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Determination of urinary concentrations of pseudoephedrine and cathine after therapeutic administration of pseudoephedrine-containing medications to healthy subjects: implications for doping control analysis of these stimulants banned in sport*

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Due to its stimulatory effects on the central nervous system, and its structural similarity to banned stimulants such as ephedrine and methamphetamine, pseudoephedrine (PSE) at high doses is considered as an ergogenic aid for boosting athletic performance. However, the status of PSE in the International Standard of the Prohibited List as established under the World Anti-Doping Code has changed over the years, being prohibited until 2003 at a urinary cut-off value of 25 μg/ml, and then subsequently removed from the Prohibited List during the period 2004–2009. The re-consideration of this position by the World Anti-Doping Agency (WADA) List Expert Group has led to the reintroduction of PSE in the Prohibited List in 2010. In this manuscript, we present the results of two WADA-sponsored clinical studies on the urinary excretion of PSE and its metabolite cathine (CATH) following the oral administration of different PSE formulations to healthy individuals at therapeutic regimes. On this basis, the current analytical urinary threshold for the detection of PSE as a doping agent in sport has been conservatively established at 150 μg/ml

Keywords: pseudoephedrine; cathine; doping; threshold; WADA

Introduction

Pseudoephedrine (PSE) is a sympathomimetic amine with stimulant properties and vasoconstriction capacity possessing a mechanism of action which relies principally on its agonistic effect on the sympathetic nervous system and its indirect activation of the α-adrenergic receptors. Pharmacologically, PSE is mainly used as a nasal decongestant and is commonly found in many over-the-counter (OTC) cold, allergy, and influenza medications. PSE can also be found in dietary supplements and products containing ephedra-derived alkaloids.[1,2]

PSE is a diastereomer of ephedrine and a precursor of methamphetamine (and thus is used as a key ingredient for the illicit manufacture of this street drug). Stimulants such as ephedrine and methamphetamine, and other substances with similar chemical structure or similar biological effect(s) (e.g. methylephedrine, cathine (CATH, norpseudoepherine), amphetamine), are included in the Prohibited List and banned in competition. The Prohibited List is the World Anti-Doping Agency (WADA)international standard that is updated and published annually to designate the doping substances and methods that are prohibited in sport. Ephedrine, methylephedrine, and CATH, a minor metabolite of PSE, are considered prohibited when their concentration in urine is greater than the established threshold (10 μg/ml for ephedrine and methylephedrine, and 5 μg/ml for CATH).

The action of PSE on the central nervous system presumably results in respiratory stimulation, an increase of heart rate, muscle contraction and blood flow to skeletal muscles, reduced fatigue, and an enhanced sensation of euphoria, and these effects are sought by some athletes for the purpose of increasing athletic performance with supratherapeutic doses of PSE.[3] However, there is limited evidence of an ergogenic effect by

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PSE, in particular when administered at therapeutic doses, and thus doubt has been cast on whether it can be effectively used as a doping agent. For example, it has been shown that (1) the ingestion of 60–120 mg PSE caused no increase in aerobic capacity,[4] (2) a single 60 mg dose of PSE did not enhance selected cardiopulmonary variables during submaximal or maximal exercise,[5] (3) a single administration of 120 mg PSE had no ergogenic effect during prolonged high-intensity exercise,[6] (4) a single 1 mg/kg or 2 mg/kg dose of PSE had no effect on time-to-exhaustion tests,[7] (5) an acute administration of two, 60 mg PSE tablets did not result in an ergogenic effect in high-intensity exercise performance by young men or women,[8] (6) a single administration of a 60 mg PSE pill did not affect anaerobic cycling performance and aerobic cycling efficiency,[9] and (7) maximal therapeutic dosing of PSE (60 mg four times daily) did not affect sub-maximal endurance running.[10] However, a different picture emerges when the doses of administered PSE are higher than the recommended therapeutic amounts. At a single, 180 mg dose, the consumption of immediate-release PSE resulted in an increase of isometric knee extension strength, better lung function and improved peak power during short-term maximal cycle performance in young men.[3] The ingestion of the same dose of PSE 60 min. before the onset of high intensity exercise improved cycling time-trial performance in well-trained male athletes.[11] Also, a single administration of 2.5 mg/kg PSE (approximately 170 mg total dose) resulted in an improvement of 1500-m running performance by male athletes.[12]

Notwithstanding the prohibited status of its main metabolite and other structurally similar substances, PSE's status on the Prohibited List has changed over the years. Until 2003, PSE was forbidden if detected in concentrations greater than the urinary cut-off value of 25 μg/ml. This analytical threshold value, initially established by the International Olympic Committee (IOC), led to several positive doping cases, with athletes often claiming that it resulted from the inadvertent use of OTC cold preparations. Studies have later shown that PSE urinary concentrations following a therapeutic dosing of PSE may indeed surpass this threshold value in some individuals.[13,14]

In 2004, PSE was removed from the Prohibited List and placed in the WADA Monitoring Program, which includes substances that are not on the Prohibited List, but which WADA wishes to monitor in order to detect patterns of misuse in sport, if any. Statistics from the WADA Monitoring Program from 2004 to 2009 showed a steady increase in the number of samples with high urinary concentrations (greater than 75 μg/ml) of PSE.[15] In addition, there was clear evidence of PSE abuse in specific sports and regions of the world, which showed clusters of samples with PSE in concentrations many times greater than would be expected from a therapeutic dose. During this period, the evaluation of anti-doping test results for CATH (in concentrations higher than the established urinary threshold of 5 μg/ml) became problematic in the presence of PSE, in that the CATH values may have resulted from the administration of PSE, a permitted drug. Consequently, in such cases there was limited legal possibility to pursue potential dopers for an anti-doping rule violation for CATH in the presence of PSE.

There are few reported studies on the urinary excretion of PSE and/or CATH following the administration of PSE at therapeutic doses in healthy individuals.[6,13–16] In these studies, different PSE therapeutic preparations and administration regimes were employed, and the number of subjects involved was generally limited. Chester *et al*. administered multiple doses of 60 mg PSE-HCl following a 36-h therapeutic regime to 16 male volunteers.[13] Strano-Rossi and colleagues reported the administration of two different PSE preparations (60 mg immediate- or 120 mg sustained-released formulations) in different dosing combinations to nine subjects.[14] Deventer *et al*., in turn, administered a single-dose of 240 mg PSE over a 24-h period to six healthy volunteers based on one of three PSE modalities: (1) 2 × 120 mg sustained-release, (2) 1 × 240 mg sustained-release, or (3) 4 × 60 mg immediate-release formulation.[15] In the latter study, even though the total daily dose administered (240 mg) constitutes the maximal therapeutic dose for PSE, only the 1 × 240 mg administration reflects a recommended therapeutic regime, since the other two preparations should be administered as single 1 × 120 mg or 1 × 60 mg doses every 12- or 4–6 h, respectively.

