Research article

Drug Testing and Analysis

Received: 29 June 2012 Revised: 12 August 2012 Accepted: 17 August 2012 Published online in Wiley Online Library: 13 September 2012

(www.drugtestinganalysis.com) DOI 10.1002/dta.1413

Detection of PPARδ agonists GW1516 and GW0742 and their metabolites in human urine

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Peroxisome proliferator-activated receptor-δ (PPARδ) agonists are the drug candidates with potential performance-enhancing properties, and therefore their illegitimate use in sports should be controlled. To simulate the metabolism of PPARδ agonist GW0742, *in vitro* reactions were performed which demonstrated that the main metabolic pathway is oxidation of the acyclic divalent sulfur to give the respective sulfoxide and sulfone. After being characterized by liquid chromatography–mass spectrometry (LC-MS), these metabolites were evaluated in urine samples collected after a controlled excretion study. For comparative purposes, GW1516 excretion study was also performed. It has been shown that GW1516 and GW0742 are best monitored as the sulfone metabolites which are detectable in urine using LC-MS/MS based procedure up to 40 and 20 days after a single oral dose of 15 mg each, respectively. The unmetabolized compounds are measurable only for a short period of time and at low ng/ml level. The sulfoxide-to-sulfone ratio for both GW1516 and GW0742 changed irregularly in the range of 1:3 to 1:15 depending on time elapsed after administration with a tendency of increasing the ratio with time. The other important finding was that the abundance of GW0742 and its metabolites in urine is about ten times lower than in case of GW1516.

Keywords: GW1516; GW0742; PPARdelta agonist; anti-doping analysis

Introduction

Peroxisome proliferator-activated receptor-δ (PPARδ) agonists are considered as potential performance-enhancing drugs and are therefore prohibited by the World Anti-Doping Agency (WADA) in professional sports.[1] This class of compounds reportedly increases expression of multiple skeletal muscle genes [2] and, when combined with exercise training and/or AMP-activated protein kinase agonist AICAR, may lead to improved endurance due to an increased use of fatty acids as the source of energy.[3–5] One of the most prominent and well-studied examples, GW1516 (or GW501516), which is a drug candidate to treat dyslipidaemia,[6,7] is currently available on the clandestine supplement market.[8] The other PPARδ agonist termed as GW0742 is a fluorinated analog of GW1516 which could produce similar effects to the organism[9] and should therefore be monitored for the purposes of doping control. At present, GW0742 is studied for its neuroprotective,[10] antithrombotic[11] and anti-inflammatory properties,[12–14] and may also find its way to elite sports.

Up to now there is only one study devoted to the identification of GW1516 urinary metabolites[15] where it has been shown that its oxygenated counterparts are good analytes to include into the liquid chromatography-mass spectrometry (LC-MS) based routine doping control procedures. The authors synthesized and fully characterized sulfoxide and sulfone metabolites of GW1516 applying high resolution/high accuracy mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. Later, Thomas *et al.*[16] reported the comprehensive plasma screening procedure which allowed the detection of unmetabolized GW1516 at low ng/ml level. The detection of GW0742 or its metabolites in humans has not yet been reported. In addition, no information is available on how long GW1516 and GW0742 could be detected in urine if voluntarily administered.

Therefore, the aim of present study was to investigate the metabolism of GW0742 *in vitro* and then, under the conditions of a controlled excretion study, evaluate the detectability of both GW1516 and GW0742 to pinpoint the most valuable metabolites for doping control analysis.

Materials and methods

Reagents

Water and methanol of gradient grade or better were purchased from Biosolve (Valkenswaard, the Netherlands) and Merck (Darmstadt, Germany), respectively. Diethyl ether and acetone were obtained from Medkhimprom (Moscow, Russia). β-Glucuronidase from *E. Coli* K12 (solution in 50% glycerol) was purchased from Roche Diagnostics (Mannheim, Germany) and used as supplied. Methyltestosterone and mefruside were from LGC Standards (Wesel, Germany). All other chemicals (potassium carbonate, potassium hydrogen carbonate, potassium phosphate monobasic, sodium phosphate dibasic, sodium sulfate, formic acid) were obtained from Sigma-Aldrich (St Louis, MO, USA).

