Accurate Serum Apolipoprotein A-I and B Measurement Using the Agilent 1290 Infinity LC and 6490 Triple Quadrupole LC/MS System

Application Note

Authors

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Abstract

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) based analytical method was developed for simultaneous measurement of apo A-I and B in serum using stable isotope dilution (SID) and multiple reaction monitoring mass spectrometry (MRM-MS). An Agilent 1290 Infinity LC coupled to an Agilent 6490 Triple Quadrupole LC/MS System was used. Serum was digested by a sample preparation procedure using 6 mol/L urea for denaturation of the proteins and 1 µg trypsin for 0.5 µL serum to yield multiple signature peptides for apo A-I and B. The signature peptides can be accurately measured in both normo- and hypertriglyceridemic sera.

Direct calibration was performed by using value-assigned, matrix-based serum pools. Interrun imprecision was < 8.6 % for the apo A-I peptides and < 6.4 % for the apo B peptides. In conclusion, accurate and simultaneous quantitation of serum apo A-I and B was achieved with only minimal sample volume, using an Agilent 6490 Triple Quadrupole LC/MS System with iFunnel Technology.

Agilent Technologies

Introduction

A common topic of cardiovascular research is the measurement of serum cholesterol, high- and low-density lipoproteincholesterol (HDLc and LDLc). Methods for HDLc and LDLc measurement are difficult to standardize because of the heterogeneity of HDL and LDL particles. These analyses can also be inaccurate due to interferences caused by hypertriglyceridemic samples. A potential alternative to HDLc and LDLc measurement could be their apolipoprotein counterparts. Apolipoproteins (Apo) A-I and B are the most prominent proteins of HDL and LDL particles and the ratio of Apo B/Apo A-I is useful for clinical research[1]. This application note explores the multiplex quantitation of serum apo A-I and B by LC/MS/MS.

Liquid chromatography coupled to mass spectrometry (LC-MS/MS) has shown to provide sensitive, selective, and direct quantitation of apolipoproteins by measurement of signature peptides obtained after tryptic digestion[2–5]. In addition, the high level of specificity afforded by LC-MS/MS might prevent interferences in dyslipidemic serum, which is found with other types of analysis.

A quantitative LC-MS/MS method for apo A-I and B has been developed using the Agilent 1290 Infinity LC System and the 6490 Triple Quadrupole LC/MS System with iFunnel Technology. This method uses a small sample volume (0.5 µL) and is able to detect multiple signature peptides from apo A-I and B and potentially other apolipoproteins.

Experimental

Sample preparation

Human serum samples were diluted 28-fold with 50 mmol/L ammonium bicarbonate (ABC), pH 8 (5 µL + 135 µL). Apolipoproteins were solubilized from lipoprotein particles and denatured with 6 mol/L urea. Disulfide bonds were reduced with 9.1 mmol/L dithiothreitol (DTT) and alkylated with 21.5 mmol/L iodoacetamide (IAA). Digestion was performed with 1 µg sequence grade trypsin (Promega) per sample, after dilution of urea with ABC to a final concentration of 0.76 mol/L (Table 1).

Table 1. Sample preparation steps for serum apo A-I and B measurement.

Normal pool serum/value assigned calibrators				
		Final concentrations in the mixture		
Denaturation—Reduction 30 minutes 56 °C		Reduction	Alkylation	Digestion
	Volume (µL)	63	80	500
Sample/ABC	14			
Internal standard mix* (A-I and B)	7	635 nmol/L A-I and 32 nmol/L B		80 nmol/L A-I and 4 nmol/L B
9 mol/L Urea/ABC	37	6.0 mol/L urea		
0.115 mol/L DTT/ 9 mol/L urea/ABC	5	9.1 mmol/L DTT		0.76 mol/L urea
Alkylation 30 minutes RT (dark)				
0.101 mol/L iodoacetamide	17		21.5 mmol/L	
Digestion 20 hours 37 °C				
50 mmol/L ABC	390			
Trypsin	30			33.3 mg/L
Total assay volume	500			
Formic acid 10 % (v/v)	15			
Final volume	515			

Serum samples were diluted 28× in 50 mmol/L ammonium bicarbonate buffer pH 8 (ABC).
*Internal standards: a mixture of apo A-I and B SIS peptides with final concentrations of 80 and 4 nmol/L, respectively, are added.

