

OVERVIEW

Purpose

Testosterone is a steroid hormone from the androgen group. In both males and females, it plays key roles in health and well-being. The purpose of this work was to develop a specific and robust method for the determination of testosterone in human EDTA K₂ plasma using LDTD technology method offering very short analysis time and very high throughput and comparing it to LC/MS/MS analysis.

Instrumentation and Analytical Method Overview

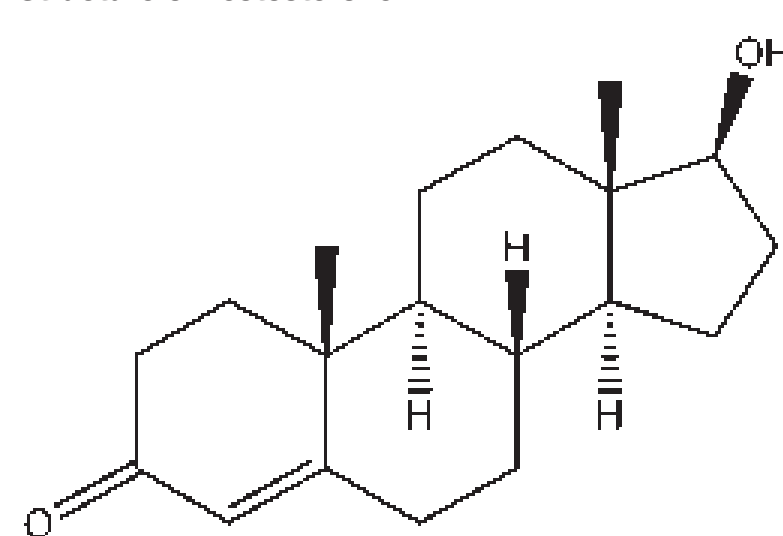
- Testosterone is extracted from human plasma EDTA K₂ using a solid phase extraction method
- Calibration range : 1 ng/mL to 200 ng/mL
- Analysis is performed using classic LC/MS/MS and atmospheric pressure chemical ionization (APCI)
- Analysis is also performed using LDTD/MS/MS analysis : Laser diode thermal desorption coupled with atmospheric pressure chemical ionization (APCI)

Results Overview

- Both LC/MS/MS and LDTD/MS/MS method shows :
 - Excellent linearity over the calibration range (r = 0.9989)
 - Within-run accuracy between 99.2 and 103.1 %
 - No matrix effect successfully demonstrated
 - No co-administrated drugs interferences
 - No hemolysis effect successfully demonstrated
- The LC/MS/MS method shows a higher selectivity as compared to the LDTD/MS/MS method
- The sample-to-sample run time is 12 time lower with the LDTD/MS/MS method (30 sec as compared to 6.0 min)

ANALYTICAL METHOD

Chemical Structure of Testosterone



Testosterone Extraction

Sample preparation

- 200µL of matrix sample
- 800µL of testosterone-d5 (Internal Standard) in 0.1 N HCl and 0.1N HCl solution for the blanks
- Mixed adequately on vortexer

SPE activation

- Elute 1 mL of Methanol by gravity
- Elute 1 mL of Milli-Q type Water by gravity

Sample extraction on SPE

- Load sample and elute by gravity

SPE washing

- Elute 1 mL of 0.1 N HCl
- Elute 1 mL of Methanol/Water (70/30) mixture
- Both elution performed by centrifugation at 300 RPM for 2 min.

Sample elution

- Elute sample with 1 mL of Methanol by gravity

Sample preparation for MS analysis

- Evaporate to dryness at 50°C under 15psi of air
- Reconstitute with 100µL of Methanol/Water (75/25)
- Filtrate using Nanosep filter 0.2µm
- Transfer 2.5µL onto LazWell™ to perform LDTD/MS/MS analysis
- Transfer 30µL, evaporate to dryness and reconstitute with 200µL of Methanol/Water (75/25)
- Inject 30µL to perform LC/MS/MS analysis

Stripped matrix

- To Human plasma, add activated coal (2% w/v) and agitate for 12 hours

On-Column and LazWell™ deposited sample

- Both technique allows to analyses of 5 pg to 1000 pg on-column or transferred into a well for thermal desorption

RESULTS

Method Calibration

This assay was validated over a nominal range of 1 ng/mL to 200 ng/mL. The linearity was excellent for both methods (Table 1).

