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In-vitro metabolism of GW1516 and implementation of its major metabolites in routine doping controls

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Abstract

The PPARδ-agonist GW1516 is categorized as a gene doping substance by the World Anti-Doping Agency due to its upregulation of genes associated with oxidative metabolism and a modified substrate preference potentially resulting in an improved athletic performance. To enable the detection of abuse in sport via analysis of urine specimens, the phase-I and -II metabolism of GW1516 was simulated by an *in-vitro* assay. Two oxygenated metabolites (sulfoxide and sulfone) were identified as main phase-I metabolites as well as one hydroxylation and the respective monoglucuronides of GW1516 and the oxygenated metabolites as phase-II metabolites. As the sulfoxide and the sulfone were both excreted in urine, they were chosen as target analytes and successfully implemented in routine doping control procedures.

Introduction

In 2008, the peroxisome-proliferator-activated receptor (PPAR)δ-agonist GW1516 (also referred to as GW501516, Fig. 1a) hit the headlines as "exercise pill" in the press due to its drastically performance enhancing effects shown in animal experiments: The administration of 2-5 mg/kg/d to laboratory rodents resulted in changes in muscle fiber composition to more Type-I, slow-twitch fibers as well as a modified substrate preference shifting from carbohydrate to lipid consumption. Furthermore, reduced susceptibility to weight gain and an increased endurance performance by approximately 70% compared to control cohorts in presence of exercise stimuli was observed[1]. Due to these results, GW1516 was selected as a

drug candidate for the treatment of obesity and associated diseases, which has completed phase II and IV clinical trials regarding dyslipidemia and the regulation of the lipoprotein transport in metabolic syndrome conditions[2-4]. However, this drug candidate is not only interesting for the treatment of diseases but has also high abuse potential in sports due to its upregulation of genes associated with oxidative metabolism as well as an increased lipid consumption[1] and has therefore been classified as doping substance under paragraph M3 of the prohibited list by the World Anti-Doping Agency since January 2009[5]. To enable the development of a detection method for this compound, its mass spectrometric behavior was characterized[6,7] and a first analytical approach established which targets the intact drug in plasma samples[6]. However, the most frequently provided doping control samples are still urine specimens, and thus, the knowledge and characterization of urinary metabolites is of particular interest. In the present report, the phase-I and -II metabolism of GW1516 were elucidated using an *in-vitro* assay with human liver microsomal fraction and subsequently, the oxygenated phase-I metabolites being the most abundant ones were implemented into sports drug testing programs.

Materials and Methods

Chemicals and reference substance. Potassium carbonate, potassium hydrogen carbonate, potassium phosphate monobasic trihydrate, sodium phosphate dibasic, ammonium acetate, acetic acid and ethyl acetate were purchased from Merck (Darmstadt, Germany) and magnesium chloride hexahydrate, deuterium oxide (D_2O), sodium phosphate monobasic, D-saccharic acid 1,4-lactone monohydrate (SL) and uridine-diphospho-glucuronic acid trisodium salt (UDPGA) from Sigma (Deisendorf, Germany). Methanol and acetonitrile (both HPLC grade) were obtained from VWR (Leuven, Belgium) and Honeywell (Seelze, Germany) respectively, *t*-butyl methyl ether from AppliChem (Darmstadt, Germany) and β–glucuronidase (*E. coli*) as well as nicotinamide adenine dinucleotide phosphate (NADPH) from Roche Diagnostics (Mannheim, Germany). The reference substance GW1516 was obtained from AXXORA (Lörrach, Germany) and human liver microsomal fraction was from BD Gentest (Woburn, MA, USA) as pooled mixture of different individual donors (protein content 20 mg/mL). Deionized water used for aqueous buffers and solutions was of Milli-Q grade.

