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Improved bioassays using a local effect, such as muscle paralysis, as an endpoint

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ABSTRACT

Drug potency testing consumes many animals, botulinum neurotoxin (BoNT) testing being perhaps the most notorious example. To avoid 50% lethal dose determination, the so-called digital abduction score (DAS) and other BoNT induced local paralysis assays were developed. This paper reveals that a simple mathematical expression — the Bateman's equation used in many pharmacokinetic data analyses — can describe in full detail the time dependence of the BoNT induced local paralysis; the equation hence allows robust interpolation and extrapolation, as well as integral effect (AUC), and its dose dependence, evaluation. The equation is moreover a convenient tool for experimental planning and for extracting, from experimental data, the parameters that characterise BoNT potency. Most important, one can generally reduce the number of animals needed to gain reliable results at least 20–33% (and possibly 50% or even 75%) by analysing and modelling the time course of a local effect (such as muscle paralysis) with the equation, rather than just by averaging the maximum observed effect size at one point in time.

1. Introduction

Regrettably, some pharmaceutical active ingredients are tested in animals also after having gained marketing approval, and not just during the preceding research and development phase. A particularly notorious example is the most toxic known substance — botulinum toxin ("BoNT") — for which the prevailing international standard is the mouse 50% lethal dose ("LD50") assay, progress in the field notwithstanding (Sesardic and Gaines, 2007). I therefore use toxicological results measured with BoNT to illustrate in a nonlimiting fashion this paper main conclusions.

For a valid 50% lethal dose determination, the covered dilution range must encompass the LD50 value. In practice, this means that only around 10% of the employed animals survive the highest and 90% of the animals survive the lowest tested dose, from which they suffer nonetheless. As the number of dilution steps and the number of animals taken per concentration point affect the test method precision (Adler et al., 2010), using sixty (60) animals per LD50 datum is rather common.

The BoNT specific EMA regulations permit use of alternatives. Without prejudice, EP Monograph 2113 ('Botulinum Toxin Type A for Injection') suggests three such possible assays: "*an endopeptidase assay in vitro, an ex vivo assay using the mouse phrenic nerve diaphragm, and a mouse bioassay using paralysis as the endpoint*". The US FDA also does not prescribe any particular test for BoNT potency testing. It merely specifies the performance criteria that any BoNT potency assay must meet to qualify as an alternative to LD50 determination (Adler et al., 2010).

The recent expert meeting on the current scientific and legal status of alternative methods to the LD50 determination for botulinum neurotoxin potency testing identified three desiderata in BoNT potency testing: "Refinement, Reduction, and Replacement" (Adler et al., 2010). This short contribution demonstrates that one can meet the first two of these goals by simply applying a known mathematical analytical method to the recorded paralysis data. As an added benefit, the method allows a *robust parameterisation* of such data, and of other experimental results having similar time dependence. By varying the underlying mathematical expression, if necessary, one can apply the method to other kind of temporal profiles.

The presently established, BoNT-related paralysis tests include the digital abduction score ("DAS") assay in mice (Sugiyama et al., 1975; Aoki, 1999, 2001, 2002) or rats (Broide et al., 2013), the mouse flaccid paralysis assay (Sesardic et al., 1996; Jones et al., 2006), and the rat muscle force assay (Pickett et al., 2008). In this note, I focus on DAS, as the oldest and the most extensively published amongst these assays. However, the paper conclusions and recommendations apply to other local tests as well, *mutatis mutandis*.

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Arguably, one can describe most, if not all, *local pharmacodynamics* with a simple (here: bi-exponential, or Bateman's) equation. To check the supposition, one applies the putative data describing equation to an existing, robust, data set. Subject to good agreement (cf. Fig. 1), one can then use the equation for further statistical evaluation of the resulting model parameters, in the stead of analysing the underlying original data. I posit that this generally diminishes the needed number of experimental animals, and vindicate the conclusion using some original, new DAS data (cf. Fig. 2).

2. Materials and methods

All independently measured DAS results reported in this work involved a commercially available BoNT/A neurotoxin and were fully compliant with the Animal Welfare Act.

In a DAS assay, the experimentalist suspends a tested animal, if needed repeatedly, to elicit a characteristic startle response, in which the animal extends its hind limbs. Simultaneously, the animal abducts its hind digits to the extent affected by a locally applied BoNT (Sugiyama et al., 1975; Aoki, 1999). The commonly used DAS scoring (on a five-point scale: 0 = normal to 4 = maximum reduction in digit abduction) involves one hind limb, for which (treatment blinded) observer(s) assign(s) an (integer) abduction value (for illustrations see Aoki, 2001 and Broide et al., 2013). Experimentalists experienced in the assay can also treat and reliably assign DAS for both hind limbs with 0.25 unit resolution, animal survival permitting.

DAS determination normally starts one day ($t \sim 24$ h) after a BoNT injection into a peripheral muscle, which is in mice *Musculus gastrocnemius*. Further DAS readings follow (typically, on the days 2, 3, 4, 7, 9, 11, 14, 16, 18, 21, see Fig. 1 for examples). The primary endpoint is the maximum observed DAS value, which is in mice normally seen at $t = 2 \pm 1$ days. Some researchers (Ruegg et al., 2009) also determine the day on which DAS undercuts a certain threshold (e.g., DAS ≤ 0.4), to quantify BoNT bioactivity duration. The standard deviation ("*SD*") of both results decreases only moderately, with a square root of animal number per test group, *n*, as only one or two experimental read-outs contribute to the chosen endpoint. Pertinent publications imply that $n = 10$ provides a decent dose *vs*. effect response curve (Aoki, 1999; Broide et al., 2013). The latter allows assignment of intramuscular 50% effect dose, which the original assay proponents had defined as the dose ensuring DAS = 2 (derived by linear or (semi)logarithmic curve fitting; Aoki, 2001). Such inter- and extrapolation method potentially exploits just some of the available experimental DAS values.