In general, all of these studies demonstrated a large inter-individual difference in the urinary concentrations and the pharmacokinetics of excretion of PSE and CATH following the administration of PSE, which could be attributed to differences in urinary flow and pH. Thus, the elimination half-time of PSE in urine increases with increasing urinary pH.[6,17] The previous urinary threshold for PSE, 25 μg/ml, and the existing threshold for CATH, 5 μg/ml, can be greatly exceeded in certain individuals following the therapeutic intake of PSE a few hours prior to urine collection. Conversely, such thresholds were not reached after the administration of supratherapeutic doses of PSE in other subjects. In addition, the concentrations of excreted PSE and CATH do not necessarily correlate, indicating inter-individual differences in both the capacity to metabolize PSE into CATH and their excretion in urine.

Considering this scientific evidence and in conjunction with the high concentrations of urinary PSE recently detected in doping control samples as part of the WADA Monitoring Program following the withdrawal of PSE from the Prohibited List, it is logical to conclude that some athletes in some countries and in some sports were using high doses of PSE as a doping aid. For these reasons, WADA decided to reintroduce PSE on the Prohibited List as a banned stimulant (in-competition). However, to do so, additional PSE administration studies were needed to re-assess the range of urinary concentrations of PSE and CATH excreted by normal individuals following the therapeutic administration of different formulations of PSE (60 mg fast-release or 120 mg sustained-release) and to consequently define an analytical threshold for the detection of doping with PSE. The results of such studies, leading to the re-introduction of PSE in the 2010 Prohibited List at a urinary threshold of 150 μg/ml, are presented and discussed here.

Experimental

Two clinical studies, differing in the number of subjects enrolled and study design, were conducted. The clinical phases of the studies were performed at Anapharm Clinical Research Facilities in Canada (Quebec City, QC and Toronto, ON) and the analysis for quantification of PSE and CATH in study urine samples was conducted at the Canada WADA-accredited anti-doping laboratory (Laboratoire de Contrôle du Dopage INRS-Institut Armand-Frappier, Laval, Canada).

All clinical work was conducted in compliance with Good Clinical Practice (GCP) rules in accordance with ICH E6 Guidelines, local regulatory requirements, and the principles enunciated in the Declaration of Helsinki. Subjects were required to read and sign a

Subject Information and Consent Form (ICF), which described the study objectives, clinical research work, post-study procedures, and potential associated risks. The clinical study protocols, any relevant associated documents, and ICFs were reviewed and approved by the Anapharm Institutional Review Board prior to subject screening and enrolment. Letters of non-objection from the Canadian authorities were also obtained. The trials did not start until the investigators had obtained favourable written approvals from an Independent Ethics Committee for the study protocols and other study documents.

Throughout the studies, the subjects were monitored for adverse events. There were no serious or significant adverse events reported. Clinical laboratory tests (haematology, biochemistry, and urinalysis) were performed for each subject at the time of the screening and post-study procedures. Electrocardiograms, vital signs measurements (blood pressure, heart rate, respiratory rate, and oral temperature), and physical examinations were performed at the time of screening only. All final laboratory test results were within normal limits or judged clinically to be not significant.

Clinical Trials (CT)

Study 1 (CT-1)

The first study consisted of a randomized, open-label, two-way crossover study of the urinary excretion profiles of PSE and CATH from PMS-Pseudoephedrine® 60 mg tablet (Pharmascience Inc., Montreal, Canada) or Sudafed™ Decongestant 12-hours 120 mg Extended-Release Caplet (Pfizer Inc., Mississauga, Canada).

Sixteen healthy, adult subjects (eight male, eight female), members of the community at large, and judged eligible for the study after assessment against demographic and clinical inclusion and exclusion criteria, were enrolled.

In treatment A, subjects received four single oral doses of one tablet of PMS-Pseudoephedrine® daily every 6 h for two consecutive days. In treatment B, two single oral doses of one Sudafed™ decongestant capsule were administered daily every 12 h for two consecutive days. In each treatment, the total daily dose was 240 mg of PSE. There was a washout period of seven days or more between the last dose of the first treatment and the first dose of the second administration period.

A total of 20 urine samples were taken per period from each subject (within 15 minutes prior to the first dose of study medication and at 2, 4, 6, 8, 10, 12, 14, 16, 24, 26, 28, 30, 32, 34, 36, 38, 40, 48, and 52 h post-first dose). Any urine voided between collection times was collected and the exact time of collection was recorded. The samples were collected at room temperature and then placed at 4 °C until the end of the processing. Two aliquots of 15 ml (when possible) from each sample were collected, dispensed into sterile polypropylene screw cap tubes and stored at −20 °C until shipment to the INRS analytical laboratory.

Study 2 (CT-2)

The second study consisted of an open-label, one-way study of the urinary excretion profiles of PSE and CATH following the administration of Sudafed® decongestant 12-h 120 mg Extended-Release Caplet (McNeil Consumer Healthcare, Guelph, Canada). For this study, 30 healthy, adult subjects (15 male, 15 female) were enrolled, following a similar protocol for subject selection as in CT-1.

The administration regime was the same as treatment B of CT-1. However, urine samples were collected up to 108 h post first-dose (within 15 min prior to the first dose of study medication and at 2, 4, 6, 8, 12, 16, 24, 28, 32, 36, 40, 48, 52, 56, 60, 64, 72, 76, 80, 84, 88, 96, 100, 104, and 108 h after the first dose). Also, in contrast to study CT-1, the CT-2 urine samples corresponding to a given collection period were pooled into one single sample (when possible): from 15 min before to 15 min after the scheduled 2-h collection intervals, up to 30 min following 4-h intervals and up to 1 h after the scheduled 8-h periods. For the 108-h post first-dose time point, a single urine sample was collected. For pooled samples, individual times of urine collection were recorded.

After collection, samples were stored and transported to the INRS laboratory following the same procedure utilized in the CT-1.

Sample analysis

Chemicals and reagents

PSE.HCl (two different lot numbers) and diphenylamine (DPA) were purchased from Sigma-Aldrich (Oakville, ON, Canada). CATH.HCl was supplied by National Measurement Institute (Sidney, Australia) and Heumann Pharma (Nürnberg, Germany). Etafedrine.HCl was a gift from Hoechst Marion Roussel (Laval, QC, Canada). Methanol (HPLC grade), tert-butyl methyl ether (TBME, distilled in glass), ethyl acetate (HPLC grade), hexane (distilled in glass) were purchases from Caledon (Georgetown, ON, Canada). Sodium chloride (NaCl) and anhydrous sodium sulfate were supplied by VWR Canlab (Ville Mont-Royal, QC, Canada) and potassium hydroxide (KOH) pellets were from Fisher Scientific (Whitby, ON, Canada).

Samples from CT-1

Calibration curve and quality control (QC) samples

Methanolic standard stock solutions were prepared at 0.1 mg/ml for CATH, 1 and 2 mg/ml for PSE, 0.1 mg/ml for etafedrine (ISTD for CATH quantitation), and 4 mg/ml for DPA (ISTD for PSE quantitation) by diluting each reference standard in methanol. The solutions were kept at −20 °C until use.