Table 1 Calibration Curve Parameters

	LC/MS/MS	LDTD/MS/MS
R ²	0.9994	0.9989
Slope (count/concentration)	0.0451	0.0551
y-absciss	0.0001	0.0470

Within-Run Accuracy and Precision

The within-run accuracy and precision was evaluated on quality control and on standards. Both method shows excellent accuracy (from 99.18 to 103.13 %) at low and high testosterone concentrations (Table 2). The precision at low concentration is lower for the LDTD-MS/MS method but within acceptable range.

Table 2 Within-Run Accuracy and Precision of Quality Controls and Back-Calculated Standards for Testosterone

	LC/MS/MS				LDTD/MS/MS			
	CS1	QC1	CS4	CS8	CS1	QC1	CS4	CS8
N	6	6	6	6	6	6	6	6
Mean	1.02	3.02	40.08	199.44	1.03	3.08	40.68	198.37
SD (+)	0.03	0.09	0.71	2.07	0.09	0.10	1.53	4.21
CV (%)	3.43	2.97	1.77	1.04	8.68	3.29	3.76	2.12
% Nominal Conc.	101.83	100.56	100.19	99.22	103.13	102.44	101.69	99.18

Co-Administered Drugs Interferences

Common drugs interference was evaluated on fortified low concentration quality control. None of the ten (10) tested potentially interfering drugs affects the quantitation of testosterone in human plasma (Table 3).

Table 3 Co-Administered Drugs Interference on Testosterone Quantification in Human Plasma

Co-Adminstrated drugs	LC/MS/MS		LDTD/MS/MS	
	QC1 (ng/ml) 3.00	% Nominal Conc.	QC1 (ng/ml) 3.00	% Nominal Conc.
Acetaminophen	3.04	101.44	3.16	105.33
Acetylsalicylic acid	3.03	100.89	2.98	99.33
Caffeine	3.04	101.33	3.09	103.00
Dimenhydrinate	2.99	99.66	2.82	94.00
Dextrometorphan	2.87	95.78	2.94	98.00
Diphenhydramine	3.02	100.53	3.00	100.00
Heparin	3.04	101.33	3.09	103.00
Ibuprofen	3.03	101.11	2.90	96.67
Nicotine	3.02	100.78	2.80	93.33
Pseudoephedrine	3.02	100.30	2.89	96.33

Results shown represent the mean of 3 replicates

Hemolysis Effect

Doping Human plasma with 2% of hemolysed blood does not affect the Testosterone quantification as shown in Table 4.

Table 4 Hemolysis Effect on Testosterone Quantification in Human Plasma

	LC/MS/MS		LDTD/MS/MS	
	QC1 (ng/ml) 3.00	% Nominal Conc.	QC1 (ng/ml) 3.00	% Nominal Conc.
BLK	2.36	78.67	2.54	84.67
QC1	3.01	100.33	3.02	100.67

1 Calculated from LLOQ mean response
2 Calculated from IS mean response

Testosterone Extraction Recovery

Recovery from high standards for analyte and internal standard using LC/MS/MS method was respectively 106.69 % and 78.05 % (Table 5). Using LDTD/MS/MS method, the same samples showed recovery of 86.17 % and 70.33 % for analyte and internal standard respectively (Table 5). Higher signal variation was observed with the LC/MS/MS method (about 18 % based on area CV values) as compared to the LDTD/MS/MS method (about 4 %). This difference in analyte recovery seems to be due to variable instrument response although analyte/IS peak area ratio is stable.

