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Determination of cross-reactivity of poly- and monoclonal antibodies for synthetic cannabinoids by direct SPR and ELISA

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ABSTRACT

One of the main reasons for the rise in popularity of synthetic cannabinoids (SCs) is their ability to remain unrecognized in conventional drug screenings. Due to their structural diversity, caused by the constant introduction of new substances to circumvent legal regulation, antibodies with a wide range of cross-reactivity are necessary for the establishment of a reliable immunological based drug test. Therefore, high-quality binding data are needed to select promising antibody candidates for further development.

In this study, we carried out a direct surface plasmon resonance (SPR) method and evaluated its suitability for the characterization of antibody–SC interactions. The cross-reactivity of 22 SCs with three polyclonal antibodies, raised against JWH-018 haptens with different attachment positions of the linker, and two commercial available monoclonal antibodies were determined. These results were compared with the commonly used competitive enzyme-linked immunosorbent assay (ELISA). It could be demonstrated, that direct SPR and competitive ELISA show comparable specificity results for the majority of the measured compounds. However, the reduced manual labor, the real-time analysis and the high information content about the binding events of SPR compared to ELISA, showed that SPR is a valuable tool during the development of antibodies against synthetic cannabinoids, currently the largest group of new psychoactive substances.

1. Introduction

Synthetic cannabinoids (SCs) were first detected as active ingredients in herbal smoking mixtures in the end of the year 2008 [1] and swiftly became the predominant group of compounds within the new psychoactive substances (NPS) worldwide [2]. In less than a decade, the number of synthetic cannabinoids listed by the United Nations Office on Drugs and Drug Addiction (UNODC) increased from 31 in 2009, over 154 in 2013 to 241 in 2016 [2–4]. The popularity and motivation for the consumption of SC can be explained by several reasons: (i) psychoactive effects similar to cannabis, (ii) curiosity and easy availability via internet shops, (iii) new SCs were usually not covered by national narcotic laws, which in turn was widely misinterpreted that these compounds are legal, and (iv) in case of misuse, the high likelihood of remaining undetected by common drug screenings [5]. These reasons have contributed to a fast spinning spiral of national banning of new appearing compounds, followed by a quick replacement of the existing SCs by slightly modified successor compounds by the manufacturers.

Hence, at least 28 (including USA, Japan, UK, China and Germany) countries have recently modified the “common practice” of individually banning synthetic cannabinoids by adopting new laws (or modifying existing laws) towards a more generic regulation of structurally related compound classes. These changes will allow the control of new and so far unknown SCs based on structural similarity to a defined SC subclass and a set of defined structural modifications [4]. The future will show how this legal action is suitable to avoid the constant circumvention of laws, through successive structural modifications of the SCs.

The identification and quantification of SCs and their metabolites in biological matrices, including serum [6], blood [7], plasma [8], urine [9], oral fluid [10] and hair [11] is mainly performed by chromatographic techniques coupled to mass spectrometric detection, such as GC–MS, LC–MS/MS or LC–HRMS.

Currently, urine is the most widely used matrix for the detection of SCs in human specimen. The main advantages of urine analysis over blood samples are the non-invasive sample

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collection, usually higher concentrations of the analytes and a longer detection window [12]. There are, however, some disadvantages with urine as sample for drug screenings, for instance the inability to determine a very recent SC use, the possibility of adulteration, the knowledge of the drug's metabolites and the need of enzymatic or acidic hydrolysis of the conjugated hydroxylated metabolites prior to analysis [13–17]. Currently, little attention is given to oral fluid as another promising matrix for workplace and roadside drug testing for SCs. Due to the facts that oral fluid is fast and easy to obtain, it is suitable for the identification of a recently consumed drug without the invasive sample collection of blood and the possibility to detect unchanged SCs instead of metabolites. However, fast and simple workplace or roadside testing usually requires immunological based test systems.

Immunoassays are cost-effective, high-throughput methods to rapidly distinguish positive from negative specimens in an initial screening. Hence, immunoassay based methods could represent a simple, cheap and fast screening approach to reduce the number of SC samples, which have to be confirmed by labor intensive techniques in clinical, forensic or workplace screening programs.

So far, only a modest number of immunoassay based methods are described in literature for the detection of SCs in urine [18–24] and so far only one for detection in oral fluids [25]. The most comprehensive study was undertaken by Castaneto et al. [20]. 20,017 authentic urine specimens were screened with the drugs of abuse V (biochip array DoA-V; Randox Laboratories Ltd., Crumlin, UK), which combines three polyclonal antibodies against JWH-018, one against JWH-250 and seven antibodies against other designer drugs. The initial biochip screening was accompanied by LC–MS/MS confirmatory analyses of 1432 presumptive positive and 1069 negative samples. The DoA-V showed promising results of high cross-reactivity (for 22 of 33 SCs and 37 of 42 metabolites) and >85% sensitivity, specificity and efficiency, respectively. Evaluations of the Immunalysis K2 Spice homogeneous enzyme immunoassay (HEIA) for identifying SCs in authentic urine samples by Barnes et al. [19] and Kronstrand et al. [21] gave high sensitivity (92/98%) and specificity (87/82%), but did not show cross reactivity against a wide range of SC metabolites or more recent SCs.

