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The prevalence of trimetazidine use in athletes in Poland: excretion study after oral drug administration

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Stimulants, together with anabolic androgenic steroids, are regarded as one of the most popular doping substances in sport. Owing to a great variety of these substances and new designer drugs being introduced to the market, each year the World Anti-Doping Agency (WADA) updates the list of substances and methods prohibited in sport. On 1 January 2014, a new doping agent - trimetazidine (TMZ) - was added to the WADA Prohibited List. TMZ, a substance prohibited in competition, is classified in the S6b Specified Stimulant Group. TMZ is used as a well-known cardiologic drug with confirmed biochemical and clinical activity. According to knowledge of the pharmacology and mechanism of TMZ action, TMZ can be used by athletes to improve physical efficiency, especially in the case of endurance sports. This study presents the phenomena of TMZ use by Polish athletes involved in anti-doping control in the WADA-accredited laboratory in Warsaw (Poland) between 2008 and 2013. Samples were taken from the athletes of such disciplines as cycling, athletics, and triathlon. Moreover, the elimination study of TMZ has been conducted to establish the change of TMZ concentration in urine sample after oral administration of a single or double (during the long-term therapy) dose. TMZ was monitored in urine samples by gas chromatography-mass spectrometry-nitrogen phosphorus detection (GC-MS-NPD). Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: trimetazidine; sport; monitoring; excretion study; doping

Introduction

Trimetazidine (TMZ) [1–(2.3.4-trimetoxybenzyl)-piperazine] as an analogue of piperazine can be classified to the specified stimulant substances and its structure is shown in Figure 1a.[1] TMZ is a crystalline white powder and it exhibits slight hygroscopic properties. TMZ as dihydrochloride has good water solubility and poor solubility in alcohol.[2]

TMZ is an effective and well-tolerated anti-anginal drug that has protective properties against ischemia-induced heart injury by shifting cardiac energy metabolism from fatty acid oxidation to glucose oxidation.[3–6] It leads to the process of restoring coupling between glycolysis and carbohydrate oxidation and results in adenosine triphosphate production with a smaller amount of oxygen consumption. High doses of TMZ suppress halothane-adrenaline arrhythmia. Moreover, the use of high TMZ doses leads to the maintenance of electrolyte homeostasis in myocardium by protecting the cardiac cells from the accumulation of calcium and sodium ions in ischemic hearts.[1,7–9] It is also known that long-term treatment with TMZ alleviates the myocardial accumulation of the oxidation reactive form and prevents lipid per-oxidation.[10] It improves the activity of the superoxide dismutase (an antioxidant enzyme) and relieves the oxidative stress phenomena.[11,12] TMZ used in standard pharmacological therapy and in a domiciliary exercise programme improves a functional performance in patients with peripheral arterial diseases.[13,14] Although TMZ is mainly applied to prophylaxis and therapy of cardiovascular diseases, it is also used in the case of patients with ischemic heart diseases and stable angina or acute coronary syndromes.[15–17]

According to the Polish Center of Drug Information (Wroclaw, Poland) the code number of TMZ is C01EB15. TMZ is present in such medical products on the Polish market as Adexor, Cyto-Protectin MR, Metazydyna, Preductal, Preductal MR, Protevasc SR, Setal MR, Trimeductan MR and Trimetaratio.[18–20] On the label of the product, Preductal MR, the serum half-life of TMZ is estimated at about 7 h in the case of young people and 12 h in the case of people over 65. Following the oral administration of TMZ the maximum serum concentration occurs after approximately 5 h after ingesting one dose. The plateau value of TMZ concentration is achieved after 60 h after starting the permanent therapy of TMZ.[21] The possible side effects of TMZ are mainly the symptoms from the gastrointestinal tract, exhaustion, and weakness. Moreover, TMZ may cause such Parkinson's symptoms as muscle tremors, increasing muscle tension or gait disorder.[22–24]

