Influence of Pain Killers on the Urinary Anabolic Steroid Profile

Supplementary data

Anna Stoll[a], Michele Iannone[b], Giuseppina De Gregorio[b], Xavier de la Torre[b], Francesco Molaioni[b], Francesco Botrè[b,c], and Maria Kristina Parr[a,*]

[a]Freie Universitaet Berlin, Institute of Pharmacy, Pharmaceutical and Medicinal Chemistry (Pharmaceutical Analyses), Koenigin-Luise-Strasse 2+4, 14195 Berlin, Germany, anna.stoll@fu-berlin.de, maria.parr@fu-berlin.de

[b]Laboratorio Antidoping FMSI, Largo Giulio Onesti 1, Rome 00197, Italy, micheleiannone14@gmail.com, degregorio.giuseppina@gmail.com, xavier.delatorre@fmsi.it, molaioni@gmail.com, botref@gmail.com

[c]'Sapienza' University of Rome, Department of Experimental Medicine, Viale Regina Elena 324, Rome 00161, Italy, francesco.botre@uniroma1.it

* Corresponding author.

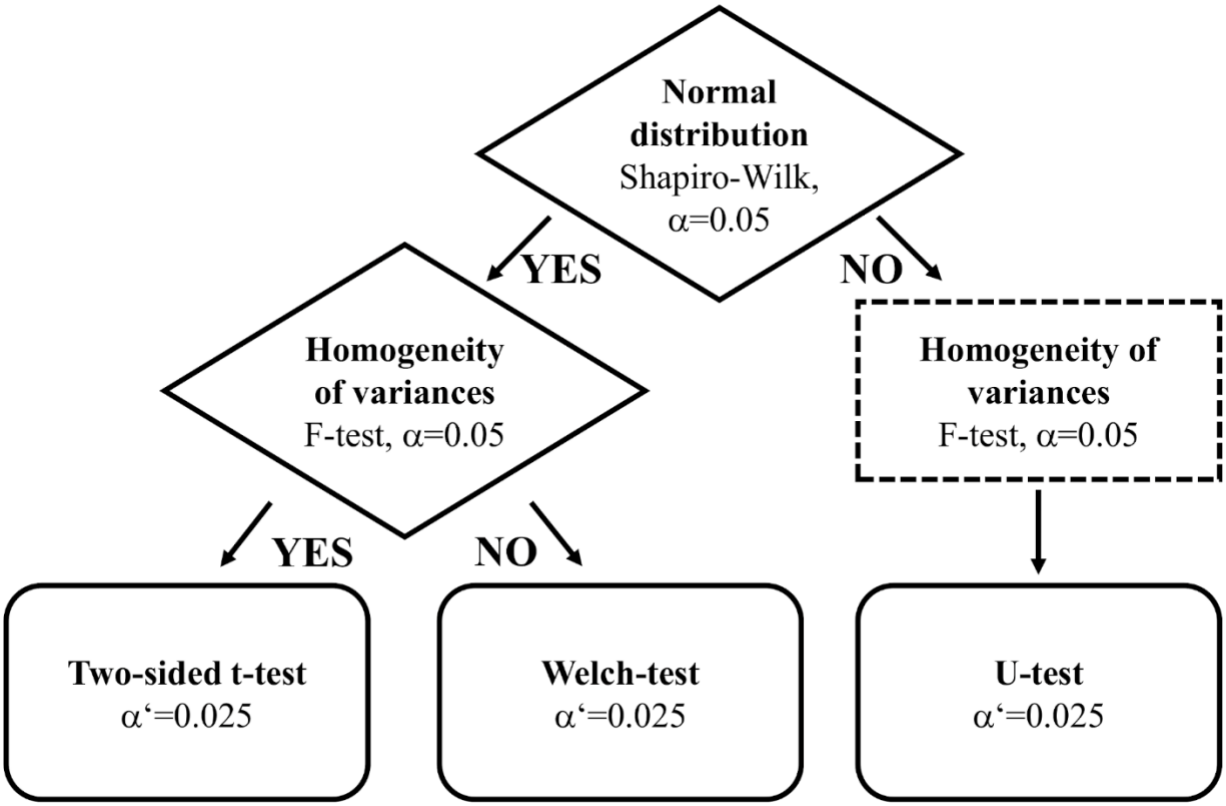
Prof Dr Maria Kristina Parr

Freie Universitaet Berlin, Institute of Pharmacy, Pharmaceutical and Medicinal Chemistry