Trypsin cleaves the apolipoproteins at arginine (R) and lysine (K) and produces the signature peptides selected for the detection of apo A-I and B (Table 2). Known concentrations of stable-isotope labeled standard (SIS-) peptides (synthesized in-house) were added to each sample to correct for any loss during sample work-up. Three serum-based, value-assigned calibrators, prepared according to CLSI C37-A, were used for the direct quantitation of serum apo A-I and apo B. Calibrators are traceable to WHO-IFCC standards. Target values are: 1.15 g/L and twice 1.60 g/L (same concentration for levels 2 and 3) for apo A-I and 0.76, 0.80 and 1.29 g/L for apo B.

Data Acquisition

Chromatographic separation was performed on an Agilent 1290 Infinity Liquid Chromatography (UHPLC) system using a 15 minute gradient from 5 to 95 % (v/v) MeOH/water + 0.05 % (v/v) formic acid on a ZORBAX Stable Bond C18 column 2.1 × 50 mm, 1.8 µm at a flow rate of 0.2 mL/min. Detection was performed using the Agilent 6490 Triple Quadrupole LC/MS System. A dynamic MRM (dMRM) method was created to detect specific precursor and product ions (quantifier and qualifier) of the selected signature peptides inside their retention time window. In addition to a specific MRM transition, the retention time and the ratio of quantifier and qualifier product ions are used to assure the specific detection of the signature peptides. Furthermore, triggered MRM (tMRM) was used to measure additional product ions for comparison to a library spectrum of the synthetic standard peptide, expressed by a match score (MS) (Table 2).

Signature peptides were selected from relevant literature[2–5] and the Peptide Atlas Best SRM transition list. By measuring the endogenous peptide relative to the SIS peptide and by using the serum-based calibrators, quantitation of serum apo A-I and B is done using four different signature peptides from apo A-I and B.

Method performance

Completeness of tryptic digestion and peptide formation in normo- and hypertriglyceridemic samples was investigated by a time-course digestion experiment up to 28 hours of trypsin digestion. Linearity and recovery were evaluated by mixing the serum-based level 1 and 3 calibrators with concentrations of apo A-I (1.15, 1.26, 1.38, 1.49, and 1.60 g/L) and apo B (0.76, 0.89, 1.03, 1.16, and 1.29 g/L).

Instrumental variation was assessed by 5-fold measurement of one sample for the two serum pools NPS1 and NPS2. Intra- and interrun variation were determined by 5 independent work-ups in one day (n = 5) and for triplicate work-ups prepared on 5 different days (n = 15).

Table 2. Quantifier (Prod 1) and Qualifier (Prod 2) ion MRM transitions for the selected signature peptides (including SIS-peptide) of Apo A-I and Apo B in dMRM-mode.