Table 5 Blank Sample Selectivity on Testosterone and on Testosterone-d5 (IS)

	LC/MS/MS			LDTD/MS/MS		
	Analyte Area	IS Area	Area Ratio (Analyte/IS)	Analyte Area	IS Area	Area Ratio (Analyte/IS)
CS8 (ng/ml) 200						
Post-extraction sample	N	6	6	6	6	6
doping	Mean	1183759	1326128	6842172	6143043	1.11
	SD (+)	77741	85918	274835	200852	0.02
	CV (%)	6.57	6.48	4.02	3.27	1.79
Pre-extraction sample	N	6	6	6	6	6
doping	Mean	1262936	134555	5895723	561682	10.50
	SD (+)	230779	24498	180975	24298	0.77
	CV (%)	18.27	18.21	3.07	4.33	2.11
Recovery (%)	106.69	78.05*		86.17	70.33*	

* Concentration factor of 0.13 applied on IS

Methods Selectivity and Sensitivity

The selectivity from charcoal stripped blank samples is different between the LC/MS/MS and the LDTD-MS/MS methods (Figure 1). Results shows that the blanks analyzed by the LC/MS/MS method were interfering at about 1.5 % as compared to 30 % for the LDTD-MS/MS method (Table 6). However, the interference on IS between the two methods are lower then 2 %. This results suggest that the thermal desorption process generate higher background level at the typical testosterone MS/MS transition. However, comparable background influence is observed at the Internal Standard MS/MS transition.

Table 6 Blank Sample Selectivity on Testosterone and on Testosterone-d5 (IS)

	LC/MS/MS		LDTD/MS/MS	
	Analyte Area	IS Area	Analyte Area	IS Area
CS8 (ng/ml) 200				
BLK	N	6	6	6
	Mean	54	125	12353
	SD (+)	31	55	3040
	CV (%)	56.80	44.20	24.61
CS1 (ng/ml) 1.00	N	6	6	6
	Mean	6945	157398	374245
	SD (+)	826	21689	3956
	CV (%)	11.90	13.78	10.57
% Interference		0.78	0.08	33.01

*Concentration factor of 0.13 applied on IS

Note for table 7 and 8: Endogenous testosterone level quantification was performed despite the fact that the concentration level was lower than the LLOQ values. The actual working concentration range of the LC/MS/MS method (40 pg/mL to 8000 pg/mL) was modified to match the working concentration range allowed by the blank selectivity of the LDTD/MS/MS method.

Table 7 LC and LDTD Matrix Effect Quantification

Matrix Identification	LC/MS/MS			LDTD/MS/MS				
	*MEC	Nominal (ng/ml)	**Measured (ng/ml)	% Nominal Conc.	*MEC	Nominal (ng/ml)	**Measured (ng/ml)	% Nominal Conc.
ME01	0.24	3.24	3.12	96.30	0.46	3.46	3.20	92.41
ME02	0.18	3.18	2.97	93.40	0.26	3.26	3.25	99.58
ME03	0.11	3.11	2.94	94.53	0.22	3.22	3.06	95.14
ME04	0.09	3.09	2.82	91.26	0.12	3.12	3.08	98.72
ME05	0.23	3.23	3.14	97.21	0.32	3.32	3.64	109.76
ME06	0.16	3.16	3.02	95.57	0.45	3.45	3.42	99.15
ME07	0.20	3.20	3.10	96.88	0.39	3.39	3.35	98.92
ME08	0.27	3.27	3.13	95.72	0.55	3.55	3.42	96.44
ME09	0.23	3.23	2.94	91.02	0.25	3.25	3.10	95.62
ME10	0.18	3.18	3.09	97.17	0.27	3.27	3.52	107.75
Mean				94.91				99.35
CV (%)				2.44				5.49

*Mean Endogenic Contribution. All samples mean performed on 3 replicates. **3 ng/ml added to matrix

Table 8 Endogenous Level Quantification (ng/ml)

	LC/MS/MS			LDTD/MS/MS		
	Unstripped matrix ZS-NS	QC1 Stripped (ng/ml) 3.00	QC1 Unstripped (ng/ml) 3.49	Unstripped matrix ZS-NS	QC1 Stripped (ng/ml) 3.00	QC1 Unstripped (ng/ml) 3.87
N	6	6	6	6	6	6
Mean	0.492	3.09	3.60	0.870	3.09	3.77
SD (+)	0.048	0.06	.09	0.10	0.15	0.15
CV (%)	9.83	1.81	2.48	11.1	4.71	4.04
% Nominal Conc.		103.00	103.15		103.00	97.42

Sample-to-Sample Run Time

The thermal desorption process allows very fast Testosterone analysis as shown in Figures 1-3. Sample to sample desorption is achieved in 30 sec for the LDTD compare to a total analysis time of 360 sec for the classic LC/MS/MS (12 times faster).

