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Horse metabolism and the photocatalytic process as a tool to identify metabolic products formed from dopant substances: the case of sildenafil

Claudio Medana,[a]* Paola Calza,[b] Valeria Giancotti,[a] Federica Dal Bello,[a] Emanuela Pasello,[a] Marco Montana[a] and Claudio Baiocchi[a]

Two horses were treated with sildenafil, and its metabolic products were sought in both urine and plasma samples. Prior to this, a simulative laboratory study had been done using a photocatalytic process, to identify all possible main and secondary transformation products, in a clean matrix; these were then sought in the biological samples.

The transformation of sildenafil and the formation of intermediate products were evaluated adopting titanium dioxide as photocatalyst. Several products were formed and characterized using the HPLC/HRMS^n technique. The main intermediates identified in these experimental conditions were the same as the major sildenafil metabolites found in *in vivo* studies on rats and horses. Concerning horse metabolism, sildenafil and the demethylated product (UK 103,320) were quantified in blood samples. Sildenafil propyloxide, de-ethyl, and demethyl sildenafil, were the main metabolites quantified in urine. Some more oxidized species, already formed in the photocatalytic process, were also found in urine and plasma samples of treated animals. Their formation involved hydroxylation on the aromatic ring, combined oxidation and dihydroxylation, N-demethylation on the pyrazole ring, and hydroxylation. These new findings could be of interest in further metabolism studies. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: sildenafil; metabolism; doping control; photocatalysis; HRMS

Introduction

Sildenafil (1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1-H-pyrazole [4,3-d] pyrimidin-5-yl)-4- ethoxy] phenylsulfonil]-4-methylpiperazine citrate) is employed in horse racing because of its action at the pulmonary level: it is known that sildenafil increases exercise capacity during hypoxia, at both low and high altitudes.[1] This drug can increase athletic performance in conditions of low oxygenation, countering the lowered performance occurring in conditions of hypoxia.[2,3] It also has a stimulating effect on the sympathetic nervous system.[4] The primary pharmacological action of sildenafil is due to its selective inhibition of phosphodiesterase type 5.[5–7]

Knowledge of biotransformation products is important in anti-doping analyses, since the presence of metabolites is proof of the administration of the prohibited substance: identification of possible metabolic structures may be of considerable use, to direct the search towards these analytes during antidoping controls. Metabolic studies on sildenafil have been done on rats, rabbits, dogs, horses, and man.[8–13] Three main metabolites have been found in plasma and urine samples: desmethyl-sildenafil, de-ethyl sildenafil, and sildenafil-propyloxide. In the blood, the demethylated product UK 103,320 is the main metabolite, both in man and animals. In addition, N,N-dealkylation on the piperazine ring (UK 150,564), aliphatic hydroxylation (M6), oxidation on the piperazine ring, and N-demethylation on the pyrazole ring (UK 95,340) also occur. With regard to horse metabolism, sildenafil and UK 103,320 have been quantified in blood samples; sildenafil propyloxide, de-ethyl and demethyl sildenafil are the main metabolites in urine samples.[12]

This study utilized a photocatalytic process to artificially produce chemically modified products, which may reasonably be assumed to be similar to those formed by the metabolic system of living organisms. This methodology approach has already been used successfully in the case of dexamethasone[14] and buspirone.[15] The photocatalytic process, through the production of OH radicals,[16–19] may produce compounds similar to those formed through a phase I metabolic route. The use of a simulative process is useful to determine new metabolic products that can be sought in anti-doping controls. An advantage of the simulative process is that it enables one to work on an easier matrix (ultra pure water) than urine or plasma, and to utilize higher concentrations than those found *in vivo*.

* *Correspondence to: Claudio Medana, Department of Analytical Chemistry, University of Turin, via P. Giuria 5, 10125 Torino, Italy. E-mail: claudio.medana@unito.it*

a *Department of Analytical Chemistry, University of Turin, Torino, Italy*

b *Department of Analytical Chemistry and NIS Centre of Excellence, University of Turin, Torino, Italy*

Techniques to investigate sildenafil and/or its metabolites in several biological matrices have been reported.[20–27] Sildenafil and its metabolites may be detected using gas chromatography-mass spectrometry (GC-MS)[28] or liquid chromatograph-mass spectrometry (LC-MS).[29] Because of the four ionization sites present, as described by Gobry *et al.*[30] the most suitable analysis technique to identify and characterize the main transformation products formed from sildenafil is high performance liquid chromatography (HPLC) coupled with mass spectrometry.

All transformation products originating through sildenafil degradation were characterized by HPLC/HRMS^n using an Orbitrap mass analyzer and then sought in the *in vivo* experiments. In this study, two horses were treated with sildenafil and the metabolic products were sought in both urine and plasma samples.

Materials and methods

Materials and reagents

Sildenafil citrate was extracted from Viagra® (Pfizer). Experiments were carried out using TiO_2 Degussa P25 (Frankfurt, Germany), as photocatalyst. Trichloroacetic acid and chloroform were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade water was from MilliQ System Academic (Millipore, Milan, Italy). Acetonitrile (Scharlau AC0331 Supergradient HPLC grade) was filtered through a 0.45 μm filter before use. Pentane (95%), 2-propanol, ammonium acetate and chloroform (99.8%) were from Merck (Milan, Italy).

Treatment of horses

Four hundred mg of sildenafil were given to a filly and to a gelding. The weight of each animal was approximately 400 kg (the dose thus being the equivalent of 1 mg/kg body weight). Blood samples were taken at progressive times from 0 min to 3 h 30 min (Table 5), while urine samples were collected until 28 h after administration (Table 4). The used procedures were as humane as possible and complied with the international guidelines for animal care.

Irradiation procedures

Irradiation was performed using a Philips (Monza, Italy) TLK/05 lamp (40 W/m^2) with maximum emission at 360 nm. Irradiation experiments were carried out in Pyrex glass cells containing sildenafil (20 mg/l) and TiO_2 (200 mg/l). The temperature reached during irradiation was 38 ± 2 °C.

Sample preparation

Urine and plasma samples were subjected to liquid/liquid extraction. Urine samples were equilibrated at 25 °C and extracted with a chloroform:2-propanol:pentane mixture (70:10:20 *v/v*).

Plasma samples (1 ml) were treated with 1 ml 0.6 M trichloroacetic acid to precipitate proteins and then extracted with chloroform:2-propanol (95:5 *v/v*). After centrifugation at 6000 rpm for 5 min, the organic phases were dried under nitrogen.