In-vitro metabolism. *In-vitro* metabolism study samples were prepared according to standard

protocols[8]. Briefly, the incubation mixture (total volume of 100 µL) contained 10 µM GW1516, 5 mM NADPH, 5 mM SL and 10 µg human liver microsomal preparation in 50 mM phosphate buffer with 5 mM $MgCl_2$ (pH 7.4). The incubation was conducted at 37 °C with continuous shaking and, for phase-I metabolism, terminated after 2 h by adding 100 µL of ice-cold acetonitrile. For subsequent phase-II metabolism, additional 5 µg human liver microsomal preparation and 10 mM UDPGA were added to the incubation mixture for glucuronidation before the incubation was continued for further 2 h. As negative controls, incubations were made without GW1516 and human liver microsomal fraction, respectively. After termination of the enzymatic reaction by the addition of ice-cold acetonitrile, all samples were centrifuged (10 min, 10,000×*g*) and the supernatant transferred to a fresh Eppendorf tube and evaporated *in vacuo*. The dry residue was reconstituted in 20 µL of acetic acid (2%) and the target analytes were twice extracted into 500 µL of ethyl acetate. The combined organic layer was evaporated to dryness and reconstituted in 50 µL of methanol and 5 mM ammonium acetate buffer (pH 3.5) (1:1, *v/v*) prior to LC-MS(/MS) analysis.

Liquid chromatography-(tandem) mass spectrometry (LC-MS(/MS)). GW1516 and its metabolites were characterized using a Thermo Accela LC coupled to a Thermo Exactive mass spectrometer (Bremen, Germany). The LC was equipped with a Thermo Scientific Hypersil Gold column (2.1 x 50 mm, particle size 1.9 µm) and 0.1% formic acid (A) and acetonitrile (B) as solvents. The flow rate was set to 200 µL/min and the gradient decreased from 90% A to 20% A within 12 min and then to 0% A in further 3 min, maintaining there for 2 min followed by re-equilibration. The MS, operated in positive ionization mode, was calibrated using the manufacturer's calibration mixture (seven reference masses) and mass accuracies <5 ppm were accomplished for the period of analysis. With a capillary temperature of 290 °C and an ionization voltage set to +3.5 kV, three MS settings were used: a) full scan MS from *m/z* 50-2,000 at a resolution of 25,000 (full width at half maximum), b) full scan MS (*m/z* 50-2,000, resolution set to 10,000) with higher energy collision-induced dissociation (HCD) set to 20 V or c) to 50 V. The curved linear ion trap was supplied with nitrogen, obtained from a CMC nitrogen generator (Eschborn, Germany). MS^n experiments from selected precursor ions were conducted using identical LC conditions but with an LTQ-Orbitrap mass spectrometer (Thermo, Bremen, Germany). This setup but with D_2O and acetonitrile as solvents was also used to differentiate hydroxylated and oxygenated products.

Urine sample preparation. The sample preparation was performed in accordance to established doping control screening procedures allowing a rapid implementation of the new target analyte into existing protocols[9]. Briefly, 1 mL of urine, fortified with 20 ng of methyltestosterone as internal standard, was buffered to pH 7.0. After addition of 20 µL of β-glucuronidase, the sample was hydrolyzed for 1 h at 50 °C followed by liquid-liquid extraction at pH 9.6 with 4 mL of *t*-butyl methyl ether. The organic layer was transferred into a fresh glass tube and evaporated to dryness. The dry residue was reconstituted in 100 µL of water/acetonitrile (4:1, *v/v*) and a total of 10 µL was injected into the LC-MS(/MS) system.

Authentic urine specimens. Proof-of-concept for the applicability of the developed assay was obtained by analyzing urine specimens from a healthy male individual after a one-time oral application of 2 mg of GW1516. Written consent of the volunteer as well as approval by the ethical committee was obtained and the samples were prepared and analyzed using the above reported approach.

Results and Discussion

The mass spectrometric behavior of GW1516 under positive ESI and CID conditions was reported in detail elsewhere[6,7]. The most abundant product ion at *m/z* 257 is attributed to a homolytic cleavage of the S-12–C-13 thioether bond (-197 Da, Figure 1a), further characteristic product ions were observed at *m/z* 396 (-58 Da) corresponding to an elimination of oxiran-2-one/glyoxal as well as at *m/z* 256 and 188 which are derived from the fragment at *m/z* 257 by subsequent release of a hydrogen atom or a trifluoromethyl group, respectively.

These characteristic product ions were employed for screening *in-vitro* metabolism samples for the presence of metabolites and identifying the locations of modifications.

