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SHORT COMMUNICATION

Forensic application and evaluation of a commercially available pregabalin immunoassay test in serum on an Olympus AU480

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Abstract

Misuse of pregabalin and its forensic relevance is steadily increasing. The aim of this study was to evaluate the usability of the commercially available ARKTM Pregabalin II Assay (ARK Diagnostics) for serum analysis of forensic samples. Overall, 156 samples were tested by both the immunoassay and a validated liquid chromatographic–tandem mass spectrometric (LC-MS/MS) method. Sensitivity was 100%, and specificity was 98.7% (n = 79 positive cases confirmed by LC-MS/MS in a range of 380–37,000 ng/mL). A good correlation (R^2 = 0.73) could also be shown between quantitative immunoassay and LC-MS/MS results. In conclusion, the assay shows excellent reliability for screening of forensic serum samples.

KEYWORDS
immunoassay, liquid chromatography, mass spectrometry, pregabalin, sensitivity

1 | INTRODUCTION

Misuse of pregabalin, an analog of the neurotransmitter gamma-aminobutyric acid (GABA), and its forensic relevance is steadily increasing.[1] Originally designed for the treatment of epilepsy, pregabalin is predominantly used to treat neuropathic pain and partial seizure disorders, but it is also approved for use in the treatment of fibromyalgia in the United States and management of generalized anxiety disorder (GAD) in the European Union (EU).[2,3] At the time of market launch in the EU in 2004, the potential for drug abuse or physical dependence for pregabalin was assessed to be low. A clinical study with 15 recreational alcohol/sedative users had found that pregabalin in therapeutic doses of 200–450 mg did not produce the same responses as diazepam, indicating that the drug did not have the profile of a prototypic drug of abuse.[4] However, in later clinical trials in patients with central neuropathic pain and in patients with GAD, euphoria as an adverse event was reported to be common for pregabalin. Assessments of withdrawal symptoms in clinical trials of GAD showed a profile similar to that of lorazepam, especially in the 600 mg/day dosage. Therefore, in 2014, the European Medicines Agency has changed abuse, misuse, and dependence from a potential risk to an identified risk.[4] Use of nontherapeutic dosages in drug users has been reported ever since then.[1,5] In Germany, pregabalin has risen to be the fifth often misused substance class after opiates, benzodiazepines, cannabis, and alcohol.[6] Besides euphoria, further side effects of therapeutic and nontherapeutic pregabalin use are dizziness, somnolence, and confusion.[2,3] This could lead to a limitation of fitness to drive. Therefore, pregabalin is important to be detected in forensic blood serum samples in forensic laboratories. Liquid chromatographic–mass spectrometric (LC-MS) unknown screening methods mostly focus on the detection of basic drugs, whereas drugs with acidic properties like pregabalin can be lost during sample preparation. However, specific and sensitive detection of pregabalin using solid-phase extraction[7,8] or our herein described method using protein precipitation has been described. An immunoassay could be useful in screening on this relevant substance, giving the possibility of high-throughput analysis without sample preparation.

When using immunochemical methods for a preliminary test according to the guidelines of the German Society of Toxicological and Forensic Chemistry (GTFCh), the relevant analyte concentrations

obtained by a chromatographic method must show positive immunochemical results for the relevant substance, which have to be documented by an appropriate validation procedure.[9] Sensitivity should be nearly 100% (at least 90%), and the rate of false positives simultaneously should be as low as possible due to costs and time. The aim of this study was to evaluate the usability of the commercially available ARK Pregabalin II Assay (ARK Diagnostics), which was originally designed for clinical urine screening, for serum analysis of forensic samples.

2 | MATERIAL AND METHODS

2.1 | Samples

Serum samples collected mainly for forensic purposes were used for the study. All samples were tested in the Institute of Forensic Medicine, Mainz (Germany), with immunoassay as well as with LC-MS/MS. Overall, 156 samples were tested.