Sequence [amino acids] [a]	Prec	Prod 1	Prod 2	CE	Rt	Ratio	MS
Apolipoprotein A-I				V	min	%	%
DYVSQFEGSALGK[52-64]	700.8	1023.5	808.4	19	7.9	70	99
DY**V**SQFEGSALGK[52-64]	703.8	1023.5	808.4	19	7.9	-	-
VQPYLDDFQK[121-130]	626.8	1025.5	228.1	18	5.3	95	92
VQPYLDDFQK[121-130]	629.8	1025.5	234.1	18	5.3	-	-
AKPALEDLR[231-239]	506.8	813.4	716.4	14	3.6	8	99
AKPA**L**EDLR[231-239]	510.3	820.4	723.4	14	3.6	-	-
QGLLPVLESFK[240-250]	615.9	819.5	299.2	20	11.0	47	95
QGLLPV**L**ESFK[240-250]	619.4	826.5	299.2	20	11.0	-	-
VSFLSALEEYTK[251-262]	693.8	940.5	853.4	20	11.0	41	98
VSFLSA**L**EEYTK[251-262]	697.3	947.5	860.4	20	11.0	-	-
Apolipoprotein B							
FPEVDVLTK[3791-3799]	524.3	900.5	450.8	20	8.0	10	82
FPE**V**DVLTK[3791-3799]	527.4	906.5	453.8	20	8.0	-	-
TGISPLALIK[220-229]	506.8	741.5	654.5	13	10.0	46	95
TGISP**L**ALIK[220-229]	510.3	748.5	661.5	13	10.0	-	-
SVSLPSLDPASAK[642-654]	636.3	1085.6	885.5	15	7.9	12	92
SVSLPS**L**DPASAK[642-654]	639.8	1092.6	892.5	15	7.9	-	-
TEVIPPLIENR[950-960]	640.9	838.5	443.2	15	8.5	38	97
TEVIPP**L**IENR[950-960]	644.4	845.5	443.2	15	8.5	-	-

[a] Stable-isotope labeled amino acid residues are underlined: V = Val+6 ($^{13}C_5$, $^{15}N_1$) and L = Leu+7 ($^{13}C_6$, $^{15}N_1$); CE: collision energy; Rt: retention time. Ratio indicates the average percentage of the qualifier to quantifier (prod 2/prod 1*100) and MS: match score comparison to reference spectrum for pool sera and calibrators.

Results

Peptide selection

MRM transitions described in the literature were used for screening of apo A-I and B signature peptides in a normal pool serum digest. Samples were analyzed in MRM mode with continuous measurement of all transitions during the 15-minute chromatographic run with CE at 20 and 15 V. For the peptides VQPYL and DYVSQ (apo A-I), and VSALL and FPEVD (apo B), CE values of 18, 19, 24, and 20 V were used based on optimization with the standard peptides.

The most intense peptides DYVSQ, VQPYL, AKPAL, QGLLP, and VSFLS (apo A-I) FPEVD, TEVIP, TGISP, and SVSLP (not shown) (apo B) were selected for further evaluation of the apo A-I and B quantitation. For the selected peptides, optimal collision energies were established by measurement of the synthesized peptides at CE ranging from 10 to 28 V. CE with optimal responses for quantifier product ion were chosen for the dMRM method (Table 2).

