Supporting Information for:

A Rapid Method for Direct Quantification of Antibody Binding-site Concentration in Serum

Erwin G. Abucayon,[§, ‡] Connor Whalen,[§, Ψ, ¥] Oscar B. Torres,[§, ‡, Φ] Alexander J. Duval,[§, ‡, ¶] Agnieszka Sulima,[†] Joshua F. G. Antoline,[†] Therese Oertel,[§, Ψ, €] Rodell C. Barrientos,[§, ‡, £] Arthur E. Jacobson,[†] Kenner C. Rice,[†] and Gary R. Matyas*[§]

§Laboratory of Adjuvant and Antigen Research, U.S. Military HIV Research Program, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, Maryland 20910, United States

†Drug Design and Synthesis Section, Molecular Targets and Medications Discovery Branch, Intramural Research Program, National Institute on Drug Abuse and the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Department of Health and Human Services, 9800 Medical Center Drive, Bethesda, Maryland 20892-3373, United States

‡Henry M. Jackson Foundation for the Advancement of Military Medicine, 6720A Rockledge Drive, Bethesda, Maryland 20817, United States

ΨOak Ridge Institute of Science and Education, Oak Ridge, Tennessee 37831, United States

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Quantitation of Antibody Binding Affinity and Binding-site in Sera by ED-UPLC-MS/MS

The binding affinity (Kd) of polyclonal antibody in sera to 6-AM was determined following published procedure. [1] Briefly, the drug binding profile of the polyclonal antibody in sera samples was measured through equilibrium dialysis (ED). The sera were diluted with 0.05% BSA in DPBS, pH 7.4 (ED buffer) containing 5 nM of 6-acetylmorphine-D_3 (6-AM-D_3). Aliquots of 100 µL were seeded into sample chambers (right) while the corresponding buffer chambers (left) of the rapid equilibrium dialysis (RED) plate were loaded with 300 uL of ED buffer. The plate was incubated at 4°C and 300 rpm for 24 h in a thermomixer. A 90 µL aliquot from sample and buffer chambers were pipetted into 1 mL recovery vial for LC-MS/MS analysis. The detailed LC-MS/MS method has been previously described [2].

The Kd values were determined via competition ED as described in the literature. [1] Briefly, serum sample was diluted with 5 nM of 6-AM-D_3 in ED buffer at a serum dilution that yielded 40-70% binding in the serum binding experiments. The buffer chambers were filled with ED buffer that contains an increasing concentration of competitor drug (6-AM; final concentration, 0 nM to 40 nM) while the sample chamber contains the sera samples diluted in 5 nM 6-AM-D_3. Half maximal inhibitory concentration (EC50) was interpolated using four parameter logistic curve (4 PL; plot of % inhibition vs. concentration of competitive inhibitor). The % inhibition values were obtained and were used to calculate K_d values. The binding-site concentration to 6-AM was estimated from the K_d values as described in the previous report.

6-AmHap-mAb production and purification

(a) Animal study for hybridoma generation: All animal studies were conducted under an approved animal use protocol in an Association for Assessment and Accreditation of Laboratory Animal Care International-Accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals. All experiments that involved animals followed the principles stated in the Guide for the Care and Use of Laboratory Animals, 8th edition. [3]A previously described vaccine composed of a heroin hapten conjugated to tetanus toxoid (TT-6-AmHap) and adjuvanted with liposomes containing monophosphoryl lipid A adsorbed on aluminum hydroxide was used to immunize mice. [4] Female BALB/c mice (n = 10 per group) were immunized intramuscularly (*i.m.*) on weeks 0, 3, 6, and 14 with 50 µL of the vaccine formulation as described. [4] The hybridoma was generated from mice that were

immunized with a dose comprised of 10 μg of TT-6-AmHap, 20 μg of synthetic monophosphoryl 3-deacyl lipid A (3D-PHAD, Avanti Polar Lipids, Inc.) in ALF43, and 30 μg of aluminum in aluminum hydroxide (Alhydrogel) in DPBS pH 7.4. ALF43 contained DMPC/ DMPG/cholesterol/3D-PHAD at a molar ratio of 9:1:7.5:1.136; the molar ratio of phospholipids/3D-PHAD was 8.8:1.

