

Chemiluminescence based immunoassay for the detection of heroin and its metabolites

Materials and Methods

Generation of anti-MAM antibody and labeling with HRP

Antibody generation was done in young four to six weeks old New Zealand white rabbits by using 250 µg MAM-BSA as an immunogen with complete adjuvant followed by incomplete adjuvant in the subsequent three boosters and used for immunoassay development after purification by affinity chromatography as discussed previously.²⁰ Labeling of anti-MAM antibodies with HRP were done in order to develop sensitive assay for the detection or quantification of the target analyte. Briefly, 2 mg/mL solution of HRP was prepared in 500 µL of DW and 100 µL of freshly prepared 0.1 M sodium periodate was added to it. The solution was stirred for 20 minutes at RT. The modified enzyme was dialyzed against 1 mM sodium acetate buffer, pH 4.5 O/N at 4°C. pH of the dialyzed HRP solution was adjusted to 9.5 by adding 10 µL of 0.2 M sodium carbonate buffer, pH 9.5. 4 mg of anti-MAM antibody was dissolved in 500 µL of 10 mM sodium carbonate buffer, pH 9.5 and immediately added to the HRP solution. The mixture was incubated for 2 h at RT. 50 µL of freshly prepared sodium borohydride solution (4 mg/mL) was added, and stirred intermittently over period of 2 h at 4 °C. The bound IgG-HRP molecules were purified by using gel filtration column Sepharose CL-6B (approx 1.5 × 85 cm) in PBS. After labeling RZ value was calculated to know the number of HRP molecules per protein. RZ value is the ratio of the absorbance at 403 nm to the absorbance at 275 nm. This value is an expression of the ratio of heme to protein content. The absorbance was determined at 275 nm and 403 nm and positive fractions (both A275 and A403 peaks coincided) were pooled, dialyzed in PBS (pH 7.4) and stored at -20°C. BSA at 1mg/ mL was added to the complex to provide long term stability.

Indirect chemiluminescence inhibition assay

For chemiluminescence based competitive assay, anti-MAM antibody labeled with HRP was used. ELISA plates were coated with 5 µg/mL conjugated hapten (MAM-OVA) prepared in carbonate buffer (pH 9.6) and incubated O/N at 4 °C. Blocking was done with 10% skim milk in PBS (pH 7.4) followed by incubation for 1 h at 37 °C. Standard drug samples were spiked in pretreated urine samples, prepared as stated above and mixed with equal volume of anti-MAM

antibody labeled with HRP (1:1000 in PBS). The plates were incubated for 2 h at 37°C and washed thoroughly with PBS, followed by addition of West Pico substrate (100 µL/well) for 30 min at 37°C and counts were taken using luminometer (Molecular Devices, USA). The % reactivity was calculated with the target analyte. Data analysis was done by normalizing the absorbance unit with following formula:

$$\% B/B_0 = A - A_{\text{excess}} / A_0 - A_{\text{excess}} \times 100$$

Where A = absorbance value of sample; A_0 and A_{excess} = absorbance value at zero and excess analyte concentration.

The % reactivity of related analogues were calculated on the basis of standard calibration curves in the range of pg/mL to sub ng/mL. After normalizing the data by % B/B_0 transformation, the specific hapten concentration yielding 50% inhibition was used to calculate the % reactivity according to formula:

$$\% \text{ Reactivity} = H/C \times 100,$$

where, H and C are concentrations of standard hapten and cross reacting hapten/analogue at 50% B/B_0 .

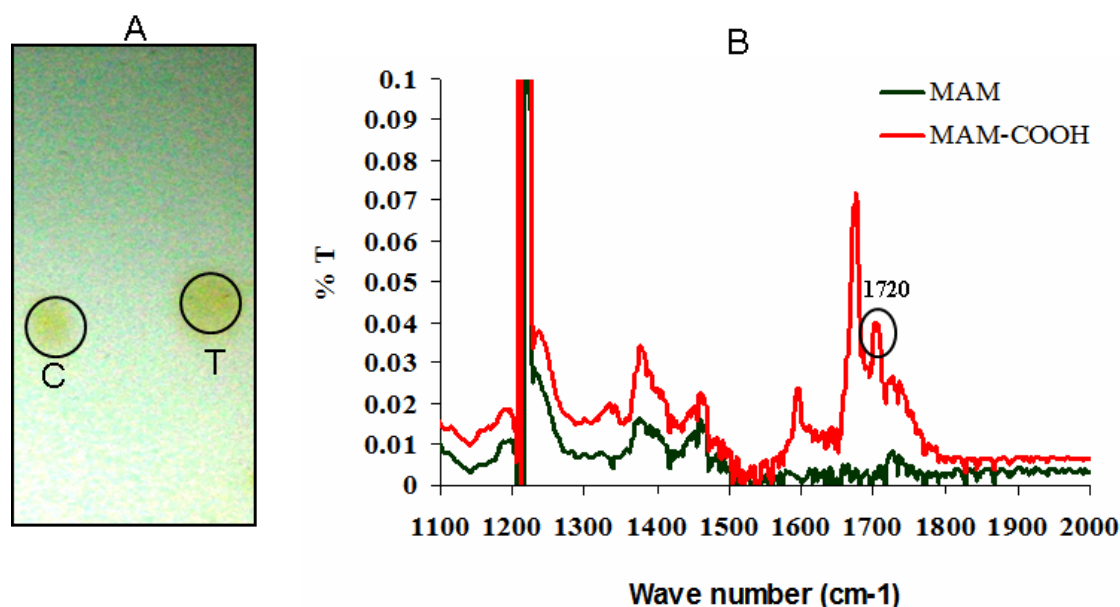


Fig. S1. [A] TLC of MAM (C= control) and MAM-D (or MAM-COOH) (T=test) where the R_f value was found to be 0.13 in test sample. [B] FT-IR Spectrum of MAM and its derivative MAM-COOH. MAM-COOH shows a peak at 1720 cm^{-1} corresponds to the C=O stretch and C–O stretch of the –COOH group.

Table S1: Determination of Hapten density by Mass spectrometry method (MALDI-TOF)

Conjugate (Protein:Hapten) Ratio	MASS SPECTROMETRY MALDI-TOF		
	Observed Mass (KD)	Change in Mass after conjugation (ΔM)	No. of MAM molecules per BSA molecule
Control (1:0)	66,455.0	0	0
M1 (1:20)	66,901.91	446.91	1
M2 (1:40)	67,613.11	1,158.11	3
M3 (1:80)	68,776.18	3,321.18	7
M4 (1:100)	71,430.82	4,975.82	14
M5 (1:200)	70,719.51	4,264.51	12