CHROMATOGRAMS AND DESORPTION PROFILES

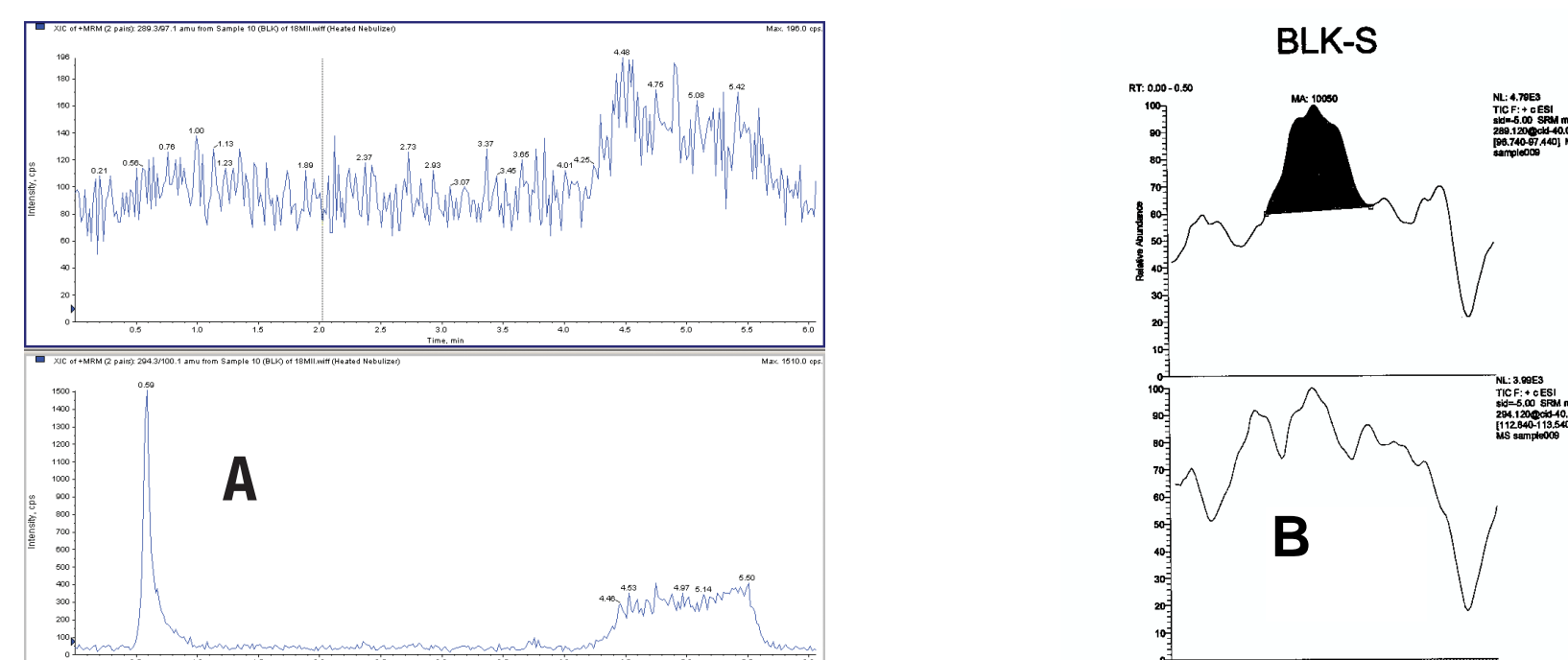


Figure 1 Representative chromatogram of Testosterone blank human EDTA K₂ plasma analyzed by A) LC/MS/MS and B) LDTD/MS/MS

