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GENERAL

BUDESONIDE TREATMENT OF PROFESSIONAL ATHLETES AND ANTI-DOPING TESTING – CASE STUDIES

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Abstract: According to the World Anti-Doping Agency (WADA) Prohibited List, glucocorticosteroids are prohibited in competition and only when administered by oral, intravenous, intramuscular or rectal routes. Up to now, in order to differentiate whether glucocorticosteroids were administered by one of the prohibited routes or not, a specific reporting limit for urinary concentrations of parent compounds and their metabolites was established at 30 ng/mL. Additionally, the new specific regulation starting from 1 September 2014 for budesonide have been introduced that the 6β-hydroxybudesonide shall be targeted. Budesonide is a glucocorticosteroid used mainly by inhalation for asthma management. Interestingly, anti-doping laboratory statistics show that budesonide adverse analytical findings (AAF) constitute almost 50% of all reported glucocorticosteroid AAFs, even though budesonide possesses a very low systemic activity which may cause performance enhance effects. This work presents the results of five studies of controlled budesonide administration carried out on professional athletes. The samples were analyzed by using a quantitative HPLC/MS/MS method for 16α-hydroxyprednisolone, the most abundant budesonide metabolite in urine. Our data clearly show that inhalation of budesonide at least 12 h before a competition at therapeutic doses leads to appearance of the main budesonide metabolite in concentrations exceeding prior reporting limit for this compound. Therefore, our work strongly supports recent WADA decision not to target the main budesonide metabolite using the same reporting limit as for other glucocorticosteroids.

Keywords: doping, budesonide, sport, LC/MS/MS

Anti-doping research was initiated by Polish pharmacist Alfons Bukowski, who in 1910 developed a method to detect alkaloids in horse saliva (1). Since then, the area of anti doping-testing expanded a lot. One of the often discussed topics is asthma treatment in respect to doping control. Presented data below strongly support recent World Anti-Doping Agency (WADA) decision changing the way of detecting doping with budesonide.

Nowadays, the exercise-induced asthma and bronchial hyper responsiveness are very prevalent, particularly among those who compete in endurance sports. Most of them require glucocorticosteroid treatment in order to effectively compete with healthy individuals. On the other hand, glucocorticosteroids may enhance sport performance, when administered systemically at high doses by alleviating pain, causing euphoria or reducing fatigue perception (2). To address the conflict between permission for therapeutic use of glucocorticosteroids and prevention of their abuse for doping purposes, WADA prohibited use of glucocorticosteroids in competitions by oral, intravenous, intramuscular, or rectal routes only (3, 4). However, their uses through other routes, which are typical for asthma or rhinitis treatment, such as inhalation or intranasal are allowed accordingly. A specific reporting limit for urinary concentrations of glucocorticosteroid parent compounds and their metabolites was established at 30 ng/mL (5) as a tool to differentiate whether a given substance was administered by a prohibited or permitted route. In addition, WADA published

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recently the new specific regulation (starting from 1 September 2014) for budesonide that the 6β-hydroxybudesonide shall be targeted (6).

Budesonide is a substance belonging to the glucocorticosteroid class. It has high topical activity with reduced systemic side effects resulting from extensive hepatic metabolism to compounds of low biological activity such as 16α-hydroxyprednisolone, 6β-hydroxybudesonide, and many others (7). Budesonide metabolism is catalyzed mainly by the CYP3A4 and CYP3A5 monooxygenases belonging to the cytochrome P450 super family of enzymes. The activity of these enzymes with respect to a given compound may be affected by multiple factors, including genetic polymorphism, gender, age, and application of other xenobiotics (8). All of the mentioned variables may underlie the possible substantial inter individual differences in elimination rates of budesonide.