The human liver microsomes (HLM) pooled from 150 mixed gender donors, as well as regenerating system solution A of reduced nicotinamide adenine dinucleotide phosphate (NADPH-A), regenerating system solution B of NADPH (NADPH-B), and phosphate buffer (0.5 M, pH 7.4) were obtained from BD Gentest (Woburn, MA, USA).

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Solid-phase extraction (SPE) was carried out on the columns filled with a non-polar C8 / cation exchanger sorbent (Bond Elute Certify, 130 mg × 3 ml, Varian Inc., Lake Forst, CA, USA). GW1516 was received from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), GW0742 was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada).

Urine samples

Urine samples were collected from two volunteers who administered single dose of 15 mg GW1516 (male, aged 31) and GW0742 (male, aged 34) after the approval from the local ethical committee at the Institute of Sport. Both individuals were not professional athletes, and signed the informed consent form before commencing the administration study. Urine samples were collected for 45 and 25 days in case of GW1516 and GW0742, respectively. Initially, 14 days collection period was assumed but this was adjusted due to the slow excretion kinetics of both compounds, as noticed when performing the present study. A certified laboratory negative control urine was used as a blank urine whenever necessary.

Preparation of urine samples

Urine samples were prepared according to the protocol for non-volatile conjugated compounds used in our laboratory. Briefly, to 3 ml of urine was added 1 ml of phosphate buffer (0.8 M, pH 6.5) containing 30 µl of β-glucuronidase and 0.6 µg of methyltestosterone (when semiquantitative analysis was carried out, mefruside was used as internal standard). After incubation at 57 °C for 60 min and the addition of carbonate buffer (3 M, pH 10), the samples were extracted with 5 ml of diethyl ether in the presence of Na_2SO_4. Extraction recovery experiments were carried out at pH values 3.3, 5.4 (acetate buffer added instead of carbonate buffer), 6.4 (no carbonate buffer added), 9.5 (carbonate buffer added), and 12.9 (solid potassium carbonate used instead of carbonate buffer). When urinary free fraction was analyzed, addition of the enzyme and incubation steps was omitted. Following the evaporation of organic layer at 70 °C in a solid state heater, the residue was reconstituted in 60 µl of methanol and then 40 µl of water were added before transfer to a vial for the LC-MS/MS analysis.

In vitro experiments

The enzyme reactions were performed in 1.7-ml Eppendorf tubes according to the protocol provided by the manufacturer. Briefly, the reaction mixture contained 10 µl of GW1516 and GW0742 solution in acetonitrile (1 mg/ml), 710 µl of deionized water, 200 µl of phosphate buffer (0.5 M, pH 7.4), 50 µl of NADPH-A and 10 µl of NADPH-B. After incubation at 37 °C for 5 min the samples were mixed gently prior to the addition of 20 µl of HLM, followed by incubation at 37 °C for 1 h under continuous agitation. The overall reaction was terminated by the addition of 100 µl of ice-cold acetonitrile. The reaction mixture was centrifuged at 15 000 g for 10 min. The supernatant was transferred into a fresh tube, and acetonitrile was evaporated at reduced pressure resulting in mostly aqueous specimens, which were further purified by SPE. To this end, the SPE columns were conditioned with 3 ml of methanol and 3 ml of deionized water. After sample addition, the columns were washed twice with 3 ml of deionized water. The analytes were eluted with 3 ml of methanol. The eluent was evaporated to dryness under nitrogen flow followed by reconstitution of the residue in 100 µl of water/methanol (1:1, *v:v*) and LC-MS/MS analysis.

