A GENERIC KIT-BASED APPROACH FOR LC-MS/MS QUANTIFICATION OF URINARY ALBUMIN FOR CLINICAL RESEARCH

Mary E. Lame, Caitlin M. Dunning, and Erin E. Chambers
Waters Corporation 34 Maple Street Milford, MA 01757

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INTRODUCTION

Albumin (~MWT 66.5 kDa) is the most abundant protein in blood.[1] Its presence in urine is often one of the first signs of kidney damage. Thus, it has become an important biomarker for renal disease.[2-4] As a result, accurate measurement in urine is of high interest in drug discovery and clinical research.

Under normal kidney function, urinary albumin levels are quite low (< 30 mg/day), but following renal injury albumin levels in urine can exceed 300 mg/day.[2-4] Existing affinity-based methods for urinary albumin quantification include: immunoturbidimetric, ELISA and radioimmunoassay.[5-8] While these immunoassay (IA) methods are sensitive and simple to execute, poor reagent reproducibility, lack of standardization, cross-reactivity, limited linear dynamic range, and other short-comings have led to increased interest in LC-MS based methods. With its many benefits (e.g., multiplexing, selectivity, dynamic range and fast method development), LC-MS is widely accepted for protein quantification. However, this workflow can be complex and time consuming, often taking 24 hours to achieve analytically sensitive and accurate quantification.

This work describes the accurate quantification of urinary albumin over 3.5 orders of magnitude (0.1—500 µg/mL), which is completed in <4 hours using commercially available digestion and peptide purification kits and generic protocols from only 15 µL of sample. This first standardized analytical method is shorter (9X) and more sensitive (50X) than published methods.[3,8]

METHODS

Sample Preparation

Human serum albumin (HSA) was spiked into normal human urine. Urine samples (15 µL) were prepared for LC-MS analysis using the ProteinWorks eXpress Digest Kits and Protocols*. After digestion, peptides were cleaned-up using the ProteinWorks µElution SPE Clean-up Kit and Protocol.

LC-MS Conditions

LC-MS/MS quantification of signature peptides was performed using a Waters Xevo TQ-XS triple quadrupole MS (ESI+). Chromatographic separation was achieved using an ACQUITY UPLC system with an ACQUITY UPLC Peptide BEH C18, 300Å , 1.7 µm, 2.1 mm x 150 mm column and 0.1% formic acid in water and acetonitrile mobile phases. A total of 11 HSA tryptic peptides (highlighted in blue, Figure 1), and 2 MRM transitions per peptide, were monitored for quantification. MS conditions are summarized in Table 1.

MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFK**DLGEENFK**ALVLIAFAQYLQQCPF
EDHVK**LVNEVTEFAK**TCVADESAENCDKSLHTLFGDK**LCTVATLR**ETYGEMADCCAKQE
P ERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKK**YLYEIAR**RHPYFYAPELLF
FAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQGLKCASLQKFGERAFKAWAV
ARLSQRFPK**AEFAEVSK**LVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLK
ECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVGSKDVCKNYAEAKDVFLGMFLYEYAR
RHPDYSVVLLLRLAK**TYETTLEK**CCAAADPHECYAK**VFDEFKPLVEEPQNLIK**QNCELFE
QLGEYK**FQNALLVR**YTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDCLSVF
LNQLCVLHEKTPVSDRVTKCCTESLVNGRPCFSALEVDETYVPKEFNAETFTFHADICTL
SEKERQIKK**QTALVELVK**HKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKK**LV**
AASQAALGL

Figure 1. Amino acid sequence of human serum albumin[9]; Tryptic peptides used for quantification are highlighted in blue.

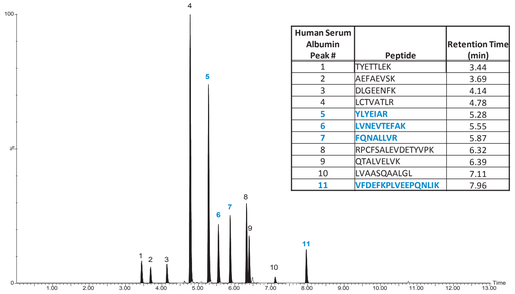
Peptide	Precursor Charge State	MRM Transition	Collision Energy (eV)	Product Ion Identification
YLYEIAR	[M+2H]2+	464.25 > 277.16	22	[1H+]1/b2
		464.25 > 651.35	22	[1H+]1/y5
FQNALLVR	[M+2H]2+	480.79 > 276.13	22	[1H+]1/b2
		480.79 > 685.44	25	[1H+]1/y6
LVNEVTEFAK	[M+2H]2+	575.31 > 694.38	25	[1H+]1/y6
		575.31 > 937.46	25	[1H+]1/y8
VFDEFKPLVEEPQNLIK	[M+3H]3+	682.37 > 712.44	25	[1H+]1/y6
		682.37 > 970.52	25	[1H+]1/y8
AEFAEVSK	[M+2H]2+	440.42 > 201.09	22	[1H+]1/b2
		440.42 > 680.36	22	[1H+]1/y6
LCTVATLR	[M+2H]2+	467.25 > 274.12	22	[1H+]1/b2
		467.25 > 660.40	22	[1H+]1/y6
DLGEENFK	[M+2H]2+	476.23 > 229.12	22	[1H+]1/b2
		476.23 > 723.33	22	[1H+]1/y6
TYETTLEK	[M+2H]2+	492.75 > 265.12	22	[1H+]1/b2
		492.75 > 720.38	23	[1H+]1/y6
QTALVELVK	[M+2H]2+	500.81 > 488.31	22	[1H+]1/y4
		500.81 > 587.38	25	[1H+]1/y5
LVAASQAALGL	[M+2H]2+	507.30 > 189.12	22	[1H+]1/y2
		507.30 > 712.40	22	[1H+]1/b8
RPCFSALEVDETYVPK	[M+3H]3+	637.64 > 244.17	22	[1H+]1/y2
		637.64 > 961.46	25	[1H+]1/b8