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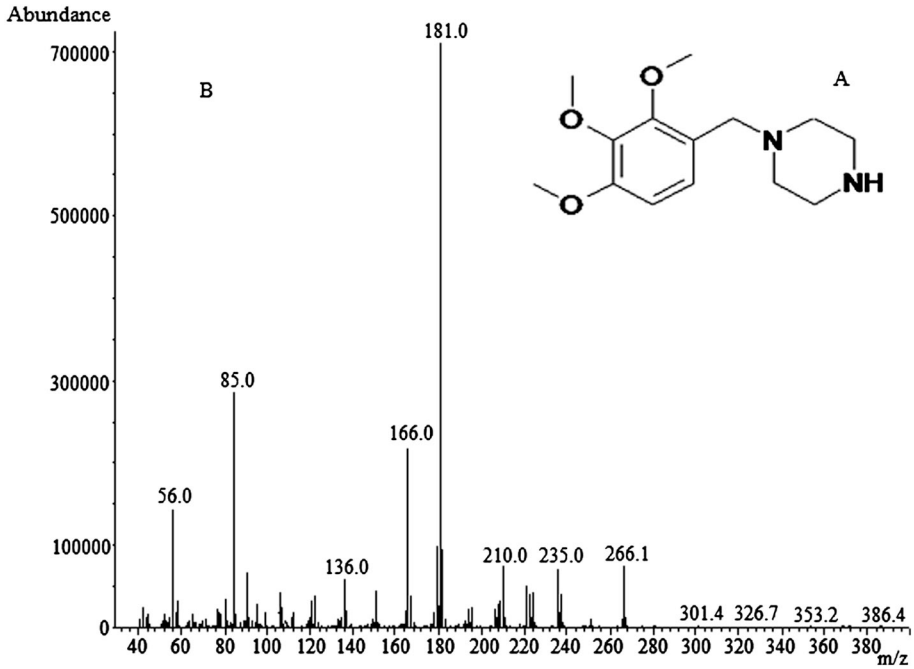

Figure 1. The structure of trimetazidine (A) and its mass spectrum (B).

In clinical studies, the concentration of TMZ was monitored in plasma.[25–28] This is due to the necessity of controlling a TMZ concentration in blood in the case of patients with cardiac problems. In anti-doping control, urine is preferred to analysis because of the non-invasive sampling process. Recently, Sigmund *et al.*[29] published a paper on the detection of TMZ and its major metabolites in urine.

Taking into account the pharmacological knowledge of TMZ, the mechanism of TMZ action, and emerging patterns of the use of drugs with TMZ, the World Anti-Doping Agency (WADA) included TMZ on the Prohibited List published on 1 January 2014.[30] Yet, in 2005, the WADA-accredited laboratory in Warsaw (Poland) sent to the Polish Commission Against Doping in Sport written notification on the common use of TMZ, as well as buflomedil and tramadol, in cyclists.

This study presents the phenomena of TMZ use by Polish athletes in the last few years. TMZ has been monitored by gas chromatography-mass spectrometry-nitrogen phosphorus detection (GC-MS-NPD) during routine analyses held in the Department of Anti-Doping Research in Warsaw between 2005 and 2013. Additionally, the elimination study of TMZ has been conducted to determine the TMZ concentration changes in urine after oral administration.

Experimental

Chemicals and reagents

TMZ has been obtained for validation purpose and elimination study as a reference standard from TRC (North York, Ontario, Canada) and a drug Cyto-Protectin RM (35 mg), respectively. Diphenethylamine (DPA) has been obtained from Sigma (Saint Louis, Missouri, USA). Sodium hydroxide and anhydrous sodium sulfate have been purchased from POCH (Gliwice, Poland). T-buthylmethyl ether (TBME) has been obtained from RATHBURN Chemicals Ltd. (Walkerburn, Scotland) Liquid chromatography-mass spectrometry (LC-MS) grade water was delivered by J.T. Baker (Center Valley, Pennsylvania, USA).

Stock solution of TMZ was prepared at the concentration of 1 mg/mL in water and DPA stock solution was prepared at the concentration of 150 μg/mL in methanol. Both of the stock solutions and working solutions were stored at the temperature of −20 °C.

