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Trypsin diminishes the rat potency of polio serotype 3

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ABSTRACT

This study addresses observations made in view of testing in practice the guideline in the European Pharmacopoeia (EP) on omitting the rat potency test for release of polio containing vaccines. In general, use of the guideline is valid and the D-antigen ELISA can indeed be used as an *in vitro* alternative for the *in vivo* test. However, the set-up of the ELISA is crucial and should include detection of antigenic site 1 in polio serotype 3 as destruction of that site by trypsin results in a reduced rat potency. Antigenic site 1 in polio serotype 2 may also be modified by trypsin, but the cleavage of viral protein 1 (VP1) did not affect the rat potency. Therefore, any antigenic site, except site 1, can be used for detection of polio serotype 2. It is advised to include testing of the effect of trypsin treatment in the EP-guideline. This allows polio vaccine manufacturers to check whether their in-house ELISA needs improvement.

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1. Introduction

Vaccines such as the inactivated polio vaccine (IPV) and the combination vaccine of diphtheria toxoid and tetanus toxoid (DT-IPV) contain formalin-inactivated poliovirus serotype 1–3.

The vaccines include intact poliovirus particles, so-called D-antigen (D-ag), which are composed of a capsid containing 60 copies of the capsid proteins VP1-4, and RNA at the inside. Administration of D-ag results in a virus neutralizing immune response providing protection against poliomyelitis [1].

Heating intact virus or vaccine results in loss of D-antigenicity [2–6]. Heating at 56 °C for 15 min destroys D-antigenicity completely [7]. These heated particles have C-antigenicity and are immunogenic, but the induced antibodies are not virus neutralizing [8].

For release of polio vaccines, determination of the D-antigen concentration (ELISA) and testing of the immunogenicity (rat potency test) of the polio component is important.

The set-up of the sandwich ELISA [7] is not specified in the European Pharmacopoeia (EP), so for example the primary antibody may be either a monoclonal antibody (mAb) or a polyclonal mix (pAb). Typically, the relative standard deviation (RSD) of the ELISA is <15%.

The rat potency test [9,10] is used to measure the immunogenicity in rats which is done by determination of the virus neutralizing antibody response against poliovirus after a single immunisation of undiluted and serially diluted vaccine (5 groups and 10 rats/group). The measured rat potency is a value expressed relative to the reference, a trivalent mixture of serotype 1–3, and calculated according to the EP parallel line method. In the calculation, fixed nominal D-antigen concentrations are used and as a consequence a difference in the dose response curves between sample and reference translates to a rat potency that differs from 1, a substantial D-ag loss (>90%) will result in a rat potency of ~0. The test variation is considerable and on average the 95% confidence intervals are between 50 (lower limit, LL) and 200% (upper limit, UL) of the rat potency.

According to the guideline in the European Pharmacopoeia (EP) [11] it is possible to omit the rat potency test and rely exclusively on the more accurate D-antigen ELISA for release of inactivated poliovirus containing vaccine. This suggests the existence of at least

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some relationship between the *in vitro* and the *in vivo* test, despite the considerable test variation in the rat potency test.

The EP-guideline states that before relying on the ELISA for release analysis, it should be demonstrated that the ELISA and the rat potency test can discriminate between potent and sub-potent vaccine.

In this study, an example is described on the preparation of an experimental inactivated polio vaccine that remains potent in the ELISA, but is sub-potent in the rat potency test. These observations were made by using trypsin-treated IPV samples. Trypsin is used in the polio production process for removal of host cells from microcarriers [12]. Hence such an experiment appears relevant within the context of polio production.

To check whether there would be more examples of discrepancies between *in vitro* and *in vivo* analysis, also the experiments as outlined in the EP-guideline were performed, *i.e.* the effect of dilution with heated vaccine (C-antigen, C-ag) and heat treatment of potent vaccine on test results from ELISA and rat potency analysis.

The results are discussed within the scope of omitting the rat potency test for polio containing vaccines as outlined in the EP [11] and recommendations for improving the ELISA are presented.

2. Materials and methods

2.1. Vaccine preparation

IPV and international reference (PU91-01; type 1–3; 430-95-285 DU/mL) [7] were produced under cGMP conditions according to a routine production process [12,13]. Briefly, Vero cells were cultivated on microcarriers (Cytodex 1, GE Healthcare) suspended in fermentors, followed by infection with wild-type strains (Mahoney, MEF-1 or Saukett). Virus was purified by filtration (clarification followed by ultrafiltration, both Millipore), gel permeation chromatography on Sepharose CL-6B (GE Healthcare) and ion exchange chromatography on either DEAE Sepharose Fast Flow or DEAE Sephadex A-50 (both GE Healthcare) in a phosphate buffer. To the processed virus, M199 and glycine (final concentration 5 g/L) were added and the fluid was filtered through a 0.22 μm pore size filter (Millipak-200, Millipore) prior to inactivation. Inactivation was performed using 0.025% formalin during 13 days at 37 °C according World Health Organization (WHO) requirements [14].