Calibration curves containing both PSE and CATH were prepared by adding precise amounts of methanolic standard stock solutions to 10-ml blank urine sample to obtain calibrators spiked at 5, 10, 25, 50, 100, 250 and 500 µg/ml of PSE and 0.3, 0.5, 1, 2.5, 5, and 10 µg/ml of CATH. Methanolic volumes exceeding 5% of total volume were evaporated under nitrogen at room temperature before adding the blank urine.

Three levels of QC samples were prepared as described for the calibration curve by using reference materials for PSE (a different batch from Sigma-Aldrich) and CATH (Heumann Pharma) different from the ones employed for preparation of the calibration curves. QCs were spiked at 25, 100, and 300 µg/ml of PSE and at 0.5, 2.5, and 7.5 µg/ml of CATH.

Sample preparation

Each batch of samples (typically two subjects per batch) was prepared along with a blank urine sample, the calibrators of the calibration curve and quality control (QC) samples. To 2 ml of urine, 50 µl of DPA (4 mg/ml) and 20 µl of etafedrine (100 ng/µl) were added. The samples were prepared by adding 200 µl of KOH 5N, approximately 1.2g of NaCl (to saturation of the sample) and 2 ml of TBME, mixed for 30 s and centrifuged for 10 min. One millilitre of the organic layer was transferred into a test tube and evaporated to dryness under nitrogen at 40 °C. The residue was reconstituted in 100 µl of TBME for GC/NPD analysis.

Samples from CT-2

Calibration curve and QC samples

Methanolic standard stock solutions were prepared at 1 mg/ml for CATH, 10 mg/ml for PSE and 0.4 mg/ml for DPA (ISTD) by diluting each reference standard in methanol. The solutions were kept at −20 °C until use.

Calibration curves containing both PSE and CATH were prepared by adding precise amounts of methanolic standard stock solutions to 10 ml blank urine samples to obtain calibrators spiked at 10, 25, 50, 100, 250, 350, and 500 µg/ml of PSE and 3, 5, 8, 10, 15, and 20 µg/ml of CATH.

Three levels of QC samples were prepared as described for the calibration curves, but using reference materials of PSE and CATH from different suppliers. QCs were spiked at 25, 200, and 400 µg/ml of PSE and at 5, 10, and 18 µg/ml of CATH.

Sample preparation

Each batch of samples (typically two subjects per batch) was prepared along with a blank urine sample, the calibrators of the calibration curve and QC samples (duplicate). To 0.5 ml of urine, 25 µl of DPA (0.4 mg/ml) were added. The samples were prepared by adding 50 µl of KOH 5N, about 0.3g of NaCl (to saturation of the sample) and 1 ml of TBME, mixed for 30 s and centrifuged for 10 min. The organic layer was transferred into a 2-ml glass vial containing a thin layer of anhydrous sodium sulfate, capped and mixed for absorption of residual water.

Instrumental analysis

All the analyses were carried out with an Agilent 6890 GC/NPD system equipped with dual injector and detector. Samples were injected in split mode (split ratio 10/1, volume: 3 µl). Separation was achieved with DB-5 columns (15m × 0.25 mm i.d. × 0.25 µm film thickness, Agilent). Operating temperatures were as follow: injector −250 °C; oven from 100 °C (held for 1 min) at 10 °C/min up to 200 °C and at 20 °C/min up to 300 °C (held for 4 min); detector −325 °C. Detector flows were set to: hydrogen at 3.0 ml/min and air flow at 60 ml/min with electrometer offset adjusted to obtain an area of about 150–200 units for etafedrine (CT-1 samples) or 80–100 units for DPA (CT-2 samples). Helium was used as the gas carrier at a constant pressure of about 17 psi (adjusted for DPA retention time of 7.0 min). Limits of quantification (LOQ) were determined as 0.25 µg/ml for CATH and 1 µg/ml for PSE for CT-1 analysis and 3 µg/ml (CATH) and 10 µg/ml (PSE) for the measurement of CT-2 samples.

Calibration curve acceptance criteria were as follows: linear regression with correlation greater than 0.990 for both curves and QC measured values within 20% of theoretical values. QC analyses covering the quantitation range were within 90–95% of theoretical values. A blank urine sample was injected after the highest PSE calibrating point to verify the absence of carry-over in each batch.

Inter-assay QC variation

CT-1	CATH 0.5	CATH 2.5	CATH 5.0	PSE 25	PSE 100	PSE 300
mean (n = 16)	0.61	2.53	7.91	25.77	99.13	294.89
STD	0.03	0.13	0.55	1.47	4.35	11.94
CV (%)	4.9	5.3	6.9	5.7	4.4	4.0
Mean precision (%)	118.0	101.0	105.5	103.1	99.1	98.3

CT-2	CATH 5.0	CATH 10	CATH 18	PSE 25	PSE 200	PSE 400
mean (n = 60)	4.8	9.2	17.5	26.3	201.2	389.3
STD	0.3	0.4	0.5	1.3	8.7	8.4
CV (%)	6.2	4.2	3.0	4.9	4.3	2.2
Mean precision (%)	96.0	91.8	97.5	105.0	100.6	97.3

Pharmacokinetic and statistical methods

PSE urine pharmacokinetics was characterized using a non-compartmental analysis of the urine data after oral intake and performed by WinNonlin Professional software version 4.1 (Pharsight Corporation, Sunnyvale, CA, USA) obtaining the following parameters: Max_rate (h) or midpoint of collection interval associated with the maximum observed excretion rate, Max_rate (µg/h) or maximum observed excretion rate, Ae_{0_last} (mg) or recovery from 0 to last time rate, Ae_{∞} (mg) or recovery from 0 to infinity, Ae_{∞_ext} (%) or percent extrapolated from Ae_{0_last} to Ae_{∞}, and Ae (mg) or observed recovery. Cmax (µg/ml) or maximum concentrations observed, $Cmax_{POST}$ (µg/ml) or maximum concentrations observed after the last dose, Tmax (h) or time when the maximum PSE concentrations were attained, $Tmax_{POST}$ (h) or time when the maximum concentrations after the last dose were observed were also assessed. Dose fractions Fe_obs (%), Fe_obs_total (%) and Fe_{∞} (%) were calculated from the recovery of PSE, the recovery of PSE and CATH, and the recovery of PSE from 0 to infinity (Ae_{∞}/Dose), respectively.

The plasma elimination constant Kel (h^{-1}) and renal excretion constant Ke (h^{-1}) for PSE were calculated using the following equation:

$$dU/dt = ke \times Dose \times e^{-Kel \times t_{mid}} \quad (1)$$

Where *dU/dt* is the excretion rate and t_{mid} is the time midpoint of the collection intervals.

The metabolic constant Km (h^{-1}) was obtained from the difference of kel and ke rate constants and the metabolic fraction Fm (%) as the ratio of Km/Kel.

Descriptive and ANOVA statistical analyses were performed with SPSS Statistics v.17.0 package.