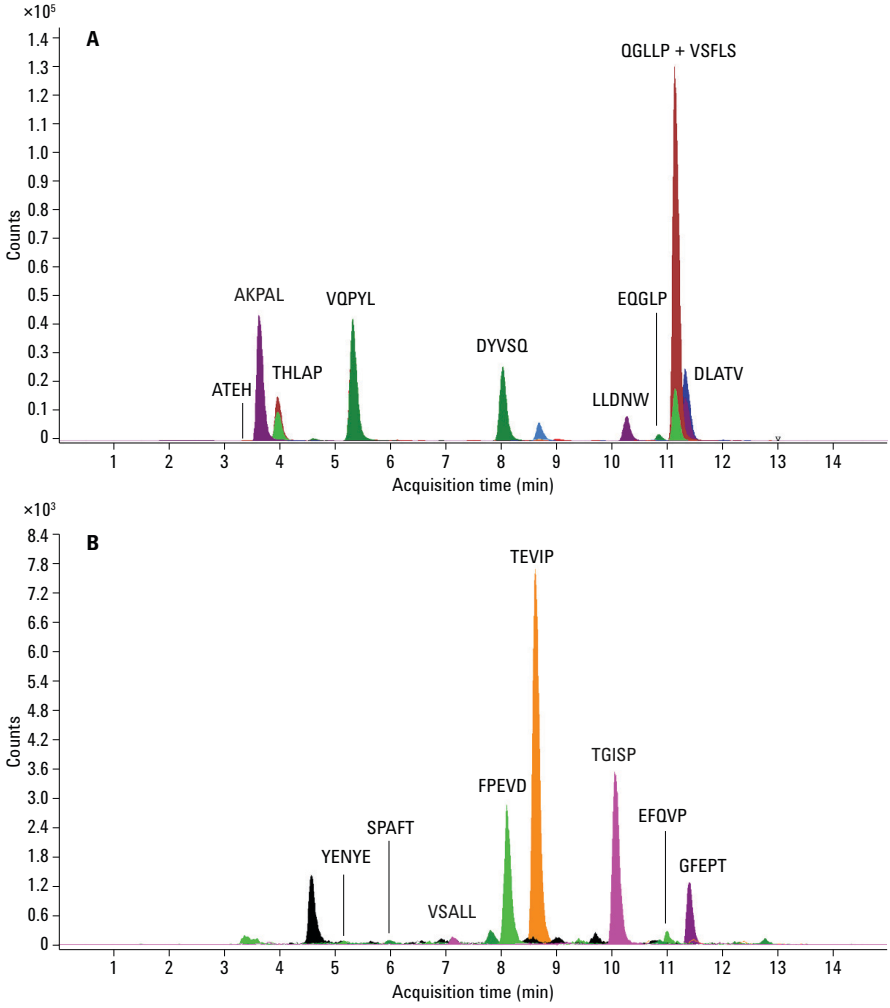

Figure 1. A)Ten apo A-I peptides (scale 1.4 × 105) and B) eight apo B peptides (scale 8.4 × 103) are identified by their MRM transitions in a tryptic digest of normal poolserum.

Completeness of peptide formation

Peptide formation in serum digests in normotriglyceridemic and hypertriglyceridemic serum samples (n = 2) is presented in Figures 2 and 3 showing examples for the measurement of the peptides AKPAL, TGISP, QGLLP, and TEVIP.

The formation kinetics of the apo A-I and B peptides in hypertriglyceridemic sera are similar to those of normotriglyceridemic sera. Complete digestion of apo A-I was observed for the peptides AKPAL, QGLLP, and VSFLS within 4–8 hours, whereas VQPYL and DYVSQ showed slower production. For apo B, maximal production of the peptides SVSLP, TEVIP, and TGISP was obtained within 4 hours and for FPEVD within 20 hours. DYVSQ was not used due to poor reproducibility.

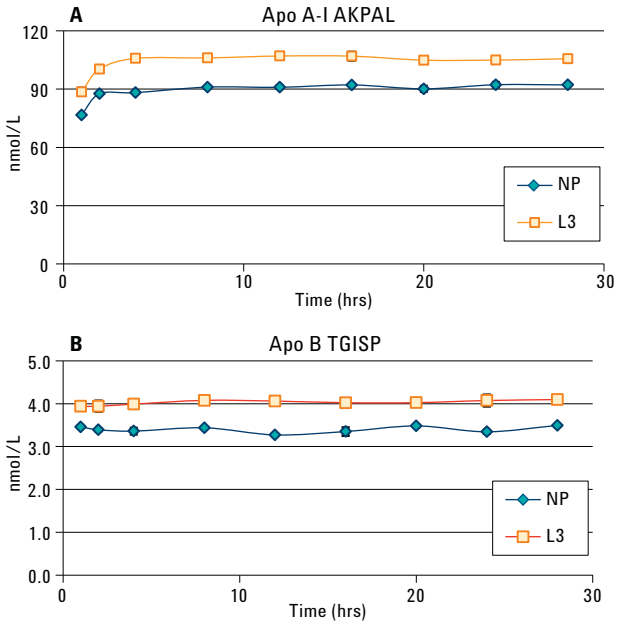

Figure 2. A) Concentration of the peptides AKPAL (apo A-I) and B) TGISP (apo B) at various time points of tryptic digestion for normal poolserum (NP) and for the value-assigned calibrator Level 3 (L3).