Table 1. Final MS conditions for HSA tryptic peptides, including precursor and fragment ions; Primary tryptic peptides used for quantification are highlighted in blue.

RESULTS

Peptide	Curve (µg/mL)	Weighting	Linear Fit (R^2)	Mean % Accuracy	% Accuracy Range
YLYEIAR	0.1-500	1/X	0.998	100.0	92.3-114.2
FQNALLVR			0.999		91.4-112.9
LVNEVTEFAK			0.997		91.8-111.8
VFDEFKPLVEEPQNLIK			0.998		93.1-113.0

Table 2. Linear dynamic range and standard curve statistics for the 4 primary albumin tryptic peptides: YLYEIAR,FQNALLVR, LVNEVTEFAK, and VFDEFKPLVEEPQNLIK used for quantification. Urine samples were digested and extracted protein quantification digestion and peptide purification kit.



Human Serum Albumin Peak #	Peptide	Retention Time (min)
1	TYETTLEK	3.44
2	AEFAEVSK	3.69
3	DLGEENFK	4.14
4	LCTVATLR	4.78
5	YLYEIAR	5.28
6	LVNEVTEFAK	5.55
7	FQNALLVR	5.87
8	RPCFSALEVDETYVPK	6.32
9	QTALVELVK	6.39
10	LVAASQAALGL	7.11
11	VFDEFKPLVEEPQNLIK	7.96

Figure 2. UPLC chromatographic separation of albumin tryptic peptides, digested in human urine (50 µg/mL).

Peptide	Albumin QC overspike concentration (µg/mL)	Mean (N=3) % Accuracy	% CV	Mean (N=3) % Accuracy	% CV	Mean (N=3) % Accuracy	%CV
		Urine Lot #1		Urine Lot #2		Urine Lot # 3	
YLYEIAR	0.000	100.0	0.5	100.0	4.4	100.0	4.0
	0.150	96.6	1.7	101.9	2.9	103.0	1.7
	0.400	92.2	0.4	103.1	3.5	101.7	1.2
	2.000	87.2	1.4	104.9	2.0	102.7	4.4
	20.000	85.3	0.7	97.0	8.1	99.8	4.0
	80.000	94.6	3.9	101.0	5.3	99.6	4.1
	400.000	103.9	0.8	103.8	5.7	100.9	3.0
FQNALLVR	0.000	100.0	5.0	100.0	1.9	100.0	4.0
	0.150	99.2	0.6	101.7	2.3	101.0	1.0
	0.400	93.6	1.2	104.3	5.7	103.8	0.7
	2.000	88.2	2.0	101.8	4.4	100.8	1.5
	20.000	85.8	0.3	98.8	6.4	97.8	5.7
	80.000	96.3	1.6	101.7	2.4	99.1	1.7
	400.000	101.1	1.0	102.8	3.9	99.3	2.3
LVNEVTEFAK	0.000	100.0	4.7	100.0	4.6	100.4	3.2
	0.150	95.0	2.0	102.5	2.8	103.8	1.9
	0.400	91.5	1.5	105.3	1.4	103.1	3.1
	2.000	86.7	0.8	106.4	0.4	107.5	0.5
	20.000	85.6	0.6	98.0	6.1	98.4	3.0
	80.000	89.8	4.0	99.6	4.7	100.1	2.0
	400.000	103.5	2.2	100.7	3.3	100.1	0.9
VFDEFKPLVEEPQNLIK	0.000	100.0	2.0	100.0	2.4	100.0	4.3
	0.150	94.9	3.7	103.3	3.0	102.0	1.3
	0.400	90.1	1.6	106.3	3.3	102.9	1.3
	2.000	86.5	1.0	107.1	2.9	103.9	3.5
	20.000	86.7	0.5	98.9	7.5	101.2	2.5
	80.000	95.1	4.9	104.2	2.7	98.7	2.6
	400.000	101.4	1.4	102.5	3.7	99.5	1.2

Table 3. Summary of the QC sample statistics (precision and accuracy) in Urine Lots #1-3 for the 4 primary tryptic peptides used to quantify albumin.

