise control

Participants were asked to refrain from exercise between 24 and 48 h prior to each experimental trial. Twenty-four hours prior to each experimental trial, participants attended the laboratory to complete a standardised training ride 60 min in duration at a fixed power output that elicited $\sim 60\% \dot{V}O_{2peak}$ (heart rate: 120 ± 5 beats·min⁻¹). Participants were then provided with a standardised snack and recorded their diet during the 24-h period prior to the first experimental trial. This diet was replicated for the second experimental trial, and in order to further minimise variation in pre-trial metabolic state a standardised meal was consumed 3 h prior to arriving at the laboratory for the experimental trial, after which no food was consumed. Fluid was encouraged and *ad libitum* until 3 h prior to the experimental trial. For further detail on dietary standardisation see (Mündel et al., 2019). This standardisation ensured that urine specific gravity (1.010 ± 0.007) and body weight (76.6 ± 9.3 kg) on arrival to the laboratory were not different between trials (both $P > 0.50$), data that reflect hydration status.

2.5. Nicotine/placebo administration

Approximately 10 h prior to each experimental trial, a staff member not involved with the research project placed a patch on the participant between the right shoulder blade and the spine. The patch was either a nicotine patch (7 mg 24-h⁻¹, Habitrol, Novartis, New Zealand) or a placebo patch (orthoptic eye patch 63.5 mm \times 45.7 mm, Nexcare, 3 M, New Zealand). Following the second experimental trial, participants were fully de-briefed. The independent staff member was only aware that they were administering intervention A (NIC) or B (PLA), with results remaining blinded to the author until data collection was complete, after which disclosure was made.

2.6. Experimental procedure

Following the pre-trial control described above, participants arrived at the laboratory after which a blood sample was obtained from the antecubital vein. Participants then changed into their cycling shorts and

top, shoes and socks whilst also providing a urine sample. Participants then completed 90 min of exercise, composed of cycling at a power output equivalent to 55% of $\dot{V}O_{2peak}$ (185 ± 21 W) for 60 min followed by the completion of an individualised set amount of work (458 ± 43 kJ) as quickly as possible, which was calculated as the equivalent of 30 min of cycling at 75% $\dot{V}O_{2peak}$. For further details on the protocol see (Watson et al., 2005). Water (5 mL·kgbw⁻¹) was provided to the participants in aliquots every 15 min in order to minimise variance in total body water between trials; water intake (412 ± 164 mL) and sweat rate (1651 ± 584 mL) were not different between trials (both $P > 0.79$). A final blood sample was obtained immediately following the trial.

The duration and intensity of exercise was chosen to mimic typical training or competition for a variety of sports.

2.7. Blood/urine sampling and analyses

Venous blood samples were obtained from an antecubital vein into a 4 mL vacutainer tube containing clot activator (Becton–Dickinson, Plymouth, UK). Following inversion, the tube was allowed to clot at room temperature for 30 min before being centrifuged (Eppendorf, Hamburg, Germany) at 4 °C for 10 min at 805 g. Serum was removed, aspirated into 500 μ l aliquots and frozen at -80 °C for later analyses using high-performance liquid chromatography (HPLC). The mid-stream urine sample was collected into a 10 mL Sterilin™ tube (ThermoFisher Scientific, North Shore, New Zealand) and immediately frozen at -80 °C for later analyses using HPLC.

Sample preparation, extraction and analysis by HPLC were based on previous methodology (Massadeh, Gharaibeh, & Omari, 2009) and performed in duplicate. The HPLC system (Shimadzu Prominence 20 Series) consisted of a DGU-20AS Prominence degasser, SIL-20AC Autosampler, SPD-M20A Diode array detector and a CTA-20 A column oven with a Phenomenex Luna 5 μ C18 (2) 100 A 150 \times 4.6 mm column attached. Operating conditions were as per the method used by (Massadeh et al., 2009) with a limit of detection for nicotine and cotinine of 7 ng mL⁻¹.