Budesonide is mainly used through inhalation in asthma management and through nasal administration in therapy of allergic rhinitis (9). Its oral application is limited to the treatment of inflammatory bowel disease (10), which is an example of a

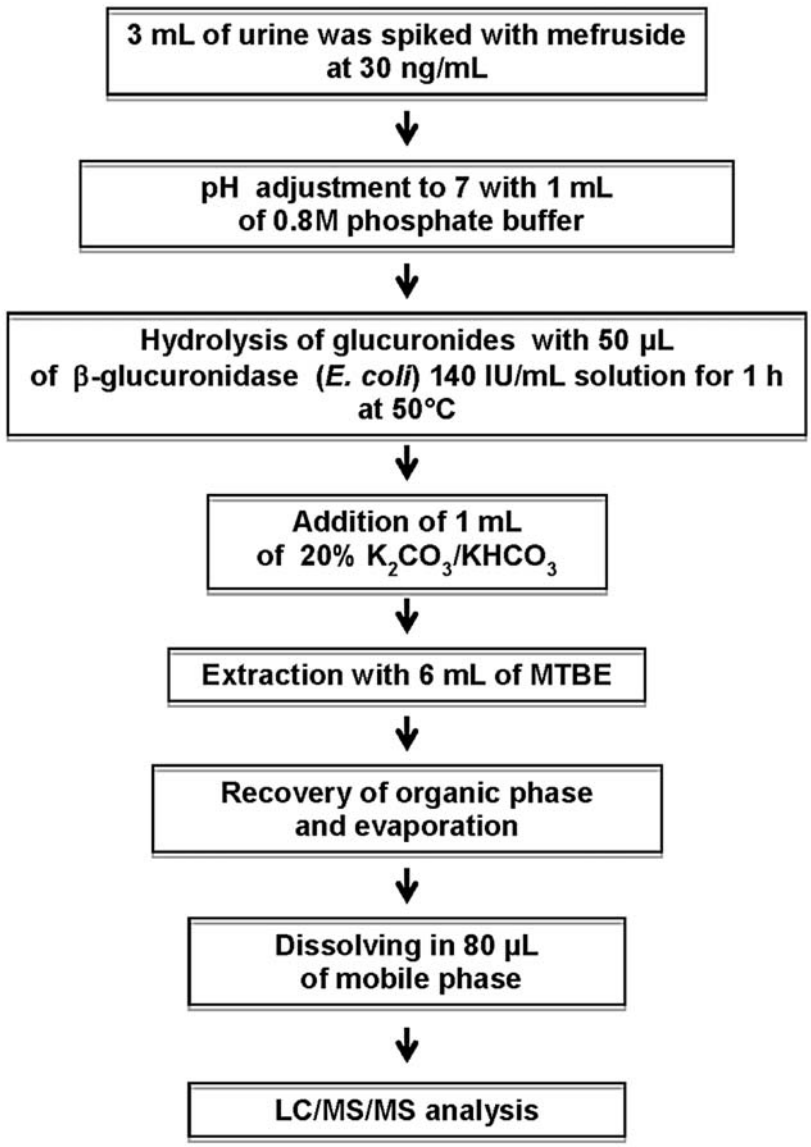

Figure 1. Schematic representation of sample preparation procedure

Table 1. Time-points [h] of sample collection during the controlled studies.

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Before drug administration	-	-	-	-	-13
	-15	-	-	-	-12
	-11	-	-	-	-11.5
	-1	-	-	-	-9.5
First controlled administration of the drug	0	-	0	-	0
After drug administration	5	3.5	2	3.5	1.5
	8	7	5	5.5	6
	9	11.5	15	11.5	7
	-	19	17.5	18.5	-
	-	26.5	21	23.5	
	-	-	-	24.5	-

therapy prohibited by WADA unless permission for its use for therapeutic purposes (called Therapeutic Use Exemption, TUE) is granted (11). However, it seems that there is no indication to grant TUE for budesonide use through permitted administration routes. Thus, before the new regulation was introduced, the detection of the parent compound or budesonide metabolites in urine at concentrations exceeding 30 ng/mL would have result in an anti-doping rule violation even though an athlete strictly followed WADA's recommendations. In recognition of this potential problem, WADA introduced a procedure of controlled administration study (CAS) designed in such a way that it reproduces conditions of post-competition sample collection as closely as possible, including dosage and the extent of physical activity (12). Urine samples are obtained at different time points (before and after administration) and concentrations of the substance and/or its metabolites are monitored. If the results of such a study clearly indicate that the use of budesonide in therapeutic doses through a non-prohibited route can produce adverse analytical findings for a given athlete, the allegations of doping are dismissed.