Liquid chromatography

Chromatographic separation followed by MS analysis was run on a Phenomenex (Castel Maggiore, BO, Italy) Synergi C18 column, 150 × 2.0 mm, 3 μm particle size, using an Ultimate 3000 HPLC instrument (Dionex, Milan, Italy). Injection volume was 20 μl and flow rate 200 μl/min. Gradient mobile phase composition was adopted: 5/95 to 100/0 in 21 min acetonitrile/ammonium acetate 0.1 mM. Chromatographic analysis for phenol derivatives was done on a RP C18 column (Lichrochart, Merck, Milan, Italy, 12.5 cm × 0.4 cm, 5 μm packing) using an HPLC instrument with two high-pressure pumps (L-6200 and L-6000) and an UV-Vis detector (L-4200) (Merck Hitachi, Darmstadt, Germany).

Mass spectrometry

An LTQ Orbitrap mass spectrometer (Thermo Scientific, Rodano, Italy), equipped with an atmospheric pressure interface and an electrospray ionization (ESI) ion source, was used.

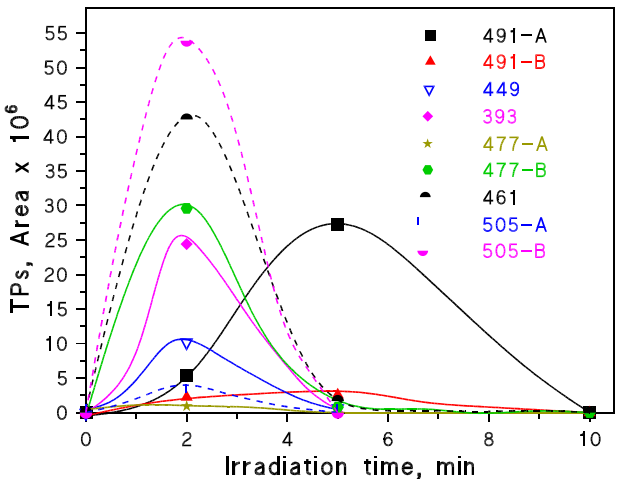


Figure 1. Formation profiles of the transformation products detected for sildenafil; the dotted curves relate to the ordinate axis on the right of the graph.

Table 1. List of main $[M+H]^+$ and fragments from MS and MS^n spectra obtained from sildenafil

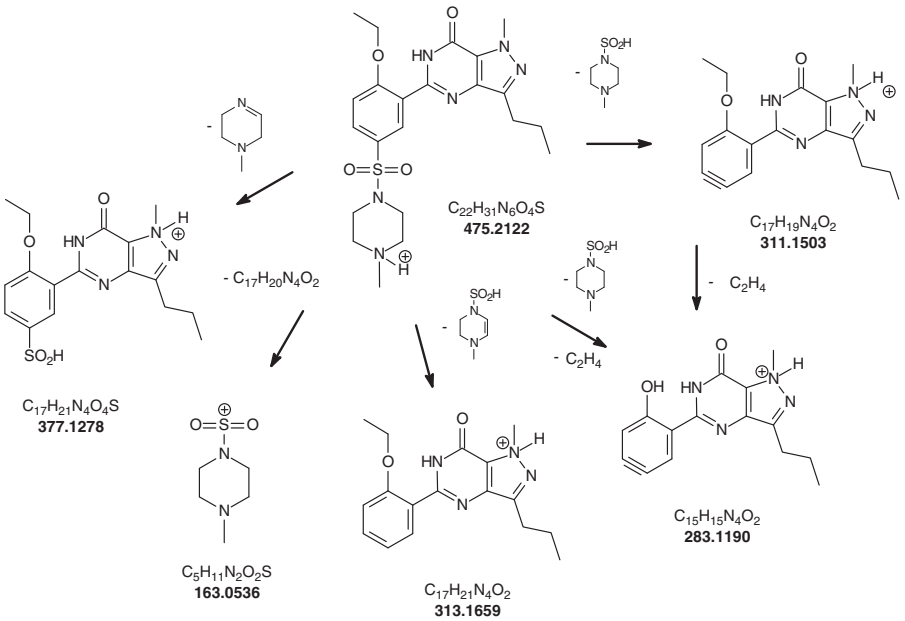
$[M+H]^+$	MS^2	MS^3
475.2122	311.1503 (100) $C_{17}H_{19}N_4O_2$	283.1190 (100) $C_{15}H_{15}N_4O_2$
	377.1278 (93) $C_{17}H_{21}N_4O_4S$	331.0859 (100) $C_{15}H_{15}N_4O_3S$
		321.1581 (71) $C_{17}H_{20}N_4O_2$
		349.0965 (66) $C_{15}H_{17}N_4O_4S$
		341.1067 (44) $C_{17}H_{17}N_4O_2S$
		359.1172 (17) $C_{17}H_{19}N_4O_3S$
		328.1530 (14) $C_{17}H_{20}N_4O_3$
		284.1267 (10) $C_{15}H_{16}N_4O_2$
	313.1659 (39) $C_{17}H_{21}N_4O_2$	285.1346 (100) $C_{15}H_{17}N_4O_2$
		284.1268 (13) $C_{15}H_{16}N_4O_2$
	283.1190 (37) $C_{15}H_{15}N_4O_2$	255.1240 (100) $C_{14}H_{15}N_4O$
		256.0958 (47) $C_{13}H_{12}N_4O_2$
		254.0798 (20) $C_{13}H_{10}N_4O_2$
		255.0879 (13) $C_{13}H_{11}N_4O_2$
	329.1615 (18) $C_{17}H_{21}N_4O_3$	-
	299.1145 (18) $C_{15}H_{15}N_3O_4$	-
	163.0536 (15) $C_5H_{11}N_2O_2S$	-
	297.1352 (13) $C_{16}H_{17}N_4O_2$	-
	312.1593 (13) $C_{17}H_{20}N_4O_2$	-
	391.1441 (11) $C_{18}H_{23}N_4O_4S$	-

The LC column effluent was delivered to the ion source, using nitrogen as both sheath and auxiliary gas. The source voltage was set to 4.2 kV. The heated capillary temperature was maintained at 275 °C. The acquisition method used had previously been optimized in the tuning sections for the parent compound (capillary, magnetic lenses, and collimating octapoles voltages) in order to achieve maximum sensitivity. The main tuning parameters adopted for the ESI source were 7.00 V for capillary voltage and 80 V for tube lens. Full-scan spectra were acquired in the range 50–1000 *m/z*. MS^n spectra were acquired in the range between ion trap cut-off and precursor ion *m/z* values. Mass accuracy of recorded ions (vs. calculated) was ± 0.001 u (without internal calibration).