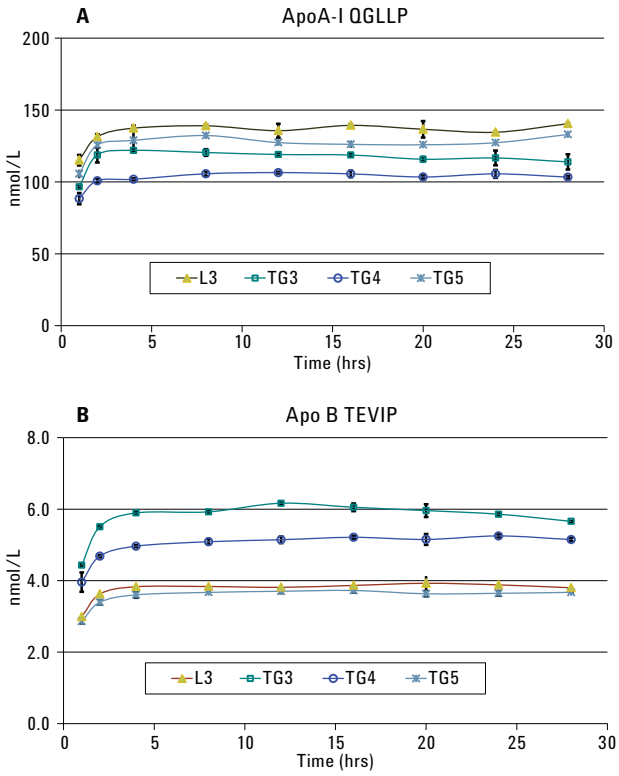

Figure 3. Concentration of the apo A-I and B peptides A) QGLLP and B) TEVIP at various time points of tryptic digestion in hypertriglyceridemic sera TG 3, 4, and 5 and in the level 3 (L3) calibrator.

Linearity and recovery

Linearity was found for the apo A-I and B peptides as displayed in Table 3 and Figure 4.

Recovery ranged between 92.6 to 103.1 % for the apo A-I peptides and between 94.4 to 105.6 % for the apo B peptides (Figure 5).

For the LC/MS/MS quantitation of apo A-I and B all three levels of the value assigned calibrators were used in further measurement of normal pool sera and hypertriglyceridemic samples.

Reproducibility

Reproducibility of the apo A-I and B measurements in normal pool sera (NPS) is presented in Table 4.

For NPS 1 and 2, interrun CVs for the four apo A-I peptides were between 4.2 and 8.6 %, and between 4.2 and 6.4 % for the four apo B peptides (n = 15).

Minimal interrun CVs (< 5 %) were obtained for the apo A-I peptide AKPAL and the apo B peptides TEVIP and TGISP.

Table 3. Linearity of signature peptides in triplicate samples

Protein	Peptide	R^2
A-I	VSFLS	0.93
A-I	QGLLP	0.95
A-I	VQPYL	0.98
A-I	AKPAL	0.97
B	SVSLP	0.97
B	FPEVD	> 0.99
B	TEVIP	> 0.99
B	TGISP	> 0.99