Koenigin-Luise-Strasse 2+4,

14195 Berlin, Germany

maria.parr@fu-berlin.de



Supplementary Figure 1. Decision tree for choice of significance test with lozenges indicating decisions, rectangles indicating processes on way to final result and rounded rectangles indicating final output generating processes; dashed lines indicating processes performed but not necessary for following step

Determination of urinary concentrations of ibuprofen and its metabolites

Experimental

Materials

In addition to the materials mentioned in the main document, acetic acid from Honeywell Fluka, (Milan, Italy), sodium acetate from Sigma Aldrich (Milan, Italy), 2-hydroxy-ibuprofen, 2-carboxyibuprofen as reference substances and probenecid as internal standard (all from Sigma Aldrich, Milan, Italy) were used.

Urine sample preparation

Aliquots of 1 ml of urine, 50 µl of internal standard (probenecid at 50 ng/ml), 30 µl of β-glucuronidase and 500 µl PBS 0.8 M were mixed and heated for 1 hour at 55 °C. After addition of 500 µl sodium-acetate buffer pH 5 and 5 ml tert-butyl-methyl-ether (TBME), liquid-liquid extraction was performed. The organic phase was then treated like described in the main document.

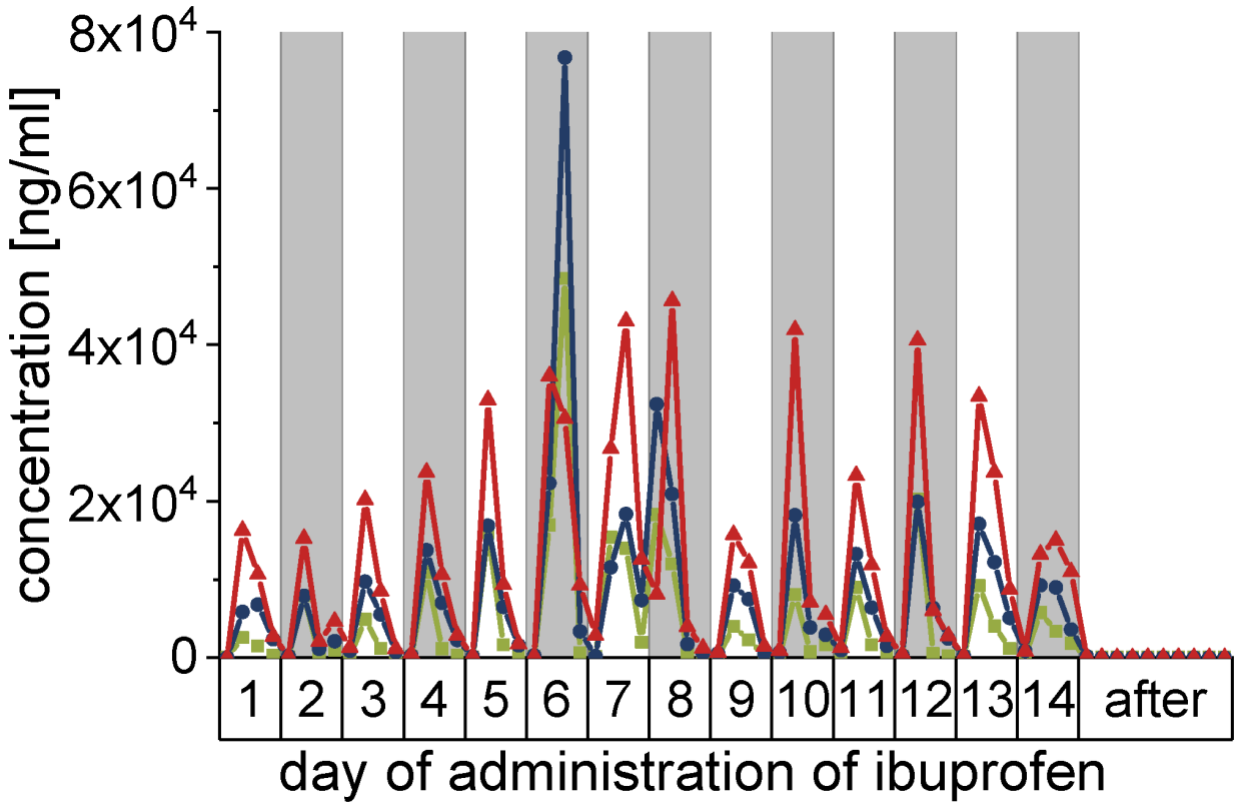
Instrumental

Measurement of ibuprofen and its metabolites in urine samples on GC–QQQ

The instrumental setting was the same as described for the analysis of steroids from urine samples. Aberrantly, for the analysis of ibuprofen and its metabolites the oven program was: 85°C initial temperature hold for 1 min, 15°C/min to 320°C. Transitions for analysed compounds are given in Supplementary Table 1. Selectivity, linearity, repeatability, recovery and limit of detection (LOD) were tested (data not shown).