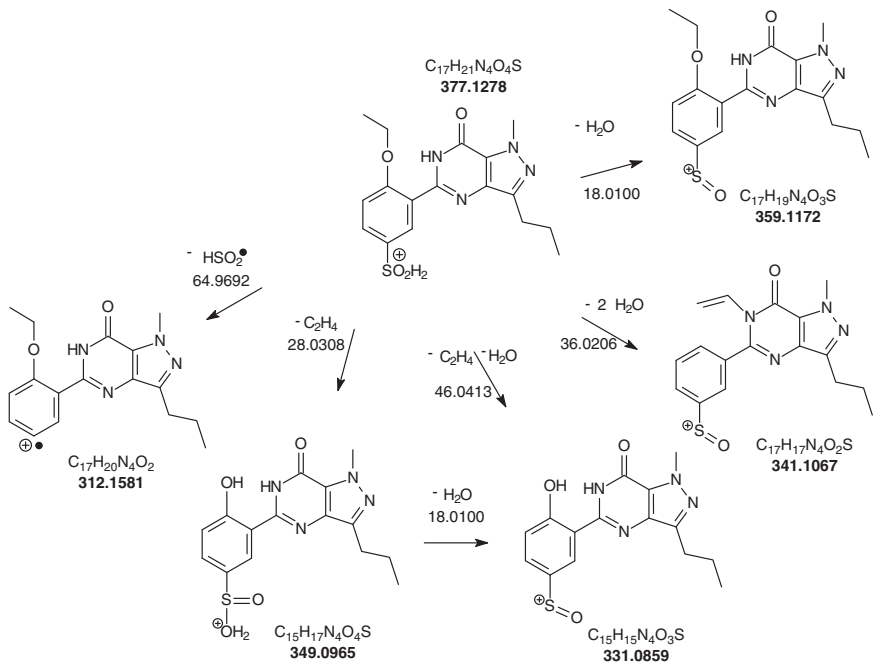
Results and discussion

Laboratory simulation

Laboratory simulation was initially performed on sildenafil in ultrapure water, in the presence of TiO_2 as photocatalyst. The parent drug is easily degraded and completely disappeared within 10 min of irradiation. In parallel with the disappearance of sildenafil, a number of transformation products (TPs) were

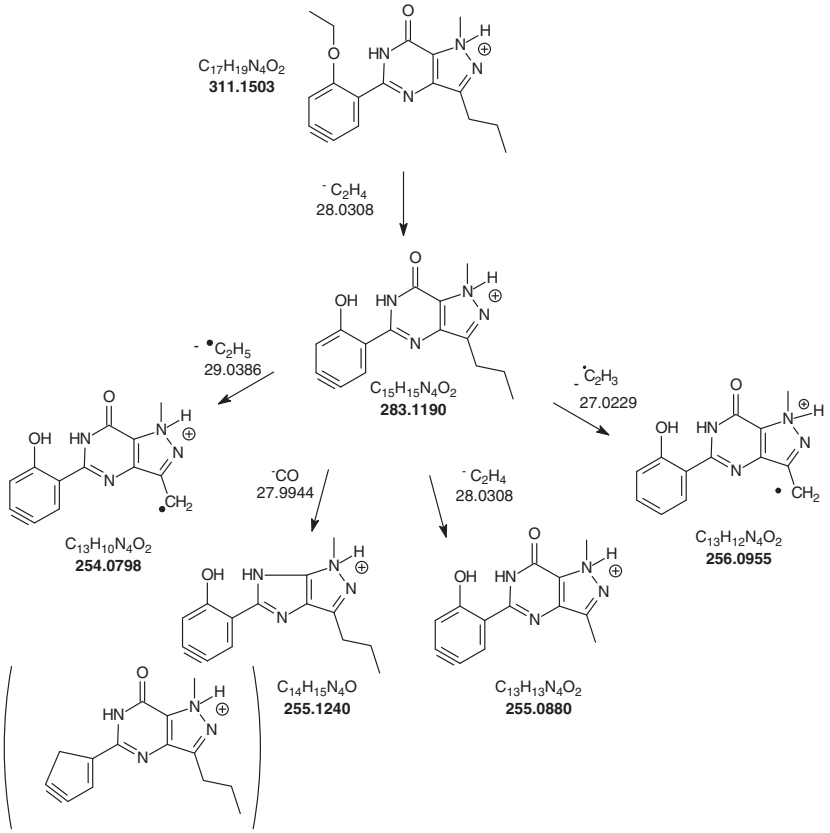


Scheme 1. Sildenafil ($[M + H]^+$ 475.2122) MS^2 fragmentation pathways.

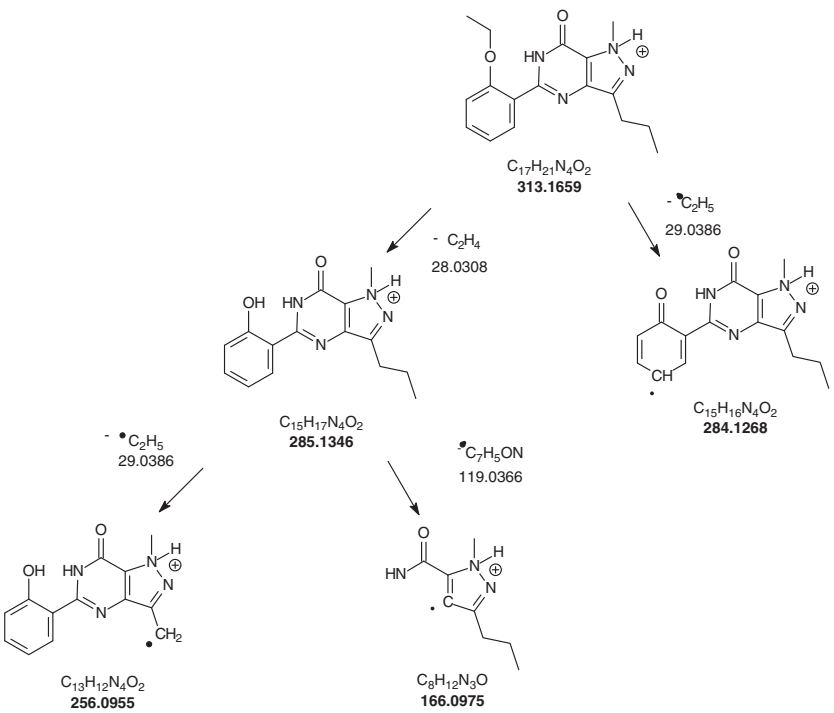


Scheme 2. MS^3 key product ions formed from sildenafil MS^2 ion at *m/z* 377.1278.

identified. Their sequential evolution is shown in Figure 1 and will be discussed under study of transformation products. TPs were characterized by different *m/z* ratios, as well as by several peaks corresponding to an equal *m/z* value, as shown in Figure 1. This finding is in agreement with the non-selectivity of the active species involved in the photocatalytic process, i.e. OH radicals.[31]



Scheme 3. Key fragmentation pathways followed by the sildenafil second generation precursor ion at *m/z* 311.1503.