Two major phase-I metabolites (M1 and M2) were observed and furthermore one of low abundance (M3). Both, M1 and M3 were found to have a molecular weight of 469 Da and the elemental compositions of the protonated molecules were determined by high-resolution MS with $C_{21}H_{19}O_4NF_3S_2$ for both metabolites (Table 1) whereas M2 with a mass of 485 Da and an elemental composition of $C_{21}H_{19}O_5NF_3S_2$ corresponds to the further addition of another oxygen atom (Table 1). Comparing the product ion spectra, these ones of M1 and M2 are similar to that of the parent compound with the main characteristic fragments at *m/z* 256 and 257 respectively corresponding to an unaltered thiazol and trifluoromethylphenyl moiety of the molecule[10]. On the contrary, the MS/MS spectrum of M3 shows a completely different

fragmentation pattern with the loss of water as main fragment (*m/z* 452, data not shown).

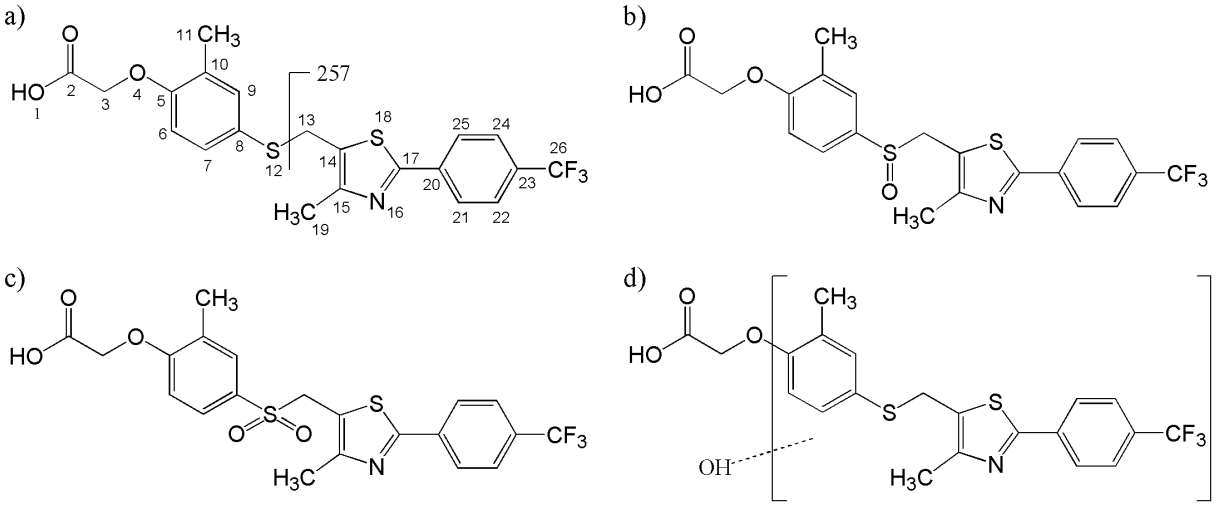

Figure 1: Structures of a) GW1516 (mol. wt. = 453) with the line indicating the main fragment ion at *m/z* 257, and its phase-I metabolites b) M1 (mol. wt. = 469), c) M2 (mol. wt. = 485) and d) M3 (mol. wt. = 469).

However, the determination of exact masses and elemental compositions does not allow a differentiation between hydroxylations and oxygenations; therefore, LC-MS analyses using D_2O/acetonitrile as solvents were performed at which all hydrogen atoms of hydroxyl groups are supposed to exchange with deuterium[11]. GW1516 is incremented by two mass units as expected (Figure 2a) corresponding to an H/D exchange of the hydrogen atom of the carboxyl function as well as the charge introduced by the ionization process. In the corresponding MS/MS spectrum (Figure 2b), all characteristic product ions were observed incremented by 1 respectively 2 mass units, depending on whether the carboxyl function is still present in the molecule, except for the fragments at *m/z* 256 and 288,which have been retained unchanged.

The mass of M1 was, comparable to that of GW1516 itself, only increased by two mass units corresponding to an oxygenation as the presence of a new hydroxyl function was excluded by no additional H/D exchange. With the main characteristic fragment at *m/z* 256 being unaltered, the modification has to be located at the substituted thiophenol residue of the molecule (Figure 2c & d). For M2, analogously to M1, no additional H/D exchange was observed either and the main characteristic fragments indicate also an unaltered thiazol and trifluoromethylphenyl moiety (data not shown).