Results

CT-1

The mean urine concentration of PSE (µg/ml) vs time (h) data and pharmacokinetic parameters obtained in CT-1 are summarized in Figure 1 and Table 1. The absolute (considering all participating subjects) maximum and minimum concentrations of urine PSE observed during this study are also represented in Figure 1. The observed mean total PSE eliminated unchanged in urine (Fe_obs) was 58.42 ± 2.24% and 54.51 ± 2.46% after treatments A and B, respectively. These values increase to 73.99 ± 3.07% and 68.49 ± 2.80% (Fe_{∞}) when the excretion extrapolated to infinity (Ae_{∞}) was taken into account. In this study, the extrapolated percentage of recovery (Ae_{∞_ext}) from the last time observed value (Ae_{0_last}) to the infinity (Ae_{∞}) was less than 20%, showing a sufficient reliability in the extrapolation from the last observed time point to infinity. The maximum rates of PSE excretion (Max_rate) were 12.30 ± 0.28 mg/h and 11.36 ± 0.44 mg/h at 29.5 ± 2.30 h and 32.38 ± 1.73 h (Tmax_rate) with treatments A and B, respectively. The plasma elimination constant (Kel) and half-life ($t_{1/2\,z}$) calculated from urine in the treatment A group were 0.0611 ± 0.0039 h^{-1} and 4.78 ± 0.81 h, whereas

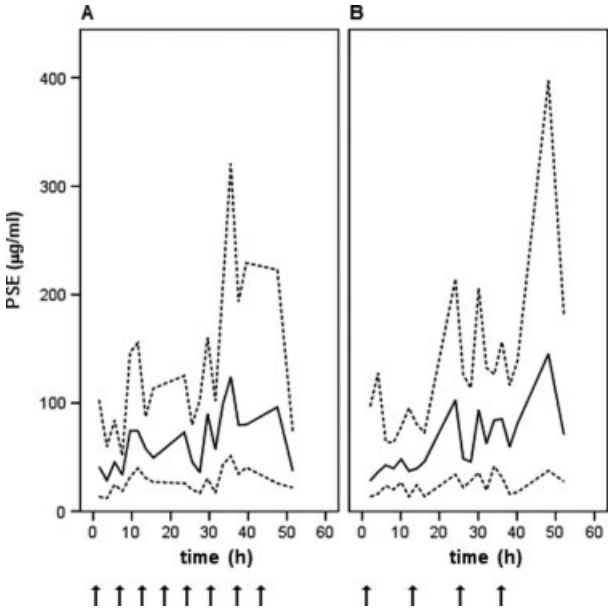

Figure 1. Urinary PSE mean (solid line), absolute maximum and absolute minimum (dotted lines) concentrations (µg/ml) observed after treatment A (left figure) and B (right figure) in the CT-1 study. Arrows indicate the time of administration (h) of 60 mg (treatment A) or 120 mg (treatment B) of PSE.

statistically different values were obtained in the treatment B group ($0.0449 \pm 0.0027\ h^{-1}$ and 7.66 ± 1.05 h; $p<0.05$). The urinary excretion constant (Ke) obtained after treatment A is slightly smaller than after treatment B ($0.0467 \pm 0.003\ h^{-1}$ vs $0.0480 \pm 0.018\ h^{-1}$; $p<0.05$) and no relevant differences were observed in the metabolized fraction (Fm) and rate of metabolism (Km). Slight but significant gender differences were seen in the amount excreted from 0 to the last measured urine concentration Ae_{0-last} (337.43 ± 14.26 mg in females *vs* 300.95 ± 12.00 mg in males; $p<0.05$) and in the metabolized fraction Fm (24.80 ± 2.95% in females *vs* 33.51 ± 2.43 in males; $p<0.05$).

The mean PSE Cmax drug concentrations in urine after PSE administration were 170.42 ± 12.98 µg/ml (range 96.4 to 313.4 µg/ml) and 173.96 ± 18.85 µg/ml (range 83.4 to 389.9 µg/ml) with a Tmax median value of 36 h and 44 h after treatments A and B, respectively. The individual concentrations of PSE in urine (µg/ml) in males and females throughout the testing period after the administration of treatment A and B are shown in Figure 2. Urinary concentrations above 150 µg/ml were mainly observed between 20 and 52 h after the first dose administration in both treatments. Following the last administered dose at 36 h the final maximum peak urine concentration is represented by $Cmax_{POST}$, whereas $Tmax_{POST}$ is the time when this peak has been observed. $Cmax_{POST}$ values were greater after treatment B (152.58 ± 21.93 µg/ml vs 113.83 ± 14.31 µg/ml; $p<0.05$) and were observed at longer $Tmax_{POST}$ with median values of 12 h (8 h observed with the treatment A). At the last time point of sample collection (16 h after the last administered dose) two individual values with concentrations of PSE above 150 µg/ml were observed from female volunteers after treatment B (Figure 2).

The mean, absolute maximum and minimum urine concentrations of CATH (µg/ml) vs time (h) data and pharmacokinetic parameters obtained in CT-1 are summarized in Figure 3 and Table 1. The observed mean CATH eliminated in urine (Fe_{obs}) was 1.94 ± 0.20% and 1.93 ± 2.17% of the dose after treatments A and B, respectively. The individual urine CATH concentrations (µg/ml) in males and females after the administration of treatment A and B in the CT-1 are shown in Figure 4. Values above the WADA-established cut-off level of 5 µg/ml for CATH are mainly located between 20 and 52 h after the first administered dose. At the last time point of sample collection (16 h after the last administered dose) five individual values are above the cut-off established for CATH, corresponding mostly to treatment B.

No gender differences and no period effect were observed in the statistical analysis of the pharmacokinetic parameters obtained in CT-1 except for the gender differences observed in Ae_{0-last} and Fm as mentioned above.

CT-2

The mean, absolute maximum and minimum urine concentrations of PSE (µg/ml) vs time (h) data and pharmacokinetic parameters obtained in CT-2 are summarized in Figure 5 and Table 2. The observed mean total PSE eliminated unchanged in urine (Feobs) was 61.85 ± 1.86%. Taken into account the extrapolated percentage of drug excreted from the last value of excreted dose (Ae_{0_last}) to the infinity (Ae_{∞_ext}) this value increases to 65.14 ± 2.64% (Fe_{∞}), which is in accordance with the values obtained in the CT-1 study with the formulation B. The maximum rate of PSE excretion (Max_rate) was 31.49 ± 22.57 mg/h at 25.9 ± 2.57 h (Tmax_rate). The plasma elimination constant (Kel = $0.0573 \pm 0.0021\ h^{-1}$) and half life ($t_{1/2\,z}$ = 7.43 ± 0.84 h) calculated from urine were in accordance with the values obtained in CT-1. Mean values obtained for the urinary excretion constant (Ke), rate of metabolism (Km) and fraction metabolized (Fm) show no relevant differences with those calculated in the CT-1 ($0.0385\ h^{-1}$, $0.0188\ h^{-1}$ and 32.82%, respectively). No gender differences were seen in the amount excreted from 0 to the last measured urine concentration Ae_{0_last} (311.15 ± 16.18 mg in females and 313.60 ± 8.07 mg in males), and in the PSE metabolized fraction Fm (32.92 ± 3.01% and 32.70 ± 1.70% in females and males, respectively).

