



Ultra-High Throughput ADME Screening Using LDTD Methodology

Authors

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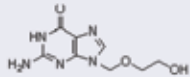
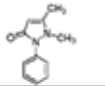
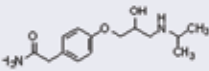
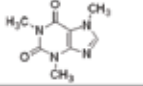
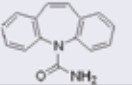
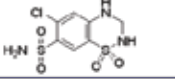
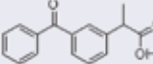
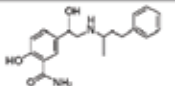
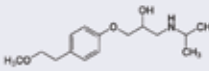
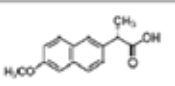
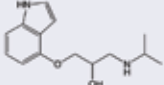
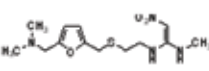
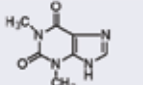
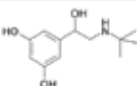
Introduction

An ultra-high throughput sample analysis for ADME assays has been developed using the Laser Diode Thermal Desorption (LDTD) interface with tandem mass spectrometry (MS/MS). LDTD does not require chemical matrix to assist compound ionization as Matrix-Assisted Laser Desorption/Ionization (MALDI) does. Thus, LDTD has an advantage over MALDI for the analysis of small molecules without the interference background peaks generated from the chemical matrix added. Plasma stability and Caco-2 assays were conducted to examine the throughput, sensitivity, and quantitation using LDTD/MS/MS. Unlike LC/MS/MS, no LC separation is needed for LDTD/MS/MS, thus reducing the sample analysis time to 10 sec per sample. Two sets of compounds with different functional groups and chemical properties were selected to test for the plasma stability (set 1: 12 compounds) and Caco-2 analysis (set 2: 14 compounds). The study samples were analyzed by both LDTD/MS/MS and LC/MS/MS. The results from these two methodologies were compared and reported.

Materials

Human plasma was obtained from BioChemed Services, Winchester, VA. Caco-2 cell stock was purchased from ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with non-essential amino acid and fetal bovine serum. Cells were seeded onto 24-well Transwell polycarbonate membranes (Corning Costar, Acton, MA) and were used at passage 40-60. Twenty six test compounds and all chemicals were purchased from Sigma-Aldrich, Milwaukee, WI. Organic Solvent was purchased from Fisher Scientific, Pittsburg, PA.

Caco-2 Test Compounds

Compound	Chemical Formula	Exact MW	Structure	MS Detection Mode	
				LC/MS/MS	LDTD
Acyclovir	C ₈ H ₁₁ N ₅ O ₃	225.09		ES+	Positive
Antipyrine	C ₁₁ H ₁₂ N ₂ O	188.09		ES+	Positive
Atenolol	C ₁₄ H ₂₂ N ₃ O ₃	280.17		ES+	Positive
Caffeine	C ₈ H ₁₀ N ₄ O ₂	194.08		ES+	Positive
Carbamazepine	C ₁₅ H ₁₂ N ₂ O	236.09		ES+	Positive
Hydrochlorothiazide	C ₇ H ₈ ClN ₃ O ₄ S ₂	296.96		ES+	Positive
Ketoprofen	C ₁₆ H ₁₄ O ₃	254.09		ES-	Positive
Labetalol	C ₁₉ H ₂₄ N ₂ O ₃	328.18		ES+	Positive
Metoprolol	C ₁₅ H ₂₅ NO ₃	267.18		ES+	Positive
Naproxen	C ₁₄ H ₁₄ O ₃	230.09		ES-	Positive
Pindolol	C ₁₄ H ₂₀ N ₂ O ₂	248.15		ES+	Positive
Ranitidine	C ₁₃ H ₂₂ N ₄ O ₃ S	314.14		ES+	Positive
Theophylline	C ₇ H ₈ N ₄ O ₂	180.06		ES+	Positive
Terbutaline	C ₁₂ H ₁₉ NO ₃	225.14		ES+	Positive