Sample preparation

The sample was prepared following the routine screening procedure used in the WADA-accredited laboratory in Warsaw (Poland) for stimulants and narcotics for many years. The urine sample of 5 mL was fortified with 20 μL of 150 μg/mL DPA (an internal standard) and adjusted pH to 9 10 with 200 μL of 5 M sodium hydroxide solution. Next, an anhydrous sodium sulfate of about 3 g was added and the sample was extracted with 1.3 mL of TBME by shaking for 20 min. Subsequently, the sample was centrifuged for 6 min and frozen. The organic phase was collected and 2 μL of the ethereal phase was injected into the GC-MS-NPD system. The samples preparation has been similar to that presented in the paper published by Sigmund *et al.*[29]

Instrumental analysis

Analyses by GC-MS-NPD system were performed with Agilent Technologies (Santa Clara, CA, USA) 6890 N gas chromatograph coupled to Agilent Technologies 5975B inert XL mass spectrometer with single quadrupole mass spectrometric detector equipped with EI ion source and nitrogen-phosphorus detector. Sample ingredients in the GC-MS system were first evaporated at 280 °C, separated according to the suitable GC temperature programme and then ionized at 230 °C. The chromatographic separation has been achieved by using a HP-5MS column (200 mm x 12 μm x 0.33 μm), constant flow of 1.2 mL/min helium carrier gas and splitless module. The GC temperature programme involved following temperatures: 70 °C for 1 min, 18 °C/min ramp to 230 °C, 230 °C for 1 min, 50 °C/min ramp to 280 °C, and 280 °C for 4 min. Mass spectrum data have been acquired by using a scan mode

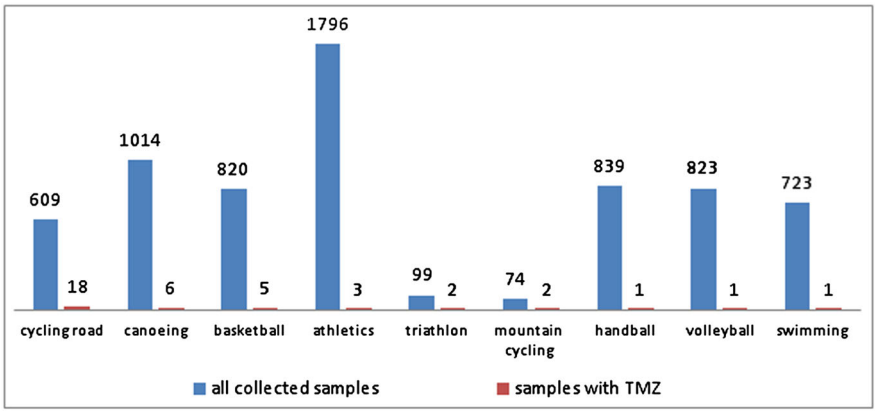

Figure 2. The number of samples with TMZ in different disciplines of sport 2008-2013.

with full scan spectra from *m/z* 30 to 400. The monitoring ions of TMZ were at *m/z*: 266, 181, 166 and 85 (Figure 1b). The base ion at *m/z* 181 has been used for the purpose of TMZ determination.

Validation

The validation procedure followed the Internal Standard for Laboratories of the World Anti-Doping Code and Eurachem guidelines.[31,32] The following validation parameters as linearity range, limit of detection (LOD), limit of quantification (LOQ), extraction recovery, precision, accuracy, and carry-over have been considered.

The calibration curve was the relationship between the ratio of peak areas for TMZ and DPA, and the concentration of TMZ. The TMZ calibration solutions, a set of blank urine samples fortified with the TMZ stock standard solution, covered the wide range of concentrations: 50 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL, 2000 ng/mL, 4000 ng/mL, and 6000 ng/mL. The correlation coefficient (R^2) was calculated for four curves that consisted of three measurements for each concentration point. The values of LOD and LOQ were based on the example of one calibration curve. LOD was calculated as the ratio of three times standard deviation of the area ratio for the calibration standard solution with the lowest concentration, to the slope of the calibration curve. LOQ was obtained as the ratio of ten times standard deviation of the lowest concentration to the calibration curve slope. Precision was defined as a relative standard deviation, while accuracy was determined by the value of relative error of a TMZ concentration. The calculation of the relative standard deviation was based on the quality control samples (blank urine samples fortified with the TMZ stock standard solution) analysis repeated ten times. Recovery was evaluated as the ratio of the peak areas for the determinations of TMZ in the standard solution of 2000 ng/mL TMZ after an extraction and in the standard solution of the same TMZ concentration without any further preparation (treated as 100 % recovery of TMZ).