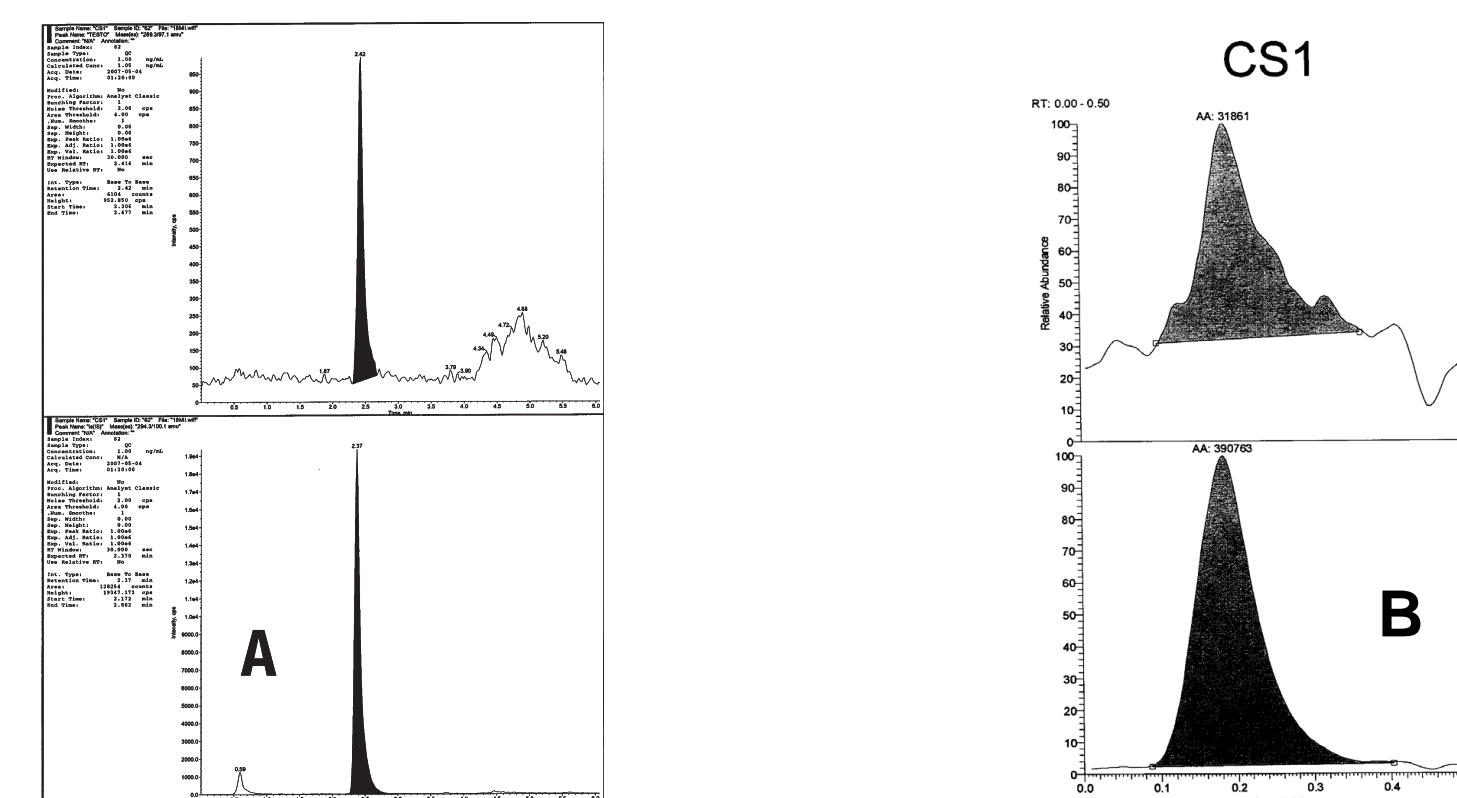


Figure 2 Representative chromatogram of the lower limit of quantitation containing Testosterone (1 ng/mL) in human EDTA K₂ analyzed by A) LC/MS/MS and B) LDTD/MS/MS