Scheme 4. Key fragmentation pathways followed by the sildenafil second-generation precursor ion at 313.1659.

Sildenafil multistage mass spectrometry study: MS^2

The sildenafil MS^2 and MS^3 fragmentation study showed several distinctive losses, that were carefully considered in identifying the unknown compounds formed during the photo-induced degradation of the drug. The MS^2 spectrum presents the product ions summarized in Table 1, and attributed to the product ions shown in Scheme 1. ESI-MS spectra are described in literature;[32,33] MS^n fragments identification was confirmed by high resolution study of neutral losses.

The main product ions were formed through simple piperazine moiety loss (*m/z* 377.1278) or through cleavage of the C-S bond, with the formation of two complementary ions at *m/z* 311.1503 and 163.0536.

Sildenafil multistage mass spectrometry study: MS^3

The MS^2 ions were subjected to further fragmentation, and the MS^3 spectra product ions, with their relative intensities, are shown in Table 1. Some of the MS^3 product ions played key roles in identifying unknown compounds, and require further discussion.

The second-generation precursor ion at *m/z* 377.1278 follows the pathway described in Scheme 2: the product ions at *m/z* 349.0965 and 331.0859 are formed through ethylene loss, or through concerted loss of ethylene and water, while the ion at *m/z* 312.1581 originates from homolytic breakage of the C-S bond and the release of the $HSO_2^{\cdot}$ radical. The product ion at *m/z* 341.1067 is formed through the loss of two water molecules.

The other second-generation precursor ion at *m/z* 311.1503 follows the pathway shown in Scheme 3: the main product ion (*m/z* 283.1190) is formed by the loss of ethylene. Its subsequent fragmentation pathways are both homolytic (ethyl or ethylene radical losses) and heterolytic (loss of ethylene or carbon monoxide).

The fragmentation pathway proposed for the precursor ion at *m/z* 313.1659 is shown in Scheme 4: also in this case the loss of the ethyl moiety might proceed *via* homo- or hetero-bond cleavage. It is noteworthy that the even electron species at 285.1346 *m/z* gives two odd electron product ions.

Characterization of sildenafil transformation products from irradiation study

Nine species were recognized, along with the disappearance of sildenafil; analyzing their MS^2 and MS^3 spectra in depth enabled a molecular structure to be assigned to all of the transformation products (TPs).

MS^n study of transformation products formed through photocatalytic degradation of sildenafil and already identified in metabolic studies

Four degradants coincide with TPs already reported as resulting from the metabolic transformation of sildenafil[12] and their main MS^2 and MS^3 product ions are shown in Table 2.

Two isobaric species at *m/z* 491.2073, labelled **491-A** and **491-B**, were formed. The difference of 15.9950 u (unified atomic mass units) compared to sildenafil closely matches the formation of its hydroxyderivatives.

491-A generates the ions at *m/z* 393.1236 (loss of piperazine) and *m/z* 449.1608 (loss of the propyl chain), thus excluding hydroxylation of these two moieties.

Hydroxylation would presumably involve the ethoxy group on the aromatic ring. In the MS^3 spectrum of *m/z* 393.1236, the lack of a loss of C_2H_6O (− 46.0413 u) supports the proposed structure.

491-B generates the following ions:

Table 2. Transformation products formed through degradation of sildenafil by the photocatalytic process and already identified in metabolic studies

$[M+H]^+$	t_R (min)	MS^2	MS^3
449.1971	**9.69**	418.1548 (100)	311.1509 (100)
$C_{20}H_{29}N_6O_4S$		$C_{19}H_{24}N_5O_4S$	$C_{17}H_{19}N_4O_2$
			377.1283 (32)
			$C_{17}H_{21}N_4O_4S$
			361.1337 (17)
			$C_{17}H_{21}N_4O_3S$
			283.1195 (10)
			$C_{15}H_{15}N_4O_2$
		392.1393 (89)	311.1506 (100)
		$C_{17}H_{22}N_5O_4S$	$C_{17}H_{19}N_4O_2$
			364.1077 (46)
			$C_{23}H_{14}N_3O_2$
			283.1193 (23)
			$C_{15}H_{15}N_4O_2$
		313.1664 (47)	285.1350 (100)
		$C_{17}H_{21}N_4O_2$	$C_{15}H_{17}N_4O_2$
		311.1508 (29)	283.1195 (100)
		$C_{17}H_{19}N_4O_2$	$C_{15}H_{15}N_4O_2$
		361.1335 (27)	332.0944 (100)
		$C_{17}H_{21}N_4O_3S$	$C_{23}H_{12}N_2O$
			284.1273 (13)
			$C_{15}H_{16}N_4O_2$
		432.1703 (22)	311.1507 (100)
		$C_{20}H_{26}N_5O_4S$	$C_{17}H_{19}N_4O_2$
		406.1549 (13)	-
		$C_{18}H_{24}N_5O_4S$	
461.1965	**10.13**	313.1664 (100)	285.1351 (100)
$C_{21}H_{29}N_6O_4S$		$C_{17}H_{21}N_4O_2$	$C_{15}H_{17}N_4O_2$
		311.1509 (99)	283.1194 8100)
		$C_{17}H_{19}N_4O_2$	$C_{15}H_{15}N_4O_2$
		377.1284 (84)	331.0866 (100)
		$C_{17}H_{21}N_4O_4S$	$C_{15}H_{15}N_4O_3S$
			312.1588 (72)
			$C_{17}H_{20}N_4O_4$
			349.0972 (57)
			$C_{15}H_{17}N_4O_4S$
			341.1073 (44)
			$C_{17}H_{17}N_4O_2S$
		283.1195 (58)	255.1245 (100)
		$C_{15}H_{15}N_4O_2$	$C_{14}H_{15}N_4O$
491.2073	8.88	473.1969 (100)	375.1124 (100)
$C_{22}H_{31}N_6O_5S$	(**491-A**)	$C_{22}H_{29}N_6O_4S$	$C_{17}H_{19}N_4O_4S$
			311.1506 (41)
			$C_{17}H_{19}N_4O_2$
			281.1036 (33)
			$C_{13}H_{17}N_2O_3S$
			377.1281 (22)
			$C_{17}H_{21}N_4O_4S$
		393.1236 (17)	365.0917 (100)
		$C_{17}H_{21}N_4O_5S$	$C_{15}H_{17}N_4O_5S$
		449.1608 (16)	-
		$C_{19}H_{25}N_6O_5S$	
	10.75	404.1392 (100)	311.1511 (100)
	(**491-B**)	$C_{18}H_{22}N_5O_4S$	$C_{17}H_{19}N_4O_2$

