Validation of a Phase I and Phase II Metabolic Stability Assay in Subcellular Fractions

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Objective

A liver microsomal metabolic stability assay was validated to evaluate both phase 1 and phase 2 enzyme metabolism using model compounds. In addition, the assay was evaluated by testing a set of structurally unrelated compounds for metabolic stability.

Methods

Incubations were performed in 1 ml 100 mM potassium phosphate buffer with HLM (BD Cat. No. 452161), or human S9 (BD Cat. No. 452961), and 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride (for NADPH-dependent metabolism). Incubations were also performed with 2 mM uridine 5'-diphosphoglucuronic acid, and 25 µg per mg protein of alamethicin (for UGT metabolism) in the same buffer. The assay was conducted in 2 mL 96-well polypropylene plates (Axygen Scientific, Inc.) on a custom-made thermal block/circulator system (MéCour Temperature Control) ***(Figure 2)***. At 0, 10, 20, and 30 minutes, 100 µL samples were removed and combined with 400 µl of acetonitrile (containing internal standard) to stop the reaction. Samples were centrifuged and analyzed by LCMS. Validation assays were performed using testosterone (20 µM) and 7-Hydroxy 4-trifluoromethylcoumarin (HFC, 25 µM) as probes for NADPH-dependent and UDPGA-dependent metabolism, respectively. After validation of the method, various model compounds were selected to reflect structural diversity and a wide range of microsomal metabolic stability.

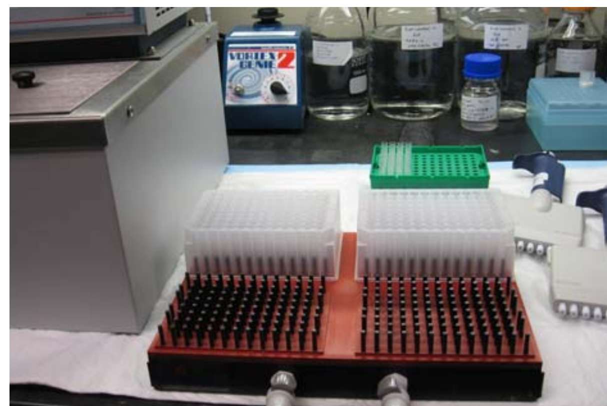
1 Validation Results of a 96-well Microsomal Metabolic Stability Method

Step 1			$t_{1/2}$ [min] replicate 1	replicate 2	mean of duplicates	average of both methods	Deviation of individual values from average: validation result	acceptance criteria	Pass / Fail
Testosterone	HLM (1 mg/ml)	Tube	9.7	10.1	9.9				
Testosterone	HLM (1 mg/ml)	Plate 1	11.0	10.9	11.0	10.5	**5%**	≤ 20%	**Pass**

Step 2.1			$t_{1/2}$ [min] replicate 1	replicate 2	mean of duplicates	average of both methods	% CV: validation result	acceptance criteria	Pass / Fail
Testosterone	HLM (1 mg/ml)	Plate 1	11.0	10.9	11.0				
Testosterone	HLM (1 mg/ml)	Plate 2	11.2	11.2	11.2	11.1	**1%**	≤ 20%	**Pass**
HFC	HLM (0.05 mg/ml)	Plate 1	42.1	40.1	41.1				
HFC	HLM (0.05 mg/ml)	Plate 2	48.5	45.2	46.8	43.9	**8%**	≤ 20%	**Pass**

Step 2.2			time point closest to 50% loss	% remaining replicate 1	replicate 2	average	% CV: validation result	acceptance criteria	Pass / Fail
Testosterone	HLM (1 mg/ml)	Plate 1	10 min	52%	50%				
Testosterone	HLM (1 mg/ml)	Plate 2	10 min	58%	56%	54%	**7%**	≤ 20%	**Pass**
HFC	HLM (0.05 mg/ml)	Plate 1	30 min	58%	58%				
HFC	HLM (0.05 mg/ml)	Plate 2	30 min	66%	61%	61%	**6%**	≤ 20%	**Pass**

2 Thermal Block with 96-well plates



Incubations were performed in 96-well polypropylene deep-well plates (Axygen Scientific, Inc.) on a thermal block/circulator system (MéCour Temperature Control). Customized pins extend between the individual wells to ensure uniform heat transfer, which reduces edge effects common with flat heating elements.