Methods

SAMPLE PREPARATION FOR PLASMA STABILITY STUDY

- A group of 12 compounds were incubated with human and rat plasma at 5 μM for 0, 10, 30, 60 and 120 minutes at 37 °C, respectively.
- At each time point, 100 μL of plasma sample was taken and was added to 400 μL of 1 μM carbutamide/ACN solution in a 96-deep well plate.
- After protein precipitation, supernatants were evenly split for analysis.
- For LC/MS/MS analysis, supernatants were diluted with the mobile phase A (95/5 $\text{H}_2\text{O}/\text{ACN}$, 0.1% formic acid) prior to analysis.
- For LDTD/MS/MS analysis, 2 μL of supernatants were transferred into a 96-Lazwell plate. The solvent was allowed to evaporate at room temperature (less than 2 minutes) before the analysis.

SAMPLE PREPARATION FOR CACO-2 PERMEABILITY STUDY

- Cell culture media was removed and replaced with Hank's balanced salt solution (HBSS) at pH 7.4.
- A group of 14 compounds were prepared in HBSS (see Compound Table) and applied to Caco-2 System.
- Receiver side was 5% bovine serum albumin in HBSS. The apical volume was 0.2 mL and the basolateral volume was 1.0 mL.
- The studies were of 60 minutes duration, and 120 μL of samples were taken from the receiver side at 15 minutes intervals. The permeation was in the apical-to-basolateral (A-to-B).
- 35 μL of sample was added to 70 μL of 0.5 μM carbutamide/ACN solution in a 96-deep well plate for protein precipitation. The supernatants were evenly split for analysis.
- For LC/MS/MS analysis, supernatants were dried and reconstituted with the mobile phase A (95/5 $\text{H}_2\text{O}/\text{ACN}$, 0.1% formic acid) prior to analysis.
- For LDTD/MS/MS analysis, see "Sample Preparation for Plasma Stability Study"

LC/MS/MS

- Column: Phenomenex Hydro-RP C18 (50 x 2.0 mm, 4 μm)
- Column Heater: 40°C

PLASMA STABILITY

- Mobile Phase A: 95/5 $\text{H}_2\text{O}/\text{ACN}$, 0.1% formic acid
Mobile Phase B: 10/90 $\text{H}_2\text{O}/\text{ACN}$, 0.1% formic acid



Methods continued

CACO-2 PERMEABILITY (POSITIVE MODE)

- Mobile Phase A: 1% formic acid in water
- Mobile Phase B: 1% formic acid in acetonitrile (ACN)

CACO-2 PERMEABILITY (NEGATIVE MODE)

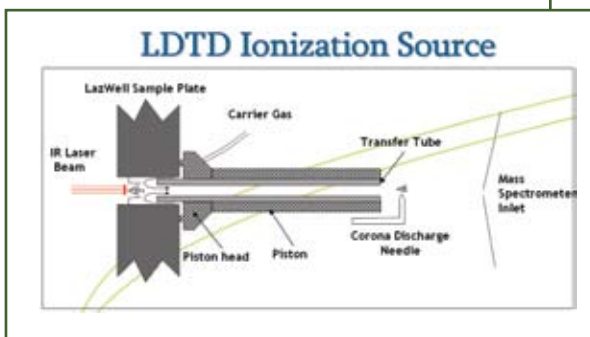
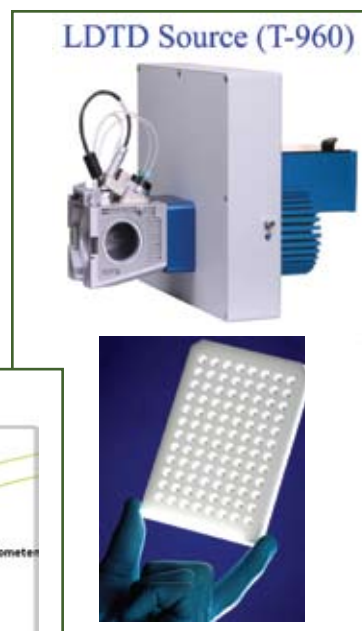
- Mobile Phase A: 10 mM ammonium acetate, 0.1 % triethylamine in water
- Mobile Phase B: 10 mM ammonium acetate, 0.1 % triethylamine in ACN
- Injection Volume: 10 μ L
- Gradient (default gradient shown below was used for most compounds and was modified for some compounds)