LC-MS/MS and LC-Orbitrap MS

In case of LC-MS/MS analyses, the system comprised an Acquity liquid chromatograph (Waters, Milford, MA, USA) coupled to a TSQ Vantage triple quadrupole mass spectrometer (ThermoFisher Scientific, San José, CA, USA). Waters Acquity BEH C18 column (100 mm × 2.1 mm, particle size 1.7 µm) maintained at 60 °C and protected by a Vanguard BEH C18 column (20 mm × 2.1 mm) was used for separation. Injection volume was 5 µl. The mobile phase flow rate was set to 0.35 ml/min. The elution program started from 0.5-min isocratic step at 95% of 0.1% formic acid in water (A) and 5% of 0.1% formic acid in methanol (B) followed by linear increase to 95% of B within 4.5 min, hold at 95% of B for 2.5 min and then re-equilibration until the end of analysis (10 min).

The heated electrospray (ESI) ion source (HESI II) was used for ionization. Positive and negative ions were detected in the full-scan, MS/MS (product ion scan) and selected reaction monitoring (SRM) modes. The collision gas pressure was 0.2 Pa (or 1.5 mTorr, argon 99.9995% obtained from NII KM, Russia). Sheath and aux gas pressure (nitrogen of 99.9% purity from a Peak Scientific nitrogen generator, Inchinnan, Scotland, UK) was set at 55 and 35 arbitrary units, respectively. The vaporizer and capillary temperatures were set at 370 and 300 °C, respectively, with a spray voltage of 4000 V. In the MS/MS experiments, the collision energy (CE) was ramped from 5 to 45 eV to select the optimal value for every transition.

To evaluate *in vitro* data, the system consisting of a high resolution mass spectrometer Exactive (Thermo Scientific, Bremen, Germany) coupled to an Accela liquid chromatograph (Thermo Scientific, Sane José, CA, USA) with the identical column and gradient elution program was used. The mass spectrometer operating in positive and negative ionization mode was calibrated using the calibration mixture containing caffeine, the tetrapeptide MRFA and Ultramark with a total of seven reference masses to provide the mass accuracy of 5 ppm. The data were collected in full scan mode over *m/z* range of 100–2000 *Da* at resolution (*R*) of 100 000 @ *m/z* 195.0882 (FWHM), or applying higher-energy collision-induced dissociation (HCD) performed at 20 and 50 V ($R = 50\ 000$). All other relevant MS parameters were identical to that of TSQ Vantage.

Results and discussion

In vitro metabolism of GW1516 and GW0742

An *in vitro* experiment was undertaken to establish SRM transitions and retention times for selective and sensitive determination of both PPARδ agonists in human urine, as currently no synthetic reference material of their metabolites is commercially available. Incubation of PPARδ agonist GW1516 with human liver microsomes resulted in formation of two oxygenated metabolites, sulfoxide (one oxygen atom added to the acyclic sulfur) and sulfone (two oxygen atoms added). The product ion mass spectra and high resolution / high accuracy full scan data obtained for both metabolites of GW1516 were identical to that reported by Thevis *et al.*[15] (data not shown). Being a fluorine-substituted analog of GW1516, GW0742 was expected to metabolize in a similar way. Indeed, two metabolites were identified formally corresponding to the addition of one and two oxygen atoms, respectively. As seen from Figure 1A, deprotonated molecule of GW0742-sulfoxide has the same fragmentation pattern as GW1516: the most abundant peak at *m/z* 212 comes from the cleavage of the bond between acyclic sulfur and aliphatic carbon with subsequent elimination of glyoxal to give the second ion at *m/z* 154.