(Continued to next page)

Table 2. *(Continued)*

$[M+H]^+$	t_R (min)	MS^2	MS^3
		377.1283 (36) $C_{17}H_{21}N_4O_4S$	331.0865 (100) $C_{15}H_{15}N_4O_3S$ 341.1073 (50) $C_{17}H_{17}N_4O_2S$ 312.1584 (51) $C_{17}H_{20}N_4O_2$ 349.0970 (42) $C_{15}H_{17}N_4O_4S$
		473.1966 (20) $C_{22}H_{29}N_6O_4S$	-
		179.0490 (5) $C_5H_{11}N_2O_3S$	-

(1) *m/z* 404.1392: loss of C_4H_9NO, which fits the contraction of the hydroxylated piperazine ring
(2) *m/z* 473.1966: loss of a water molecule
(3) *m/z* 377.1283: this ion was also formed from sildenafil fragmentation, and closely matches the hydroxylation on the piperazine moiety

This species coincides with the metabolic product identified in blood samples by Walker *et al.*[12]

Analogously, the species at *m/z* 449.1971, ascribed to the dealkylation product, and that at *m/z* 461.1965 (demethylated sildenafil), were already found in previous metabolism studies, and are shown in Figure 2.[12,13]

MS^n study of sildenafil transformation products formed through photocatalytic degradation only

In addition to these TPs already found in metabolism studies, several new TPs were formed only during the photocatalytic treatment. Their MS^2 and MS^3 spectra showed the formation of the product ions in Table 3.

Two isobaric species at *m/z* 477.1917, labelled **477-A** and **477-B**, were formed.

Looking closely at the species **477-A**, it follows the fragmentation pathway described in Scheme 5 (top). MS^2 produces a single ion at *m/z* 459.1817, by the loss of a water molecule. The MS^3 product ions had the same peculiar losses described for sildenafil, all with the difference of 2 u. This could be explained by assuming a demethylation on the piperazine ring, followed by a hydroxylation on the propyl chain. The formation of the ion at *m/z* 281.1038, through the loss of an ethylene molecule from the ethoxy group, confirms the absence of a hydroxy group on the ethoxy moiety.

The MS^2 spectrum for **477-B** shows two ions:

1. *m/z* 449.1970: loss of a carbon monoxide molecule
2. *m/z* 459.1817: loss of a water molecule

The species **477-B** was tentatively attributed to the structure shown in Scheme 5 (bottom), where a demethylation on the piperazine ring and a further hydroxylation on the same ring have occurred.

The TP at *m/z* 393.1233 is formed through detachment of piperazine and a further hydroxylation. The proposed structure is shown in Scheme 6.

In the MS^2 spectrum, *m/z* 365.0919 was the main product ion, formed through loss of an ethylene molecule. This ion is easily

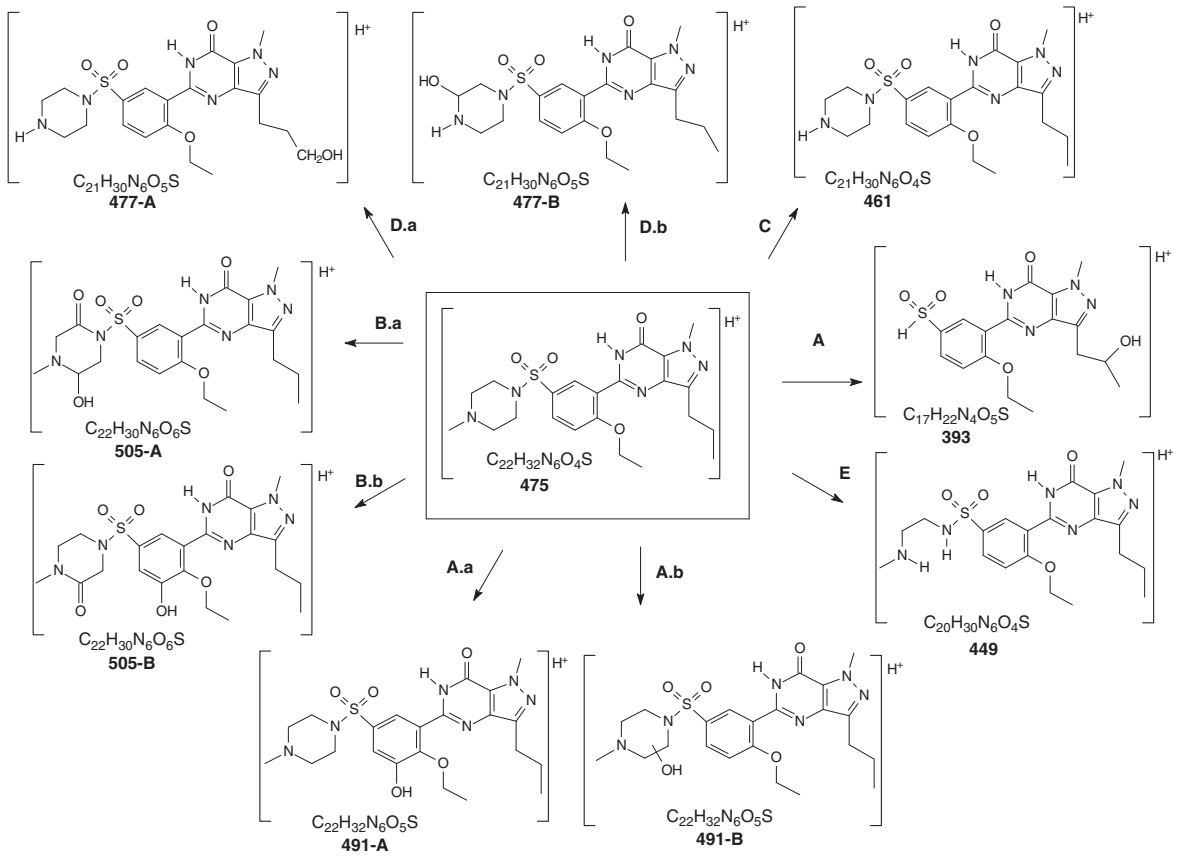


Figure 2. Proposed transformation pathways followed by sildenafil (protonated forms).

