Use of Liver Homogenates for Rapid Generation of Phase I Metabolites to Facilitate Characterization of Emerging Drugs of Abuse by High Resolution Liquid Chromatography-Mass Spectrometry

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INTRODUCTION

Normally, liver S9 and/or microsomal fractions are used to produce drug metabolites for preliminary *in vitro* metabolism investigations and the development of targeted mass spectrometric methods.

However, liver S9 and microsomal fractions are

- Not readily available for many species
- Production thereof is tedious, time-consuming and specialized equipment is needed.

Therefore, simple, rapid and cost-effective *in vitro* strategies that can promptly respond to analytical challenges in the control and monitoring of emerging illegal drug use in the livestock sector are required.

The aim of this work was to investigate the potential of bovine liver homogenates to rapidly generate and characterise phase I metabolites of selective androgen receptor modulator (SARM) compounds.

The class of SARMs are anabolic agents and as such, pose a potential for abuse in human as well as animal sports and agricultural farming. Their use in feed compositions has been patented worldwide[1]. In Europe, the use of any substance with hormonal actions in stock farming is prohibited[2]. Adverse analytical findings with various SARMs were reported in humans[3-5] and equine[6] emphasizing the importance of the identification of metabolites as target analytes in control analysis.

METHODOLOGY

Isolation of Liver Fractions

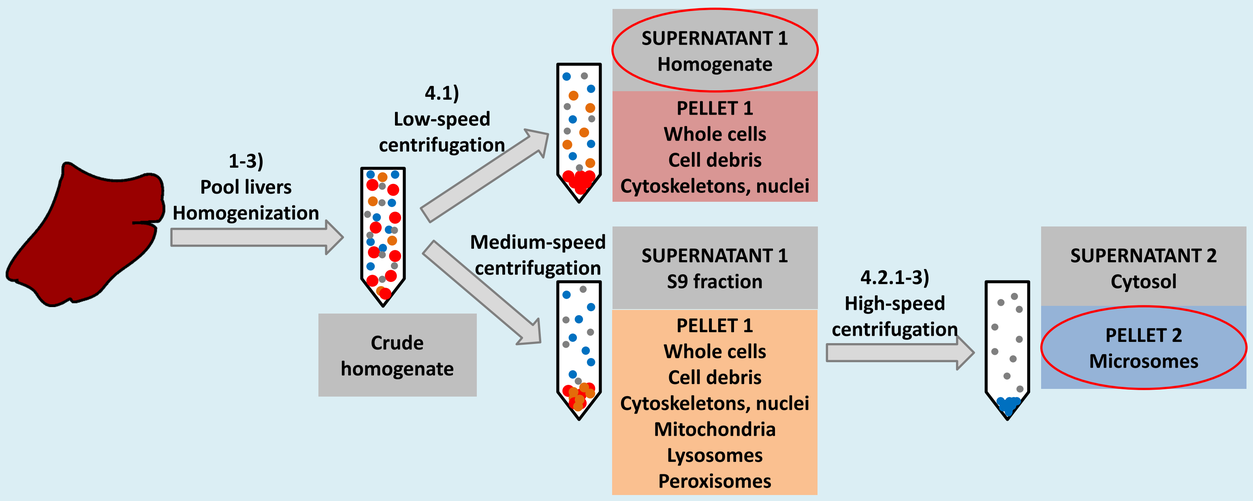
1) Bovine livers (n=6) were randomly collected at slaughter
2) Tissues were perfused/washed with ice-cold PBS and finely minced
3) Liver pieces were pooled and 3 volumes of homogenization buffer (50 mM Tris, 150 mM KCl and 2 mM EDTA pH 7.4 at 4 °C) were added

4.1) Mixture was homogenized followed by a low-speed centrifugation step at 1000 × g_{max} for 5 min at 4 °C resulting in homogenate

4.2.1) Mixture was homogenized followed by a medium-speed centrifugation step at 15 700 × g_{max} for 20 min at 4 °C and a subsequent high-speed centrifugation step of the supernatant at 105 000 × g_{max} for 60 min at 4 °C resulting in the microsomal pellet

4.2.2) Microsomal pellet was suspended in 150 mM KCl, 2 mM EDTA and high-speed centrifugation step was repeated

4.2.3) Microsomal pellet was resuspended in 250 mM sucrose



The protein concentration of bovine liver homogenate and microsomal fractions was determined with the colorimetric bicinchoninic acid assay.