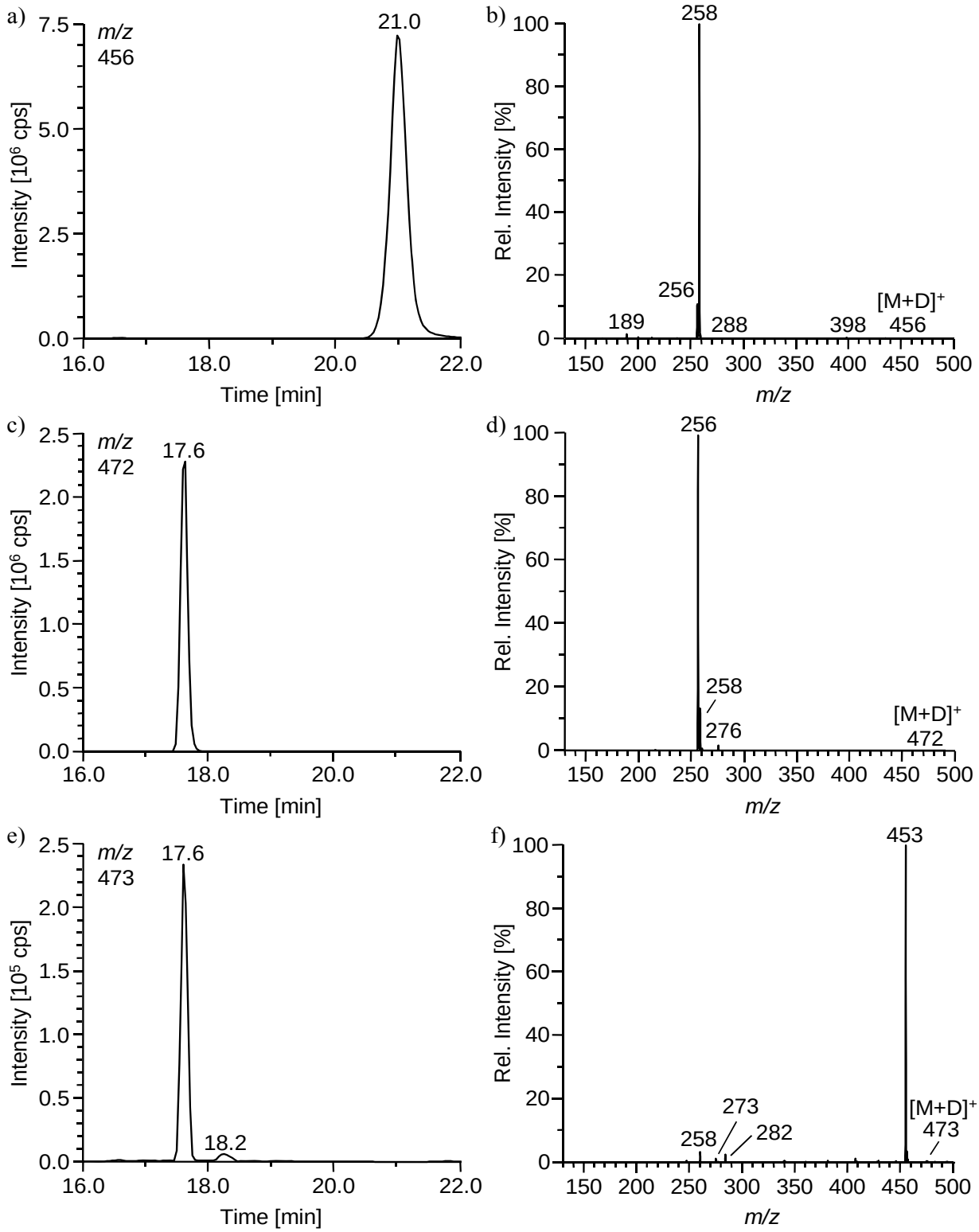

Figure 2: LC-MS/MS chromatograms employing D_2O/acetonitrile as solvents with the corresponding MS/MS spectra of GW1516 (*m/z* 456, RT 21.0 min, corresponding to two H/D exchanges, a) & b)), M1 (*m/z* 472, RT 17.6 min, equal also to two H/D exchanges, c) & d)) as well as M3 (*m/z* 473, RT 18.2 min, correlating with three H/D exchanges, e) & f)).