Table 3. Transformation products formed from degradation of sildenafil, only by the photocatalytic process

$[M+H]^+$	t_R (min)	MS^2	MS^3
477.1917	8.80	459.1817 (100)	375.1127 (100)
$C_{21}H_{29}N_6O_5S$	(**477-A**)	$C_{21}H_{27}N_6O_4S$	$C_{17}H_{19}N_4O_4S$
			309.1351 (98)
			$C_{17}H_{17}N_4O_2$
			311.1508 (67)
			$C_{17}H_{19}N_4O_2$
			281.1037 (49)
			$C_{15}H_{13}N_4O_2$
	12.48	449.1970(100)	418.1548 (100)
	(**477-B**)	$C_{20}H_{29}N_6O_4S$	$C_{19}H_{24}N_5O_4S$
			392.1393 (85)
			$C_{17}H_{22}N_5O_4S$
			313.1664 (50)
			$C_{17}H_{21}N_4O_2$
			311.1508 (28)
			$C_{17}H_{19}N_4O_2$
			361.1335 (26)
			$C_{17}H_{21}N_4O_3S$
			432.1703 (25)
			$C_{20}H_{26}N_5O_4S$
			406.1549 (12)
			$C_{18}H_{24}N_5O_4S$
		459.1817 (24)	311.1508 (100)
		$C_{21}H_{27}N_6O_4S$	$C_{17}H_{19}N_4O_2$
			283.1194 (27)
			$C_{15}H_{15}N_4O_2$
393.1233	12.53	365.0921 (100)	256.0959 (100)
$C_{17}H_{21}N_4O_5S$		$C_{15}H_{17}N_4O_5S$	$C_{13}H_{12}N_4O_2$
			285.1350 (35)
			$C_{15}H_{17}N_4O_2$
			299.1142 (29)
			$C_{15}H_{15}N_4O_3$
			283.1193 (20)
			$C_{15}H_{15}N_4O_2$
			269.1036 (16)
			$C_{14}H_{13}N_4O_2$
505.1861	12.62	487.1760 (100)	311.1507 (100)
$C_{22}H_{29}N_6O_6S$	(**505-A**)	$C_{22}H_{27}N_6O_5S$	$C_{17}H_{19}N_4O_2$
			377.1281 (9)
			$C_{17}H_{21}N_4O_4S$
	13.60	459.1817 (100)	311.1508 (100)
	(**505-B**)	$C_{21}H_{27}N_6O_4S$	$C_{17}H_{19}N_4O_2$
			283.1194 (26)
			$C_{15}H_{15}N_4O_2$
		393.1235 (21)	365.0918 (100)
		$C_{17}H_{21}N_4O_5S$	$C_{15}H_{17}N_4O_5S$
		477.1919 (20)	459.1817 (100)
		$C_{21}H_{29}N_6O_5S$	$C_{21}H_{27}N_6O_4S$

subjected to radical breakage. The contemporary formation of the structurally-diagnostic ion at *m/z* 269.1038, through loss of formaldehyde, and at *m/z* 256.0960, through loss of an HSO_2 radical and acetaldehyde, places the OH group on C3 of the propyl chain, rather than on the methyl group. The precursor ion at *m/z* 365.0919 was also subjected to:

- Loss of H_2SO_2 with formation of the ion at *m/z* 299.1144.
- Loss of H_2SO_3 with formation of the ion at *m/z* 283.1195.
- Loss of HSO_3 radical with formation of the ion at *m/z* 285.1351.

Two isobaric species at *m/z* 505.1869, labelled **505-A** and **505-B**, were formed. The difference compared to sildenafil is 29.9740 u, which is reasonably due to a combined double hydroxylation and oxidation process.

The **505-A** MS^3 spectrum showed the formation of two product ions at *m/z* 311.1507 and 377.1281, already formed from sildenafil fragmentation through piperazine loss, thus implying that both hydroxylation and oxidation involved the piperazine ring.

505-B presumably follows the fragmentation pathway shown in Scheme 7.

The species **505-B** is characterized by oxidation on the piperazine ring and hydroxylation, supported by the fragmentation pathway shown in Scheme 7. The absence of the likely loss of C_2H_6O induced us to hypothesize that the hydroxylation position could be close to the ethoxy-phenyl moiety. The key ions are:

- *m/z* 459.1814: loss of a water molecule and CO; in MS^3 it produces the ions at *m/z* 311.1508 and *m/z* 283.1194, both known from sildenafil MS^n spectra analysis.
- *m/z* 393.1235: loss of keto-piperazine ring. MS^3 shows ethylene loss.
- *m/z* 477.1919: loss of CO through contraction of the piperazine ring. Successively, a water molecule is lost from the aromatic ring, with formation of the ion at *m/z* 459.1808.

Transformation pathways

Figure 2 shows all the species found through the photocatalytic process. The sildenafil transformation routes are:

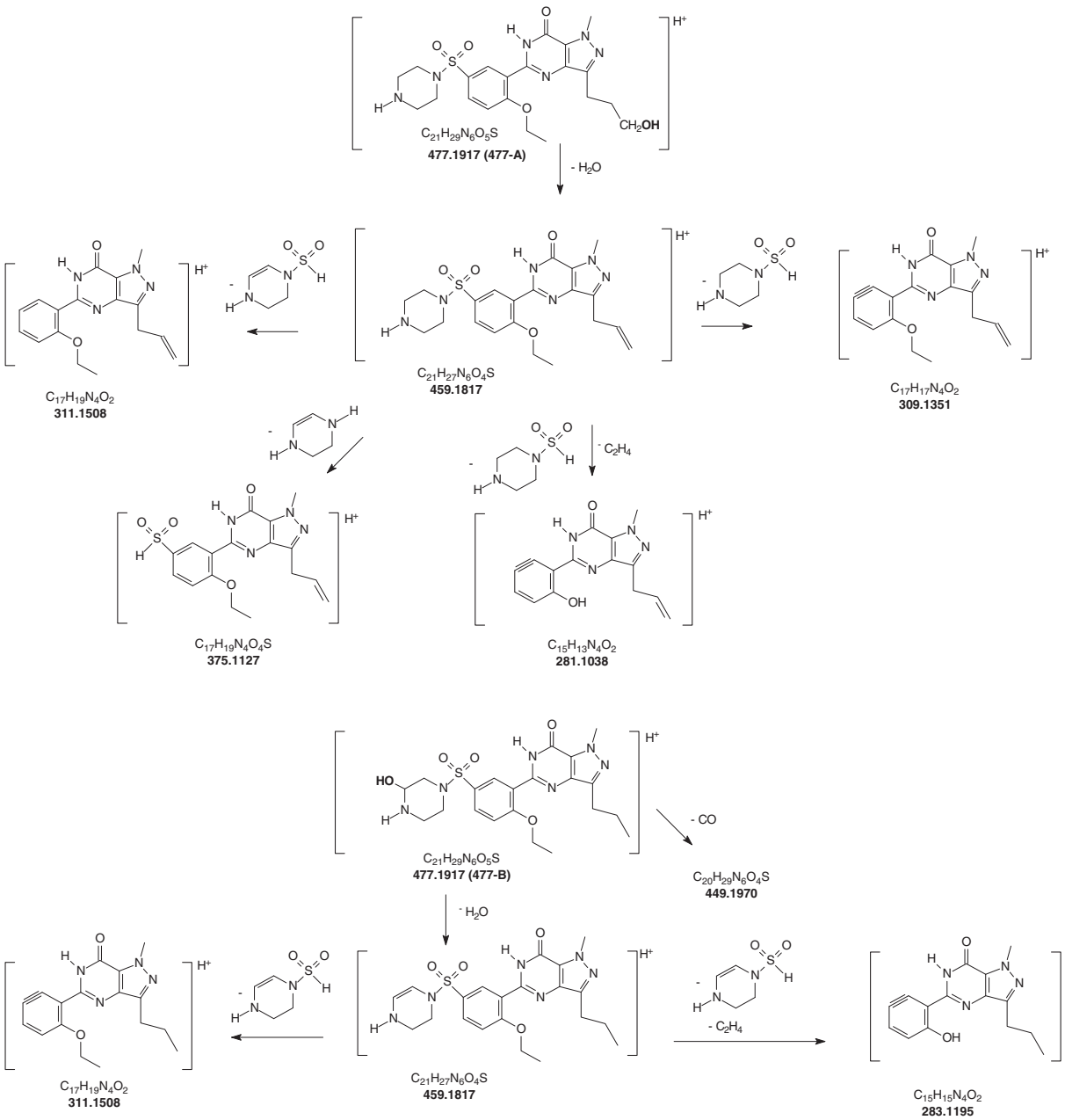
1. Hydroxylation (TPs **393**; **491-A** and **491-B**): pathways **A, A.a** and **A.b**
2. Dihydroxylation (TPs **505-A** and **505-B**): pathways **B.a** and **B.b**
3. Demethylation (TP **461**): pathway **C**
4. Demethylation and hydroxylation (TPs **477-A** and **477-B**): pathway **D.a** and **D.b**
5. De-ethylation (TP **449**): pathway **E**

Analysis of horse urine and plasma

The biological samples were subjected to the procedure described in section 'Treatment of horses' and analyzed by HPLC/HRMSn.

A C_{max} of 36.6 ng/ml after 2 h has been reported for sildenafil,[12] dropping to 2 ng/ml after 10 h; the main metabolite had a C_{max} of 9.3 ng/ml after 2 h, reduced to 0.5 ng/ml after 10 h.

In our experiments (chromatographic peak areas are shown in Tables 4 and 5), sildenafil was only excreted in small amounts as the non-metabolized compound. We found a C_{max} value of 17.1 ng/ml in plasma and of 11.8 ng/ml in urine. In urine samples, the maximum concentration of all metabolites was found in U1, sampled 7–12 h after sildenafil administration. The main metabolism products were the species at *m/z* **477-A** and 449.1971. Thus, demethylation followed by oxidation appear to be the main transformation route. These data are similar to those obtained by De Kock *et al.*[13] In both cases the metabolites **477-B**, *m/z* 449.1971, and *m/z* 491.2073, prevail. Interestingly, in addition to sildenafil and these metabolic products, already identified in other studies,[12,13] five new species – **491-A**, **477-A**, **477-B**, **505-A, 505-B** – were



Scheme 5. MS^2 and MS^3 fragmentation pathways for the TP species **477-A** (top) and **477-B** (bottom).

identified. The less abundant species were TPs **477-B**, **505-A**, and **505-B** and a species at *m/z* 461.1965. The low concentration of the two species **505-A** and **505-B** may account for why they have not previously been found in urine matrix.

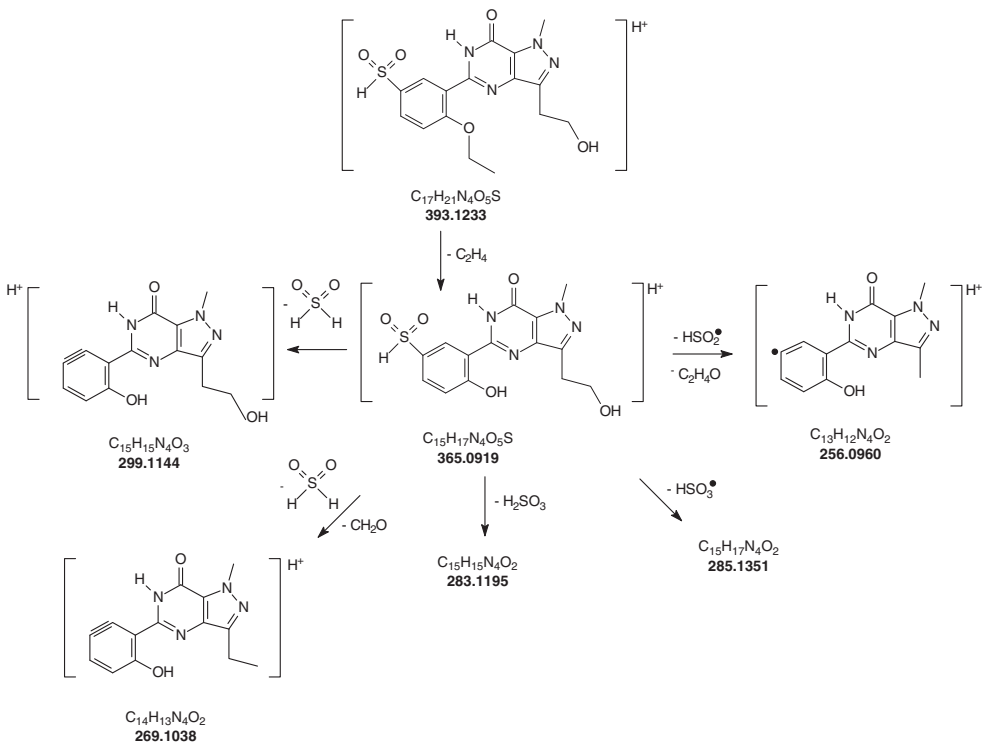
In the blood samples, alongside unmetabolized sildenafil and the species at *m/z* 461, the two new metabolic products **491-A** and **505-B** were also found (Table 5). As expected, the quantity of unchanged sildenafil was lower in the urine than in the plasma, and consequently a larger quantity of the metabolites was present in the urine.