Time (min)	% B	Flow (mL)
0.0	10	0.6
1.5	90	0.6
2.0	90	0.6
2.1	10	0.6
2.5	10	0.6

- API 4000 (Applied Biosystem/Sciex)
- MRM under an electrospray positive or negative mode

LDTD/MS/MS

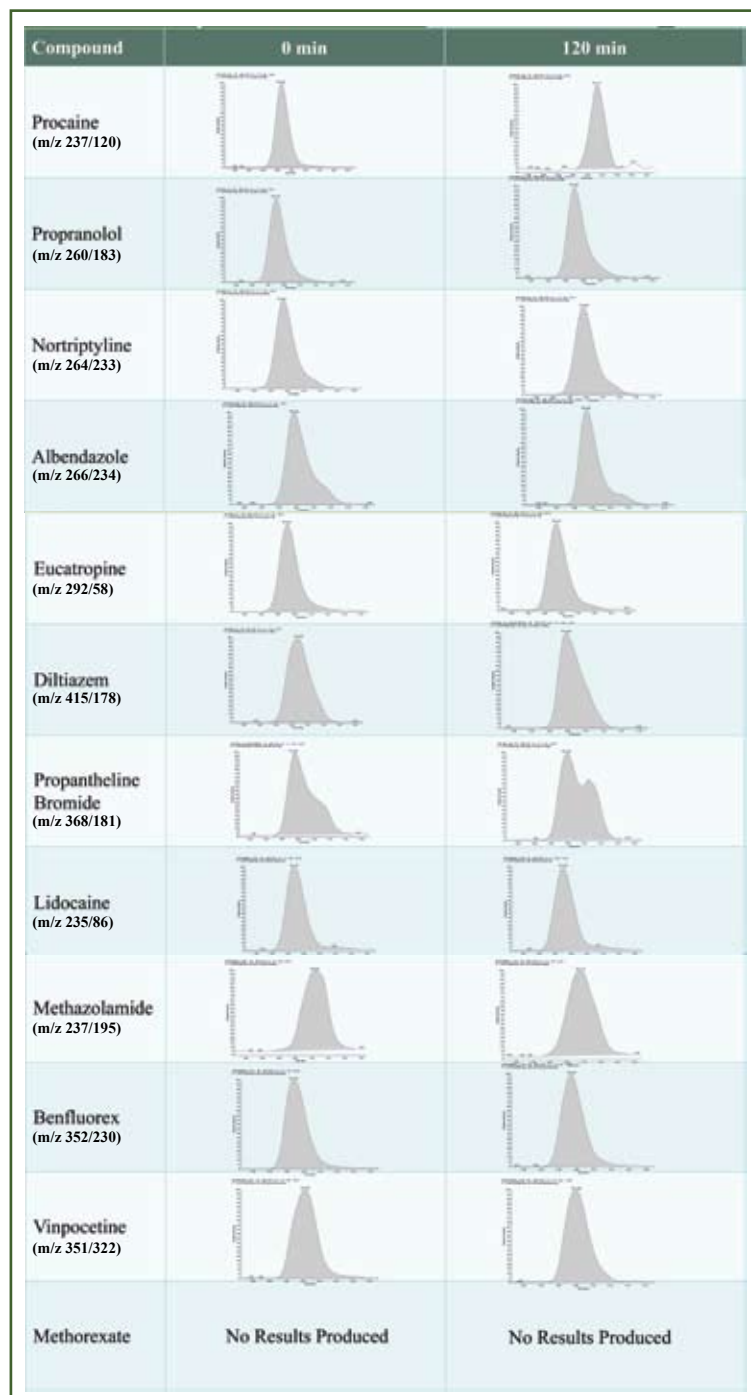
- A Phytronix LDTD source (model T-960) on Thermo TSQ Vantage triple quadrupole mass spectrometer
- Two micro-liters of supernatants were deposited onto the LazWell plate.
- Dry the solvent on the LazWell plate prior to LDTD/MS/MS analysis