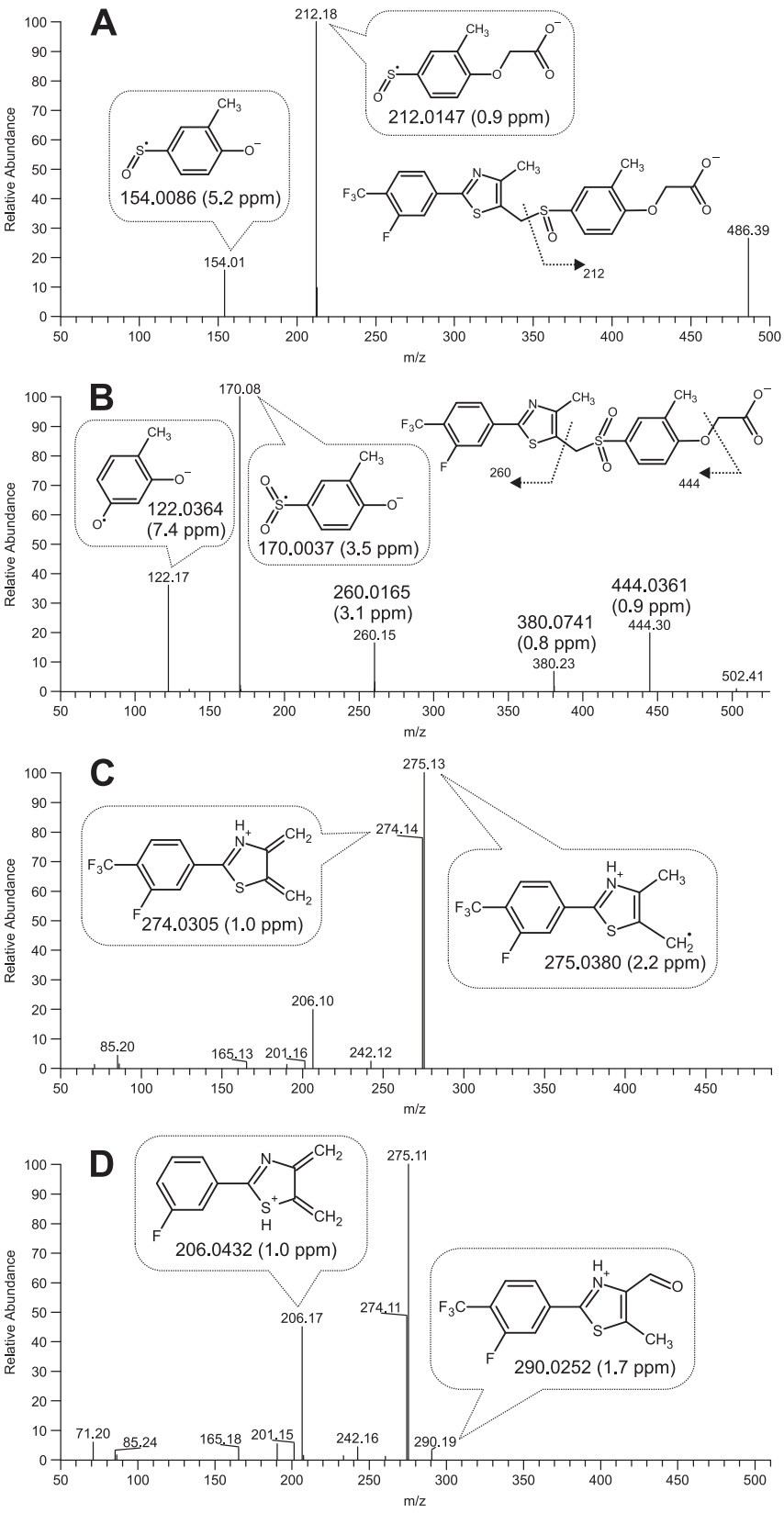

Figure 1. Product ion mass spectra of GW0742-sulfoxide (*A*: ESI–; *C*: ESI+) and GW0742-sulfone (*B*: ESI–; *D*: ESI+) produced at 15, 30, 35 and 35 eV, respectively.

In case of GW0742-sulfone (Figure 1B), the latter ion is shifted to *m/z* 170 (+16 *Da*, oxygen atom). Upon elimination of glyoxal from the deprotonated molecule the ion at *m/z* 444 is formed which further cleaves sulfur dioxide to give the ion at *m/z* 380. The left part of molecule corresponds to the ion at *m/z* 260, or 2-[3-fluoro-4-(trifluoromethyl)phenyl]-4-methyl-1,3-thiazole. Interestingly, the

ESI– mass spectra of GW1516 and GW0742 sulfone metabolites both contain the ion at *m/z* 122, which is probably formed after rearrangement involving one of the sulfone oxygen atoms and the methyl group of 1,3-thiazole. The theoretical mass of this ion, 122.0373 *Da*, is in good agreement with *m/z* 122.0364, as measured using Orbitrap mass spectrometer under the HCD conditions.

