HDL and Total Cholesterol analysis by LDTD-MS/MS in 7 seconds per sample with cross-validation data from a clinical laboratory using real patient serum samples

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OVERVIEW

Purpose

- High-throughput quantification of HDL and Total Cholesterol in serum.

Method

- Specific HDL separation.
- A saponification reaction & Liquid-Liquid extraction was used for the Cholesterol analysis.
- Quantification:
  - Linearity: $r^2 > 0.99$, over the calibration range (15.6 to 500 mg/dL)
  - Accuracy ranging from 88.1 to 108.3 % using area ratio value
  - Precision ranging from 6.2 to 11.8 % using area ratio value
- **Samples were analyzed with a run time of 7 seconds using LDTD-MS/MS system**

INTRODUCTION

High concentration of cholesterol in blood is associated with heart disease while HDL removes fats and cholesterol from cells and transports it back to the liver. Cholesterol level in blood is a frequently analyzed parameter that requires a high-throughput method due to the quantity of samples involved in routine analysis. HDL separation is achieved and all the samples are then treated in the same way for the extraction. A cross validation is made with data obtained from an external clinical laboratory to compare with the LDTD results.

LDTD® Ionization Source:

The LDTD uses a Laser Diode to produce and control heat on the sample support **(Figure 1)** which is a 96-well plate**.** The energy is then transferred through the sample holder to the dry sample which vaporizes prior to being carried by a gas in a corona discharge region. High efficiency protonation and strong resistance to ionic suppression characterize this type of ionization, and is the result of the absence of solvent and mobile phase. This allows for very high throughput capabilities of 7 seconds sample-to-sample analysis time, without carry over.

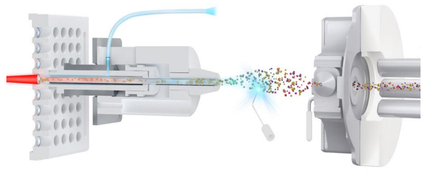

Figure 1 Schematic of the LDTD ionization source

METHOD

HDL Separation Procedure

Working solution 1: 1:1 ratio of Dextran Sulfate 20 g/L and Sodium Azide 0.5 g/L
Working solution 2: 1:1 ratio of $MgCl_2$ 0.7 mol/L and Sodium Azide 0.5 g/L

HDL Separation:
100 µL serum sample
10 µL working solution 1
10 µL working solution 2
Vortex
Incubate 30 min at room temperature
Centrifuge 30 min at 1500 g
Use supernatant as sample for cholesterol analysis

Extraction Procedure (cholesterol analysis)

10 µL sample (total cholesterol) or HDL supernatant (or water for a standard)
100 µL EtOH (or curve solution for standard)
12 µL KOH (9N)
Incubate 1h at 60°C
390 µL NaOH (1N)
400 µL Internal Standard (7 µg/mL Cholesterol-d7 in MeOH)
Vortex
2 mL Hexane
Vortex 30 seconds
Transfer 4 µL of the upper layer in LazWell™ plate
Analyze after complete solvent evaporation

METHOD

LDTD Parameters

- Laser power pattern :
  - Increase laser power to 45 % in 3.0 s
  - Maintained for 2 s.
  - Decrease laser power to 0 %
- Carrier gas flow : 3 L/min (Air)

MS Parameters

- APCI (+)
- MRM mode
- CE = 30
- Cone = 20
- Cholesterol: 369 → 161
- Cholesterol-d7: 376 → 161

Instrumentation

- LDTD model: WX-960
- MS: Waters® Xevo® TQ MS

Figure 2 LDTD system on Xevo TQ-S MS

RESULT

Linearity results

Table 1 shows the calibration curve results of three different validation runs. A calibration curve (15.6-500 mg/dL) has been prepared in water and analyzed in triplicate. It is necessary to prepare the calibration curve with water as cholesterol is endogenous in human plasma. **Figure 4** presents a typical calibration curve for cholesterol. The desorption peak shape is shown in **Figure 3.**

	R^2
Run 1	0.9981
Run 2	0.9973
Run 3	0.9915

Table 1 Inter-run curve parameters

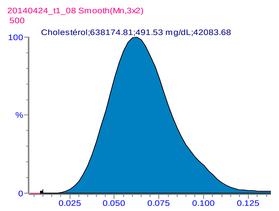

Figure 3 Typical Desorption peak

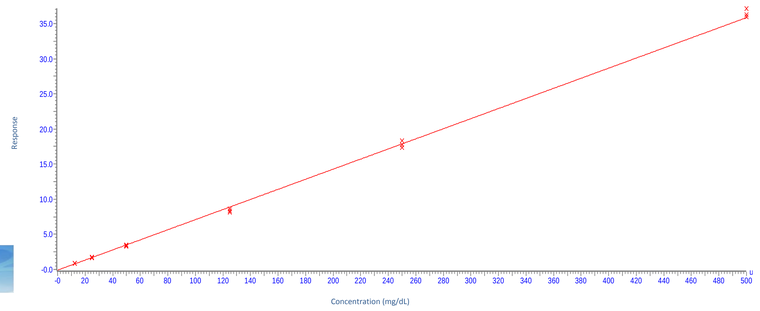

Figure 4 Typical Cholesterol standard curve

Carry over

Carry over was evaluated by analyzing 3 blanks after the highest standard. Blank peak areas were evaluated against the mean peak area value of the lower standard to determine the interference percentage. **Table 2** shows the % Interference value of the 3 blanks.