Results

Concentrations of ibuprofen and its main metabolites (2-hydroxyibuprofen and carboxyibuprofen) were determined in samples of the male volunteer. No ibuprofen or metabolites were detected in randomly selected samples before the application study or in samples collected after the administration period, while ibuprofen and both metabolites were detectable in at least one sample per day during the whole administration period (peak concentrations mostly in the urine collected at 1 pm, Supplementary Figure 2).



Supplementary Figure 2. Measured concentrations of ibuprofen and its metabolites in urine samples collected from male volunteer. Concentrations (corrected for specific gravity) of ibuprofen (green), 2-hydroxy-ibuprofen (blue) and carboxy-ibuprofen (red) are plotted for each sampling point

Supplementary Table 1: Transitions of Ibuprofen and its metabolites after derivatization

Analytes	**Precursor ion [m/z]**	**Product ion [m/z]**	**Collision energy [eV]**
Ibuprofen mono-O-TMS	263	191; 129; 193	30; 30; 30
2-Hydroxy-ibuprofen bis-O-TMS	351; 261; 351	159; 206; 261	30; 30; 30
Carboxy-ibuprofen bis-O-TMS	336; 365; 365	118; 173; 221	30; 30; 30

Supplementary Table 2: Transitions for the steroid profiling on the GC–MS-MS system

Analytes (all as bis-O-TMS-derivatives)	**Precursor ion [m/z]**	**Product ion [m/z]**	**Collision energy [eV]**
Testosterone	432	209; 196	20; 30
Epitestosterone	432	209; 196	20; 10
Androsterone	419; 434	329; 419	10; 10
Etiocholanolone	419; 434	329; 419	10; 10
5α-Androstane-3α,17β-diol	256	185; 198	10; 10
5β-Androstane-3α,17β-diol	241; 256	159; 199	20; 10
5α-Dihydrotestosterone	434	405; 127	5; 20
Testosterone-d_3	435	209; 196	20, 30
Epitestosterone-d_3	435	209; 196	20; 30
Androsterone-glucuronide-d_4	423	231; 333	20; 10
Etiocholanolone-d_3	424	221; 334	20; 10
5α-Androstane-3α,17β-diol-d_3	259	188; 244	10; 5
5β-Androstane-3α,17β-diol-d_3	246; 261	164; 199	20; 10
17α-Methyltestosterone	301; 446	169; 301	20; 5

*Supplementary Table 3: P-values for significant differences at α'=0.025 for the male and female volunteer and the different steroids and ratios included in the steroid profile. 5a-DHT and T/E is not reported, as no significant difference was observable. Groups classified as B/D: before compared to during administration of ibuprofen, D/A: during compared to post administration; Gray fields (-) indicate, that this test was not performed; red fields indicate no significant difference (n.s.); white fields indicate significant differences: orange writing and *: significant difference at α'=0.025, blue writing and **: very significant difference at α'=0.005, green writing and ***: highly significant difference at α'=0.0005*