To further support the proposed structure of GW0742 metabolites, they were subjected to the positive ESI. The fragmentation of protonated GW0742-sulfoxide (Figure 1C) resulted in completely different mass spectrum with the ion peaks corresponding to 2-[3-fluoro-4-(trifluoromethyl)phenyl]-4,5-dimethyl-1,3-thiazole and its cleavage products. Interestingly, the product ion mass spectrum of protonated GW0742-sulfone (Figure 1D) is essentially the same except that a minor ion at *m/z* 290 instead of *m/z* 292 is formed. An additional evidence of the suggested structure of GW0742 metabolites is that their product ion mass spectra are similar to those reported by Thevis[15] but shifted by 18 *Da* (fluorine substitution). Postulated structure of the most abundant ions at *m/z* 274, 275 and 206 is consistent with the high resolution mass spectrometry data obtained in the HCD mode. The chromatographic and other relevant mass spectral data for GW1516, GW0742 and their metabolites are summarized in Table 1.

Administration studies with GW1516 and GW0742

When working with *in vitro* synthesized metabolites, both positive and negative ionization modes were used, and the positive ESI provided better ionization efficiency. However, in case of urine analysis negative ionization mode was preferred due to considerably lower background from the urinary matrix.

Furthermore, the excretion studies were necessary to investigate how long the oxygenated metabolites of these PPARδ agonists can be detected in urine and whether it is sufficient to monitor only parent compounds for the purposes of doping control. In total, 40 urine samples were collected from each volunteer (for each drug). These urine samples were then processed according to our routine procedure for the non-volatile conjugated compounds; the free fraction was also analyzed. It was noted that enzymatic hydrolysis with β-glucuronidase generally increased the abundance of the metabolites by a factor of 2 to 5, but in some urine samples this was nullified by the elevated ionization suppression. It is also worth mentioning that due to the acidic nature of the compounds in question one may anticipate better extraction yield at low pH values. However, the recovery of GW1516 and GW0742 was found to be only slightly dependent on pH, changing from 83 to 92% and from 73 to 81%, respectively, when pH was decreased from 12.9 to 3.3. Therefore, the extraction at weakly alkaline conditions (pH 9.5) was considered as acceptable in our experiments.

The amount of GW1516-sulfone in urine was notably higher than that of the sulfoxide with a ratio of 3:1 to 15:1 (roughly estimated as the ratio of peak areas corresponding to the most intense SRM transitions for each metabolite). The ratio increased with time indicating that the abundance of the sulfoxide metabolite decreased faster than that of the sulfone. The unmetabolized GW1516 turned out to be an inadequate analyte as its peak concentration reached *ca.* 10 ng/ml only for a short period after