The mean PSE Cmax values in urine after PSE administration in the CT-2 study were 99.52 ± 5.61 µg/ml at a median Tmax value of 30 h. The mean Cmax PSE urine concentrations (µg/ml) in males and females throughout the testing period are shown in Figure 5. Urine mean Cmax concentrations above 150 µg/ml were mainly observed in females between 46 and 56 h after the first dose administration. Individual values plotted after the last PSE administration at 36 h (Figure 6) show that all the urine PSE concentration values in males were below this cut-off while in the female group, one concentration value was above this cut-off at $Tmax_{POST}$ 48 h (i.e. 12 h after the last dose).

The pharmacokinetic parameters of CATH obtained in CT-2 are summarized in Table 2. The observed mean CATH eliminated in urine (Feobs) and percent of drug excreted in urine were less than the corresponding values observed in the CT-1 with the same administered dose and formulation. Cmax and $Cmax_{POST}$ concentrations were also less when compared to treatment B of the CT-1 study and most of the samples were below the cut-off established for CATH.

No gender differences were observed in the statistical analysis of CT-2.

Discussion

In the present paper, two PSE administration studies were conducted in normal individuals with the purpose of establishing

Table 1. Pharmacokinetic parameters of PSE and CATH after the 1 × 60 mg, four times daily for two days (treatment A) or after 1 × 120 mg, twice daily for two days (treatment B) obtained in the clinical trial CT-1

		A mean	es	IC95%	B mean	es	IC95%
PSE							
Tmax_rate	h	29.5	2.30	23.75–35.25	32.38	1.73	28.70–36.06
Max_rate	mg/h	12.30	0.28	11.71–12.89	11.36	0.44	10.41–12.30
$t_{1/2\,z}$	h	4.78	0.81	3.04–6.52	7.66*	1.05	5.41–9.90
Cmax	µg/ml	170.42	12.98	142.76–198.09	173.96	18.85	133.79–214.14
Tmax	h	36			44		
Cmax $_{POST}$	µg/ml	113.83	14.31	83.31–144.35	152.58*	21.93	105.83–199.34
Tmax $_{POST}$	h	8			12		
Ae	mg	278.12	11.86	252.78–30.45	264.03*	1.092	240.76–287.30
Ae_{∞}	mg	355.16	147.44	323.74–386.59	329.97	130.06	302.12–357.81
Ae_{∞_ext}	%	3.59	0.89	1.67–5.51	9.75	1.73	6.05–13.44
Ae_{0_last} **	mg	342.43	147.13	311.09–373.79	295.95*	101.66	274.28–317.62
Fe_obs	%	58.42	2.24	53.64–63.20	54.51*	2.46	49.26–59.75
Feobs_tot	%	60.38	2.29	55.49–65.27	56.44	2.51	56.44–61.79
Fe_{∞}	%	73.99	3.07	67.44–80.54	68.49	2.80	62.53–74.46
Fm **	%	26.00	3.07	19.45–32.56	32.29	2.52	26.91–37.66
Kel	h^{-1}	0.0622	0.0039	0.054–0.070	0.0449*	0.0027	0.039–0.051
Ke	h^{-1}	0.0467	0.0029	0.041–0.053	0.0480*	0.0185	0.009–0.088
Km	h^{-1}	0.0167	0.0023	0.012–0.022	0,0152	0,0019	0,011–0.019
CATH							
Cmax	µg/ml	6.73	1.04	4.51–8.95	6.75	0.76	5.13–8.36
Tmax	h	36			44		
Cmax $_{POST}$	µg/ml	5.46	1.06	3.20–7.73	5.55	0.86	3.71–7.40
Tmax $_{POST}$	h	12			12		
Ae	mg	9.50	0.87	7.64–11.37	9.56	0.87	7.70–11.42
Fe_obs	%	1.94	0.2	1.54–2.34	1.93	0.17	1.54–2.31

* = $p<0.05$ between treatments A and B, no period effect (ANOVA).
** = $P<0.05$ slight but statistical sex differences in Fm and Ae_{0_last}.
Tmax and Tmax POST are expressed as median values.
Tmax_rate (h): Midpoint of collection interval associated with the maximum observed excretion rate.
Max_Rate (µg/h): maximum observed excretion rate.
Ae_{0_last} (mg): recovery from 0 to last time rate calculated from the area under the urinary excretion rate curve from time 0 to the last rate.
$t_{1/2\,z}$ (h): terminal half-life calculated from the first order constant associated with the terminal (log-linear) portion of the curve and estimated via lineal regression of time *vs* log concentration.
Ae_{∞} (mg): recovery from 0 to infinity calculated from area under the urinary excretion rate curve extrapolated to infinity.
Ae_{∞_ext} (AURC_% Extrap) (%): percent extrapolated from last area under the urinary excretion rate to Ae_{∞}.
Cmax (µg/ml): maximum urine PSE concentrations observed.
Cmax $_{POST}$ (µg/ml): maximum urine PSE concentrations observed after the last dose.
Tmax (h): time when the maximum PSE concentrations was observed.
Tmax $_{POST}$ (h): time when the maximum PSE concentrations was observed after the last dose.
Ae (mg): observed recovery of PSE or CATH.
Fe_obs (%): fraction observed of PSE or CATH relative to the dose of PSE recovered in urine.
Feobs_tot(%): dose fraction observed of PSE and CATH recovered in urine.
Fe_{∞} (%): dose fraction of unaltered PSE excreted in urine from 0 to infinity calculated from Ae_{∞} /Dose.
Kel (h^{-1}): elimination rate constant of PSE calculated from the slope terminal (log-linear) portion of the curve of the rate of excretion vs mid point time.
Ke (h^{-1}): urinary excretion rate constant of PSE obtained from the intercept of the linear regression curve of the rate of excretion vs mid point time.
Km ((h^{-1}). metabolic constant of PSE calculated as the difference between Kel and Ke rate constants.
Fm (%): fraction of PSE metabolized calculated as Km/Kel.

analytical urinary cut-off values to distinguish therapeutic from non-therapeutic use of this stimulant in sport.

In line with previous findings described in the literature,[6,13–15] these studies have shown large inter-individual differences in the excreted urinary concentrations of PSE and CATH at any sample collection time point, irrespective of the PSE administration treatment followed (Tables 1 and 2, Figures 2, 4, and 6). Moreover, there was no correlation between the levels of urinary concentrations of PSE and CATH observed. The individual urinary Cmax for PSE and CATH determined for different study subjects were reached at different time points in the course of the administration regimen. Therefore, in order to establish a unique analytical cut-off to reflect the maximum concentrations of PSE excreted in urine following a therapeutic administration of PSE-containing medications, and taking into account that stimulants on the Prohibited List are banned during the competition period only (in-competition), the time elapsed since the last PSE administration ought to be considered.