This work presents for the first time the results of controlled administration studies performed on professional athletes. Our data clearly demonstrate that the administration of budesonide through permitted routes may lead to appearance in urine of 16α-hydroxyprednisolone in concentration exceeding its previous reporting limit. It also strongly supports the WADA's policy of an individual approach to such cases, based on CAS procedures.

EXPERIMENTAL

Chemicals and reagents

The standard of 16α-hydroxyprednisolone was purchased from Toronto Research Chemicals (Canada), whereas mefruside was obtained from Bayer (Germany). β-Glucuronidase *E. coli* was purchased from Roche (Germany). Potassium carbonate and potassium hydrogen carbonate were obtained from POCH (Poland). LC/MS-grade acetonitrile was purchased from Merck Millipore (Germany). Formic acid was from J.T. Baker (Netherlands), whereas methyl tert-butyl ether (MTBE) was purchased from Rathburn (Scotland). The Millipore DirectQ UV3 system (R >18 MΩ/cm, Germany) was used as the source of water.

Stock solutions of standard substances were prepared at the concentration of 1 mg/mL in methanol and stored at -20°C. Relevant working solutions were prepared in methanol and were stored at 4°C.

Urine specimens

Urine samples were collected from five athletes during the controlled excretion studies at different time-points up to 9 h post-administration in accordance with Annex 2 of WADA Medical Info Asthma 5.0 (12). The study was supervised by the staff of the National Anti-Doping Organization (NADO) Polish Commission Against Doping in Sport. All subjects provided their written consent. The athletes took the drug in accordance with the treatment course (dose, frequency, route of adminis-