Results and Discussion

REPRESENTATIVE LDTD/MS/MS THERMAL DESORPTION PEAKS





Results and Discussion continued

CACO-2 PERMEABILITY DATA

Compounds Model	Concentration (μM)	A-to-B (x10 ⁻⁶ , cm/s)		
		LDTD (Phytronix)	LC/MS/MS (TLNE)	Literature
Acyclovir	50	ND	1.1 ± 0.08	1.2
Antipyrine	50	34.7 ± 6.1	31.6 ± 3.9	48.4
Atenolol	100	0.8 ± 0.07	1.3 ± 0.1	0.9
Caffeine	10	ND	57.3 ± 11.9	50.5
Carbamazepine	10	27.1 ± 8.6	23.4 ± 2.2	18.3
Hydrochlorothiazide	50	5.5 ± 3.1	5.5 ± 1.2	4.7
Ketoprofen	10	21.4 ± 0.6	18.7 ± 0.2	21.1
Labetalol	10	2.3 ± 0.7	3.6 ± 0.9	9.3
Metoprolol	10	21.5 ± 2.2	17.2 ± 1.8	15.0
Naproxen	50	46.4 ± 6.3	32.8 ± 4	54.2
Pindolol	10	2 ± 0.3	1.5 ± 0.3	<0.5
Ranitidine	50	0.4 ± 0.06	1.1 ± 0.2	3.4
Terbutaline	10	0.18 ± 0.02	0.19 ± 0.04	16.0
Theophylline	10	ND	11.4 ± 2.3	1.4

ND: not detected

Results and Discussion continued

HUMAN PLASMA STABILITY DATA (% REMAINING)

Compound	Analysis	0 min	10 min	30 min	60 min	120 min	T _{1/2}
Procaine	LC/MS/MS	100	0.65	0.06	0.03	0.05	< 10 min
	LDTD/MS/MS	100	0.67	0.36	0.31	0.31	< 10 min
Propranolol	LC/MS/MS	100	107	79	91.7	85.8	> 4 hr
	LDTD/MS/MS	100	114	107	98.7	101	> 4 hr
Nortriptyline	LC/MS/MS	100	109	103	110	114	> 4 hr
	LDTD/MS/MS	100	106	95.6	105	110	> 4 hr
Albendazole	LC/MS/MS	100	77.9	109	94.1	108	> 4 hr
	LDTD/MS/MS	100	111	103	128	161	> 4 hr
Eucatropine	LC/MS/MS	100	71.6	51.4	33.9	9.68	34 min
	LDTD/MS/MS	100	83.9	48.2	27.5	6.79	31 min
Diltiazem	LC/MS/MS	100	78.9	89.5	78.3	87.7	> 4 hr
	LDTD/MS/MS	100	100	93.9	100	121	> 4 hr
Propantheline Bromide	LC/MS/MS	100	98.9	96.4	65.3	29.3	78min
	LDTD/MS/MS	100	79.0	75.2	73.5	34.3	82 min
Lidocaine	LC/MS/MS	100	93.3	106	104	105	> 4 hr
	LDTD/MS/MS	100	147	136	124	106	> 4 hr
Methazolamide	LC/MS/MS	100	104	111	102	107	> 4 hr
	LDTD/MS/MS	100	155	141	143	166	> 4 hr
Methotrexate	LC/MS/MS	100	126	108	103	167	> 4 hr
	LDTD/MS/MS	---	---	---	---	---	---
Benfluorex	LC/MS/MS	100	96.6	83.9	82.5	56.8	152 min
	LDTD/MS/MS	100	75.0	70.7	55.9	43.6	89 min
Vinpocetine	LC/MS/MS	100	102	107	130	127	> 4 hr
	LDTD/MS/MS	100	110	120	107	118	> 4 hr



Results and Discussion continued

RAT PLASMA STABILITY DATA (% REMAINING)