(b) Hybridoma generation: Mice were boosted again *i.v.* at week 20 with 10 μg of TT-6-AmHap, 20 μg of synthetic monophosphoryl 3-deacyl lipid A (3D-PHAD) in ALF43, and 30 μg of aluminum in aluminum hydroxide (Alhydrogel) in DPBS pH 7.4. Three days following the boost, mice were terminated, and spleens disaggregated into single-cell suspension in serum-containing culture media. B cells of the immunized mice were fused with nonproducing myeloma cells (P3X63/Ag8.653), using the ClonaCell-HY Hybridoma Kit (Stemcell Technologies). The fusion, selection and expansion were done following the experimental procedure of the kit. Once the fused hybridoma were appropriately expanded, the cells were selected based on the ability of cell culture media to bind BSA-6-AMHap in ELISA using the method described previously. [2] The cells that bound in the assay were then selected and subcloned in HAT selection media to ensure that they were monoclonal. This resulted in one clone (P1E2H6) with isotype of mouse IgG1 heavy chain and λ light chain.

The purification of mAb was confirmed by SDS-Page and ELISA for mouse antibodies against BSA-6-AmHap based on a literature procedure. [4-6] The samples were then sterilized using a 0.22 μm syringe filter and stored in aliquots at -20 °C. The protein concentration was determined to be 1.1 mg/mL using Nanodrop One (ThermoFisher). Molecular weight of the purified mAb was found to be around 147.65 kDa using MALDI-TOF (Axima MegaTOF, Shimadzu Scientific) following literature procedure. [7]

Determination of the molecular weight of mAb by MALDI-TOF

The mAb sample was first desalted in Zeba spin desalting column. Sample was then mixed with sinapinic acid (10 mg/mL) in 50:50 ACN/H_2O 0.1% formic acid (FA) and spotted on a MALDI-TOF 384-well stainless plate and loaded to the AXIMA MegaTOF instrument (Shimadzu Scientific Instruments, Columbia, MD). The instrument was calibrated using standard human serum IgG. MS were acquired using the following settings: tuning mode, linear; laser power, 60−80; profiles, 500; shots, 2 per profile. Spectra were smoothed using the Gaussian method, and masses were assigned using threshold apex peak detection method. [5]

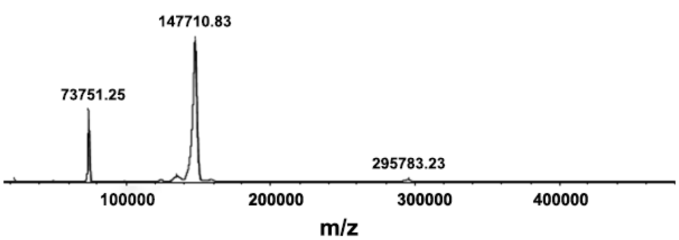

Figure S1. MALDI-TOF mass spectrometric analysis of in-house generated 6-AmHap-mAb.

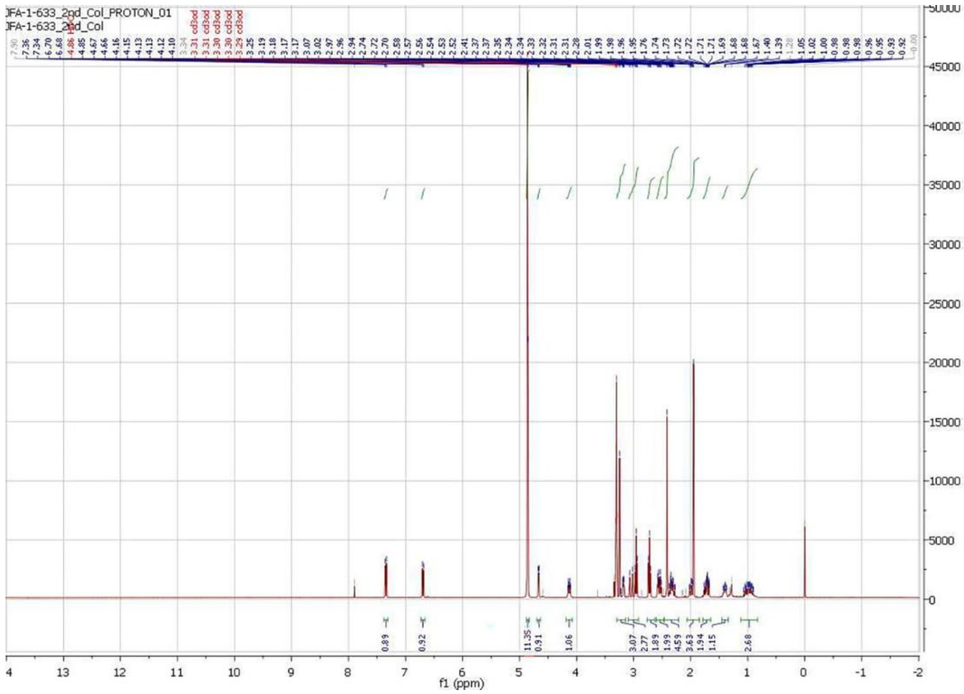

Figure S2. ^{1}H NMR spectrum of 6-AmHap-acetamide (**5**) in CD_3OD.