2.8. Data and statistical analyses

All descriptive and statistical analyses were performed with SPSS software for windows (IBM SPSS Statistics 20, NY, USA). Descriptive values were obtained and reported as means and standard deviation (SD) unless stated otherwise. Levene's test was used to ensure data did not differ substantially from a normal distribution. Data were analysed using a paired samples *t*-test, with statistical significance set at $P \leq 0.05$. In order to determine whether concentrations of cotinine or nicotine were associated with one another or body mass, Pearson's correlation coefficients were used to describe the form and strength of bivariate association for absolute values.

3. Results

Despite being able to recover both nicotine and cotinine in spiked serum and in the serum of smokers, the sensitivity for nicotine obtained by (Massadeh et al., 2009) was unable to be realised for the serum from the participants following patch administration, and, therefore only urine nicotine concentrations are reported. Nicotine and cotinine were not detected in any samples during the PLA trials.

Serum cotinine concentrations can be seen in Fig. 1A and were not different when measured immediately before and after the exercise bout ($P = 0.43$). However, when comparing serum with urine cotinine concentrations measured immediately before exercise (Fig. 1B), urine concentrations measured greater ($P < 0.01$). When comparing urinary nicotine and cotinine concentrations measured immediately before exercise (Fig. 1C), nicotine measured greater ($P = 0.05$).

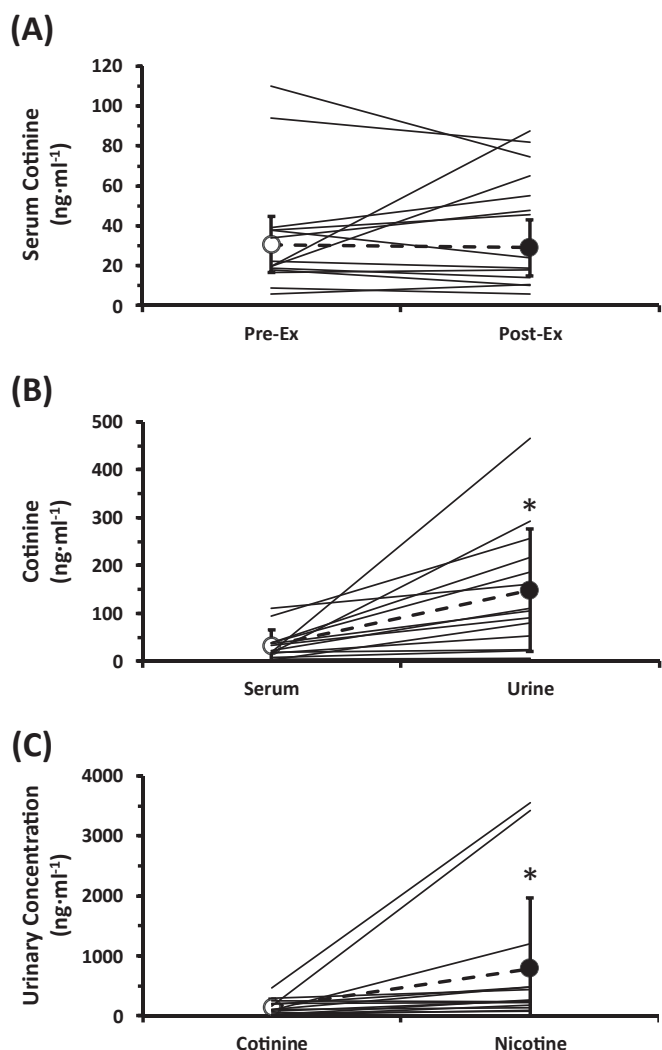


Fig. 1. (A) Serum cotinine before (Pre-Ex) and following (Post-Ex) 90-min exercise following nicotine patch treatment; (B) Serum and urine cotinine, and (C) urine nicotine and cotinine before exercise following nicotine patch treatment. Individual and mean (SD) values for $n = 14$. * indicates significant difference.