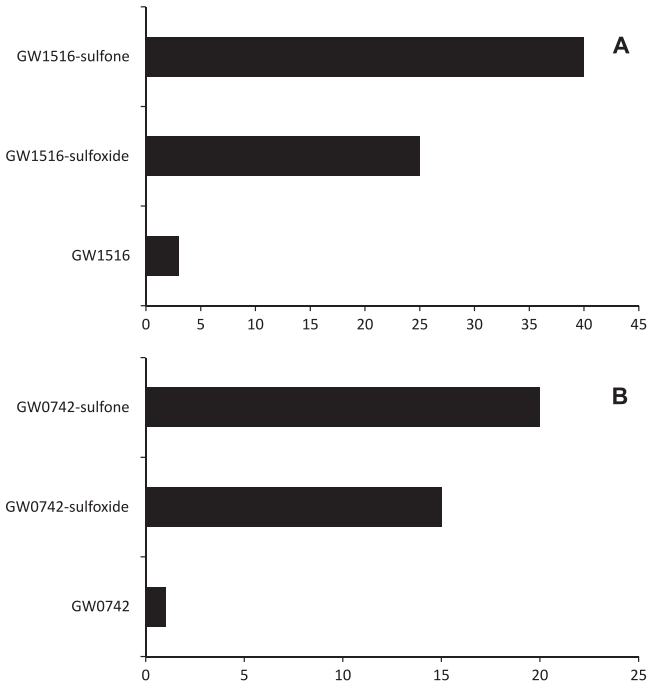

Figure 2. Detection time (days) of GW1516 (*A*), GW0742 (B) and their metabolites.

Table 1. Chromatographic and mass spectral data for GW1516, GW0742 and their metabolites. Collision energy (CE) is given in eV

Compound	Experimental mass				RT, min	SRM (CE) in ESI–
	ESI+	mass error, ppm	ESI–	mass error, ppm		
GW1516	454.0739	4.2	452.0610	1.8	6.01	452 > 394 (18) 452 > 138 (40)
GW1516-sulfoxide	470.0695	2.8	468.0555	0.9	5.34	468 > 154 (25) 468 > 212 (15)
GW1516-sulfone	486.0644	2.7	484.0508	1.7	5.40	484 > 426 (21) 484 > 170 (32)
GW0742	472.0650	3.0	470.0513	1.1	6.07	470 > 412 (19) 470 > 138 (41)
GW0742-sulfoxide	488.0599	2.9	486.0460	0.6	5.45	486 > 212 (15) 486 > 154 (25)
GW0742-sulfone	504.0550	2.6	502.0413	1.4	5.52	502 > 170 (30) 502 > 122 (40)