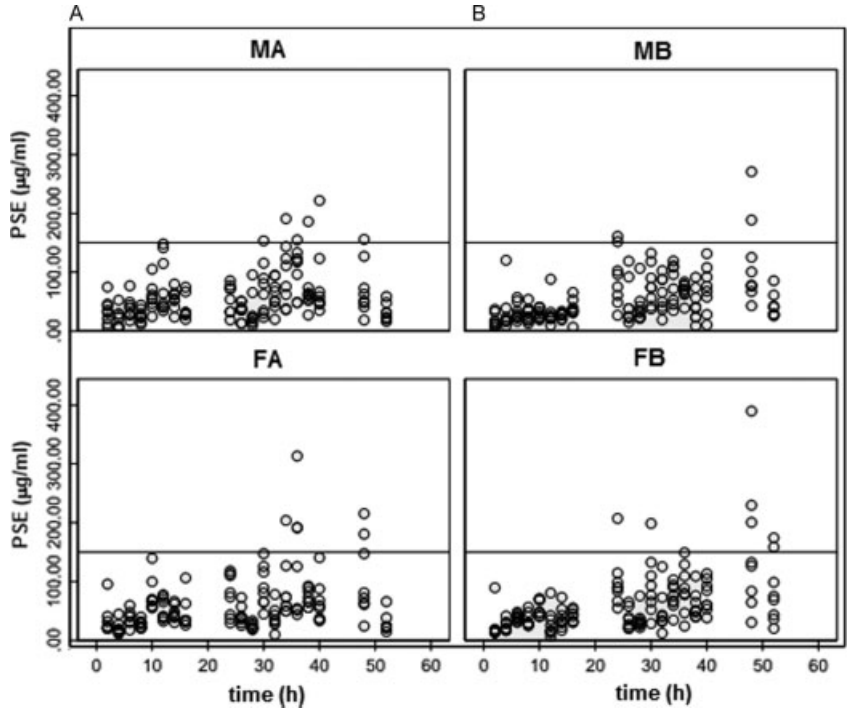

Figure 2. Individual values of urine PSE concentration (µg/ml) in males (M, upper panels) and females (F, lower panels) after administration treatments A (left panels) or B (right panels) in the CT-1. The cut-off concentration at 150 µg/ml is shown.

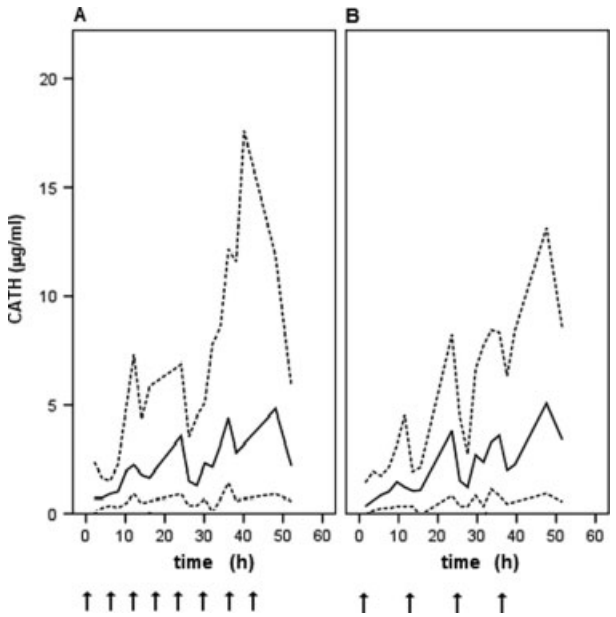

Figure 3. Urinary CATH mean (solid line), absolute maximum and absolute minimum (dotted lines) concentrations (µg/ml) observed after treatment A (left figure) and B (right figure) in the CT-1 study. Arrows indicate the time of administration (h) of 60 mg (treatment A) or 120 mg (treatment B) of PSE.

In CT-1, two different therapeutic regimes for PSE cold medications were followed in a cross-over study design: the administration of either a short-lived, 60 mg PSE preparation every 4–6 h (treatment A), or a long-release 120 mg PSE formulation every 12 h (treatment B). In each case, the drug was administered in multiple doses during two days, and the maximum daily dose was at the therapeutically recommended amount (240 mg). There was a cumulative effect of multiple dosing on the urinary concentrations of PSE and CATH during the course of the treatments. The $Cmax_{POST}$ for treatment B was higher than for treatment A, and the time to reach this peak concentration ($Tmax_{POST}$) was longer (12 h and 8 h for treatments A and B, respectively). The differences in the pharmacokinetic parameters between the A and B treatments are in accordance with the expected variations between immediate- and slow-release formulations: a decrease in the PSE elimination constant (Kel) and an increase of half-life ($t_{1/2\,z}$) and median Tmax values after the administration of a long-release preparation.

Importantly, at the last sample collection time for CT-1 (16 h post last dosage), the urinary concentrations of PSE and CATH were still quite significantly high. For some individuals, those values exceeded the established anti-doping threshold for CATH at 5 µg/ml, whereas for PSE they could be as high as 158–174 µg/ml (after treatment B). Therefore, a follow-up study was needed in which samples would be collected long after the end of the dosing regimen in order to ensure that the full excretion of the administered PSE and its minor metabolite CATH was observed. Consequently, an analytical threshold for the athletic population could be established considering not only the maximum individual urinary concentrations of PSE and CATH ($Cmax_{POST}$), but also the $Tmax_{POST}$ at which these concentrations were achieved. That is to say, the decision criterion for determining an anti-doping Adverse Analytical Finding for PSE (and its related metabolite CATH) in-competition would have to include both a concentration threshold and a minimum elapsed time period since the last PSE administration.

The CT-2 study was designed to take the findings of the CT-1 trial into consideration. The CT-2 followed the same administration protocol as the CT-1 treatment B, in which the long-lasting, PSE-containing formulation was used. This would ensure that a higher