Phase I Incubation Assay

50 mM Phosphate buffer, 5 mM $MgCl_2$ pH 7.4

Homogenate or microsomes (1 mg/mL)

Ostarine (50 µM)

NADPH (10 mM)

Incubation at 37°C for 120 min

Termination with 3 volumes of acetonitrile 1 % formic acid

Removed and dried down supernatant LLE with ethyl acetate

Organic phase was dried down, reconstituted (water:methanol 1:1)

Ostarine belongs to the class of arylpropionamide SARMs. Besides the homogenate and microsomal incubations with ostarine, blanks without NADPH (co-factor blank), without ostarine (substrate blank) and without liver fractions (enzyme blank) were included. 5 µL of the samples were injected onto the LC-MS.

Liquid Chromatography Conditions

LC system	Waters Acquity UPLC I-Class System
Column	Waters HSS T3 1.8 µm 2.1x100 mm
Column temp.	45 °C
Sample temp.	10 °C
Mobile phase A	H_2O 0.1 % formic acid
Mobile phase B	Methanol
Flow rate	0.4 mL/min
Gradient	0–1.0 min 3 % B, 1.0–7.0 min, 3–99 % B, 7.0–8.0 min 99 % B, 8.0–8.1 min 99–3 % B, 8.1–10 min 3 % B

Mass Spectrometry Conditions

MS system	Waters Xevo G2-S QTof
Source	Electrospray
Ionization mode	ESI-
Capillary voltage	1.2
Cone voltage	30 V
Desolvation temp.	450 °C
Desolvation gas	850 L/h
Source temp.	120 °C
Cone gas	50 L/h
MS Acquisition	MS^E, HE CE 20-35 V
Acquisition range	50 – 1200 *m/z*
Data management	MassLynx software V 4.1

RESULTS AND DISCUSSION

Figure 1 Overlay of the extracted ion chromatograms of the parent compound (ostarine) and all the metabolites after incubation with bovine liver homogenate

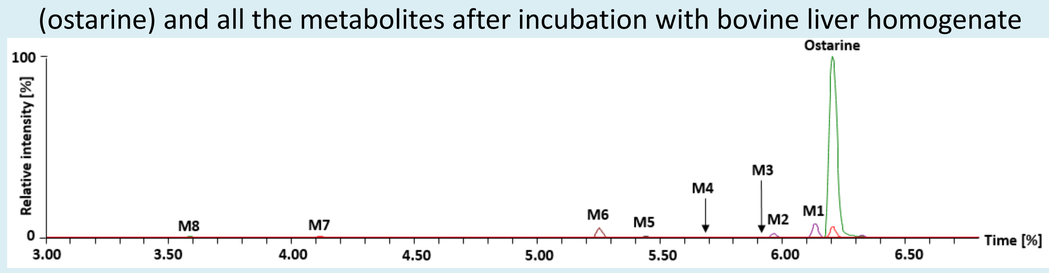


Table 1 Ostarine and its phase I metabolites, in ascending order of their RT with their product ion (PI) masses as well as main fragment ions (FI) in high energy collision mode