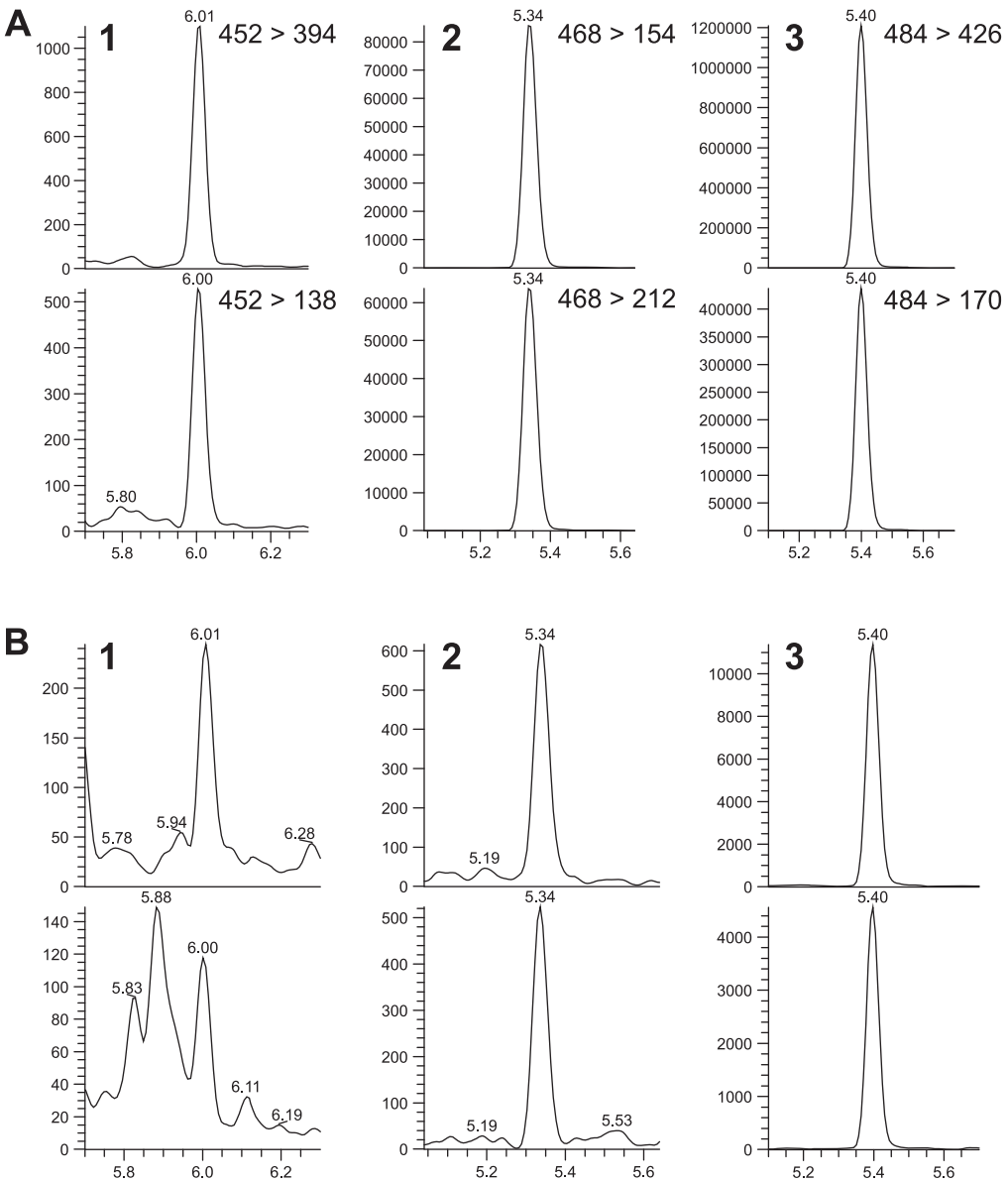

Figure 3. SRM chromatograms for GW1516 (1) and its sulfoxide (2) and sulfone (3) metabolites at 26 h (A) and 27 days (B) after administration.

administration (less than 24 h) and then decreased to the limit of detection (0.1 ng/ml) within 72 h. The concentration of parent compound was estimated using mefruside as the internal standard which was monitored using SRM transition 381 > 189. As chemical synthesis of the metabolites has not been attempted, their urinary concentration was not determined but based on the peak area one may expect about 100-fold higher amount of the oxygenated metabolites compared to the parent drug.

Direct analysis of 5-fold diluted urine was attempted to identify the glucuronic acid conjugates but this was unsuccessful, whereas unconjugated sulfoxide and sulfone were both measurable (data not shown). As can be seen from Figure 2A, the metabolites of GW1516 demonstrate exceptionally slow excretion rate and are well detectable even 30 days after single dose administration. A complete clearance of the drug took about 40 days. The chromatograms given in Figure 3 illustrate that GW1516-sulfone should be the metabolite of choice when determining this compound in urine analysis.

In case of GW0742 all aforementioned findings were essentially the same, except the fact that the maximal urinary concentration of GW0742 reached only *ca.* 0.3 ng/ml with its limit of detection being at 0.1 ng/ml. Similarly, the abundance of its metabolites was about 10-fold lower than that of GW1516. Because of this, the detection time window was almost twice as short (Figure 2B). Figure 4 shows the mass chromatograms for the urine samples collected 25 h and 9 days after administration of 15 mg GW0742, which once again demonstrates unsuitability of a screening method relying on the detection of the parent drug only.

Conclusion

For reliable detection of PPARδ agonists GW1516 and GW0742 in urine the optimal targets are the bisoxygenated (sulfone) metabolites. Recommended analytical procedure is the analysis of enzymatically deconjugated urine by LC-MS. Using this procedure, GW1516-sulfone could be detected in urine up to 40 days after a single dose of