Administration study

Four female and one male healthy volunteer in the age range of 24 53 years ingested a single tablet of the product Cyto-Protectin MR containing 35 mg of trimetazidine dihydrochloride. Additionally, one man volunteer in the age of 53, who was a patient with permanent treatment of TMZ, took the therapeutic dose of two pills of TMZ the product Cyto-Protectin MR. Both, the ethical approval from the Bioethics Committee established at the Medical University of

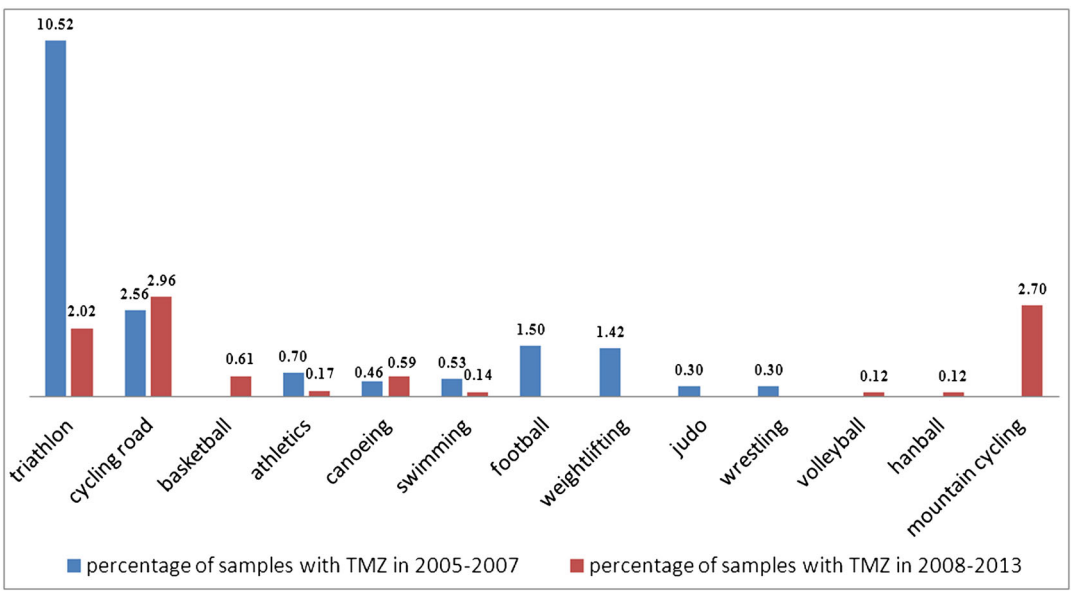

Figure 3. Comparison of the number of urine samples with TMZ between the years 2005-2007 and 2008-2013.

Table 1. Validation parameters of TMZ determination method

Calibration curve (y = a · x + b)				LOD ng/mL	LOQ ng/mL	Inter-assay precision (%)	Extraction recovery (%)
Concentration range ng/mL	a	b	R^2				
50-6000	0.00037	-0.03273	0.9934-0.9994	14	43	3.6	85

Warsaw, Poland (KB 186/2008) and the written consents of TMZ elimination study participation from the all volunteers have been obtained.

The urine sample was collected from each volunteer before taking the medicine (except for the patient case) to 1 day post- administration of TMZ. The samples were stored at 4 °C until preparation and analysis. If the concentration of TMZ in a urine sample was out of the linear concentration rage, the sample was appropriately diluted with water before the analysis.

Results and discussion

Prevalence of TMZ use by athletes through the years

The main aim of the study was to investigate the phenomenon of TMZ use by Polish athletes in the last few years. For this purpose the anti-doping control data from the WADA-accredited laboratory in Warsaw (Poland) in the period 2008-2013 were analyzed in accordance with the International Standard for Testing.[33] Additionally, the results of the study were compared with those from the previous research[34,35] based on the years 2005-2007.