Table 1: Elemental compositions and resulting diagnostic product ions of GW1516 and its phase-I and –II metabolites using high-resolution/high-accuracy mass spectrometry.

Cmp.	Precursor ion (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Product ion (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Cleaved species
phase-I							
GW1516	454.0755	$C_{21}H_{19}O_3NF_3S_2$	0.4	396.0700	$C_{19}H_{17}ONF_3S_2$	0.6	$C_2H_2O_2$
				288.0123	$C_{12}H_9NF_3S_2$	-0.0	$C_9H_{10}O_3$
				257.0482	$C_{12}H_{10}NF_3S$	0.6	$C_9H_9O_3S$
				256.0406	$C_{12}H_9NF_3S$	1.5	$C_9H_{10}O_3S$
				188.0529	$C_{11}H_{10}NS$	0.1	$C_{10}H_9O_3F_3S$
				172.0370	$C_8H_5NF_3$	1.1	$C_{13}H_{14}O_3S_2$
M1	470.0701	$C_{21}H_{19}O_4NF_3S_2$	-0.2	274.0508	$C_{12}H_{11}ONF_3S$	0.0	$C_9H_8O_3S$
				257.0481	$C_{12}H_{10}NF_3S$	0.0	$C_9H_9O_4S$
				256.0404	$C_{12}H_9NF_3S$	0.5	$C_9H_{10}O_4S$
				188.0526	$C_{11}H_{10}NS$	-1.3	$C_{10}H_9O_4F_3S$
M2	486.0654	$C_{21}H_{19}O_5NF_3S_2$	0.5	272.0353	$C_{12}H_9ONF_3S$	0.5	$C_9H_{10}O_4S$
				257.0482	$C_{12}H_{10}NF_3S$	0.7	$C_9H_9O_5S$
				256.0406	$C_{12}H_9NF_3S$	1.4	$C_9H_{10}O_5S$
				244.0404	$C_{11}H_9NF_3S$	0.9	$C_{10}H_{10}O_5S$
				188.0528	$C_{11}H_{10}NS$	-0.0	$C_{10}H_9O_5F_3S$
M3	470.0702	$C_{21}H_{19}O_4NF_3S_2$	0.0	452.0600	$C_{21}H_{17}O_3NF_3S_2$	0.8	H_2O
				272.0351	$C_{12}H_9ONF_3S$	0.0	$C_9H_{10}O_3S$
				256.0404	$C_{12}H_9NF_3S$	0.7	$C_9H_{10}O_4S$
phase-II							
Gluc.-GW1516	630.1070	$C_{27}H_{27}O_9NF_3S_2$	-0.6	454.0751	$C_{21}H_{19}O_3NF_3S_2$	-0.4	$C_6H_8O_6$
				257.0481	$C_{12}H_{10}NF_3S$	0.0	$C_{15}H_{17}O_9S$
				256.0401	$C_{12}H_9NF_3S$	-0.3	$C_{15}H_{18}O_9S$
				188.0530	$C_{11}H_{10}NS$	0.8	$C_{16}H_{17}O_9F_3S$
Gluc.-M1	646.1022	$C_{27}H_{27}O_{10}NF_3S_2$	-0.1	274.0505	$C_{12}H_{11}ONF_3S$	-1.0	$C_{15}H_{16}O_9S$
				257.0482	$C_{12}H_{10}NF_3S$	-2.2	$C_{15}H_{17}O_{10}S$
				256.0402	$C_{12}H_9NF_3S$	-0.1	$C_{15}H_{18}O_{10}S$
				188.0531	$C_{11}H_{10}NS$	1.2	$C_{16}H_{17}O_{10}F_3S$
Gluc.-M2	662.0977	$C_{27}H_{27}O_{11}NF_3S_2$	0.7	486.0647	$C_{21}H_{19}O_5NF_3S_2$	-1.0	$C_6H_8O_6$
				272.0346	$C_{12}H_9ONF_3S$	-2.1	$C_{15}H_{18}O_{10}S$
				257.0472	$C_{12}H_{10}NF_3S$	-3.3	$C_{15}H_{17}O_{11}S$
				256.0403	$C_{12}H_9NF_3S$	0.1	$C_{15}H_{18}O_{11}S$
				244.0400	$C_{11}H_9NF_3S$	-1.1	$C_{16}H_{18}O_{11}S$
				188.0529	$C_{11}H_{10}NS$	0.1	$C_{16}H_{17}O_{11}F_3S$

Consequently, common oxidation reactions at the sulfur atom (position 12, Figure 1a) were considered yielding the sulfoxide (M1) and the sulfone (M2) respectively (Figure 1b & c) and were confirmed by LC-NMR analysis after synthesis of these oxygenated derivatives[10].