Urine Lot #	Peptide	Mean (N=3) Calculated Endogenous Concentration (µg/mL)	Mean (N=3) Calculated Endogenous Concentration (nM)	% CV
1	YLYEIAR	2.520	37.9	0.5
	FQNALLVR	2.069	31.2	5.0
	LVNEVTEFAK	2.428	36.5	4.7
	VFDEFKPLVEEPQNLIK	1.486	22.4	2.0
	Mean Albumin Concentration (4-peptides)	**2.126**	**32.0**	**3.0**
2	YLYEIAR	11.544	173.8	4.4
	FQNALLVR	11.129	167.5	1.9
	LVNEVTEFAK	11.159	168.0	4.6
	VFDEFKPLVEEPQNLIK	10.241	154.2	2.4
	Mean Albumin Concentration (4-peptides)	**11.018**	**165.9**	**3.3**
3	YLYEIAR	6.668	100.4	4.0
	FQNALLVR	6.193	93.2	4.0
	LVNEVTEFAK	6.383	96.1	3.2
	VFDEFKPLVEEPQNLIK	5.715	86.0	4.3
	Mean Albumin Concentration (4-peptides)	**6.240**	**93.9**	**3.9**

Table 4. Calculated endogenous urinary albumin concentrations in 3 lots of human urine using the YLYEIAR, FQNALLVR, LVNEVTEFAK, and VFDEFKPLVEEPQNLIK tryptic peptides of HSA.

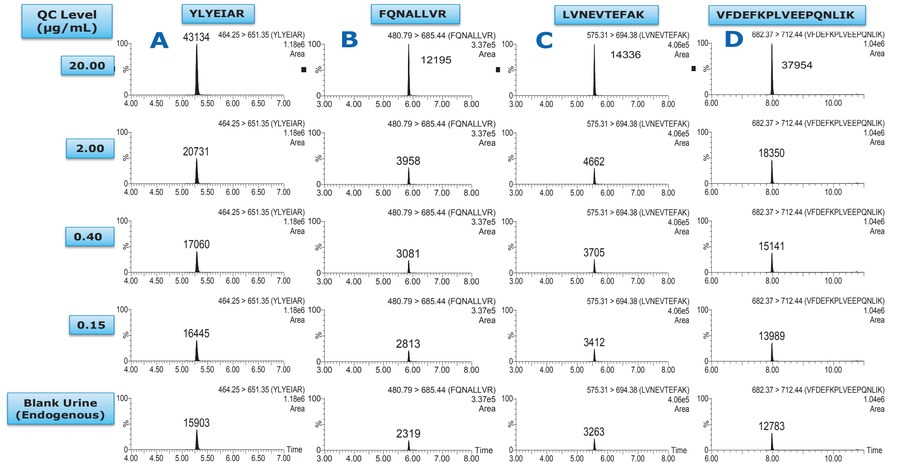


Figure 3. Representative QC chromatograms for the 4 primary HSA peptides: YLYEIAR (A),FQNALLVR (B), LVNEVTEFAK (C), and VFDEFKPLVEEPQNLIK (D) used to quantify albumin in human urine.

DISCUSSION

Using a generic kit-based approach (with simple step-wise protocols and standardized, pre-measured reagents) for digestion and subsequent peptide purification, accurate albumin quantification in urine was achieved.

- Total sample preparation time, including SPE, was <4 hours.
- UPLC chromatographic separation of albumin tryptic peptides, digested in human urine, is illustrated in Figure 2. Peak widths for all peptides were <4.5 seconds wide.
- Through direct digestion (no affinity purification) of 15 µL of urine and subsequent peptide purification, quantification limits of 0.1—500 µg/mL was readily achieved for the four primary HSA signature tryptic peptides (Table 2). In addition, standard curves were linear over 3.5 orders of magnitude with mean accuracies ranging from 91.4—114.2%.
- Employing a mixed-mode SPE clean-up step, effectively removing buffer salts and excess digestion reagents, while also concentrating the sample, significantly improved accuracy and precision of the QCs across all lots of urine tested.
- The accuracy and precision for HSA quantified in human urine (3 lots) was excellent with accuracies ranging from 85.3—107.5% and CVs <8.1%. HSA QC statistics are highlighted in Table 3, and illustrated in Figure 3 for the 4 primary HSA peptides.
- Endogenous urinary albumin concentrations were accurately quantified in three individual lots of urine and are summarized in Table 4.

CONCLUSION

Using a generic kit-based approach for digestion and subsequent peptide purification, accurate and reproducible quantification of endogenous urinary albumin was achieved. The analytical sensitivity (0.1 µg/mL), broad linear dynamic range, and selectivity of this LC-MS method reliably quantifies both low endogenous and elevated urine levels that would be expected in normal and disease populations while approaching the analytical sensitivity of immunoassays.

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