In absolute terms, body mass did not correlate with serum ($r = -0.32$, $P = 0.27$) or urine ($r < 0.01$, $P = 0.99$) cotinine or urine nicotine ($r = -0.07$, $P = 0.80$). Serum cotinine did not correlate with urine cotinine ($r = 0.27$, $P = 0.36$), however urine cotinine did correlate with urine nicotine ($r = 0.57$, $P = 0.04$).

4. Discussion

Measurement of nicotine and (or) its metabolites in biological fluids is paramount in determining if an athlete has actively consumed nicotine (e.g. nicotine replacement therapies) or nicotine-containing substances (e.g. smokeless tobacco), something that WADA is currently monitoring as it considers whether nicotine should remain part of its Monitoring Program or upgrade to be included on the List of Prohibited Substances. The important results of the present study concerning detection of nicotine use by athletes in the peri-exercise period are that 1) cotinine concentrations are not affected acutely by exercise when measured in blood (i.e. before vs. after exercise), and 2) cotinine concentrations are greater in urine than blood. Taken together, it is recommended that in order to detect whether athletes have used nicotine in the peri-exercise period, a urine sample is

collected for the determination of cotinine concentration. This appears consistent with current anti-doping efforts.

The current study observed that serum cotinine concentrations were not different when measured almost 2 h apart, separated by 90 min of exercise (Fig. 1A). This is consistent with the known reduction of hepatic blood flow by up to 80% during intense exercise (Rowell, Blackmon, & Bruce, 1964); this restricts renal clearance of nicotine and is accompanied by a limited (non-existent) urine excretion. However, Klemsdal et al. (Klemsdal, Gjesdal, & Zahlsten, 1995) have demonstrated that there is increased transdermal absorption leading to small increases in systemic nicotine concentrations during exercise whilst wearing a transdermal patch, as in the current study. Whether these (pre-to post-exercise) results would be maintained if an acute nicotine delivery were used (i.e. smokeless tobacco or electronic cigarette) prior to exercise remains to be determined.

Whilst it is recommended that concentrations of serum nicotine or urine cotinine are used to determine active use of nicotine (Benowitz et al., 2009), urine remains the preferred biological fluid (cf. blood and saliva) as it is less invasive, concentrations of analytes measure greater and provides a longer window for detection (Mündel, 2017; Benowitz et al., 2009). Furthermore, the liver represents the primary site of nicotine metabolism. In humans, seventy percent of nicotine is metabolised to cotinine by cytochrome P450 and aldehyde oxidase; less than thirty percent of nicotine that enters the liver exits unchanged, with the remaining >70% eliminated from the liver in the form of metabolites (Dhar, 2004). Therefore, urine cotinine concentration represents the optimal biomarker for detection of nicotine use in athletes over the preceding 3–4 days, something the current results support (Fig. 1B). Nevertheless, cotinine is imperfect due to individual variation in metabolism [see Benowitz et al., 2009]. Urine output affects both cotinine and nicotine excretion, although not as much as pH (Benowitz et al., 2009), and therefore factors affecting flow (e.g. fluid ingestion and/or hydration status) before, during and following exercise warrant consideration as they will affect urine concentrations.