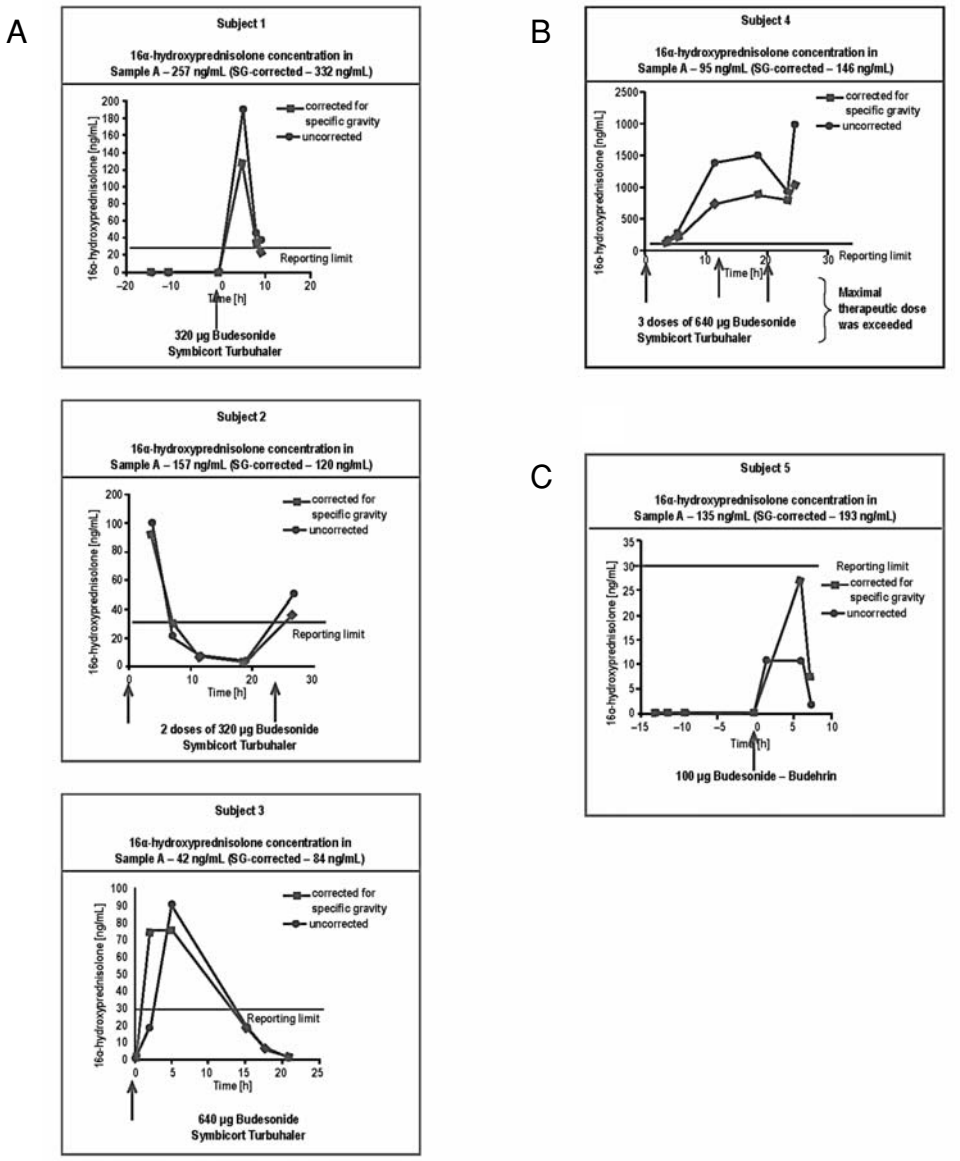

Figure 2. Experimental data obtained for controlled studies. Urinary excretion profiles of 16α-hydroxyprednisolone after administration of budesonide: by inhalation (A), inhalation and maybe oral route (B) and intranasal (C). Concentrations of 16α-hydroxyprednisolone measured in the respective "A" samples collected during in-competition doping control are provided at the top of each graph

Table 2. LC gradient employed in the study.

Time [min]	Solvent A [%]	Solvent B [%]	Solvent C [%]	Curve
0.00	40	60	0	initial
2.00	40	60	0	constant
11.50	5	95	0	linear
12.50	5	5	90	linear
16.75	5	5	90	constant
18.00	5	95	0	linear

tration) declared in their respective doping control forms. The samples before the controlled drug administration were collected only if the cessation of treatment course for at least 24 h was allowed by a medical doctor (Table 1). Moreover, the studies were conducted in a controlled setting allowing strict and independent supervision of the drug administration (route, dose, frequency, etc.) and sample collection (protocol volume, frequency). Four athletes inhaled Symbicort Turbuhaler (AstraZeneca); Subject 1 (athletics) took a single dose of 320 μg of budesonide, Subject 2 (volleyball) inhaled two doses of 320 μg with a 22 h interval, Subject 3 (cross country skiing) took two doses of 320 μg at once (Fig. 2A). Subject 4 (cross country skiing) inhaled 640 μg of budesonide (two doses of 320 μg) three times with intervals of 10 h and 9 h, respectively (1920 μg in total; Fig. 2B). Subject 5 (cycling) received 100 μg of budesonide by using the Buderhin nasal spray (GlaxoSmithKline Pharmaceuticals SA, Poland; Figure 2C). Additionally, the athletes exercised during the study to reproduce the conditions of post-competition sample collection.

Sample preparation

Urine samples were prepared as follows: 3 mL of urine was spiked with mefruside at 303 ng/mL (internal standard), and the pH was adjusted to 7 with 1 mL of 0.8 M phosphate buffer. Hydrolysis was carried out with β-glucuronidase *E. coli* (50 μL) at 50°C for 1 h and was followed by the addition of 1 mL of 20% K_2CO_3/$KHCO_3$ buffer and 6 mL of MTBE. The organic phase was then evaporated and the dry residue was reconstituted in 80 μL of mobile phase (40% of water and 60% of acetonitrile) (Fig. 1). Calibrators containing 16α-hydroxyprednisolone at concentrations of 10, 30, 60, 120, 240, 360 ng/mL with mefruside as the internal standard were prepared using blank urine (pooled).

