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Multiplex approach for an immunological detection of drug abuse: a validation study

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Aim: The aim of this study was to develop a multiplex immunoassay for nine different drugs. Therefore each reagent undergoes a stringent quality control e.g. antibodies used has to be validated with at least 2 independent methods. **Methods:** For validation Western Blot analysis and ELISA were performed. A competitive ELISA was established allowing the quantification of the drugs in sera. Appropriate controls were included for background subtraction, determination of unspecific signals and assay control. Furthermore the assay was miniaturized on a microarray, which was produced with a non-contact spotter. **Results:** For 4 out of 9 selected drugs specific antibodies could be obtained and a competitive ELISA established for quantification. Validated antibodies were characterized by no cross-reactivity to serum and no unspecific binding to other compounds. Serum samples with spiked drugs or samples from the LKA Berlin were analyzed. Each sample was performed in triplicates and each experiment was done twice at least. Limits of quantification meet the requirements of the GTFCh. First multiplex and spotting experiments were done with a satisfying result. **Conclusion:** The presented approach enables a sensitive and reliable detection method for drug abuse in sera. The validation studies are continued for the remaining drugs.

1. Introduction

Immunoassays, detection of specific antigens with the corresponding antibodies, represent a powerful tool. During the last 60 years [1-3] a remarkable development according to sensitivity and applicability could be observed. Most of all immunoassays are used in the field of allergy testing [4, 5] or diagnosis of autoimmune diseases [6, 7]. Another major application is point-of-care tests, i.e. lateral flow immunoassays for pregnancy testing [8, 9].

Developing an immunoassay always means extensive quality control especially antibody validation. Since a poor antibody performance including cross-reactivities is the major drawback of this technique, selectivity, sensitivity and stability have to be tested carefully. For these parameters, the GTFCh (Society for Toxicological and Forensic Chemistry) guideline [10] defines which requirements have to be met for new analytical methods.

Within this study the aim was to develop a multiplex immunoassay for drug screening for nine different drugs in serum. Therefore the antibodies were validated with different methods and quantitative analysis with ELISA and microarray experiments were performed. Initial experiments for multiplexing were done and for verification purposes LC-MS quantified samples of the Criminal Office of Investigation (LKA) in Berlin were analyzed. These samples served as controls for the development process.

The validation study includes the following individual steps and different methods: Quality control (QC) of the antibodies, establishment of a standard/calibration curve and verification of the method via measuring samples provided by the LKA Berlin. The illicit drugs used in this study are: Amphetamine, Methamphetamine, MDMA, Cocaine, Benzoylecgonine, PCP, Morphine, Methadone and THC.

2. Material and Methods

2.1. Experimental steps

For quality control concerning specificity standard protocols [11, 12] of Western Blot and ELISA (Enzyme-Linked-Immunosorbent-Assay) were performed. Therefore Drug-BSA conjugates were immobilized and incubated with commercial primary antibodies. The detection of binding was done using a fluorescent-labelled secondary antibody and the read-out was done with appropriate systems. For quantification purposes a competitive ELISA was established. Before the incubation with the immobilized conjugates the primary antibody was pre-incubated with defined amounts of free drug. The miniaturization on a microarray slide or in a well of a 96-MTP was realized with a non-contact spotter.

2.2. Material

Drug-BSA conjugates were purchased from Fitzgerald Industries, the free drugs dissolved in methanol were obtained from LGC-Standards and the blank serum was purchased from UTAK. For documentation following systems were used: for ELISAs a BMG LABTECH plate reader, for Western Blot analysis a Typhoon laser scanner produced by GE Healthcare and for microarrays an Axon Microarray Scanner supplied by Molecular Devices. For non-contact spotting the iTWO-400 developed by M2-Automation was used.

3. Results and Discussion

Within this study several antibodies could be validated. Based on the results of Western Blot and ELISA antibodies which showed no cross-reactivities to other drugs or assay compounds were used for further experiments (data not shown).

In the following a calibration curve for quantification was generated on microarrays and ELISAs in a competitive procedure. In figure 1 the results for MDMA are shown.

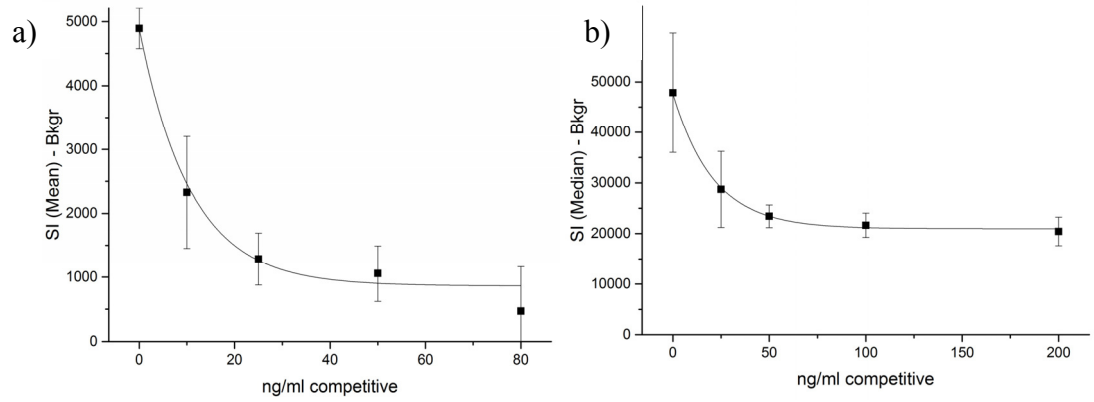

Fig. 1. A competitive assay with MDMA in a) a 96-MTP and b) on a microarray for generation of a calibration curve is shown. The microarrays were produced with a non-contact spotter. MDMA-BSA was immobilized and incubated with increasing amounts of free MDMA. With increasing concentrations of free MDMA the signal decreased until a plateau was reached.