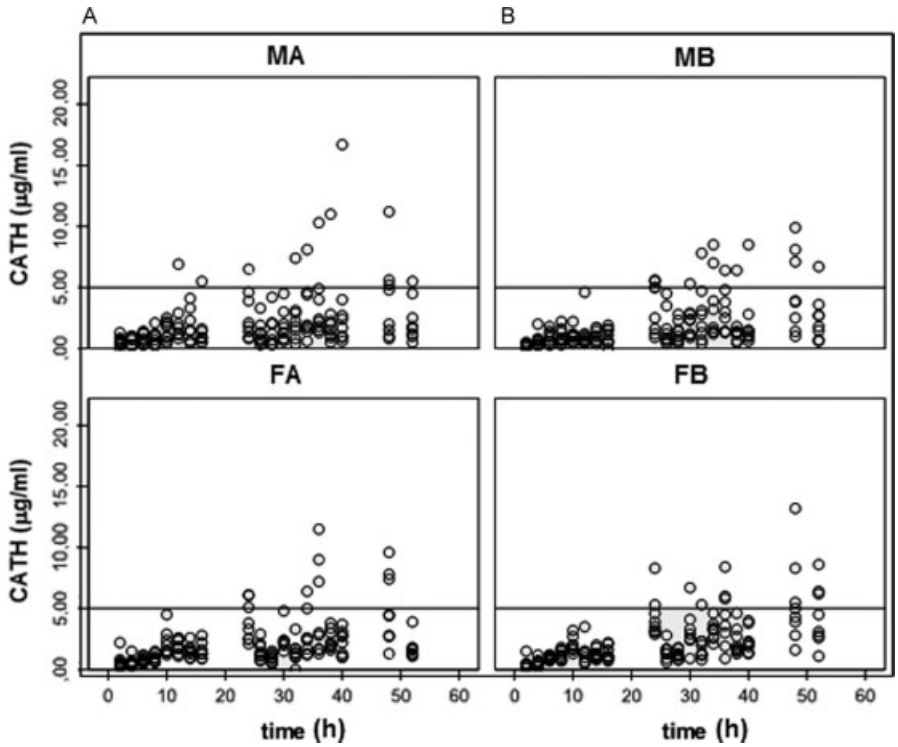

Figure 4. Individual values of urinary CATH concentrations (µg/ml) in males (M, upper panels) and females (F, lower panels) after administration treatments A (left) and B (right) in the CT-1. The cut-off concentration at 5 µg/ml is shown.

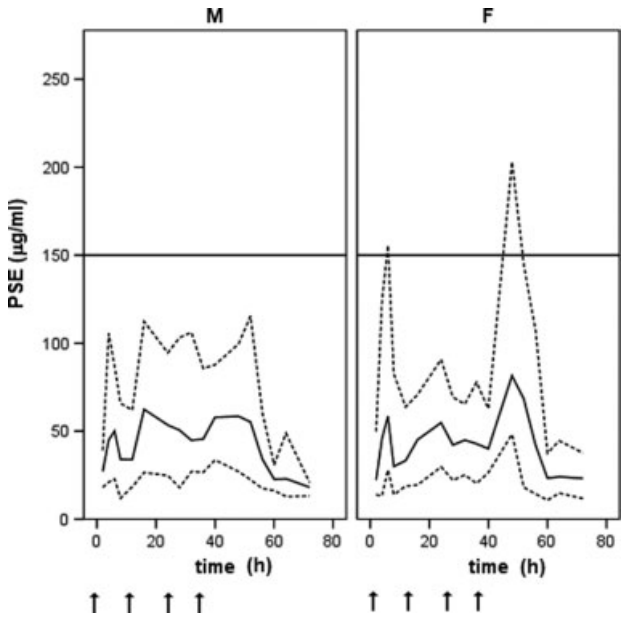

Figure 5. Urinary PSE mean (solid line), absolute maximum and absolute minimum (dotted lines) concentrations (µg/ml) observed in male (M, left panel) and female (F, right panel) volunteers after PSE administration in the CT-2. The cut- off value at 150 µg/ml is represented.

Cmax and longer PSE excretion kinetics would be considered and therefore establish a more conservative criterion for anti-doping compliance decisions.

There were, however, some differences between these two studies related to the sample collection procedure and the analytical protocols applied. Whereas in CT-1 each urine sample was collected and analyzed individually, in CT-2 the samples corresponding to a given collection interval for an individual were pooled into one single specimen. This modality of sample collection in the CT-2 study has shown an impact on the urine concentration values. The observed Cmax and $Cmax_{POST}$ concentration values for PSE and CATH were lower than those observed in the treatment B of the CT-1 study. Additionally, the Tmax value was observed earlier in the CT-2 study (30 h *vs* 44 h). Despite this, a similar pharmacokinetic behaviour in terms of 95% confidence interval of the pharmacokinetic parameters describing the fraction of the dose excreted in urine, rates of metabolism, excretion, and elimination, and half-life has been obtained.

These results suggest that both PSE and CATH urine concentrations can be different depending on the sampling and sample collection schedule. When comparing PSE and CATH urine concentrations obtained in the CT-1 and CT-2 studies, lower concentration values were observed in the latter study. Additionally, higher volumes of excreted urine (especially at 24 and 48 h) were collected in the CT-2 study (mean ± sd: 250.3 ± 164.9 ml *vs* 1008.0 ± 306.4 ml $p<0.001$ and 244.6 ± 194.9 ml *vs* 773.8 ± 292.4 ml $p<0.001$, respectively). Despite these observations, the pharmacokinetic parameters in CT-1 are in accordance with those obtained in the CT-2, which suggests that the pooled urine collections made in the CT-2 study did not significantly modify the interpretation of the pharmacokinetics of PSE and CATH.

In addition to the differences in the sample collection protocols, in the analysis of the CT-1 samples by GC/NPD, the extracted methanolic fraction was evaporated to dryness and reconstituted in a small volume of TBME (100 µl). This ensured that in most sample aliquots analyzed, the small proportion of PSE metabolized and excreted as CATH could be detected in concentration values that were above the assay's LOQ (0.25 µg/ml and 1 µg/ml for CATH

Table 2. Pharmacokinetic parameters of PSE and CATH after the intake of 1 × 120 mg, twice daily for two days obtained in the clinical trial CT-2

		mean	es	IC95%
PSE				
Tmax_rate	h	25.9	2.57	20.64–31.15
Max_rate	mg/h	31.49	22.62	nd
$t_{1/2\,z}$	h	7.43	0.84	5.71–9.15
Cmax	µg/ml	99.52	5.61	88.04–101.99
Tmax	h	30		
$Cmax_{POST}$	µg/ml	84.63	6.27	71.80–97.45
$Tmax_{POST}$	h	12		
Ae	mg	296.87	8.93	278.61–315.13
Ae_{∞}	mg	322.41	8.28	305.58–339.24
Ae_{∞_ext}	%	4.20	1.17	1.81–6.59
Ae_{0-last}	mg	312.36	9.16	293.63–331.11
Fe_obs	%	61.85	1.86	58.04–65.65
Feobs_tot	%	62.29	2.89	58.43–66.15
Fe_{∞}	%	65.14	2.64	59.73–71.55
Fm	%	32.83	1.71	29.32–36.34
Kel	h^{-1}	0.0573	0.0021	0.0530–0.0616
Ke	h^{-1}	0.0385	0.0015	0.0355–0.0417
Km	h^{-1}	0.0188	0.0013	0.0160–0.0215
CATH				
Cmax	µg/ml	2.49	0.64	1.18–3.80
Tmax	h	nd	nd	nd
$Cmax_{POST}$	µg/ml	2.36	0.64	1.05–3.67
$Tmax_{POST}$	h	nd	nd	nd
Ae	mg	2.14	0.80	0.52–3.76
Fe_obs	%	0.45	0.16	0.11–0.78