Chromatographic separation

Analytes were separated on a Waters Alliance 2695 system equipped with a Thermo Hypercarb column (100 × 2.1 mm, 5 μm) and a Thermo Hypercarb guard column (10 × 2.1 mm, 5 μm). The mobile phase consisted of 0.5% acetic acid in water (A), 0.5% acetic acid in acetonitrile (B), and 0.5% acetic acid in isopropanol (C). A stepwise LC gradient was employed at a constant flow rate of 400 μL/min at 58°C (Table 2). Samples were stored at 10°C in the autosampler prior to analysis and the injection volume was fixed at 10 μL.

Mass spectrometry conditions

Substances of interest were analyzed in a multiple reaction monitoring (MRM) mode with a Micromass Quattro Micro API mass spectrometer (Waters, USA) equipped in an ESI source. The desolvation gas flow was set at 600 L/h at the temperature of 350°C and the source temperature was 120°C. The cone flow was set at 40 L/h. The capillary voltage was fixed at 3.20 kV. The analytes were traced in a positive mode with the following selected precursor ion-product ion transitions at their respective collision energies (CE) and cone voltage (CV) settings: 16α-hydroxyprednisolone: CV 26 V, MRMs: 377.20 > 146.90 (quantitation MRM; CE 20 eV), 377.20 > 225.07 (CE 15 eV), 377.20 > 323.27 (10 eV), 377.20 > 359.13 (10 eV); mefruside: CV 25 V; MRM: 382.97 > 129.05 (CE 20 eV).

Method validation

The method was validated for selectivity, linearity, accuracy, precision, recovery, matrix effect according to the European Medicines Agency's guideline on bioanalytical method validation (13).

Selectivity

Selectivity of the method was assessed by the analysis of 10 blank urine samples. Evaluation of chromatograms recorded for three selected precursor ion-product ion transitions at the retention times of 16α-hydroxyprednisolone (±1.0 min) were evaluated.

Extraction recovery

To evaluate extraction recovery (ER), urine samples were fortified with 16α-hydroxyprednisolone at the concentration of 15 ng/mL and extracted together with one blank sample for each urine sample. The latter samples were then spiked with the analyte at the concentration of 15 ng/mL immediately prior to evaporation. The ER parameter was calculated for 8 different urine samples by the comparison of peak areas obtained for samples fortified before and after extraction.

Calibration curves

The calibration curves were constructed by plotting the peak-area ratios of 16α-hydroxyprednisolone to internal standard (IS) *vs.* concentrations using least square fit regression model.

Precision and accuracy

The precision and accuracy of the method were tested at three levels 10, 30, 120 ng/mL in five repetitions. Three independent runs were performed on

three different days. Repeatability (within-run) and reproducibility (between-run) were expressed as coefficient of variation (CV). Within-run and between-run accuracies were defined as deviation of the mean measured concentration from the theoretical concentration for all compounds.

Limit of quantitation and limit of detection

The limit of quantification (LOQ) of the method was defined as the lowest concentration where acceptable reproducibility and accuracy could be guaranteed. The limit of detection was defined arbitrarily as 1/2 LOQ.

Matrix effect

For the matrix effect evaluation, the matrix factor was calculated by the ratio of the peak area from the six different urine samples spiked after extraction, to the peak area of the standard solutions in the mobile phase at concentrations of 20 and 300 ng/mL. The IS normalized matrix factor was calculated by dividing the matrix factor of the 16α-hydroxyprednisolone by the matrix factor of the IS, (mefruside) for each sample.

RESULTS AND DISCUSSION

The quantitative method to measure 16α-hydroxyprednisolone was developed by means of HPLC/MS/MS and validated according to European Medicines Agency's guideline on bioanalytical method validation (13).

Method validation

Selectivity

Evaluation of chromatograms recorded for three selected precursor ion-product ion transitions at the retention time of 16α-hydroxyprednisolone (±1.0 min) showed the absence of any interfering components. It is also important to note that the sample preparation protocol was developed based on a highly selective screening procedure for 16α-hydroxyprednisolone that had been used for analysis of at least a few thousand urine samples.

Extraction recovery

Average extraction recovery for 16α-hydroxyprednisolone was 41.2% with standard deviation of 3.8 and was deemed satisfactory.

Table 3. Precision and accuracy established for 16α-hydroxyprednisolone.