It should be noted that:

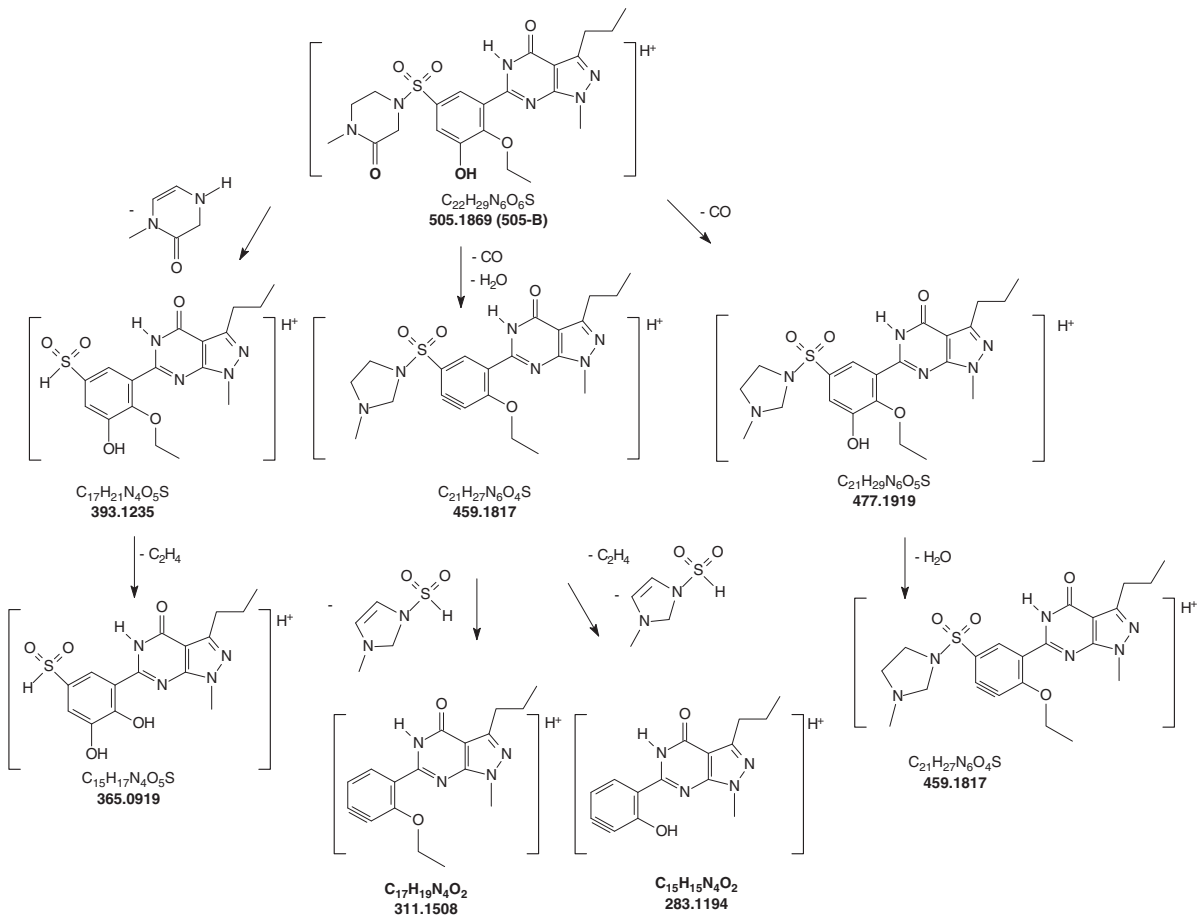
1) In sample S0 (prior to administration), neither sildenafil nor its metabolites were present.
2) The species at *m/z* 477.1917, *m/z* 449.1971 and *m/z* 393.1233 were not found in any samples.
3) Mono-hydroxylation on the aromatic ring (TP **491-A**) appears to be the preferential pathway.

Conclusions

Four sildenafil metabolic transformation pathways are known: demethylation, N,N-de-ethylation of the piperazine ring, hydroxylation of the piperazine ring, and hydroxylation of the propyl chain. In addition, through the use of a photocatalytic process, several new TPs have been identified and a mechanism of



Scheme 6. MS^2 and MS^3 fragmentation pathways for the TP species at *m/z* 393.1233.



Scheme 7. Key transformation pathways followed by TP **505-B**.

Table 4. Metabolic compounds formed from sildenafil in urine samples. Data are reported as chromatographic peak areas (signal counts). Values are averages for both animals

$[M+H]^+$	U0 (0 min)	U1 (7–12 h)	U2 (27–28 h)
	Area (10^6)	Area (10^6)	Area (10^6)
475.2122 (sildenafil)	2	7.6	1.5
491-A	0.5	68	11
449.1971	-	97	23
461.1965	-	2	-
477-A	-	310	35
477-B	-	26	3.5
505-A	-	27.5	0.3
505-B	-	13.5	0.4

Table 5. Metabolic compounds formed from sildenafil in blood samples. Data are reported as chromatographic peak areas (signal counts). Values are averages for both animals

$[M+H]^+$	S0 (0 min)	S1 (2 h)	S2 (2 h 30 min)	S3 (3 h)	S4 (3 h 30 min)
	Area (10^6)	Area (10^6)	Area (10^6)	Area (10^6)	Area (10^6)
475.2122 (sildenafil)	-	4.2	6.4	22.3	14.2
491-A	-	-	-	2.8	1
461.1965	-	-	-	0.6	-
505-B	-	-	0.4	-	-

transformation has been constructed that can explain all the observed species. The process involved comprises:

1. Hydroxylation on the aromatic ring.
2. Loss of piperazine moiety and hydroxylation of the propyl chain.
3. Di-hydroxylation of the piperazine ring.
4. Hydroxylation on the piperazine and aromatic rings.
5. Demethylation and hydroxylation on the piperazine ring.
6. Demethylation and hydroxylation on the propyl chain.

Structural attribution was achieved through HPLC/HRMSn.

All of these TPs, in addition to sildenafil, were sought in the urine and plasma of treated horses. In these matrices, in addition to sildenafil and the two metabolic products already known, five metabolites, already detected through photocatalysis, were also identified. These biomarkers of exposure could be included among the novel sildenafil metabolic products to screen for in anti-doping examination.

The methodology used is confirmed as a suitable tool to simulate biotransformations through photochemical laboratory experiments, and that will be able to suggest new structures in future metabolic studies.

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