In the MS/MS chromatogram of *m/z* 473 (Figure 2e) corresponding to an increase of three mass units in comparison to *m/z* 470, two peaks were observed. The most abundant of which at a retention time of 17.6 min was identified as ^{13}C isotope of M1 and the peak at 18.2 min as a further metabolite (M3). Having one additional H/D exchange compared with GW1516, M1

and M2 and a loss of D_2O (Figure 2f) as main characteristic fragment, a hydroxylation was assumed for this metabolite M3 (Figure 1d).

As phase-II metabolites, the monoglucuronides (Gluc.) of GW1516, M1 and M2 were identified by means of their MS/MS spectra as well as exact masses (Table 1). As an example, the product ion mass spectrum of the glucuronic acid conjugate of M2 is shown in Figure 3. Besides two dehydratisation reactions forming the product ions at *m/z* 644 and 626, the fragment at *m/z* 486 corresponding to the aglycon (M2) is observed as well as its characteristic product ions at *m/z* 272 and 256 which further substantiate the attributed structure of the glucuronide.

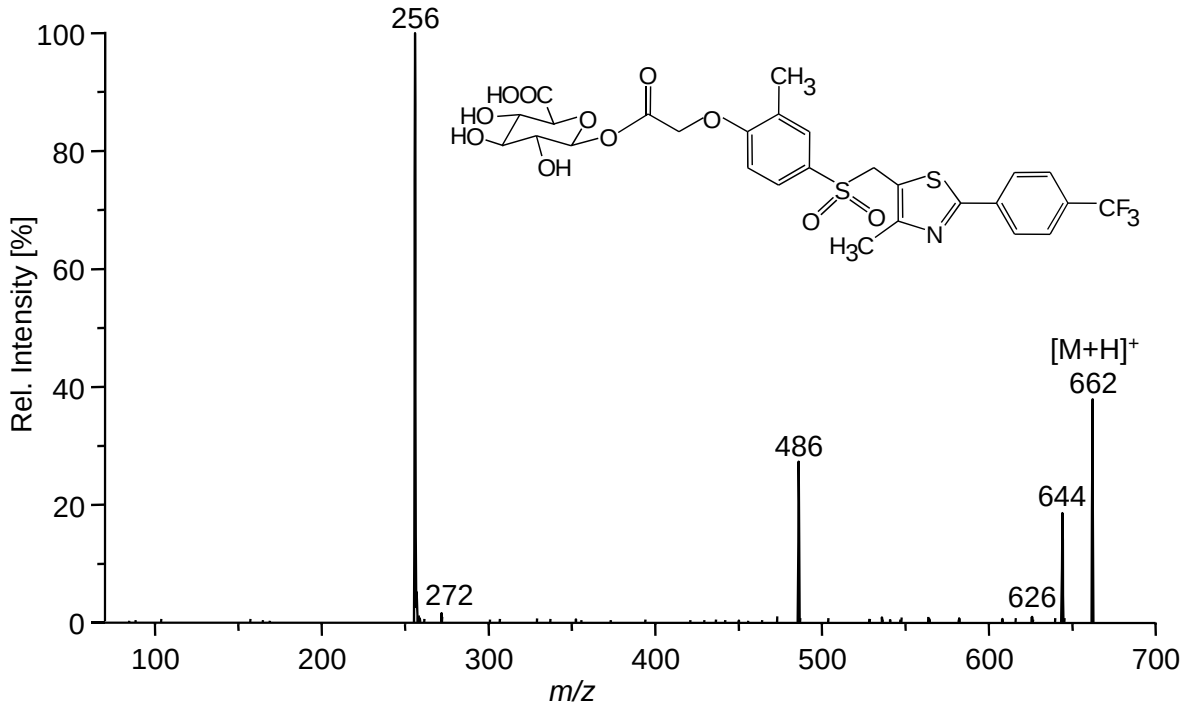

Figure 3: ESI product ion mass spectrum of the protonated monoglucuronide of M2.