Analyte	Concentration [ng/mL]	Within run (n = 5)		Between run (n = 15)	
		Accuracy [%]	CV [%]	Accuracy [%]	CV [%]
16α-Hydroxyprednisolone	10	6.2	15.5	0.4	10.3
	30	6.6	8.3	3.4	8.2
	120	4.7	4.3	6.6	5.4

Table 4. Glucocorticosteroids identified by WADA accredited laboratories in the years 2012 and 2013.

Substance	Year 2012 [% within drug class]	Year 2013 [% within drug class]
Budesonide	43.0	40.9
Prednisolone	18.4	17.6
Prednisone	16.4	16.7
Betamethasone	8.2	10.6
Dexamethasone	4.9	5.5
Triamcinolone acetonide	4.4	3.6
Methylprednisolone	4.1	4.2
Triamcinolone	0.3	0.3
Fluticasone propionate	0.3	0.6

Linearity

Calibration curves showed a satisfactory linearity in the range of 10–360 ng/mL with correlation coefficient above 0.99.

Precision and accuracy

The intra- and inter-day precision and accuracy at the corresponding quality control (QC) levels are summarized in Table 3. Repeatability (within-run precision) and reproducibility (between-run precision) were within the acceptable range of 20% for limit of quantitation (LOQ) and 15% for the other QC samples. Within-run and between-run accuracies were within 20% of the nominal values for the LOQ and 15% for the other QC samples. The results indicated that the method showed good precision and accuracy.

Limit of quantitation and limit of detection

The limit of quantification of the method was 10 ng/mL (LOQ). The limit of detection was 5 ng/mL.

Matrix effect

To overcome the potential problems connected with influence of matrix on results, the internal standard was chosen to have similar retention time with analyte and to have the ionization under similar conditions. Matrix effects were investigated, using 6 different urine samples from individual donors. This determination was done at 20 ng/mL and at 300 ng/mL for analyte and 30 ng/mL for IS. Similar matrix factors were observed for analyte fortified at 20 ng/mL and for IS – 48.7% and 55.3%, respectively, and 81.04% for analyte spiked at 300 ng/mL. The variability of IS-normalized matrix factors were deemed acceptable – 12.3% and 7.8% (20 ng/mL and 300 ng/mL).

Application in routine testing and controlled studies

Budesonide is a specially designed selective drug having high topical combined with low systemic biological activity. Even the form of the drug for oral administration, which is prohibited by WADA, has mainly a local effect in intestines and low systemic potency (14). Therefore, budesonide seems not to be a very attractive doping agent as compared with systemic glucocorticosteroids. Surprisingly, the 2012 and 2013 anti-doping laboratory statistics published on the WADA website indicated that budesonide is the glucocorticosteroid which was identified the most frequently in samples collected in competition worldwide as shown in Table 4 (15). The same trend was observed in Poland in the years 2011-2012; there were 13 adverse analytical findings for budesonide, which represented 76% of all glucocorticosterods reported. In five cases, athletes (*via* the NADO) requested a controlled excretion study to prove that breach of a reporting limit concentration of 16α-hydroxyprednisolone in their urine sample was a result of inhalation or nasal administration of therapeutic doses of budesonide. Three out of four studies showed that the levels of 16α-hydroxyprednisolone were higher than the reporting limit up to 12 h after budesonide inhalation (Fig. 2A). This is also in agreement with already published data (16, 17), which may indicate that it is a common behavior rather than an exception. In one case, the athlete declared the use of a total daily dose slightly exceeding the maximal dose recommended by the drug manufacturer. Moreover, unusually high concentrations of 16α-hydroxyprednisolone measured in the samples collected during the study may suggest the possibility that even though the athlete was supervised by a doping control officer, he had been able to take an even larger dose of the drug than declared (Fig. 2B). It is also possible that, in addition to inhalation, an oral form of budesonide had been taken because the concentrations measured were in a range noted for samples collected after oral administration (17). On the other hand, it cannot be excluded that this phenomenon can be explained by other factors affecting budesonide metabolism and/or excretion such as genetic polymorphism or interaction with other xenobiotics etc. Indeed, these aspects are currently extensively studied in the context of interpretation of anti-doping results (18, 19). Additionally, the laboratory was not provided with a blank sample collected before any administration as the athlete declared that for health reasons it is not recommended to make a break in drug administration or lower the dose before a controlled excretion study. Nasal administration of budesonide also resulted in the appearance of its main metabolite in urine; however, the concentration did not exceed the reporting limit. Nevertheless, the level of 16α-hydroxyprednisolone at 5 h post-administration was very close to the reporting limit (Fig. 2C).