2.2 | Immunoassay test

The test used was the ARK Pregabalin II Assay from ARK Diagnostics (Fremont, CA, USA). The assay was originally designed for the qualitative and/or semiquantitative determination of pregabalin in human urine using a cutoff concentration of 500 ng/mL. It is a homogeneous immunoassay based on competition between pregabalin in the specimen and pregabalin labeled with recombinant glucose-6-phosphate dehydrogenase (rG6PDH) for antibody binding sites. As the latter binds antibodies, enzyme activity decreases. In the presence of pregabalin, enzyme activity increases relative to the drug concentration. Active enzyme converts nicotinamide adenine dinucleotide (NAD) to NADH in the presence of glucose-6-phosphate, resulting in an absorbance change that is measured spectrophotometrically.[10] An Olympus AU480 (Olympus Europa, Hamburg, Germany) was used for the assay. To receive semiquantitative results, a five-point calibration curve (250–2000 ng/mL) was established. Samples with immunoassay results higher than the highest calibrator were not diluted. Two commercially available quality control (QC) samples were measured per working day (QC low: 250 ng/mL; QC high 750 ng/mL) and showed precision (relative standard deviations) of 4.27% (QC low, n = 14) and 4.84% (QC high) and bias of 8.1% (QC low, n = 14) and 1.6% (QC high), respectively. Regarding the analytical specificity, some drugs relevant in forensic toxicology were tested negative by the manufacturer at high concentrations in urine.[10] We confirmed that the analysis of the GABA analog gabapentin did not lead to a positive result even at a concentration of 5,000,000 ng/mL in serum. Because there are no legal requirements for the determination of pregabalin, we decided to choose an immunoassay cutoff of 100 ng/mL immunoassay units (IAU) corresponding to a very low pregabalin serum concentration. Therapeutic concentrations of pregabalin are described to be 2000–8000 ng/mL.[11]

2.3 | Confirmation analyses

Pregabalin (standard received from LGC, Cologne, Germany) was confirmed by a LC-MS/MS method. Briefly, 100-μL internal standard solution (including 5 ng/μL pregabalin-D_6 from Merck, Germany) was added to 100 μL of methanol (Fisher Scientific, Waltham, MA, USA). Afterward, 100 μL of serum was added, and the solution was vortexed for 30 s and centrifuged for 10 min at 3000 g. The LC-MS/MS system used consists of an Agilent (Santa Clara, CA, USA) 1290 Infinity liquid chromatograph coupled to an Agilent 6490A triple quadrupole mass spectrometer. A 5 μL of the supernatant was injected into the system. Chromatographic separation was achieved using a raptor biphenyl column (2.1 * 100 mm; Restek, Germany) and a raptor biphenyl guard column (2.1 * 5 mm; Restek, Germany). Solvent A was water (ultra LC-MS grade with 5 mmol/L ammonium formate from Roth GmbH, Karlsruhe, Germany), and Solvent B was acetonitrile with 0.1% formic acid (from Roth, Germany). Gradient was as follows: 0–4 min increase from 0% to 20% B; 4–6.5 min increase to 50% B; 6.5–7.5 min increase to 100% B; 7.5 min: decrease to 0% B, hold until the end of the run at 8.5 min. Flow was 0.3 mL/min. Pregabalin and pregabalin-d6 were measured using electrospray ionization with a gas temperature of 300°C, a gas flow of 11 L/min, a nebulizer pressure of 15 psi, a sheath gas temperature of 380°C, a sheath gas flow of 11 L/min, and the capillary in positive ionization mode (4000 V). Substances were detected in multiple reaction monitoring mode using the following ion transitions and collision energies: pregabalin: 160 → 142 (target, CE 5 V), 160 → 124 (qualifier, CE 15 V); pregabalin-d6: 166 → 148 (target, CE 5 V), 166 → 130 (qualifier, CE 15 V). Validation results were as follows: limit of detection (determined using a signal-to-noise ratio ≥3) 10 ng/mL, lower limit of quantification (showing an acceptable precision of 3.6% and bias of 8.0%) 100 ng/mL; calibration range up to 20,000 ng/mL, intraday precision 8.4% at 2000 ng/mL and 5.4% at 10,000 ng/mL; interday precision 8.4% at 2000 ng/mL and 5.4% at 10,000 ng/mL; matrix effects 95.4% at 2000 ng/mL and 92.4% at 10,000 ng/mL. Samples with concentrations higher than the highest calibrator were diluted with water and reanalyzed. Gabapentin could be detected simultaneously by multiple reaction monitoring.