RT, min	Metabolite and FIs at measured accurate masses, $[M-H]^-$, *m/z*	Calculated mass, $[M-H]^-$, *m/z*	Elemental composition	Error, ppm	Chemical structure with ESI-fragmentation patterns
6.20	**S-22**				
	PI at 388.0932	388.0915	$C_{19}H_{13}O_3N_3F_3$	- 4.4	
	FI at 269.0545	269.0537	$C_{12}H_8O_2N_2F_3$	- 3.0	
	FI at 185.0321	185.0332	$C_8H_4N_2F_3$	5.9	
	FI at 118.0297	118.0298	C_7H_4ON	0.8	
6.13	**S-22-M1 (hydroxy-)**				
	PI at 404.0845	404.0864	$C_{19}H_{13}O_4N_3F_3$	4.7	
	FI at 269.0545	269.0537	$C_{12}H_8O_2N_2F_3$	- 3.0	
	FI at 185.0321	185.0332	$C_8H_4N_2F_3$	5.9	
	FI at 134.0245	134.0245	$C_7H_4O_2N$	0	
5.97	**S-22-M2 (hydroxy-)**				
	PI at 404.0845	404.0864	$C_{19}H_{13}O_4N_3F_3$	4.7	
	FI at 269.0545	269.0537	$C_{12}H_8O_2N_2F_3$	- 3.0	
	FI at 185.0321	185.0332	$C_8H_4N_2F_3$	5.9	
	FI at 134.0245	134.0245	$C_7H_4O_2N$	0	
5.92	**S-22-M3 (bis-hydroxy-)**				
	PI at 420.0797	420.0813	$C_{19}H_{13}O_5N_3F_3$	3.8	
	FI at 150.0181	150.0193	$C_7H_4O_3N$	8.0	
5.69	**S-22-M4 (bis-hydroxy-)**				
	PI at 420.0797	420.0813	$C_{19}H_{13}O_5N_3F_3$	3.8	
	FI at 150.0181	150.0193	$C_7H_4O_3N$	8.0	
5.44	**S-22-M5 (O-dephenyl-demethyl-)**				
	PI at 257.0552	257.0543	$C_{11}H_8O_2N_2F_3$	- 3.5	
	FI at 185.0321	185.0332	$C_8H_4N_2F_3$	5.9	
5.25	**S-22-M6 (O-dephenyl-)**				
	PI at 287.0634	287.0649	$C_{12}H_{10}O_3N_2F_3$	5.2	
	FI at 185.0321	185.0332	$C_8H_4N_2F_3$	5.9	
4.12	**S-22-M7 (4-Hydroxybenzonitrile)**				
	PI at 118.0297	118.0298	C_7H_4ON	0.8	
3.59	**S-22-M8 (Bis-hydroxybenzonitrile)**				
	PI at 134.0245	134.02475	$C_7H_4O_2N$	1.9	

Ostarine and associated metabolites, including hydroxy-, bis-hydroxy-, O-dephenyl, O-dephenyl-demethyl ostarine, hydroxybenzonitrile and bis-hydroxybenzonitrile, were detected following incubations with either homogenate (**Figure 1**) or microsomal preparations (**Table 1**). The main metabolic steps were hydroxylation and O-dephenylation.

CONCLUSIONS

In the current study, the use of liver homogenate was demonstrated to

- Qualitatively produce the same metabolites as microsomes, hence, it is an effective accessible alternative approach for use within *in vitro* drug metabolism based strategies
- Result in short(er) preparation times and few(er) resources needed
- Facilitate the development of food safety focused targeted mass spectrometry detection methods

Future work will investigate the *in vitro* metabolism of new emerging SARMs, interspecies differences in SARM metabolism and relate the *in vitro* metabolism to actual *in vivo* metabolism.

REFERENCES

[1]WO 2011/119544 A1 [2]Dir 96/22/EC [3]E. Grata, L. Perrenoud, M. Saugy, N. Baume, SARM-S4 and metabolites detection in sports drug testing: A case report, Forensic Sci. Int. 213 (2011) 104–108. [4]H.D. Cox, D. Eichner, Detection of LGD-4033 and its metabolites in athlete urine samples, Drug Test. Anal. (2016). [5]B. Starcevic, B.D. Ahrens, A.W. Butch, Detection of the selective androgen receptor modulator S-4 (Andarine) in a doping control sample, Drug Test. Anal. 5 (2013) 377–379. [6]A.T. Cawley, C. Smart, C. Greer, M.L. Lau, J. Keledjian, Detection of the selective androgen receptor modulator andarine (S-4) in a routine equine blood doping control sample, (2016) 257–261.

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