Beside the required optimization concerning the linear range a quantification of drug positive serum samples was possible. Both platforms provided similar results. Furthermore sensitivity and reproducibility fulfilled the demanded values. In table 1 the results for the different drugs were summarized for the various platforms.

Tab. 1. The present results for QC and establishment of a standard curve with various methods are summarized. QC was performed with Western Blot analyses and direct ELISA. The standard curves were generated with competitive ELISA in 96-MTP and on microarray (MA). An antibody was validated when no unspecific binding with serum, other drugs or antibodies and assay components occurred.

Drug	Quality Control	ELISA	Microarray
Amphetamine	-	-	-
Methamphetamine	-	-	-
MDMA	+	+	+
Cocaine	-	-	-
Benzoylecgonine	+	+	-
PCP	-	-	-
Morphine	-	-	-
Methadone	+	+	-
THC	+	+	-

Due to unspecific binding or poor sensitivity some of the commercial antibodies had to be excluded from this study. Efforts were made to produce specific antibodies in-house.

In parallel first experiments in preparation of multiplexing were done. It should be clarified whether the administration of a mixture of drugs or antibodies will influence the general outcome of the developed assay. Therefore one or a combination of nine drugs or antibodies respectively were used in the preincubation step and compared to each other. In figure 2 the results of a representative experiment for Methadone is shown.

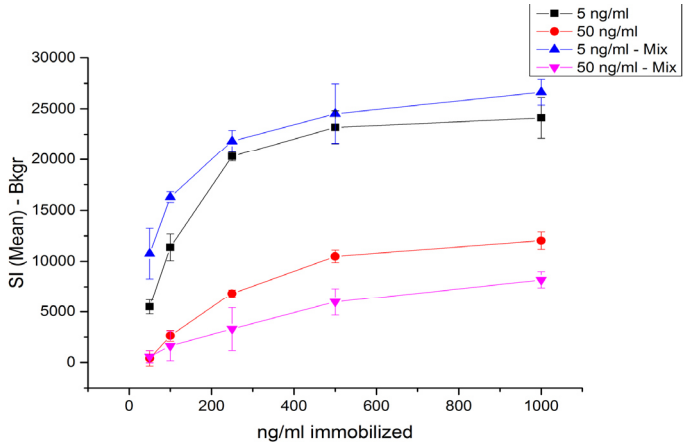

Fig. 2. Competitive ELISA with Methadone. Methadone-BSA was immobilized and incubated with 2 different amounts of free drug. The influence of the presence of 9 drugs (blue and pink) of the same concentration was analyzed. Only minor differences to the solitary presence of Methadone (black and red) were observed.

The data showed minor differences of the signal intensities when a mixture is applied, but the general ratios stayed the same.

As a final step of the assay validation authentic serum samples were measured in a competitive ELISA to verify the method. In figure 3 the results of measurements of Methadone-positive serum samples are shown. It has to be noticed that of the LC/MS measurement only qualitative data was available. Therefore only the proportions could be compared with the quantitative ELISA results.

It could be shown that the established immunological assay was able to quantify authentic serum samples in a satisfied manner.

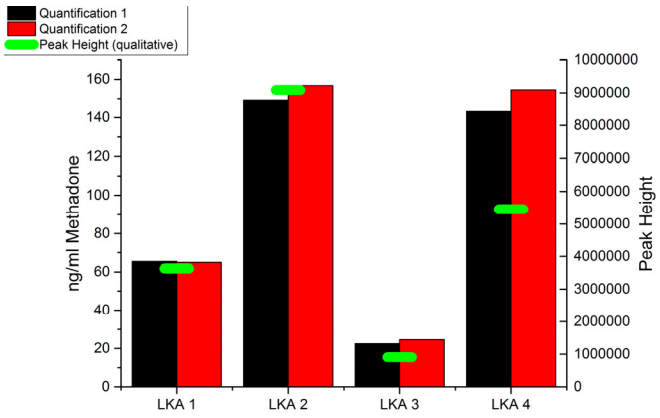

Fig. 3. Two independent quantifications of four Methadone samples are depicted. Methadone concentrations were calculated with a calibration curve and compared with qualitative LC/MS measurements. With both methods comparable results were achieved.

4. Conclusions

To sum up it was possible to validate specific antibodies and establish a competitive assay for 4 out of 9 drugs. Minor signal differences by administration of a mixture of drugs or antibodies were observed, but first successful quantifications of LKA samples with good correlations to reference data could be obtained. Furthermore a miniaturization from 96-MTP to Microarrays was done successfully. This means that a platform comparison could be realized. The next steps will include an improvement of linearity of standard curves for a more reliable quantification. Additionally the multiplexing should be extended. Finally to substantiate the assay verification further LKA samples will be quantified.

5. Acknowledgements

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6. References

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