Analysis of routine anti-doping samples performed in the Warsaw laboratory in the years 2011 and 2012 showed that about 35% of the samples containing 16α-hydroxyprednisolone exceeded the reporting limit. However, none of the samples reported as an adverse analytical finding (AAF) contained 16α-hydroxyprednisolone at concentrations that are much higher than those observed after

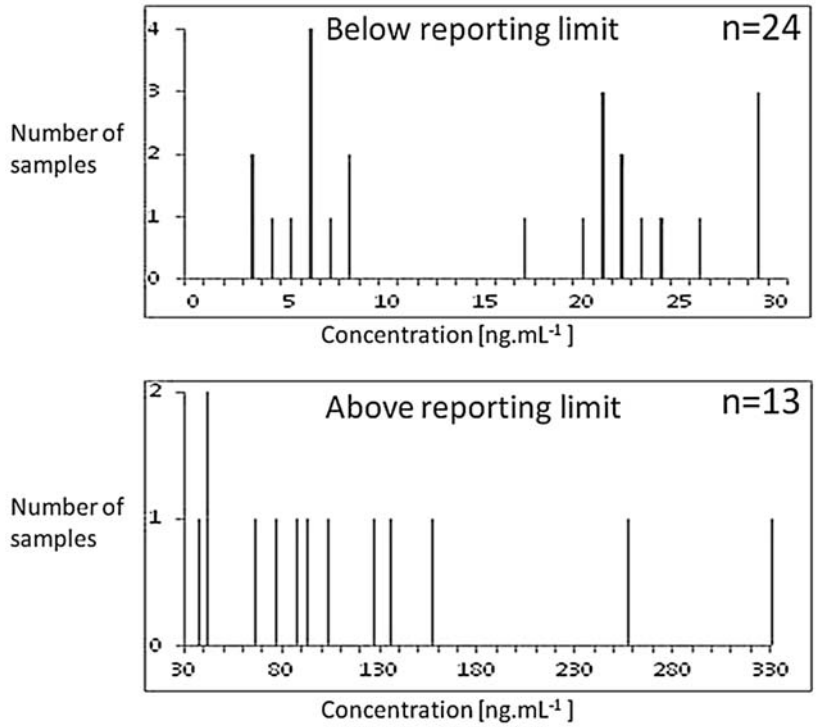

Figure 3. Distribution of 16α-hydroxyprednisolone concentrations in routine in-competition samples in the years 2011-2012 in the Warsaw Laboratory

inhalation of budesonide (Fig. 3). So, it cannot be excluded that in such cases budesonide had been administered by a non-prohibited route. To solve the problem, it is necessary to search for a specific marker of budesonide abuse by prohibited routes of administration. Indeed, in the last WADA document efective from 1 September 2014 to measure concentration of 6β-hydroxybudesonide and use the reporting limit of 30 ng/mL should discriminate between prohibited and authorized administration (6, 20).

CONCLUSIONS

Altogether, these results indicate that the use of budesonide by inhalation within 12 h before and during competition may have lead to a positive result of anti-doping testing if WADA rules effective till 1 September 2014 are applied. The only way to prove that budesonide had been taken by a non-prohibited route was a controlled excretion study. This vindicates the decision of WADA, which allows participation of athletes in controlled studies in order to prove that they did not violate anti-doping rules. Only five out of thirteen athletes positively tested for budesonide decided to take part in such a study in Poland (years 2011/12). A possible reason for that may be the substantial cost of the controlled excretion study, which in Poland is covered by the athlete. Further studies are required in order to check discrimination capability between routes of budesonide administration by using 6b-hydroxybudesonide as a marker.

Acknowledgments

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