Assessing metabolic profiles in chimeric PXB mouse with humanized livers following oral dosing of troglitazone.

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1: Introduction

In the continuing search for new chemical entities the use of chimeric mice with humanized livers are being used in the search for unexpected drug metabolites. Chimeric mice, in which the majority of the hepatocyte population of the mouse liver has been replaced by human hepatocytes, have the capacity to express human Phase I and II metabolic enzymes and hepatic transporter proteins with gene-expression profiles and phenotypes similar (up to 85%) to those of the original donor liver. To assess the viability of the chimeric Phoenix Bio (PXB) mouse in modeling human liver metabolism, troglitazone (TGZ) was dosed orally over 7 days at two dose concentrations (300 & 600 mg/kg). In pre-clinical studies TGZ showed inter-species differences in metabolism particularly in sulfation and glucuronidation pathways. The present study evaluated the metabolic profile of troglitazone and endogenous metabolites in the PXB compared to control mice (severe combined immunodeficiency - SCID) using high mass accuracy MS/MS analysis.

2: Materials and Methods

Liver extracts from SCID (control) and PXB (chimeric) mice were analyzed using a high resolution LC/MSn system (Nexera LC coupled with a LCMS-IT-TOF; Shimadzu Corporation). Both aqueous and organic extracts were analyzed using a Phenomenex Kinetex column (C18 1.7um, 2.1x100mm); aqueous components were separated at a flow rate of 0.6 mL/min, with the column maintained at 30 °C. The chromatographic system used a binary solvent system delivered as a gradient of solvent A (H_2O containing 0.1% formic acid, 10mM ammonium acetate) and solvent B (ACN containing 0.1% formic acid). The gradient conditions were: 5% B (5 min), to 35% (3 min), to 50% (22 min), to 95% (2.5 min) held for 5 min, re-equilibration 2.5 min. The solvent composition was then held at 100% B for 6.5 min after which the column was returned to 5% B over the next 2.5 min, making a total cycle time of 25 min per sample; organic extracts were separated using a different gradient method (Castro-Perez *et al.* 2010) – (mobile phase: A – water:acetonitrile (60:40) 10mM ammonium formate pH5, B - propan-2-ol:acetonitrile (90:10) 10mM ammonium formate) at a flow rate of 0.5mL/min

The LCMS-IT-TOF acquired positive and negative MS and MS^2 data using high speed polarity switching (m/z 150-1250). Profiling Solution software (Shimadzu, Japan) was applied to metabolite profiling analysis to assess the impact of changes in lipid profiles. MetID Solution software was used for a targeted metabolomics study in addition to searching for drug metabolites through similarity scoring MS^n data of potential metabolites by comparing common fragment ions to parent drug MS^n data.

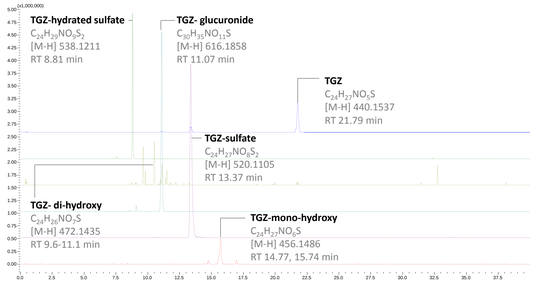
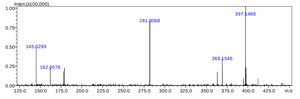


Figure 1. Liver metabolite profile of control mouse (SCID) following oral administration of troglitazone (TGZ).

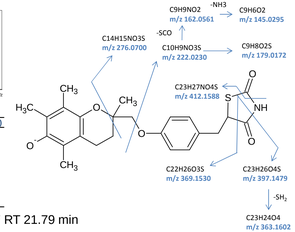
3: Results

3.1: Troglitazone metabolism

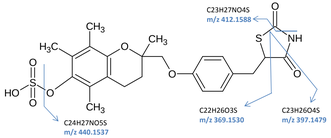
Analysis of aqueous liver extracts by accurate mass negative ion MSn enabled detection of metabolites by MetID Solution software (Fig. 1). Confirmation of troglitazone metabolites was also possible through analysis of common fragmentation data (Fig. 2).