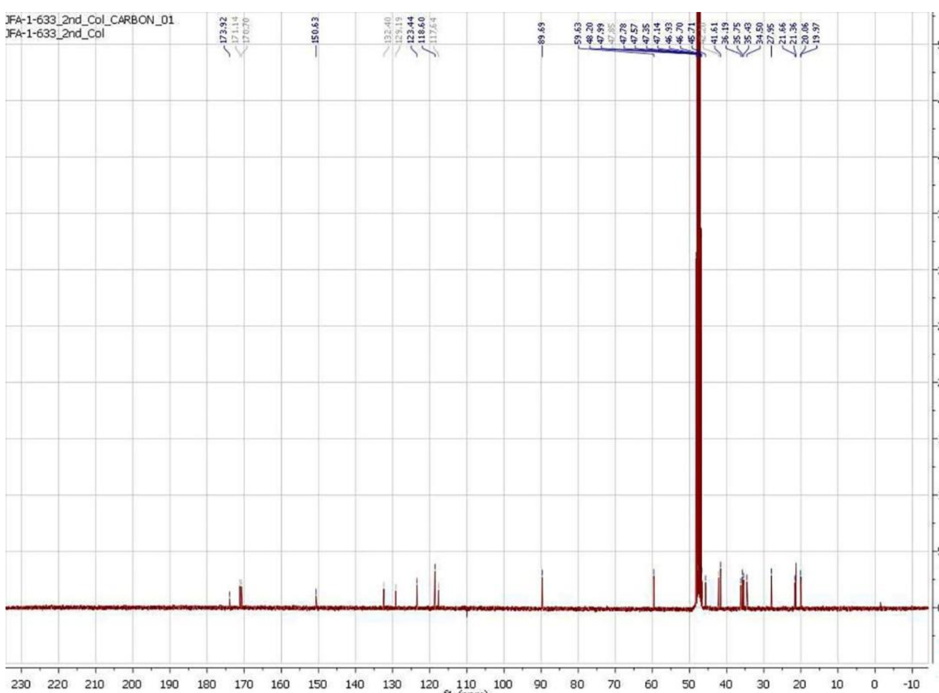

Figure S3. ^{13}C NMR spectrum of 6-AmHap-acetamide (**5**) in CD_3OD.

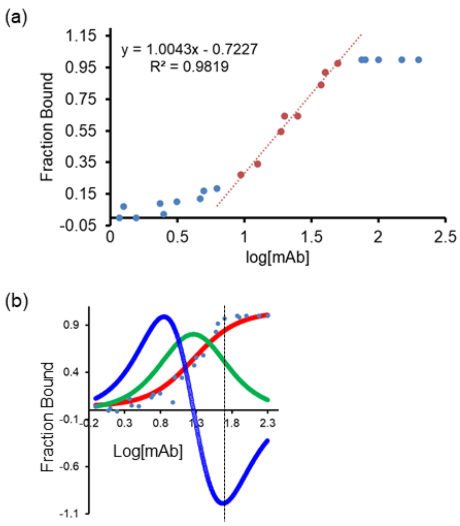

Figure S4. Representative methods for estimating antibody binding-site concentrations from the binding curves. (a) From a plot of Fraction Bound (F_B) vs. log[mAb], best-fit by linear function (y = mx + b) was generated at $0 < F_B < 1$, which was used to estimate the [antibody binding-site] at y = 1. (b) 4 PL model (red trace), first derivative plot (green trace), and second derivative plot (blue trace) of F_B as a function of log[mAb]. The lowest minimum of the second derivative plot estimates the point at x or log[mAb] where F_B started to deviate from 1.0, which corresponds to the equivalence point or the estimated antibody binding-site concentration ([tracer] = [antibody binding-site]).