The presence of TMZ in the urine samples was controlled in the routine screening analysis for stimulants and narcotics between 2008 and 2013 and the substance was identified as a free base in 39 of 16 725 samples (0.23%). In 2008, the present of TMZ was found in 8 of 2684 samples (0.30%); in 2009, in 4 of 2644 samples (0.15%); in 2010, in 4 of 2693 samples (0.15%); in 2011, in 6 of 2734 samples (0.22%); in 2012, in 9 of 2750 samples (0.32%); and in 2013, in 8 of 3220 samples (0.25%). TMZ was detected mainly in the samples taken in competition and only 10% of the collected samples were from out-of-competition. It was found frequently in the following sport disciplines: canoeing, mountain cycling, cycling road, basketball, athletics, swimming, volleyball, triathlon and handball (Figure 2). The results of similar studies carried out in the period from 2005 to 2007,[34,35] showed that TMZ was detected in 36 of 6210 samples (0.58%) and found in such disciplines of sport as: cycling road (13), athletics (7), swimming (2), triathlon (4), weightlifting (3), canoeing (2), football (3), judo (1), and wrestling (1). Although the total number of urine samples containing TMZ was slightly higher in 2008-2013 that in 2005-2007, the total number of analyzed samples in 2008-2013 was three time larger than in 2005-2007. Also the number of investigated years in our study was almost twice as long as previously. According to these facts, we observed a decreasing number of samples containing TMZ. Comparison of the number of urine samples with TMZ in different sport disciplines in the two analyzed time periods is shown in Figure 3. The increasing number of samples containing TMZ was observed in cycling, canoeing, handball, and volleyball. The number of samples with TMZ in triathlon and athletics was lower than previously. Moreover, in comparison to the previous study we did not find TMZ in the case of athletes from such disciplines as judo, wrestling, weight lifting, and football. It is worth mentioning that it is not known if the urine samples with TMZ were collected from the same athlete or different athletes.

TMZ was detected in 0.22% of all samples collected in the anti-doping control in the WADA-accredited laboratory in Warsaw (Poland) between 2008 and 2013. All stimulants detected in this time period constituted 0.55%. Methylhexanamine (40 collected samples), trimetazidine (39), amphetamine (13), and ephedrine (12) were stimulants most often detected. From January to July in 2014, only one urine sample collected in competition in the anti-doping control in the WADA-accredited laboratory in Warsaw (Poland) contained TMZ. TMZ was detected in athletics, a sports discipline with rare cases of TMZ identification. Furthermore, from 2014, the use of TMZ by athletes can be only in the case of Therapeutic Use Exemption (TUE). In the years covered by this study, none of the athletes during the doping control had declared the administration of TMZ in the context of the TUE.

Validation

The analytical method of TMZ determination in the urine samples was validated and the received parameters are shown in Table 1. As for accuracy, the maximum value of relative error (equal to 21%) was observed for the lowest concentration of TMZ (50 ng/mL). The results of relative error obtained for higher concentrations were lower than 10%.

Elimination study

The experiments of TMZ elimination in the case of the five healthy volunteers enabled us to follow the changes of TMZ concentrations after different times elapsed from the administration of a single dose (Figure 4). The differences in the shape of the obtained excretion curves can be explained by the different time of releasing the active substance in each volunteer. The maximum concentration of TMZ in urine samples occurred at about 4 h after the

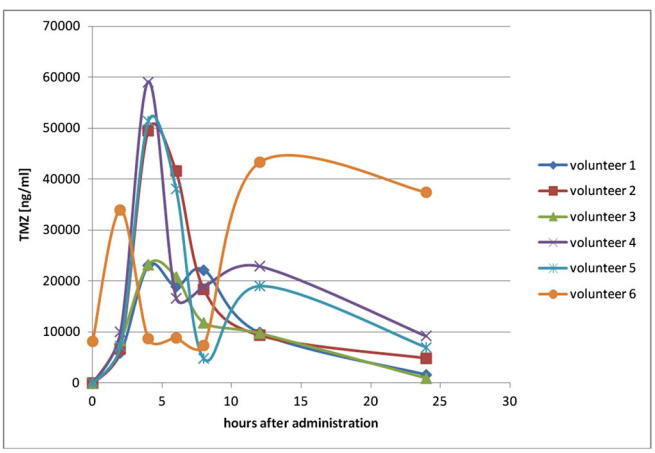

Figure 4. TMZ concentration in urine after oral administration.