Compound	Analysis	0 min	10 min	30 min	60 min	120 min	Half-Life T _{1/2}
Procaine	LC/MS/MS	100	108	105.0	88.0	81.0	> 4 hr
	LDTD/MS/MS	100	119	109	116	88.9	> 4 hr
Propranolol	LC/MS/MS	100	84.2	85.2	90.7	102	> 4 hr
	LDTD/MS/MS	100	127	95.6	96.4	114	> 4 hr
Nortriptyline	LC/MS/MS	100	93.9	95.7	90.0	95.0	> 4 hr
	LDTD/MS/MS	100	121	126	128	107	> 4 hr
Albendazole	LC/MS/MS	100	114	117	128	159	> 4 hr
	LDTD/MS/MS	100	185	161	139	151	> 4 hr
Eucatropine	LC/MS/MS	100	100.4	83.3	99.8	89.5	> 4 hr
	LDTD/MS/MS	100	113	113	120	117	> 4 hr
Diltiazem	LC/MS/MS	100	98.2	86.0	78.6	48.7	122 min
	LDTD/MS/MS	100	118	87.4	87.3	41.5	107 min
Proprantheline Bromide	LC/MS/MS	100	108.1	103.2	104.7	95.3	> 4 hr
	LDTD/MS/MS	100	127	82.9	94.6	83.2	> 4 hr
Lidocaine	LC/MS/MS	100	93.4	85.4	89.3	82.3	> 4 hr
	LDTD/MS/MS	100	135	104	109	123	> 4 hr
Methazolamide	LC/MS/MS	100	92.5	90.4	91.5	97.2	> 4 hr
	LDTD/MS/MS	100	115	109	110	100	> 4 hr
Methotrexate	LC/MS/MS	100	140.9	149.8	139.4	123.7	> 4 hr
	LDTD/MS/MS	100	---	---	---	---	---
Benfluorex	LC/MS/MS	100	90.6	76.6	53.5	23.3	61 min
	LDTD/MS/MS	100	106	86.5	55.1	25.6	67 min
Vinpocetine	LC/MS/MS	100	86.4	81.3	67.2	34.5	83 min
	LDTD/MS/MS	100	96.8	90.2	71.0	32.1	82 min

PLASMA STABILITY: The plasma stability data generated from each method matched remarkably well. With each method, we were able to distinguish compounds with a long half-life (> 4hr) from those with a moderate half-life (within 2 hr) and a short half-life (< 10 min) in both human and rat plasma. We found that the stabilities of procaine, eucatropine, diltiazem, proprantheline bromide and vinpocetin in rat plasma and in human plasma were different. The findings from both analytical methods also agreed with each other regarding the difference of half-lives of these compounds in these two species.

CACO-2 PERMEABILITY: The permeability (A-to-B) generated using Caco-2 cells matched very well when comparing literature values with experimental data (both LDTD and LC/MS/MS). No permeability data was obtained on LDTD for acyclovir, caffeine, and theophylline. The ionization of these chemical molecules using the LDTD source needs to be further investigated.

Chemistry vs. Ionization: We found that the ionization of basic compounds, such as amine



Results and Discussion continued

containing compounds are generally very good by using the LDTD/MS/MS method. The LDTD source produced strong signals for the hetero-cyclic compounds (e.g., Albendazole). Ionization of Albendazole was weak using an electrospray source on API 4000. However, for quaternary amines, the LC/MS/MS method has an advantage over the LDTD/MS/MS method. The presence of OH groups seemed to weaken the LDTD ionization.

A signal could not be obtained when using the LDTD/MS/MS for compounds possessing a carboxylic acid functional group, e.g., Cerivastatin (data not shown) and Methotrexate. Acyclovir, caffeine and theophylline could not be detected using LDTD ionization. These three compounds were all xanthine analogues.

Throughput: The total analysis time for 100 samples was less than 20 minutes by LDTD/MS/MS (10 seconds per sample). It took approximately 5 hours using a 2.5-minute LC/MS/MS method.

Conclusion

The LDTD/MS/MS technology can be applied as an ultra-high throughput screening method for the stability assessment of small molecules in plasma and for the Caco-2 permeability study. The results over 12 compounds suggest a combination of the LDTD/MS/MS method (covering 90% of all screened compounds) with the traditional LC/MS/MS method (covering the 10% uncovered by the LDTD/MS/MS) allows to increase the throughput on plasma stability screening of a factor of 6.2 times. As for Caco-2 permeability study, the LDTD/MS/MS method covered about 78% of all screened compounds. The LDTD/MS/MS analysis of compounds with a carboxylic acid functional group or compounds with a Xanthine core structure would be a challenge or limitation.