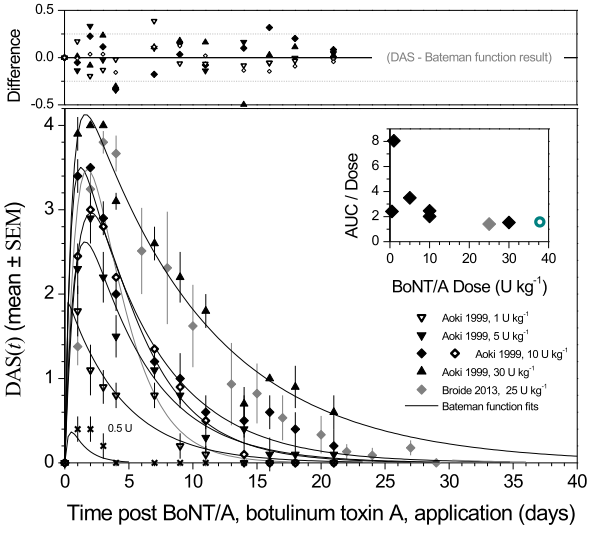

Fig. 1. Lower panel: time ("*t*") dependence of murine *M. gastrocnemius* paralysis caused by an intramuscular injection of various amounts of BoNT/A into mice ($n = 10$). Data points stem from publications of Aoki (1999) and Broide et al. (2013). Curves represent results of the corresponding, individually optimised Bateman's function, defined in eq. (*) with the characteristic parameter values given in Table 1. Inset gives the corresponding areas under the curve (AUC/day kg U^{-1}), calculated by integrating the Bateman's function to from $t = 0$ to 8.6 $t_{d,1/2}$. (Open circle corresponds to the data shown in Fig. 2.) Upper panel: Difference between all individually measured and calculated DAS results.

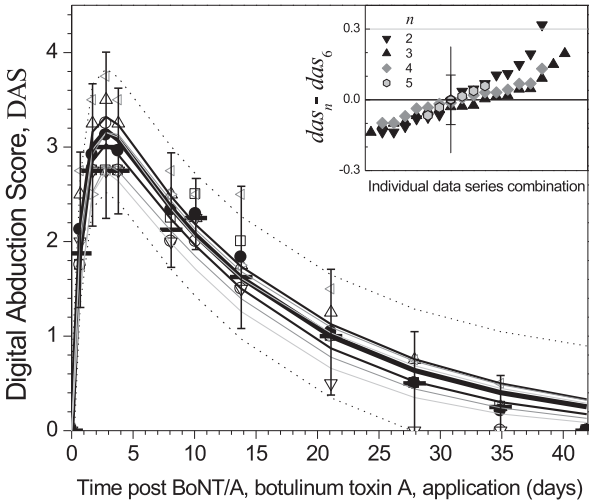

Fig. 2. Time dependence of an individual muscle paralysis, expresssed as Digital Abduction Score (DAS, open symbols, $n = 6$), resulting from an injection of a preparation of the commercially available BoNT/A into murine *M. gastrocnemius*. Horizontal dashes show the median and bullets the average DAS values. Error bars reveal the corresponding 95% confidence interval ("CI_{95}") estimate, identified with 2*SD*. The thick curve illustrates the Bateman's function (e.q. *) result that matches the latter set of values the best. The thin black, grey, and light grey curves define CI_{95} pertaining to $n = 6$, 3, and 2, respectively (using the 'worst' data selections for the latter two, see the text for more detail). The dotted curve defines prediction interval for the full data set ($n = 6$). Inset: Deviation between the optimum *das* value, calculated for $n = 6$, and the *das* values calculated for all possible data subset combinations ($n = 5$, 4, 3, and 2), ordered in sequence of increasing deviation between the two *das* values. The vertical error bar gives 2*SD* averaged around the DAS maximum for $n = 6$. The vertical line gives the corresponding average calculated from the data of Aoki (1999).

Fitting a suitable function to an experimental data set mitigates inherent scattering of the data. It can also elucidate the analysed data meaning, if the fitted function reflects a parsimonious phenomenological model of action. The following line of thoughts justifies searching for a mathematical model that fits well, and is thus suitable for analysing, local effects—including the BoNT induced local muscle paralysis assessed in a DAS assay.

Any externally induced local effect must depend on the inducer local activity (=potency × concentration = the inducer effective strength). The activity onset and decay rates are influential too. If the locally available inducer concentration changes with time, the spatial and temporal characteristics of the change play a role as well, when the changes are significant.

Phenomenologically, this resembles the case of a substance entering/gaining activity and leaving/loosing activity in a body compartment. To analyse a local effect, one may therefore use the simplest and the most popular expression for modelling drug pharmacokinetics in blood, the bi-exponential Bateman's function: $C(t) = C'[\exp(-k_e t) - \exp(-k_a t)]$ (Bateman, 1910).

In pharmacokinetics studies, the Bateman's function quantifies the time course of a first-order invasion (rate constant k_a) to and a first-order elimination (rate constant k_e) from a one-compartment body model, where $C' = (\gamma Dose/V)/(1 - k_e/k_a)$, giving $C' = (\gamma Dose/V)$ for $k_e << k_a$; the ratio $(\gamma Dose/V)$ hereby gives the effective drug concentration, with $\gamma \leq 1$ (Garrett, 1994). When $k_e > k_a$ the so-called "flip-flop" occurs, and the values of the evaluated rate constants then interchange. The condition $k_e >> k_a$ is also met for the DAS data sets explored herein. The two rates therefore effectively