Certain considerations and study limitations warrant discussion. It is important to note that collection of a urine sample should be into a pre-cleaned/siliconised polypropylene container with screw cap closure as it can then withstand breakage during handling or transportation (Dhar, 2004). Next, chromatographic analysis techniques are preferred with gas chromatography–mass spectrometry the ‘gold standard’ due to being specific, highly sensitive, able to analyse both nicotine and cotinine from a single assay and are less susceptible to interference (Dhar, 2004). This technique and operator experience were not available, and, therefore HPLC was used in the current study but at a cost of sensitivity for determining serum nicotine. Only males naïve to nicotine use were used in this exercise-based study. However, future research should determine i) if females respond differently given that they metabolise nicotine faster than men, ii) how tolerant users are influenced by withdrawal/re-introduction, and how naïve users are influenced by desensitisation, whilst iii) assessing time-course changes before and following nicotine administration in a resting state would further indicate a true exercise effect. Finally, only two time-points were sampled (pre-post exercise) as this study was part of a larger project concerning nicotine's risk for heat illness. Nevertheless, as the collection of anti-doping samples can occur both in and out of competition, future research should collect samples in the hours following exercise e.g. 1, 3 and 12 h.

In summary, the current study has shown that following ~10 h transdermal nicotine administration, exercise lasting 90 min does not affect serum cotinine concentrations, and that urine cotinine concentrations measure 8-fold greater than in serum. Thus, urine cotinine concentration is the optimal biomarker for nicotine use in athletes.

Ethical approval

The study was approved by the Massey University Human Ethics Committee: Southern A (10/73) and conformed to the standards set by

the latest revision of the *Declaration of Helsinki*, except for registration in a database, with each participant providing informed, written consent.

Author contributions

TM conceived and designed the work and obtained funding; acquired, analysed and interpreted data; wrote the manuscript.

Funding

This work was supported by the World Anti-Doping Agency (12D18TM).

Declaration of Competing Interest

The author declares that the research was conducted in the absence of any personal, commercial or financial relationship(s) that could be construed as a potential conflict of interest or inappropriately influence (bias) this work.

Acknowledgments

The author would like to thank Michelle McGrath for performing the HPLC analysis.

References

- Benowitz, N.L., Hukkanen, J., Jacob 3rd, P., 2009. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb. Exp. Pharmacol.* 192, 29–60.
- De Pauw, K., Roelands, B., Cheung, S.S., de Geus, B., Rietjens, G., Meeusen, R., 2013. Guidelines to classify subject groups in sport-science research. *Int. J. Sports Physiol. Perform.* 8, 111–122.
- Dhar, P., 2004. Measuring tobacco smoke exposure: Quantifying nicotine/cotinine concentration in biological samples by colorimetry, chromatography and immunoassay methods. *Journal of Pharmaceutical and Biomedical Analysis* 35, 155–168.
- Klemsdal, T.O., Gjesdal, K., Zahlsen, K., 1995. Physical exercise increases plasma concentrations of nicotine during treatment with a nicotine patch. *British Journal of Clinical Pharmacology* 39, 677–679.
- Massadeh, A.M., Gharaibeh, A.A., Omari, K.W., 2009. A single-step extraction method for the determination of nicotine and cotinine in Jordanian smokers' blood and urine samples by RP-HPLC and GC-MS. *J. Chromatogr. Sci.* 47, 170–177.
- Mündel, T., 2017. Nicotine: Sporting friend or foe? A review of athlete use, performance consequences and other considerations. *Sports Medicine* 47, 2497–2506.
- Mündel, T., Houltham, S.D., Barnes, M.J., Stannard, S.R., 2019. Nicotine supplementation does not influence performance of a 1h cycling time-trial in trained males. *Front Physiol* 10, 292.
- Rowell, L.B., Blackmon, J.R., Bruce, R.A., 1964. Indocyanine green clearance and estimated hepatic blood flow during mild to maximal exercise in upright man. *J Clin Invest* 43, 1677–1690.
- Watson, P., Hasegawa, H., Roelands, B., Piacentini, M.F., Loooverie, R., Meeusen, R., 2005. Acute dopamine/noradrenaline reuptake inhibition enhances human exercise performance in warm, but not temperate conditions. *J Physiol* 565 (Pt 3), 873–883.
- World Anti-Doping Agency, 2012. The 2012 monitoring Program, Montreal. www.wada-ama.org/sites/default/files/resources/files/WADA_Monitoring_Program_2012_EN.pdf.