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Validation of Mitragynine Immunoassay on Tecan Freedom Evo 75 with Randox ELISA Kit

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Background/Introduction: Mitragynine is the main active ingredient found in the *Mitragyna speciosa*, medicinal plants native to South East Asia. The leaves are often referred to as kratom. Mitragynine acts on the μ and δ opioid receptors and at high doses can produce opioid-like analgesic effects. At low doses, mitragynine can produce stimulant-like effects. There has been an increase of cases reporting Mitragynine, indicating there is a need to develop a robust screening method. The Tecan Evo 75 using the Randox ELISA kit described herein provides a sensitive and quick immunoassay screening option in whole blood for both human performance (DUI) and postmortem applications.

Objective: To validate the Randox Mitragynine ELISA kit in 96-well format in whole blood using a semi-automated Tecan Freedom Evo 75 instrument.

Methods: 96-well plates were coated with Mitragynine antibody by Randox. Whole blood samples were diluted 1:4 with diluent (150 µL sample + 450 µL diluent). 50 µL of diluted sample was added to each well followed by 75 µL of horseradish peroxidase labeled antigen (conjugate) to allow for competitive binding. Incubation time with the antibody, enzyme substrate (TMB), and stop solution (HCl) were 30, 20, and 5 minutes, respectively. These times were monitored carefully to enhance reproducibility. Optical density was measured at 450 nm by UV/VIS spectrophotometry. The cutoff for mitragynine was set to 2 ng/mL. Precision at 50% below the decision point (low – 1 ng/mL), at the decision point (cutoff – 2 ng/mL), 50% above the decision point (1.5X – 3 ng/mL), and 100% above the decision point (high – 4 ng/mL) was monitored in triplicate over 5 days for mitragynine. 26 previously analyzed samples were evaluated to determine false positive/negative rates and to assess the assay's ability to reliably detect compounds at concentrations commonly observed in routine casework. Interference was evaluated in ante- and post-mortem blood specimens. As a component of their in-house validation, Randox studied the following parameters: intra-assay precision, limit of detection, interference, and cross-reactivity. Demographics, autopsy findings, and other commonalities were evaluated in 24 mitragynine positive cases between 2014-2017.

Results: Intra-day precision (CVs) was 1.1% – 17.9 % for mitragynine. Between-day precision is shown below. All concentration points had CVs less than 20%.

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	Between-day Precision (CV%)			
Target	Low (1 ng/mL)	Cutoff (2 ng/mL)	1.5X (3 ng/mL)	High (4 ng/mL)
Mitragynine	14.9%	14.2%	11.3%	14.1%

There were no false negatives or false positives in the twenty-six previously analyzed. The cases were previously analyzed using GC/MS or LC/MS/MS. There was no interference in negative blood specimens or with commonly encountered drugs of abuse and therapeutic drugs. All subjects were Caucasian and 63% were male. The median age of males and females was 33 and 35, respectively. Both antemortem and postmortem cases were tested; however, postmortem cases accounted for 71% of the positives. Evaluation of autopsy reports revealed pulmonary edema as a common finding.

Conclusion/Discussion: We were one of the first forensic toxicology laboratories to validate the Randox Mitragynine ELISA assay. Sufficient precision around the cutoff and the lack of false positives/negatives indicate that the assay has the ability to presumptively identify or eliminate mitragynine. Since these data suggest this assay is a highly specific preliminary test for mitragynine, it is an additional tool for laboratories to increase their scope of analysis.

Keywords: ELISA, Mitragynine, Immunoassay