Performance Evaluation of Antibody and Antigen Conjugate Pair for Detection of Xylazine and its Metabolite Hydroxy Xylazine in Lateral Flow Assay

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Introduction

Xylazine is a veterinary tranquilizer and an alpha-2 adrenergic receptor agonist that is increasingly misused in combination with synthetic opioids, especially in the United States, where it has been associated with numerous overdose deaths.[1] Detection of xylazine is critical because its effects cannot be reversed with naloxone, the standard treatment for opioid overdoses.[1] In preclinical settings, alpha-2 adrenergic receptor antagonists have been researched as treatment option for xylazine overdose.[2]

Xylazine (Figure 1b) and its hydroxy metabolite (Figure 1a) can be detected from blood and urine samples of opioid users. Among the various analytical methods available, lateral flow assays remain a key approach for detecting drugs of abuse use in urine. The sensitivity of these assays is highly dependent on the optimal pairing of antibody and antigen conjugate (Figure 1c).

Medix Biochemica develops antibodies, antigens, and other critical raw materials for in vitro diagnostics (IVD) tests.

The aim of the study was to develop antibodies for the detection of xylazine and its main urine-excreted metabolite, hydroxy-xylazine, intended for use by diagnostic test developers in xylazine IVD test. To complement the monoclonal antibodies in a competitive assay format, we also developed xylazine-hapten conjugates with varying conjugation ratios. Conjugates were synthesized by using different hapten:BSA ratios in conjugation step (10:1, 15:1 and 20:1).

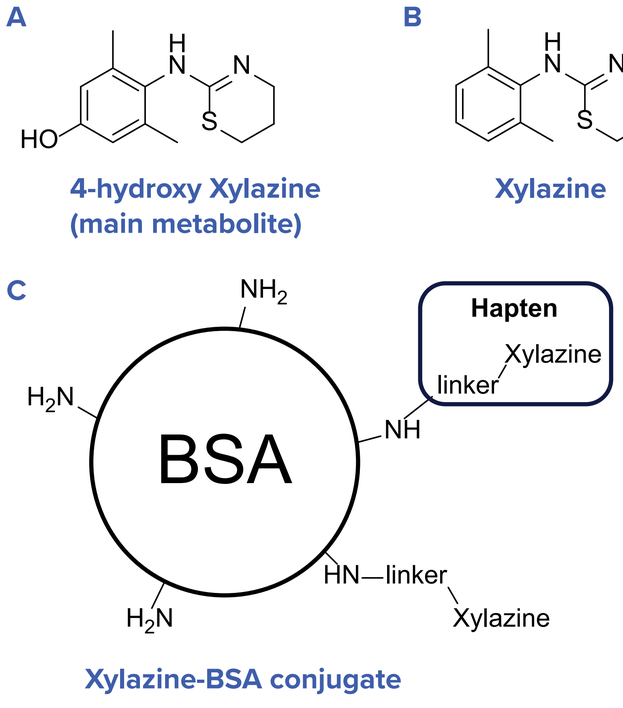

Figure 1. *a) Structure of main metabolite of xylazine.*
b) Structure xylazine
c) Xylazine-BSA conjugate and general presentation of hapten structure: antigen + linker.

Materials & Methods

To evaluate sensitivity of the mAb and conjugate pairs, the BSA-conjugates were labelled with a fluorescent Europium reporter and studied in a competitive fluorescence immunoassay (FIA) format with xylazine and 4-hydroxy-xylazine. The most sensitive pairs were further evaluated in lateral flow.

For lateral flow assay, 40nm passive gold-antibody conjugate was prepared for tested antibodies at a selected coating concentration. Each hapten conjugate was coated onto nitrocellulose membrane at a fixed coating concentration of 0.5 mg/mL. The coated membranes were dried and stored desiccated until ready for testing. For the first screening xylazine and 4-hydroxy-xylazine was diluted in phosphate-buffered saline with tween (PBST) to 20 ng/mL. This solution was used as the positive control (Pos) in the testing. PBST buffer was used as the negative control in the testing (Neg). The coated nitrocellulose membranes were laminated onto adhesive backing card along with other lateral flow pad materials. The laminated cards were cut into test sticks. Each gold-antibody conjugate was applied to the test sticks and dried.

Standard curves and urine sample testing were done for the two most sensitive pairings based on the first screening. Xylazine and 4-hydroxy-xylazine was diluted in PBST buffer in concentrations ranging from 1 to 125 ng/mL. PBST was used as a negative control. Each standard was tested in triplicate for each pairing condition. The intensity of the signals were quantified using a Detekt RDS 2500 reader and plotted against the concentration of xylazine and 4-hydroxy-xylazine to form standard curves. Five xylazine positives (Medix Biochemica, cat.no. 991-03-S-CUST) and one negative urine sample (Medix Biochemica, cat.no. 991-03-P-FNF) were tested, with measured xylazine concentrations ranging from 10 to 150 ng/mL.

Kinetics for the most sensitive xylazine mAb were measured with biotinylated xylazine-BSA conjugate attached to streptavidin sensors using Octet RED96e bio-layer interferometry (BLI).