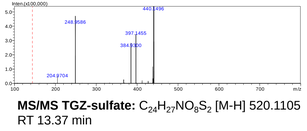


MS/MS fragments	Fragment Formula	Expected mass	Mass accuracy (ppm)
412.1581	C23H27NO4S	412.1588	-1.7
397.1488	C23H26O4S	397.1479	2.3
369.1546	C22H26O3S	369.1530	4.3
363.1577	C23H24O4	363.1602	-6.9
276.0714	C14H15NO3S	276.0700	5.1
179.0175	C9H8O2S	179.0172	1.7
162.0576	C9H9NO2	162.0561	9.3
145.0299	C9H6O2	145.0295	2.8

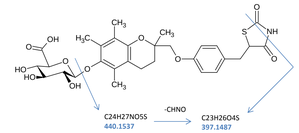


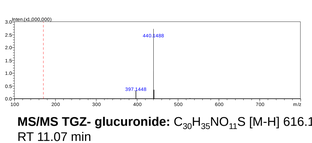
MS/MS Troglitazone: $C_{24}H_{27}NO_5S$ [M-H] 440.1537 RT 21.79 min





MS/MS TGZ-sulfate: $C_{24}H_{27}NO_6S_2$ [M-H] 520.1105 RT 13.37 min





MS/MS TGZ- glucuronide: $C_{30}H_{35}NO_{11}S$ [M-H] 616.1858 RT 11.07 min

Figure 2. Fragmentation analysis of troglitazone by accurate mass MSn data. Common fragment ions and neutral loss information consistent to troglitazone parent enabled characterization of metabolite structures.

Table 1. Averaged peak area data of troglitazone and metabolites detected in aqueous liver extracts.

Peak ID	Assignment	MS2	RT	m/z [M-H]-	SCID 600 mg	PXB 600 mg
Troglitazone	Parent	+	21.79	440.1537	2,807,994	3,898,820
M1	Di-hydroxy glucuronide		8.61	648.1756	72,254	71,725
M2	Hydrated glucuronide	+	8.40	634.1963	4,842,608	3,460,630
M3	Hydrated sulfate	+	8.81	538.1211	5,390,498	6,246,988
M4	Hydroxy sulfate	+	9.18	536.1054	25,491	28,861
M9	Di-hydroxy	+	9.63	472.1435	177,029	184,855
M10	Hydroxy glucuronide	+	9.88	632.1807	174,260	125,705
M12	Hydroxy sulfate	+	11.59	536.1054	336,482	230,705
M13	Glucuronide	+	11.07	616.1858	12,618,486	8,414,646
M15	Sulfate	+	13.37	520.1105	25,852,882	26,671,871
M16	Di-hydroxy	+	10.52	472.1435	345,952	150,980
M18	Di-hydroxy		11.12	472.1435	105,877	75,805
M27	Mono-hydroxy	+	14.77	456.1486	193,239	280,266
M30	Mono-hydroxy	+	15.74	456.1486	2,657,528	2,307,728

Peak area data comparing relative levels of troglitazone metabolites showed differences in metabolic profiles were also observed between PXB and SCID mice; consistent with metabolic profiles reported in human and mouse the sulfate conjugate being the most abundant metabolite detected while glucuronidation was greater in mouse.

3.2: Endogenous metabolite profiling

Organic liver extracts were analyzed to examine endogenous lipid differences between PXB and SCID livers. Data was aligned using Profiling Solution software (Shimadzu Corporation) and principal component analysis (PCA) was performed to examine group differences using Simca-P (Umetrics).

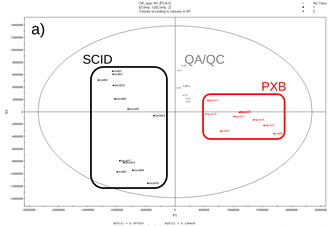
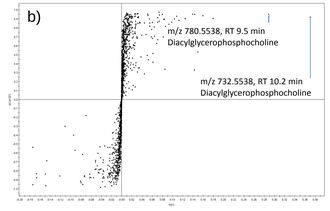


Figure 3. Statistical analysis of organic liver extracts comparing all SCID to all PXB samples.

a) PCA analysis revealed two main experimental groups (PXB and SCID) with no clear grouping associated with dosing of troglitazone. Tight clustering of QA/QC samples indicated good system stability throughout the sample analysis period.



b) OPLS-DA S-plot analysis comparing PXB to SCID enabled ions of highest significance to be identified. Two diacylglycerophosphocholine compounds (labeled) were detected at significantly higher levels in PXB mice compared to SCID.