Determination of antibody binding-site concentration

Two methods for fitting the antibody binding-site concentrations from the binding curve of ED of mAb or serum against ligand have been explored. First, regression analysis along the slope of the curve, where $0 < F_B < 1$ was fitted to a linear function,

$$y = mx + b \qquad \textbf{(1)}$$

where, $F_B = 1$, entirety of hapten-tracer is bound; m = slope of the line; x = antibody concentration (log[nM] or antibody dilution); b = y-intercept. Second, the second derivative plot of the 4 PL model. The binding curve, F_B as a function of log[antibody] or serum dilution was entered in the GraphPad Prism. This curve was modeled with 4 PL to generate the Top plateau (bound agonist/tracer, 1), Bottom plateau (unbound tracer), logEC50 (point at which ~50% of the agonist is bound), and Hill Slope (steepness of the curve) values. The points along x-axis ([antibody] or dilution) were then artificially generated by multiplying the highest concentration/dilution with 0.99 sequentially to the minimum/lowest concentration/dilution. For example, ED of 6-AmHap-mAb against 50 nM 6-AmHap-acetamide has [mAb] range of 200 nM to 0.78 nM. The highest concentration 200 nM is multiplied by 0.99, sequentially down to arrive ~0.78 nM. Their corresponding values in the y-axis (Fraction bound, F_B) were determined using the 4 PL values of the model applied in the equation below,

$$y = Bottom + \left(\frac{Top - Bottom}{1 + 10^{((LogEC_{50} - x) * Hill\ Slope)}}\right) \quad \textbf{(2)}$$

The calculated F_B was plotted against the calculated log[antibody], then the plot was converted to the first and second derivatives. In the second derivative plot, the lowest minimum estimates the point of the curve where fraction bound tracer started to deviate from F_B = 1.0 or the equivalence point, that is [tracer] = [antibody binding-site].

Table S1. Summary of determined antibody binding-site concentrations (nM) with their associated absolute and % relative errors by ED-UPLC-MS/MS at constant [6-AmHap-acetamide].

[6-AmHap-acetamide] (Theoretical conc.)	Calculation method	Trials (Calculated conc.)	Average	Absolute (% relative) error
5 nM	y = mx + b	7.20	7.19±0.02	2.19 (44%)
		7.17		
	2nd derivative	8.01	7.91±0.15	2.91 (58%)
		7.8		
25 nM	y = mx + b	25.71	25.68±0.25	0.68 (2.7%)
		25.41		
		25.91		
	2nd derivative	24.5	26.1±1.6	1.1 (4.4%)
		26.1		
		27.7		
50 nM	y = mx + b	53.71	53.46±0.36	3.46 (6.9%)
		53.20		
	2nd derivative	47.52	47.50±0.33	2.5 (5.0%)
		47.04		

Table S2. Summary of determined antibody binding-site concentrations (nM) with their associated absolute and % relative errors by ED-UPLC-MS/MS at constant [6-AmHap-mAb] as a function of [6-AmHap-acetamide].

[6-AmHap-mAb] (Theoretical conc.)	Calculation method	Trials (Calcd. conc.)	Average	Absolute (% relative) error
5 nM	y = mx + b	3.9	3.95±0.07	1.05 (21%)
		4.0		
	2nd derivative	3.7	3.85±0.20	1.15 (23%)
		4.0		
50 nM	y = mx + b	45.3	43.9±1.90	6.10 (12.2%)
		42.6		
	2nd derivative	43.4	45.9±3.50	4.10 (8.2%)
		48.4		

Table S3. Summary of determined antibody binding-site concentrations (nM) with their associated absolute and % relative errors by ED-Fluorimetry at constant [6-AmHap-Cy5] tracer.

[6-AmHap-Cy5] (Theoretical Conc.)	Calculation method	Trials (Calcd. Conc.)	Average [% CV]*	Absolute (% relative) error
5 nM	y = mx + b	5.05	4.8±0.2 [4.2%]	0.2 (4.0%)
		4.63		
		4.75		
	2nd derivative	4.4	4.2±0.2 [4.8%]	0.8 (16.0%)
		4.0		
		4.2		
25 nM	y = mx + b	22	21.2±1.6 [7.5%]	3.8 (15.2%)
		21		
		24		
	2nd derivative	20.5	22.3±1.7 [7.6%]	2.7 (10.8%)
		23.9		
		22.4		
50 nM	y = mx + b	34.9	34.3±0.8 [2.3%]	15.7 (31.4%)
		33.8		
	2nd derivative	38.9	37.7±1.7 [4.5%]	12.3 (24.6%)
		36.5		

*CV = coefficient of variation

Table S4. Comparison of measured antibody binding-site concentrations from ED of 6-AmHap-mAb against 6-AmHap-Cy5 in the presence and absence of preimmune sera.