Results

We have previously observed that the conjugation ratio can significantly affect assay sensitivity. In FIA assay no differences were seen between different conjugation ratios used with the most sensitive antibody R14501 (Figure 2) and the IC50 values for all pairs were below 1 ng/ml. On the other hand, in lateral flow significant difference in sensitivity was seen between conjugates.

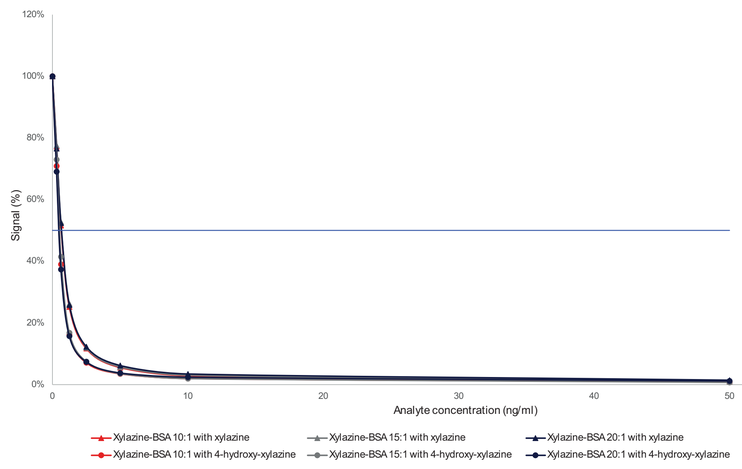

Figure 2. *Sensitivity of R14501 and xylazine-BSA conjugates 10:1, 15:1 and 20:1 in competitive FIA assay with xylazine and 4-hydroxy-xylazine.*

The standard curves for R14501 demonstrated excellent performance in lateral flow assay for both xylazine and 4-hydroxy-xylazine with xylazine-BSA 15:1 and xylazine-BSA 20:1 (Figure 3). Both pairs effectively separated xylazine negative and positive samples urine (Figure 4). In lateral flow xylazine-BSA 15:1 conjugate shows significantly better sensitivity.

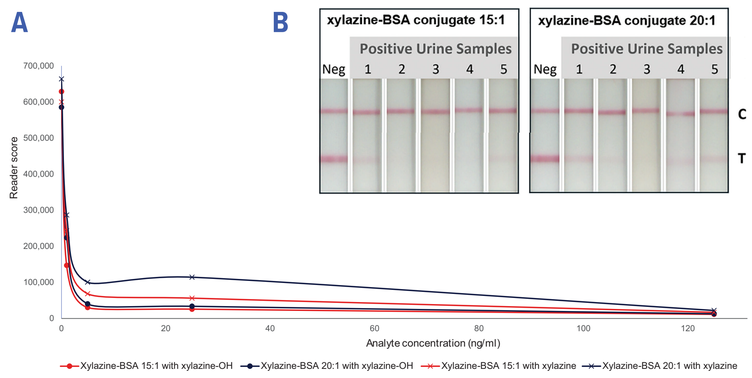

Figure 3. *a) Standard curves in lateral flow for xylazine and 4-hydroxy-xylazine with R14501 as a pair with xylazine-BSA 15:1 and xylazine-BSA 20:1.*
b) Xylazine negative and positive urine samples measured in lateral flow with R14501 as a pair with xylazine-BSA 10:1 and xylazine-BSA 20:1.

In kinetic measurements R14501 showed high affinity to xylazine-BSA 15:1 with association rate 5.2 x 10^5 1/Ms and did not dissociate under conditions used (Figure 4).

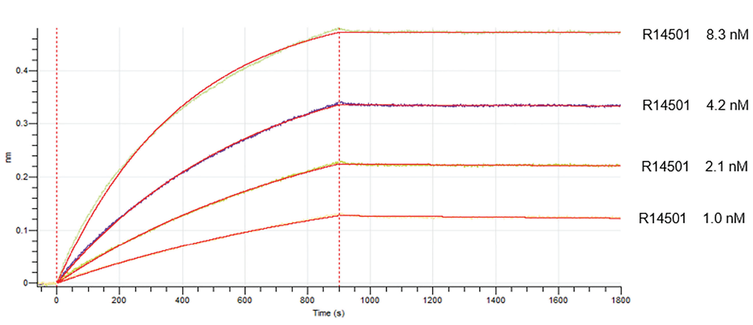

Figure 4. *Kinetic measurements of R14501 with xylazine-BSA 15:1.*

Conclusion

In this poster, we present data demonstrating how the developed monoclonal antibodies and antigen conjugates perform together in lateral flow format. Best pairs effectively separated xylazine negative and positive urine samples. In lateral flow xylazine-BSA (15:1) conjugate shows significantly better performance, which highlights the importance of optimizing the conjugate and mAb pair during the development of these IVD reagents. This pre-screening approach supports diagnostic test developers in selecting suitable components for assay development.

References

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2. Choi, S., Irwin, M. R., Noya, M. R., Shaham, Y., & Kiyatkin, E. A. (2024). Combined treatment with naloxone and the alpha2 adrenoceptor antagonist atipamezole reversed brain hypoxia induced by a fentanyl-xylazine mixture in a rat model. *Neuropsychopharmacology*, 49(7), 1104-1112.

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