	% Interference
BLK 1	0.59
BLK 2	0.53
BLK 3	0.49

Table 2 Blank interference value of carry over test

Precision/Accuracy results

Intra-run and inter-run precision/accuracy are reported in **Table 3** and **4**. Accuracies ranging from 88.1 to 108.3 % and precisions ranging from 6.2 to 11.8 % using area ratio values were obtained.

	QC-Low	QC-Med	QC-High
Conc. (mg/dL)	31.3	125.0	250.0
N	18	18	18
Mean (mg/dL)	30.7	126.8	261.8
%RSD	11.8	11.7	9.4
%Nom	92.9	108.3	91.1

Table 3 Inter-run precision and accuracy

RESULT

	LLOQ	QC-Low	QC-Med	QC-High	ULOQ
Conc. (mg/dL)	15.6	31.3	125.0	250.0	500.0
N	6	6	6	6	6
Mean (mg/dL)	13.7	31.1	132.7	267.0	511.7
%RSD	10.9	9.5	10.9	9.0	6.2
%Nom	88.1	99.5	106.1	106.8	102.3

Table 4 Intra-run precision and accuracy

Cross-validation with both HDL and Total Cholesterol

Serum samples were obtained from 6 different people. Samples were split in groups of three (N=3). Samples were analyzed using LDTD-MS/MS technology and by an accredited clinical laboratory. This lab uses a clinical chemistry system as an in vitro diagnostic test.

Technique used by the clinical lab for Total Cholesterol:

An enzyme catalyzes the hydrolysis of cholesterol ester. The free cholesterol is oxidized and hydrogen peroxide is formed. Peroxide reacts with a specific reagent and forms a chromophore that absorbs at 540 nm. Less than 20.6% of difference on 6 different serum samples was obtained. Results are showed in **Table 5**.

	LDTD-MS/MS		Clinical lab	% of difference
	Mean mg/dL	CV %	Mean mg/dL	
Woman #1	177.2	7.4	197.2	10.1
Woman #2	162.6	5.5	174.0	6.6
Woman #3	151.6	3.4	150.8	-0.5
Man #1	202.7	7.7	255.2	20.6
Man #2	227.3	5.0	224.2	-1.3
Man #3	155.2	6.3	162.4	4.4

Table 5- Cross-validation of serum samples for total Cholesterol

Technique used by the clinical lab for HDL Cholesterol:

Sample are mixed with dextran sulfate and magnesium sulfate for the LDL to form a non-reactive complex. HDL is then mixed with cholesterol oxidase and cholesterol esterase (both PEG modified) for it to be oxidized. The oxygen peroxide formed creates a colored complex with HSDA and 4-aminoantipyrine reagent that absorbs at both 600 and 700 nm. Results are showed in **Table 6.**

	LDTD-MS/MS		Clinical lab	% of difference
	Mean mg/dL	CV %	Mean mg/dL	
Woman #1	34.7	7.0	36.7	5.5
Woman #2	66.2	3.7	70.0	5.4
Woman #3	56.6	2.2	55.7	-1.6
Man #1	36.5	12.4	44.1	13.4
Man #2	38.1	2.8	37.5	-1.6
Man #3	42.4	8.9	42.2	3.9

Table 6- Cross-validation of serum samples for HDL Cholesterol

Stability verification

Following the extraction process, all samples were stored at 4°C to evaluate the wet stability of cholesterol. After 48h, all samples were analyzed. Results show that a good wet stability of 48 hours is obtained with a precision of 10.3% and an accuracy of 86.1% for the LOQ standard.

The stability of dry samples in LazWell plate was also determined. All standards and QCs are spotted, dried and kept at room temperature. After 48h, all samples were analyzed. Results show that a good dry stability of 48 hours is obtained with a precision of 13.8% and an accuracy of 91.3% for the LOQ standard.

CONCLUSION

- **Total cholesterol analysis in serum can be performed in 7 seconds by LDTD-MS/MS.**
- **Good precision and accuracy is obtained. No carryover was observed.**
- **Samples are stable at least 48 hours according to wet and dry stability tests.**
- **Cross-validation with an accredited clinical method shows less than 20.6% difference on 6 different serum samples.**

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