2.2. Antibodies

D-specific anti-type 1 (3-4E4, site 1 specific), anti-type 2 (10E8D5 site 1 specific, 3-14-4, site 2 or 3 specific) and anti-type 3 (1-12-9, site 2, 3 or 4 specific) monoclonal antibodies (mAbs) were obtained after immunization with IPV from Mahoney, IPV from MEF (10E8D5) or trypsin treated MEF (3-14-4) and trypsin treated Saukett virus (1-12-9), respectively [15]. MAbs Hyb295-17 (anti-type 1), Hyb294-06 (anti-type 2) and Hyb300-06 (anti-type 3, site 1 specific) were commercially available (BioPorto). MAb 234 (anti-type 1, site 4 specific) was a kind gift of the National Institute for Biological Standards and Control (Potters Bar, UK). MAbs 3-4E4 (type 1), 3-14-4 (type 2) and 1-12-9 (type 3) were used as detecting antibodies in the so-called D-ag ELISA (used for release by BBio).

Polyclonal sera were obtained by immunization of rabbits with IPV from Mahoney, MEF, and Saukett virus for type 1–3, respectively.

Anti-C-antigen mAbs were obtained after immunization with formic acid-treated IPV from Mahoney, heated (15 min at 56 °C) MEF virus and heated (15 min at 56 °C) Saukett virus for type 1, 2, and 3, respectively.

2.3. ELISA

D-antigen (D-ag) was quantified with a sandwich ELISA. Polystyrene 96-well plates were coated overnight at room temperature with bovine anti-polio serum and blocked with 1% BSA (Sigma–Aldrich) for 30 min at 37 °C. The plates were washed with tapwater containing 0.05% Tween 80. A series of eight two-fold dilutions of vaccine in 0.01 M PBS containing 0.05% Tween 80 was added to each plate and incubated at 37 °C for 2 h and overnight at 4 °C.

The unbound antigen was removed and the plates were washed as above. Type-specific mAbs (3-4E4, 10E8D5 and 1-12-9 for type 1, 2, and 3, respectively) were added followed by horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG (Southern Biotech). Sometimes, polyclonal (rabbit anti-IPV) antibodies were used as detecting antibodies with HRP-conjugated sheep anti-rabbit (Intravacc, Bilthoven, The Netherlands) as the final step. Use of polyclonal antibodies is indicated; in all other cases mAbs were used. D-ag units were calculated by using a four parameter curve to fit the dose-response curve of the reference preparation PU91-01.

C-antigen (C-ag) was detected in a similar assay, but with the use of type specific anti-C mAbs. Plates were coated with rabbit anti-C antibodies. As a C-ag reference PU91-01 was heated for 15 min at 56 °C. The C-ag content was assigned by definition, using 1 D-ag unit (DU) for 1 C-ag unit (CU).

2.4. Biosensor analysis

Antigenicity was also measured in a Biacore T100 (GE Healthcare), equipped with an anti-polio biosensor as described elsewhere [16]. Goat anti-mouse IgG Fc-specific (Thermo Scientific Inc), antibodies were covalently immobilized on the dextran layer of a CM3 sensorchip (GE Healthcare) by primary amine coupling, following the manufacturers recommendations (GE Healthcare). Type-specific mAbs (3-4E4, 10E8D5 and 1-12-9 for type 1, 2, and 3, respectively) were bound to the sensor, followed by IPV. The sensorchip was regenerated with 10 mM glycine-HCl and pH 1.5. Assay data were analysed by four-parameter curve fitting using the Biacore T100 evaluation software. Antigenicity was calculated relative to the international reference PU91-01.

Particle concentrations were measured in a Biacore T100 by the calibration free concentration analysis (CFCA). CFCA measurements are based on the observed binding rate during sample injection under partially or complete mass transport limited conditions. Mass transport limitations arise when poliovirus particles bind to the surface faster than it diffuses from the solution during injection and/or poliovirus particles do not diffuse fast enough from the surface during dissociation, leading to rebinding. Type specific mAbs Hyb295-17 (anti-type 1), Hyb294-06 (anti-type 2) or Hyb300-06 (anti-type 3) were bound to a goat anti-mouse IgG Fc CM3-sensorchip. Monovalent and trivalent vaccines were diluted to 1–2 μg/mL protein and injected during 36 s at two different flow rates (5 and 100 μL/min). The observed binding data were fitted to a mass transport-limited 1:1 interaction model with a known value for the mass transport coefficient (derived from the diffusion coefficient of poliovirus [1]) and an unknown variable for the analyte concentration.

2.5. Preparation of sub-optimal IPV

- D-antigen concentration in the vaccine was reduced to 33%, 50% and 67% by diluting with C-antigen (obtained by heating D-antigen 15 min at 56 °C).

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