Table 2. Concentration of TMZ in urine samples after oral administration

Time after administration hours	Volunteer 1	Volunteer 2	Volunteer 3	Volunteer 4	Volunteer 5	Volunteer 6
	ng/mL					
0	0	0	0	0	0	81845
2	5819	6668	8337	10045	7279	33959
4	23002	49552	23204	59083	51354	8711
6	18960	41528	20775	16659	38071	8872
8	22182	18382	11807	18916	4778	7381
12	9854	9379	9747	22892	19038	43275
24	1638	4873	966	9182	7018	37443
36	407	—	—	—	—	—
48	340	—	—	—	—	—
72	131	—	—	—	—	—

administration. The maximum values of TMZ concentration in the case of volunteers 2, 4, and 5 were significantly higher (49-59 μg/mL) then in volunteers 1 and 3 (about 23 μg/mL) (Table 2). However, the concentration values were not recalculated with the use of the urine specific gravity. Moreover, differences in the TMZ maximum concentrations could be the result of genetic factors that influence the speed of the elimination process. In the time period of 24 h the concentration of TMZ in urine samples was in the range of 1-9 μg/mL. Additionally, the elimination study of volunteer 1 was prolonged to three days. The concentration of TMZ after 72 h post- administration was above 50 ng/mL, i.e., 50% of the minimum required performance level (MRPL) for stimulants according to the WADA technical document.[36] The result of this study shown that the anti-doping test can be treated as adverse analytical finding after three days from taking a drug with TMZ by an athlete.

Analyzing the concentration of TMZ in the urine samples from volunteer 6, who was in the course of long-term TMZ therapy, it should be marked that the medication was ingested twice a day. Therefore, the TMZ concentration was much higher in 12 and 24 h. The shape of the elimination curve for the volunteer 6 is completely different then in the case of the healthy volunteers, but the lack of specific gravity correction of TMZ concentration does not enable us to give advantage conclusions on this field.

Examples of TMZ determination in urine samples from athletes

Determination of TMZ concentration in the urine samples taken from five athletes in the anti-doping control in the first half of 2011 was performed. The results were within the range of 16-29 μg/mL. The received concentrations of TMZ were compared to the results obtained for the investigated volunteers. It is hard to state whether these athletes took the drug as a part of the long-term therapy or to increase the efficiency of the organism during the performance. The main problem was related to the fact that it was not known how long after taking a drug the urine sample was collected.

Additionally, significant decrease of the number of samples with TMZ collected in competition in the first six months of 2014 compared to the same period of time in 2011 should be pointed out.

Conclusions

The prevalence of use of TMZ by athletes has been investigated. TMZ was detected in 0.22% of all samples collected in the anti-doping control in the WADA-accredited laboratory in Warsaw (Poland) between 2008 and 2013. Next to methylhexaneamine, TMZ was the stimulant detected most often in the investigated period of time. Urine samples with TMZ were taken from the athletes in such disciplines as cycling, athletics, basketball, handball, volleyball, triathlon, and canoeing. Cycling and canoeing are an increased risk group among the listed sport disciplines.

The excretion study has shown that the consumption of the single therapeutic dose of TMZ can lead to the maintenance of TMZ concentration in urine above 50 ng/mL up to three days. The reflection of this case in some athletes would have caused adverse analytical finding as the result of an anti-doping test. Therefore, athletes and their medical support should consider the risk of taking drugs with TMZ, not just during competition but also out of competition.

The annual modifications of the WADA Prohibited List and the subsequent introduction of new prohibited substances strengthened the world anti-doping system, as shown in the paper published by Pokrywka *et al.*[37] Hence, the insertion of TMZ on the WADA Prohibited List on 1 January 2014 should result in decreasing number of athletes using TMZ in competition.

Acknowledgements

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