3 $T_{1/2}$ and CL_{int} values determined for a set of structurally diverse compounds

	HLM+NADPH set 1 $t_{1/2}$	HLM+NADPH set 1 CL_{int}	HLM+NADPH set 2 $t_{1/2}$	HLM+NADPH set 2 CL_{int}	HLM Literature CL_{int}	HLM+UDPGA $t_{1/2}$
Diazepam	35	20	39	18	3 [Riley2005]	n.d.
Diltiazem	11	64	16	45	34 [Riley2005], 27 [Soars2003]	n.d.
Omeprazole	15	45	24	28	34 [McGinnity2000], 97 [Naritomi2001], 12 [Soars2003]	n.d.
Dextromethorphan	12	56	21	33	22 [Soars2003]	n.d.
Phenacetin	27	25	39	18	9 [Soars2003]	n.d.
Midazolam	<5	≥ 140	<5	≥ 140	160 [Obach1999]	n.d.
Rac-Propranolol	21	33	31	22	13 [Riley2005], 11 [Soars2003]	n.d. #
Verapamil	<5	≥ 140	<5	≥ 140	122 [Riley 2005], 138 [Soars2003]	n.d.
Rac-Ketoprofen *	29	24	20	34		n.d. #
Rac-Ketoprofen *	32	21	40	17		n.d. #
Alprenolol	8	85	10	69		n.d.
Nicardipine	<5	≥ 140	<5	≥ 140	1719 [Riley2005]	n.d.
Imipramine	26	27	60	12	19 [Obach1999]	n.d. #
Indomethacin	n.d.	n.d.	n.d.	n.d.		n.d. #
Buprenorphine	7	102	3	231		n.d. #
Naproxen	n.d.	n.d.	n.d.	n.d.		n.d.
Diclofenac	7	105	13	53	80 [Soars2003], 189 [Obach1999]	32 #
Gemfibrozil	15	47	6	116	47 [Andersson2004]	55 #
HFC	30	23	19	36		<5 #
Methylphenidate	n.d.	≥ 140	n.d.	≥ 140		n.d.
	Human S9 +NADPH					
Methylphenidate	15	46				

** two ketoprofen peaks observed in chromatogram consistent with separation of isomers, # known UGT substrate*
CL_{int} – in vitro intrinsic clearance in µl/min/mg, $t_{½}$ in min., n.d. – not determined due to <15% loss

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Results

The assay validation consisted of two steps: a comparison of the plate-based method with the traditional micro-centrifuge tube assay (step 1), as well as an inter-day comparison of testosterone (CYP) and HFC (UGT) metabolic stability in 96-well plates (step 2) ***(Figure 1)***. Using the validated 96-well assay, 19 structurally different compounds (1 µM) were assayed using HLM (1 mg/ml) in presence and absence of NADPH or UDPGA ***(Figure 3)***.

As expected, the compounds tested showed a large variability in CYP metabolic stability (HLM + NADPH) with 100% loss ($t_{1/2}$< 5 minutes) to < 15% loss over a 30-minute incubation period. In absence of NADPH, all compounds appeared to be stable over 30 minutes with the exception of gemfibrozil ($t_{1/2}$ = 55 minutes) suggesting the contribution of an NADPH-independent component.

Incubations in presence of UDPGA did not result in substantial metabolism, with the exception of HFC (a positive control for UGT metabolism), as well as diclofenac and gemfibrozil, consistent with latency of UGT activity or slow turnover.

Methylphenidate, a substrate of cytosolic carboxylesterase (CES1A1)[Sun2004], was stable in HLM, however, incubations with human S9 showed substantial metabolism ($t_{1/2}$ = 15 minutes).

Conclusions

- CL_{int} values were in good agreement with literature data (where available).
- Inter-day $t_{1/2}$ /CL_{int} values varied generally < 2-fold (16/19 compounds, < 2.5-fold for 19/19 compounds).
- Methylphenidate was identified as compound that can serve as a positive control for cytosolic carboxylesterase (CES1A1) in metabolic stability screening in S9.
- Nearly all compounds tested (some known UGT substrates) exhibited poor metabolism, attributable in part to latency of UGT activity indicating that UGT metabolism may be better assessed using a hepatocyte model.

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