Tmax and Tmax POST are expressed as median values.
nd = not determined.
Tmax_rate (h): Midpoint of collection interval associated with the maximum observed excretion rate.
Max_Rate (µg/h): maximum observed excretion rate.
Ae_{0_last} (mg): recovery from 0 to last time rate calculated from the area under the urinary excretion rate curve from time 0 to the last rate.
$t_{1/2\,z}$ (h): terminal half-life calculated from the first order constant associated with the terminal (log-linear) portion of the curve and estimated via lineal regression of time vs. log concentration.
Ae_{∞} (mg): recovery from 0 to infinity calculated from area under the urinary excretion rate curve extrapolated to infinity.
Ae_{∞_ext} (AURC_% Extrap) (%): percent extrapolated from last area under the urinary excretion rate to Ae_{∞}.
Cmax (µg/ml): maximum urine PSE concentrations observed.
$Cmax_{POST}$ (µg/ml): maximum urine PSE concentrations observed after the last dose.
Tmax (h): time when the maximum PSE concentrations was observed.
$Tmax_{POST}$ (h): time when the maximum PSE concentrations was observed after the last dose.
Ae (mg): observed recovery of PSE or CATH.
Fe_obs (%): fraction observed of PSE or CATH relative to the dose of PSE recovered in urine.
Feobs_tot(%): dose fraction observed of PSE and CATH recovered in urine.
Fe_{∞} (%): dose fraction of unaltered PSE excreted in urine from 0 to infinity calculated from Ae_{∞} /Dose.
Kel (h^{-1}): elimination rate constant of PSE calculated from the slope terminal (log-linear) portion of the curve of the rate of excretion vs mid point time.
Ke (h^{-1}): urinary excretion rate constant of PSE obtained from the intercept of the linear regression curve of the rate of excretion vs mid point time.
Km ((h^{-1}). metabolic constant of PSE calculated as the difference between Kel and Ke rate constants.
Fm (%): fraction of PSE metabolized calculated as Km/Kel.

and PSE, respectively). In contrast, this additional concentration step was not performed in the quantification procedure of PSE and CATH in the CT-2 study, and many samples showed non-quantifiable CATH values below the assay's LOQ (3 µg/ml and 5 µg/ml for CATH and PSE, respectively). However, these differences in protocol were anticipated and do not affect the conclusions of the study, since the defined LOQ values of the assays applied for both CT-1 and CT-2 are lower than the established analytical cut-off for CATH (5 µg/ml) and the threshold values expected for PSE. Therefore, the two assays used to analyze the samples allowed the meaningful determination of PSE and CATH concentrations for the purpose of these studies.

The CT-2 study showed that 24 h after the last PSE dose (60 h since the first administration) no subject showed urinary PSE concentrations above 50 µg/ml (Figure 6, Cmax of 37.5 µg/ml) or CATH above 5 µg/ml (Cmax of 3.7 µg/ml, not shown). The last samples producing concentration values above these thresholds corresponded to T_{POST} at 56 h (20 h after the last administration): PSE values between 53.2 and 90.1 µg/ml in samples collected from five subjects (three male, two female) with one female volunteer producing 106.9 µg/ml of PSE; one male and two female subjects with urinary CATH values at 7.5, 5.8, and 12.0 µg/ml. The higher CATH value at 12.0 µg/ml corresponded to the same volunteer that produced the high PSE concentration at 106.9 µg/ml; the

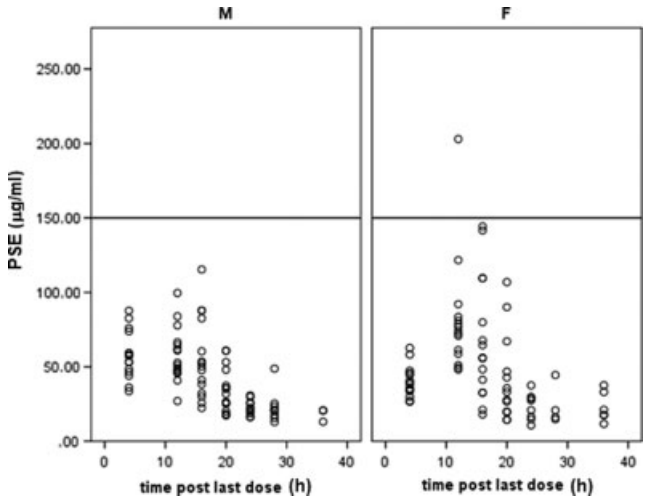

Figure 6. Individual urinary PSE concentrations (µg/ml) observed in male (M, left panel) and female (F, right panel) volunteers after the last administration in the CT-2. The cut-off value at 150 µg/ml is shown.

other two outlying CATH results corresponded to PSE values of 60.9 and 90.1 µg/ml, respectively. Concentrations of PSE above 150 µg/ml, on the other hand, were last observed for one female volunteer (the same one showing high PSE and CATH values at T_{POST} 56 h) 12 h post the last dose (T_{POST} 48 h).

Conclusions

Based on the results of these two clinical studies, the WADA List Expert Group has reintroduced PSE on the Prohibited List as a specified stimulant prohibited in-competition at a urinary threshold of 150 µg/ml. Given the wide availability of PSE-containing medicines, WADA has recommended that the reintroduction of PSE be supported by an active information/education campaign by all of its stakeholders.

There were several reasons for defining the cut-off value at 150 µg/ml. The objective was to establish the conditions under which PSE medications can be safely taken by the athletes for valid therapeutic use with no risk of returning an Adverse Analytical Finding in anti-doping tests, as well as preventing its abuse at supratherapeutic doses for enhancing athletic performance.

The high inter-individual variability of PSE excretion rates makes it almost impossible to establish a perfect cut-off value. Given the relatively low number of subjects recruited for these clinical trials ($n = 16$ for CT-1; $n = 30$ for CT-2), which limits the statistical power of any data analysis extrapolated to the general athletic population, a rather conservative approach was followed for the establishment of an anti-doping urinary threshold for PSE. The results of the CT-2 study showed that 150 µg/ml represents more than three times the maximum urinary concentration of PSE excreted by healthy individuals that have followed therapeutic PSE administration regimes up to 24 h before testing. However, a lower cut-off might not only target cheating athletes, but it could potentially result in Adverse Analytical Findings for athletes therapeutically using PSE-containing medications right before the competition period. On the other hand, a higher cut-off value would potentially miss athletes using PSE at higher doses for doping purposes. This threshold level aims at reflecting the dose - ergogenic effect relationship observed for PSE administrations. Nevertheless, WADA continues to monitor PSE data from doping control tests to further fine-tune the cut-off value, if necessary.

Since stimulants like PSE are prohibited in-competition only, WADA has further advised that athletes stop taking PSE medications at least 24 h before the competition. During the in-competition period, athletes must, in consultation with a physician, consider the use of alternative permitted medications or apply for a Therapeutic Use Exception (TUE) for the valid therapeutic application of PSE.

WADA has also clarified that the PSE analytical threshold has been established in accordance with a therapeutic regimen, taking into consideration both the dose and the frequency of PSE administration, and has advised that the uptake of a total daily dose at higher dosing frequencies than therapeutically recommended (for example, a single daily dose of $3-4 \times 60$ mg pills) constitutes a supratherapeutic administration that may lead to an anti-doping Adverse Analytical Finding.

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