MetID Solution was used to perform a targeted search of known endogenous metabolites using LipidMaps entry information from the following compound classes: phosphatidic acid, phosphatidylglycerol , phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine. The analysis enabled identification of over 80 ions that differed significantly between sample groups (concise summary: Table 2). Putative identifications were made based on mass accuracy and isotope score. Fold differences are shown between PXB and SCID at no dose (0mg/kg), high dose (600mg/kg) and for all animals averaged (0, 300 and 600 mg/kg). Although the aim of the data analysis was to identify compounds that differed between PXB and SCID mice, the data analysis also revealed subtle differences occurring possibly as a result of troglitazone dosing. Some compounds such as the glycerophosphocholine compounds were consistent in up or down regulation irrespective of dosing, hence showing most significance in S-plot analysis (Fig. 3b) due to homogenous variance averaged across all dosing groups. Conversely other lipid species exhibited differences in the fold change, although still up or down due to being PXB or SCID, show that administration of troglitazone may influence the concentration of these lipid levels.

Table 2. Endogenous metabolites identified as significantly increased (green) or decreased (red) in PXB mice compared to SCID mice at 0mg, 600mg dosing and data from all animals averaged (SCID- indicates not detected in SCID mice).

DB reference	Putative ID	Formula	Ion	m/z	RT	%RSD QA/QC	0 mg PXB / SCID	600 mg PXB / SCID	All animals PXB / SCID
LM GL0301 0065	TG(16:0/16:1(9Z)/18:3(9Z,12Z,15Z))	C53H94O6	[M+H]+	827.7323	24.35	8.2	5.72	SCID -	SCID -
LM GP01 0100395	PC(10:0/20:0)	C38H76NO8P	[M+H]+	706.5381	9.93	6.1	14.61	10.62	10.94
MID370	Glycerophosphocholine	C8H20NO6P	[M+H]+	258.1101	0.48	3.8	22.48	8.08	10.78
LM GL0301 0078	TG(16:1(9Z)/16:1(9Z)/18:3(9Z,12Z,15Z))	C53H92O6	[M+H]+	825.6967	24.35	7.0	7.92	20.67	10.46
LM GL0301 0018	TG(16:1(9Z)/14:0/18:1(9Z))	C53H94O6	[M+NH4]+	820.7389	24.12	4.8	11.02	8.59	8.76
LM GP03 0104 90	PC(14:0/18:1(11Z))	C40H78NO8P	[M+H]+	732.5558	11.21	13.3	20.48	9.47	8.76
HMDB0 0125	5-Aminoimidazole ribonucleotide	C8H14N3O7P	[M+H]+	296.0642	0.41	3.2	11.48	7.53	8.26
LM GP10 0200005	PA(O-16:0/14:1(9Z))	C33H65O7P	[M+H]+	605.4541	14.08	6.9	10.93	6.63	6.65
LM GP10 0100088	PA(13:0/22:2(13Z,16Z))	C38H73O8P	[M-H]-	685.4834	10.26	7.2	15.30	2.98	6.10
LM GP06 0100175	PE(14:0/22:2(13Z,16Z))	C41H76NO8P	[M-H]-	861.5499	10.89	4.7	4.91	4.64	5.82
LM ST05 0400015	Taurourosdeoxycholic acid	C26H45NO6S	[M-H]-	498.2895	6.60	3.2	9.79	4.34	5.41
LM GP04 0200069	PG(O-20:0/22:0)	C48H97O9P	[M+H]+	849.6943	24.00	7.1	4.83	5.57	5.37