Despite the advantage to detect parent SCs in oral fluid instead of a multitude of possible metabolites in urine, only one evaluation of an ELISA method with a polyclonal antibody raised against conjugated JWH-018 for the detection of SCs in oral fluid was performed. There, 21 out of 26 LC–MS/MS confirmed SC positive oral fluid specimen were identified by ELISA with a cut-off concentration of 0.25 ng/mL [25].

To establish an efficient and reliable immunoassay for SC screenings, it is necessary to develop antibodies that show a high degree of cross-reactivity, to ensure that a set of only a few antibodies ideally can cover a wider range of the structural modifications across several SC subclasses. During the development of such antibodies it is necessary to analyze the cross-reactivity of newly developed anti-sera or antibodies against a larger number of structurally divers SCs. These cross-reactivity data of developed antibodies is most widely obtained by enzyme-linked immunosorbent assays (ELISA). However, a number of alternative biophysical techniques have emerged potentially capable to analyze the binding of low-molecular weight compounds to macromolecular targets, including X-ray crystallography, ligand-observed NMR, isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC), differential scanning fluorimetry (DSF), microscale thermophoresis (MST), small-angle X-ray scattering (SAXS) and surface plasmon resonance (SPR) [26].

SPR-based biosensors combine the benefits of high-throughput screening, high information content about the binding events and the possibility to directly observe the antigen-antibody-binding without the need of labeling or labeling reactions. In most SPR applications biomolecules (e.g. antibodies) are bound to a gold surface of a sensor chip. Binding interactions of added compounds (e.g. antigens) are monitored in real-time by measuring the change of refractive index using polarized light [27]. Although, SPR is mainly used for measuring binding events of high molecular weight macromolecules, it was recently demonstrated that SPR can also be applied to monitor the binding of lower molecular weight compounds [28,29].

To evaluate the suitability of SPR for the characterization of antibody – SC binding, we conducted a direct binding analysis of 22 SCs and monoclonal or newly raised polyclonal antibodies based on surface plasmon resonance (SPR) to determine cross-reactivity of this major group of new psychoactive substances. To evaluate these findings, the results were compared with data obtained by competitive enzyme-linked immunosorbent assay (ELISA) which was carried out in parallel.

2. Material and methods

2.1. Experimental

2.1.1. Chemicals

All chemicals were of analytical grade purity and were purchased from Fluka (Buchs, Switzerland), Sigma–Aldrich (Steinheim, Germany) or Acros Organics (Geel, Belgium). Solvents were of high-performance liquid chromatography grade purity and were used without further purification. Monocrotaline (**25**), used as a negative control in the immunoassays and terephthalaldehyde, used as reference in quantitative 1H NMR analysis and 3,3′,5,5′-tetramethylbenzidine for ELISA detection (TMB, T8665, Ready-to-Use Kit), were obtained from Sigma–Aldrich (Steinheim, Germany). TLC plates (Polygram SIL G/UV 40 × 80 mm) and silica gel (mesh 0.04–0.063) were obtained from Macherey-Nagel (Düren, Germany).

0.1 M phosphate buffered saline (PBS, 81 mM Na_2HPO_4, 19 mM NaH_2PO_4, 135 mM NaCl, 2.7 mM KCl, pH 7.4) was used as dialysis buffer for the hapten–protein conjugates.

PBS–EtOH (8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 13.5 mM NaCl, 0.27 mM KCl, pH 7.4, modified with 10% ethanol (v/v) and 0.05% polysorbate 20 (v/v)) was used in SPR experiments as running buffer, sample dilution buffer and ELISA wash buffer, unless otherwise mentioned.

HBS-EP+ (HEPES 10 mM, NaCl 150 mM, EDTA 3 mM, polysorbate 20 0.05% (v/v) at pH 7.4) was used in SPR as immobilization buffer.

Monoclonal anti-K2 antibody 1 (**MAb1,** 1.26 mg/mL) was obtained from Arista Biologicals, Inc. (Allentown, PA) and monoclonal anti-K2-antibody 2 (**MAb2,** 6.0 mg/mL) from CalBioreagents, Inc. (San Mateo, CA). Beside general information on specificity against metabolites of JWH-018 and JWH-073 no further information on SC-specificity was available for these commercially available antibodies.

Peroxidase-conjugated antibodies for competitive ELISA were anti-sheep IgG (SAB3700702), purchased from Sigma–Aldrich (Steinheim, Germany) used for the polyclonal antisera (**PAb1-3**) and anti-mouse IgG (610-103-121) purchased from Rockland Inc. (Limerick, PA) used for both monoclonal antibodies (**MAb1** and **MAb2**).

Synthetic cannabinoids and SC-fragments (**1-24**) were previously synthesized by standard chemical procedures or isolated from herbal smoking mixtures with subsequent silica gel column purifications. Structures and purities were confirmed by NMR [30–34].

2.1.2. Nuclear magnetic resonance spectroscopy

^1H and ^{13}C NMR spectra of the haptens **1A–3B** were recorded at 600.1 and 150.9 MHz, respectively, on a Bruker Avance II