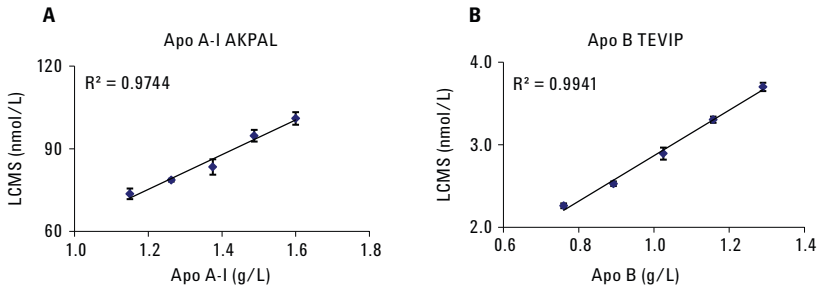

Figure 4. Linearity of the measurement for A) apo A-I between 1.15 (L1) and 1.60 g/L (L3) and B) apo B between 0.76 (L1) and 1.29 g/L (L3) as shown for the peptides AKPAL and TEVIP, respectively.

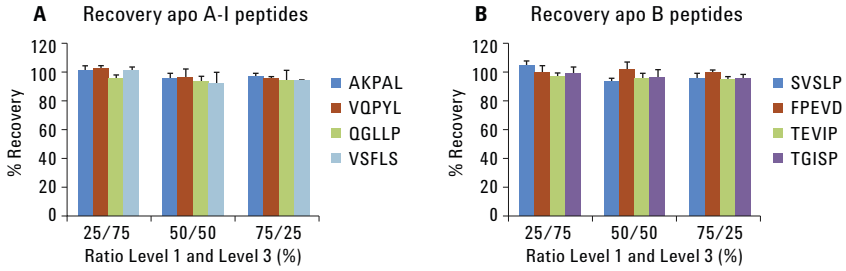

Figure 5. Recovery of A) the apo A-I peptides, AKPAL, VQPYL, QGLLP, and VSFL and B) the Apo B peptides, SVSLP, FPEVD, TEVIP, and TGISP in mixtures of the L1 and L3 calibrators.

Table 4. Instrumental, intra- and interrun reproducibility data of the apo A-I and apo B signature peptides in normotriglyceridemic sera.

	CV (%) normal pool serum (NPS 1)			CV (%) normal pool serum (NPS 2)		
Apo A-I	Instrumental	Intrarun	Interrun	Instrumental	Intrarun	Interrun
AKPAL	0.9	2.9	4.2	1.6	3.3	4.9
VQPYL	1.7	2.7	4.9	1.5	4.6	7.2
QGLLP	5.1	3.2	8.6	3.2	5.6	7.1
VSFLS	3.5	4.3	7.8	3.8	5.6	8.2
Apo B						
SVSLP	3.8	3.1	6.4	5.4	1.0	4.5
FPEVD	2.3	0.8	5.2	1.0	3.3	5.6
TEVIP	3.0	1.8	4.2	0.1	1.4	4.6
TGISP	1.0	1.6	4.3	0.7	0.2	5.1

Conclusions

A multiplexed LC/MS/MS analytical method was developed for the accurate quantitation of serum apo A-I and B with the Agilent 1290 Infinity LC coupled to the Agilent 6490 Triple Quadrupole LC/MS system.

A sample preparation protocol was developed for simultaneous measurement of serum apo A-I and B that produces signature peptides for both proteins in normo- and hypertriglyceridemic sera with similar kinetics using only 5 µL of sample (actual 0.5 µL of serum in the assay mixture) and 1 µg sequence grade trypsin (enzyme to protein ratio 1:35).

Careful selection of multiple peptides is crucial for the accurate and reliable quantitation of apolipoproteins. Calibration of the apo A-1 and B is achieved by using human, serum-based, value-assigned calibrators that undergo the entire sample preparation and measurement procedure.

Analytical performance was achieved with interrun CVs of 5–10 %.

The developed apo A-I and B research application on the Agilent 1290 Infinity LC and Agilent 6490 Triple Quadrupole LC/MS system has potential for multiplex quantitation of apo A-I and B. Further analytical validation of the application is needed, especially in dyslipidemic sera, for evaluating future potential use in routine clinical chemistry.

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Published in the USA, July 21, 2014
5991-2901EN

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