Trials	Binding-site conc. without serum (nM)	Binding-site conc. with serum (nM)
T1	5.05	5.05
T2	4.63	3.85
T3	4.75	-
Average	4.8 ± 0.2	4.45 ± 0.85

Table S5. Antibody binding-site concentration of sera from TT-6-AmHap-immunized Spague Dawley rats.

Animal ID #	ED-UPLC-MS/MS[a]	ED-Fluorimetry[b]	Difference in Means	K_d,[c] nM
1	4570.8±669.2 nM (0.34 mg/mL)	4283.6±334.8 nM (0.32 mg/mL)	287.2	0.7055
2	4928.0±811.4 nM (0.37 mg/mL)	4439.0±225.6 nM (0.33 mg/mL)	489.0	0.9186
3	1899.6±264.5 nM (0.14 mg/mL)	3350.2±170.2 nM (0.25 mg/mL)	1450.6	0.0809
4	5612.2±632.5 nM (0.42 mg/mL)	6244.0±200.0 nM (0.47 mg/mL)	631.8	0.4133
5	9215.9±239.0 nM (0.69 mg/mL)	6522.5±234.7 nM (0.49 mg/mL)	2693.4	0.2053
6	3340.2±157.1 nM (0.25 mg/mL)	4398.9±331.3 nM (0.33 mg./mL)	1058.7	0.2201
7	4341.9±789.0 nM (0.33 mg/mL)	3926.8±321.8 nM (0.30 mg/mL)	415.1	0.3660
8	4110.5±1213.5 nM (0.31 mg/mL)	3186.1±162.7 nM (0.24 mg/mL)	924.4	0.1077
% Coefficient of variation	45%	27%		

a = antibody binding-site concentrations were determined via equilibrium dialysis of sera against 6-AM-D_3 using UPLC-MS/MS; b = antibody binding-site concentrations were determined via equilibrium dialysis of sera against 6-AmHap-Cy5 using ED-Fluorimetry; c = K_d values of polyclonal antibodies in sera (week 16) to 6-AmHap by ED-UPLC-MS/MS. Concentrations in mg/mL were calculated based on the molecular weight of mAb (MW = 150 kDa) and two binding sites.

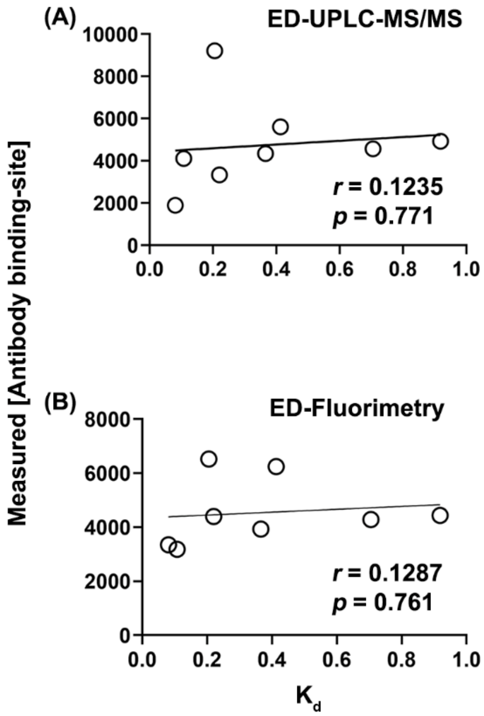

Figure S5. Correlation plots of binding affinities (K_d) of polyclonal antibodies to 6-AmHap in sera samples from TT-6-AmHap immunized rats vs. measured antibody binding-site concentrations at 5 nM tracer by (A) ED-UPLC-MS/MS and (B) ED-Fluorimetry.

Table S6. UPLC mobile phase gradient

Time (min)	% A (Water with 10 mM NH_4HCOO and 0.1% HCOOH)	% B (MeOH with 0.1% HCOOH)
-3.00 (equilibration time)	100	0.0
0.00	100.0	0.0
2.70	99.0	10.0
3.30	80.0	20.0
8.61	20.0	80.0
9.2	0.0	100.0
10.5	0.0	100

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