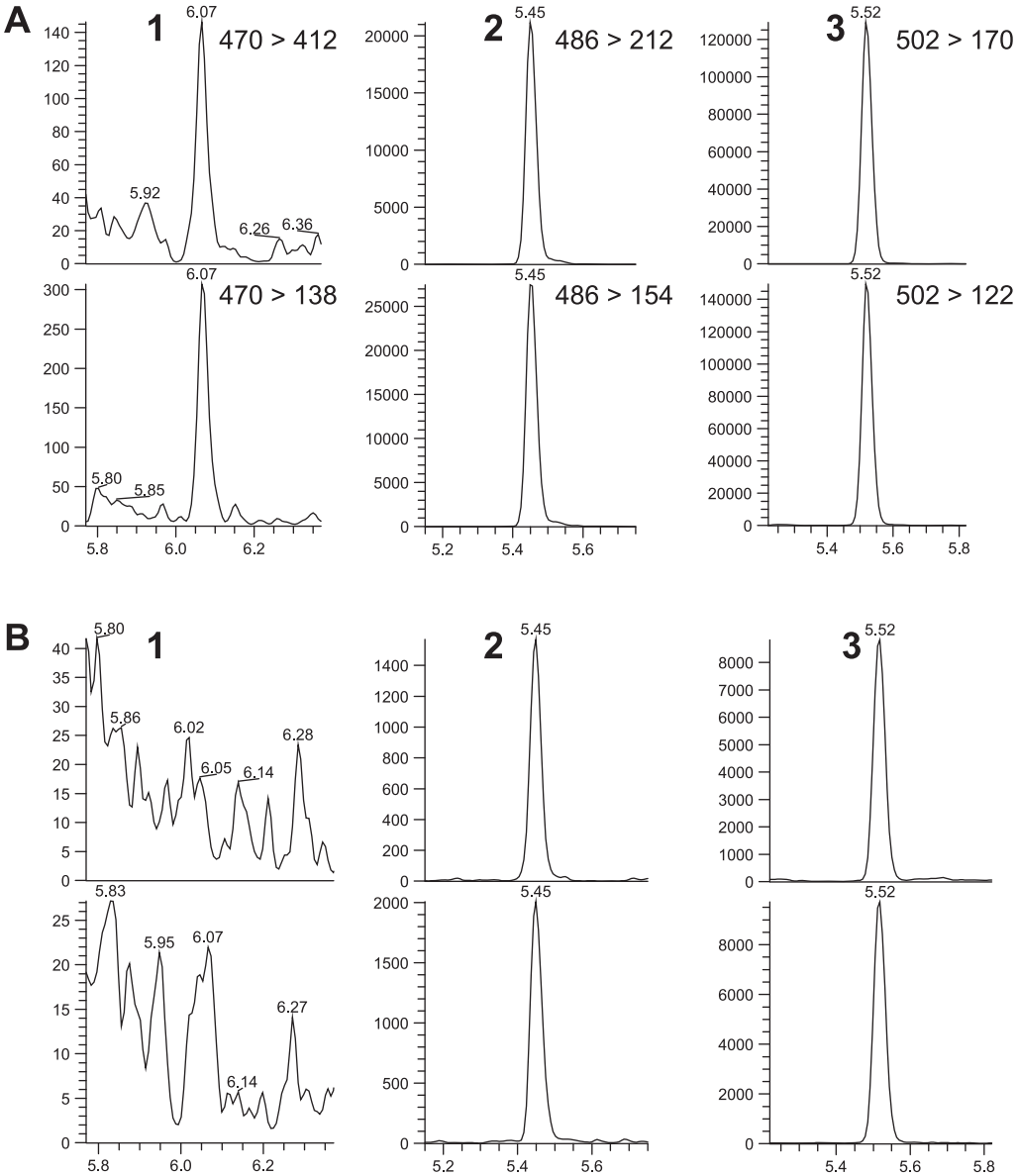

Figure 4. SRM chromatograms for GW0742 (1) and its sulfoxide (2) and sulfone (3) metabolites at 25 h (A) and 9 days (B) after administration.

15 mg. For the same dose of GW0742 the detection period of sulfone metabolite is about 20 days. This could be explained by the fact that the abundance of urinary metabolites of GW0742 is approximately ten times lower. Until the sulfone metabolites become commercially available, the excretion urine samples may be used as positive control to confirm the suspicious urine samples in doping control analysis.

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