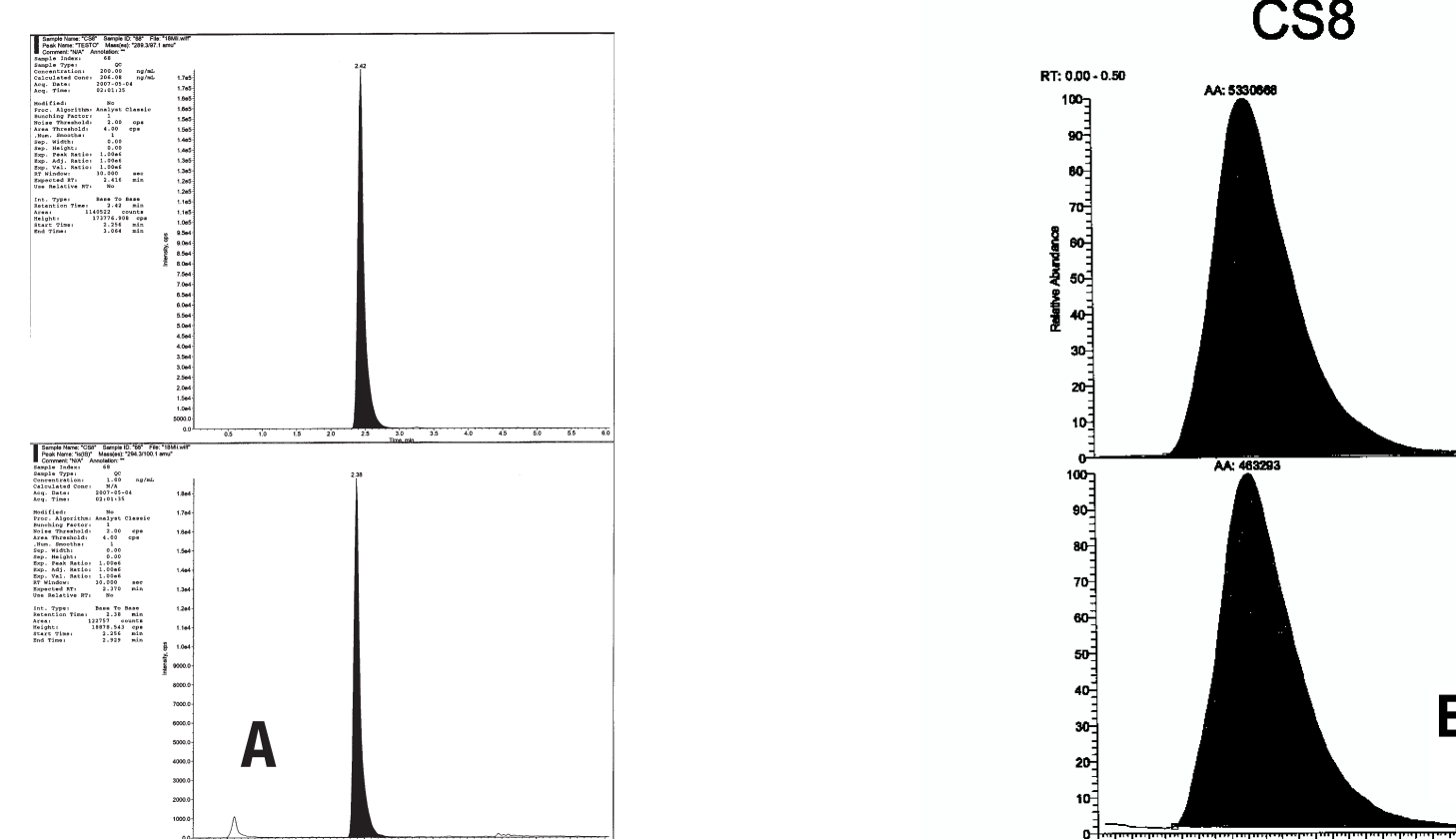


Figure 3 Representative chromatogram of the upper limit of quantitation containing Testosterone (200 ng/mL) in human EDTA K₂ analyzed by A) LC/MS/MS and B) LDTD/MS/MS

CONCLUSION

- The blank interference level for LDTD using stripped plasma samples were higher than the same analyzed with LC/MS/MS detection but acceptable at a LLOQ of 1ng/mL.
- To improve selectivity and sensitivity using plasma, further R&D work is to be performed to get the desired sample clean up from the extraction.
- All tests performed in this assay were clearly equivalent from the LC/MS/MS detection compared to LDTD/MS/MS detection.
- The comparison of liquid chromatography and Laser Diode Thermal Desorption are equivalent for the dosing of testosterone at a range of 1ng/mL to 200ng/mL
- Preliminary data using pure reference solution and different LDTD source parameters showed promising sensitivity to quantitate Testosterone down to 40pg/mL
- The runtimes achieved with LDTD detection was 30 seconds for each chromatograms compared to a total run time of 360 seconds using LC/MS/MS detection resulting an important decrease in sample analysis time (12 times faster). Reduced chance of autosampler carry over, mobile phase preparation elimination and therefore solvent consumption were also among the found benefits.