Marker of steroid profile	Groups	Male volunteer		Female volunteer	
		t-Test	U-Test	t-Test	U-Test
T	B/D	-	n.s.	-	**P = 0.0051 ***
	D/A	n.s.	n.s.	-	**P = 0.0014 ****
E	B/D	-	n.s.	.-	n.s.
	D/A	-	n.s.	-	**P = 0.0083 ***
AND	B/D	-	**P = 6.0*10$_{-4}$ ****	-	**P = 9.7*10$_{-4}$ ****
	D/A	-	n.s.	-	**P = 0.023 ***
ETIO	B/D	-	n.s.	-	**P = 8.8*10$_{-4}$ ****
	D/A	n.s.	n.s.	-	**P = 0.0023 ****
ADIOL	B/D	-	n.s.	-	**P = 5.6*10$_{-5}$ *****
	D/A	-	n.s.	**P = 3.4*10$_{-4}$ *****	**P = 8.3*10$_{-5}$ *****
BDIOL	B/D	-	n.s.	-	**P = 0.011 ***
	D/A	-	n.s.	-	**P = 5.8*10$_{-6}$ *****
5αDHT	B/D	n.s.	n.s.	-	n.s.
	D/A	n.s.	n.s.	-	**P = 0.0020 ****
AND/T	B/D	**P = 7.2*10$_{-8}$ *****	**P = 6.2*10$_{-7}$ *****	-	n.s.
	D/A	n.s.	n.s.	-	**P = 0.0015 ****
AND/ETIO	B/D	**P = 6.6*10$_{-12}$ *****	**P = 8.5*10$_{-11}$ *****	n.s.	n.s.
	D/A	**P = 5.9*10$_{-4}$ ****	**P = 1.8*10$_{-4}$ *****	**P = 7.5*10$_{-5}$ *****	**P = 1.4*10$_{-4}$ *****
ADIOL/BDIOL	B/D	-	n.s.	**P = 0.024 ***	n.s.
	D/A	-	n.s.	-	n.s.

Supplementary Table 4: Type of assay and steady state kinetic parameters from literature compared to values obtained in this study for AKR1C2 catalyzed reduction of 5αDHT

source	Assay	K_m [µM]	k_{cat}	V_{max} [µmol/mL/min]
This study	**Fluorescence-emission assay:** 0.28 µM AKR1C2, 12 µM NADPH, 3-100 µM 5αDHT (and 30 µM Ibuprofen for values in lower line) in PBS 0.1 M, pH 7.4, 37°C	13.4; SE=5.6	0.46 min^{-1}	$1.3*10^{-4}$ (SE=$1.7*10^{-5}$)
		16.0; SE=3.1	0.18 min^{-1}	$5.1*10^{-5}$ (SE=$3.3*10^{-6}$)
	GC–QToF-analysis: 0.28 µM AKR1C2, 12 µM NADPH, 3-100 µM 5αDHT (and 30 µM Ibuprofen for values in lower line) in PBS 0.1 M, pH 7.4, 37°C	10.6; SE=5.9	1.70 min-1	$4.8*10^{-4}$ (SE=$1.4*10^{-4}$)
		10.7; SE=1.0	1.09 min^{-1}	$3.0*10^{-4}$ (SE=$2.1*10^{-5}$)
Jin and Penning [1]	**Fluorescence-emission assay:** 0.17 µM AKR1C2, 12 µM NADPH, 1.5–25 µM 5αDHT in PBS 10 mM, pH 7, 25°C	2.9 ± 0.2	0.033 ± 0.002 s^{-1}	not indicated
Penning et al [2]	**Radiochemical assay:** 5 µg AKR1C2, 2.3 mM NADPH, 3–50 µM ^{14}C-5αDHT in PBS 0.1 M, pH 7, 37°C	26.0 ± 6.6	0.23 min^{-1}	6.2 nmol/min/mg ± 0.8 nmol/min/mg
Byrns et al [3]	**Radiochemical assay:** AKR1C2, 200 µM NADPH, 1.5–16.5 µM ^{14}C-5αDHT in PBS 100 µM, pH 7	4.6 ± 1.4	3.8 ± 0.3 min^{-1}	

Refrences

[1] Jin, Y. and Penning, T.M. (2006) Multiple steps determine the overall rate of the reduction of 5alpha-dihydrotestosterone catalyzed by human type 3 3alpha-hydroxysteroid dehydrogenase: implications for the elimination of androgens. Biochemistry, 45, 13054-13063.

[2] Penning, T.M., Burczynski, M.E., Jez, J.M., Hung, C.-F., Lin, H.-K., Ma, H., et al. (2000) Human 3α-hydroxysteroid dehydrogenase isoforms (AKR1C1–AKR1C4) of the aldo-keto reductase superfamily : functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. Biochemical Journal, 67–77.

[3] Byrns, M.C., Steckelbroeck, S. and Penning, T.M. (2008) An indomethacin analogue, N-(4-chlorobenzoyl)-melatonin, is a selective inhibitor of aldo-keto reductase 1C3 (type 2 3alpha-HSD, type 5 17beta-HSD, and prostaglandin F synthase), a potential target for the treatment of hormone dependent and hormone independent malignancies. Biochemical pharmacology, 75, 484–493.