2.4 | Calculation of sensitivity and specificity

Sensitivity describes the percentage of positives (positive by LC-MS/MS) correctly identified to be positive by the immunoassay. Specificity describes the percentage of negatives (negative by LC-MS/MS) correctly identified to be negative by the immunoassay.

2.5 | Tested samples

Overall, 156 samples were tested by both methods. All blood samples that came to our laboratory in February 2020 and where pregabalin analysis was requested were introduced in the study. These included plasma and serum samples. Furthermore, samples positive on the

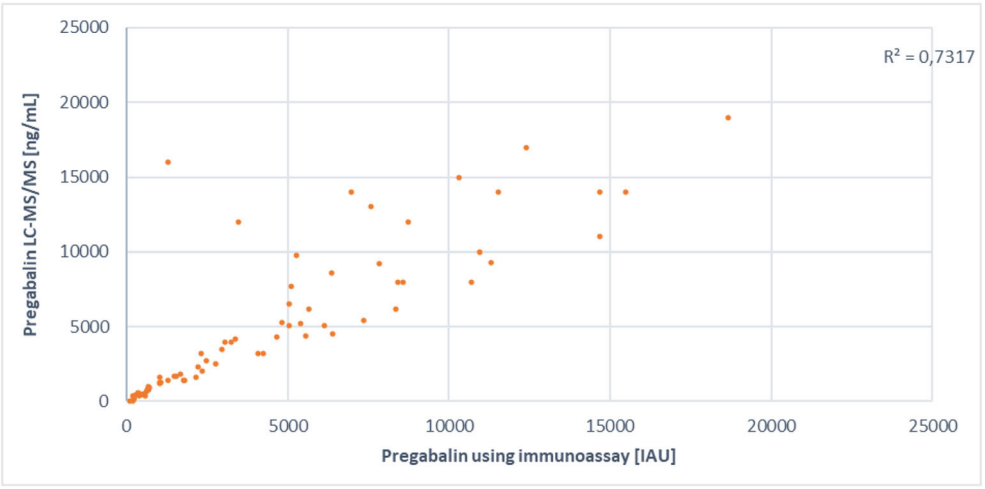

FIGURE 1 Correlation of the quantitative results received by immunoassay and received by LC-MS/MS

immunoassay and also tested by confirmation analyses were included into the study until the end of June 2020. In 15 of these cases, hemolytic blood serum was received and was treated with acetone (1:1) for protein precipitation before immunochemical analysis; the supernatant was analyzed.

3 | RESULTS AND DISCUSSION

Seventy-six samples were tested negative with immunoassay (<100 IAU). In none of these cases, pregabalin could be detected by LC-MS/MS. Eighty samples were tested to be positive with immunoassay (>100 IAU). Seventy-nine of these 80 samples were confirmed by LC-MS/MS analysis (pregabalin concentration range: 380–37,000 ng/mL). Only in one sample with an immunoassay result slightly higher than the cutoff (102 IAU) pregabalin could not be detected by LC-MS/MS. In conclusion, using the cutoff concentration of 100 IAU, a sensitivity of 100% (79/79) and a specificity of 98.7% (76/77) could be achieved. Hemolytic samples showed 100% sensitivity and 100% specificity. It has to be taken into account that the positive case with the lowest concentration was 380 ng/mL. Therefore, the given cutoff of 100 IAU only supports the assumption that a concentration of at least 380 ng/ml pregabalin could be detected with the given sensitivity.

The immunoassay also worked well semiquantitatively. Correlating concentrations of the positive cases measured by LC-MS/MS with the values received by the immunoassay, a correlation coefficient R^2 of 0.56 could be achieved. Eliminating concentrations detected by LC-MS/MS > 20,000 ng/mL, a coefficient of determination R^2 of 0.73 could be achieved (see Figure 1). However, for forensic purposes, a confirmation of the immunoassay and quantitation by LC-MS/MS or GC-MS is mandatory.

In conclusion, the assay shows excellent reliability for screening of forensic serum samples.

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