ACKNOWLEDGMENTS

We would like to acknowledge Nancy Lampron from bioanalytical department for all the technical support and help.

INSTRUMENTATION

Instruments for LDTD/MS/MS analysis

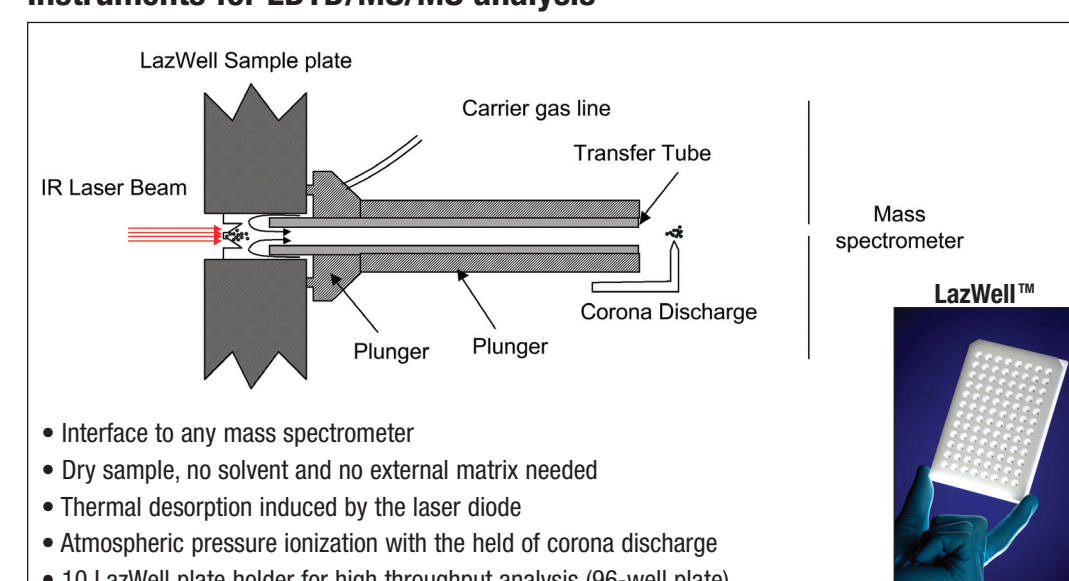


Figure 1. Schematic representation of LDTD ionization source LDTD - Phytronix Technologies model T960

Mass Spectrometer - Thermo Fisher Scientific TSQ³ QuantumTM Ultra AM

LDTD Parameters

- Laser Power Pattern
 - Hold at 0 % for 3 sec (stabilization)
 - Increase to 18 % in 9 sec
 - Hold at 18 % for 5 sec
 - Decrease to 0 % in 3 sec
 - Hold at 0 % for 3 sec
- Sample-to-Sample Time : 0.5 min.
- Carrier Gas : Air
- Carrier Gas Flow : 2.0 L/min
- Carrier Gas Temp. : 50 °C
- Corona Voltage Value : 4000 V

MS Parameters

- Collision Gas Pressure : 1.5 mTorr Argon
- Scan Time : 0.10 sec
- Q1 Width : 0.7 amu
- Skimmer offset : 5 V

Instruments for LC/MS/MS Analysis

Mass Spectrometer - MDS Sciex API 4000

LC Parameters

- Analytical Column : Waters Atlantis dC18, 75 x 4.6mm
- Mobile Phase : Acetonitrile / Water (50/50)
- Flow Rate : 1.0 mL /min
- Sample-to-Sample Time : 6.0 min.

APCI Parameters

- Heated Nebulizer, positive mode
- Nebulizer Pressure : 60 psi
- Curtain Gas Pressure : 30 psi
- CAD Gas Value : 10
- Heated Nebulizer Temp. : 600°C
- Corona Current Value : 5

MS Parameters

- DP: 80
- EP: 10
- CE: 32
- CXP: 10