LM GP10 0200004	PA(O-16:0/14:0)	C33H67O7P	[M+H]+	607.4697	17.11	6.8	6.24	5.72	5.36
LM GP01 0100508	PC(14:0/20:5(5Z,8Z,11Z,14Z,17Z))	C42H74NO8P	[M+H]+	752.5225	8.50	8.2	6.73	4.40	4.59
LM GL0301 0166	TG(17:2(9Z,12Z)/17:2(9Z,12Z)/18:2(9Z,12Z))	C55H94O6	[M+H]+	851.7323	24.38	5.7	5.91	4.36	4.56
LM GP01 0100490	PC(14:0/18:1(11Z))	C40H78NO8P	[M+H]+	732.5538	10.23	4.1	6.65	4.53	4.51
LM GP01 0100532	PC_LM GP01 0100532	C44H76NO8P	[M+H]+	778.5381	8.62	9.0	6.48	5.87	4.45
LM GP01 0100490	PC(14:0/18:1(11Z))	C40H78NO8P	[M+H]+	732.5538	10.30	3.8	6.41	4.41	4.38
LM GL0301 0140	LM GL0301 0140	C55H96O6	[M+H]+	853.7280	24.36	4.5	4.59	4.68	4.23
LM GP01 0100494	PC(14:0/18:2(11Z,14Z))	C40H76NO8P	[M+H]+	730.5381	9.21	3.7	5.55	4.39	4.18
LM GP01 0100541	PC(15:0/18:1(11Z))	C43H80NO8P	[M+H]+	746.5694	15.46	4.1	4.23	4.27	4.06
LM GP01 0100433	PC(14:0/20:5(5Z,8Z,11Z,14Z,17Z))	C44H78NO8P	[M+H]+	780.5538	9.51	3.2	2.57	2.54	2.33
LM GP01 0250125	PC(15:1(9Z)/0:0)	C23H46NO7P	[M+H]+	480.3085	5.68	4.4	-3.63	-180	-2.30
LM GP01 0100645	PC(16:0/22:5(4Z,7Z,10Z,13Z,16Z))	C46H82NO8P	[M+H]+	808.5851	10.50	14.0	-3.39	-188	-2.34
LM GP06 0100076	PI(14:0/22:4(7Z,10Z,13Z,16Z))	C45H79O13P	[M-H]-	857.5286	9.36	5.0	-2.62	-2.24	-2.38
LM GP03 0200032	PA(O-18:0/18:4(6Z,9Z,12Z,15Z))	C39H73O7P	[M-H]-	683.5030	18.30	7.6	-3.24	-2.20	-2.46
LM GL0201 0197	DG(20:3(8Z,11Z,14Z)/20:4(5Z,8Z,11Z,14Z)/0:0)	C43H70O5	[M+H]+	667.5296	18.13	2.9	-2.95	-2.43	-2.49
LM GL0301 0722	TG(18:3(9Z,12Z,15Z)/18:3(9Z,12Z,15Z)/20:1(11Z))	C59H100O6	[M+H]+	905.7593	18.37	7.0	-10.88	-1.10	-3.98
LM GP10 0111755	PC(19:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C49H86NO8P	[M+H]+	848.6364	13.07	3.5	-6.06	-3.26	-4.47
LM GP01 0116170	PC(18:3(6Z,9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C48H78NO8P	[M+H]+	828.5538	9.53	9.3	-5.62	-3.80	-4.64
LM GP01 0100788	PC(18:0/20:2(11Z,14Z))	C46H88NO8P	[M+H]+	814.6320	15.43	4.0	-5.38	-3.58	-4.71
LM GP01 0200004	PC(O-10/16:0)	C25H52NO7P	[M-H]-	508.3409	6.35	17.7	-10.58	-3.88	-4.85
LM GP01 0100788	PC(18:0/20:2(11Z,14Z))	C46H88NO8P	[M+H]+	814.6320	15.54	5.7	-26.45	-4.61	-5.38
LM GP03 0100199	PE(16:0/22:1(13Z))	C44H84NO8P	[M-H]-	818.5906	8.51	6.2	-90.23	-5.10	-5.65
LM GP01 0113028	PC(20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C50H88NO8P	[M+H]+	862.6320	14.48	4.7	-20.00	-9.37	-14.21
LM GP01 0200072	PC(O-16:0/4:0)	C28H58NO7P	[M+H]+	552.4024	7.30	6.2	-43.04	-12.65	-14.76

4: Conclusions

- Human specific troglitazone metabolism, consistent to published data, was shown from PXB mice.
- Endogenous lipid differences between PXB and SCID were detected some consistent irrespective of troglitazone dosing and others that may be influenced by troglitazone dosing.
- MetID Solution combined use of accurate mass and isotope scoring enabled greater confidence in putative metabolite identification.