After elucidation of the major metabolic pathways of GW1516 using an *in-vitro* assay, urine samples from an excretion study were analyzed to evaluate whether the *in-vitro* simulated metabolites are also excreted in urine. The presence of all three *in-vitro* identified phase-I metabolites was confirmed in these urine specimens with the oxygenated metabolites, comparable to the *in-vitro* study, being the most abundant ones (Figure 4). Therefore they were chosen as target analytes and successfully implemented in a routine doping control assay.

Employing this setup and considering therapeutic dosages of GW1516 of 2.5 mg/day[2], detection windows for the metabolites of several days are expected following cessation of the drug.

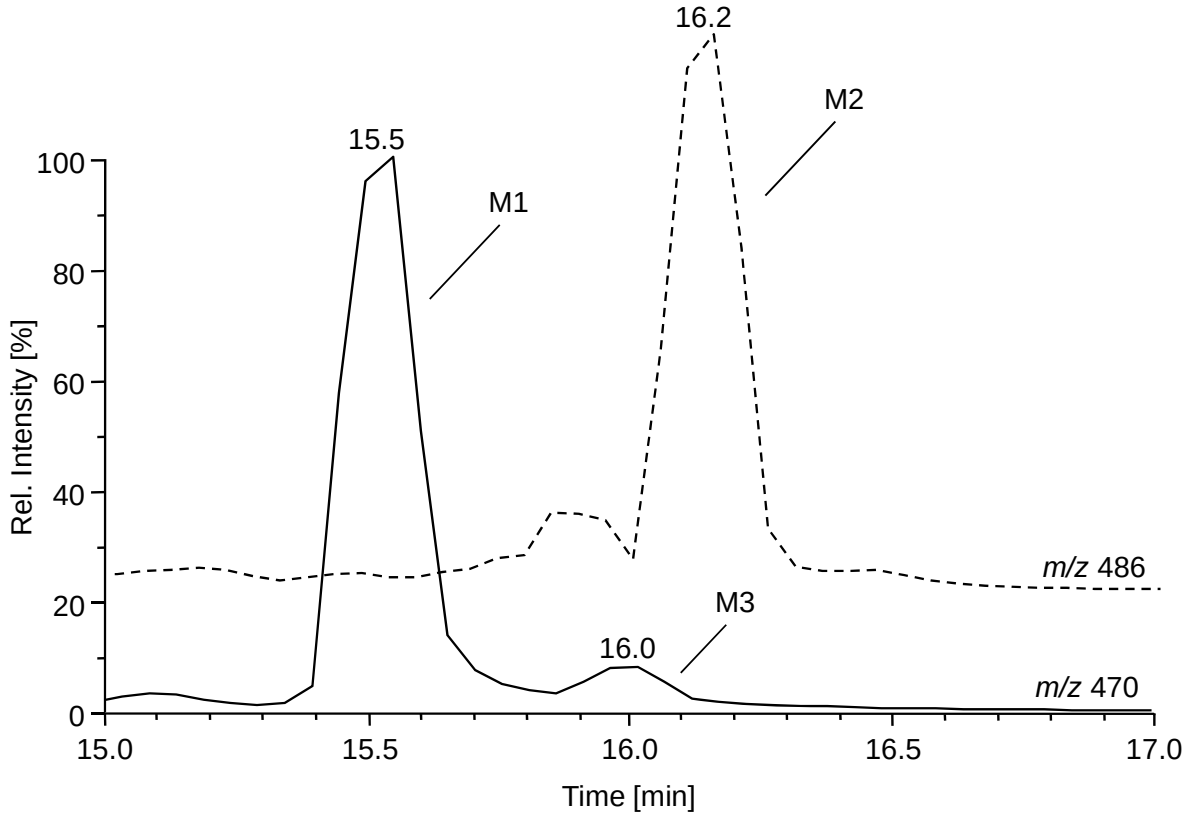

Figure 4: Product ion chromatograms of *m/z* 470 and 486 of a post-administration urine specimen.

Acknowledgements

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