A simple method to determine the elimination half-life of drugs displaying noncumulative toxicity

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ABSTRACT

The pharmacokinetic characterization of a drug, especially the determination of its biological half-life, is an essential step during the early phases of drug development. An adequate half-life is amongst the many properties needed for selecting a drug candidate for clinical trials. Conversely, drug candidates possessing inadequate half-lives may be modified or eliminated from the drug discovery pipeline altogether. Several methods exist for determining the half-lives of drugs, namely HPLC, fluorescence assays, radioassays, radioimmunoassays, and elemental mass spectrometric assays. However, all these techniques are resource and labor-intensive, and cannot be used for the high-throughput half-life determination of hundreds of drug candidates. Here, we describe TOX_{HL}: a simple technique to determine the half-lives of compounds displaying noncumulative toxicity. To calculate the half life, TOX_{HL} only relies on the survival outcomes of three experiments performed on an animal model: an acute toxicity experiment, a cumulative toxicity experiment, and a multi-dose experiment at different dosing intervals. As a proof of concept, we use TOX_{HL} to determine the peritoneal half-life of $\Omega 76$, an antimicrobial peptide. The half-life of $\Omega 76$ determined by TOX_{HL} is in good agreement with results from a standard mass spectrometric method, validating this approach.

1 Introduction

The biological half-life of a drug is the time required for half the drug to be eliminated from an organism, provided the rate of removal can be modeled as an exponential function¹. Typically, the kidneys eliminate hydrophilic drugs from circulation, while the liver acts on hydrophobic compounds, adding polar groups for later renal excretion². Drugs undergoing hepatic metabolism may also enter bile and be excreted through feces³. Proteolysis, the degradation of proteins into smaller peptides or amino acids, is also an important process for the elimination of therapeutic peptides⁴. Secondary drug removal also occurs through respiration, through the salivary glands⁵, mammary glands⁶, lacrimal glands⁷, skin⁸, and hair⁹.

A successful drug candidate should demonstrate adequate bioavailability¹⁰. The drug should remain at its site of action at a sufficient concentration, and for a sufficient time, for therapeutic effects to occur. During the early phases of drug development, measuring the half-life of a drug candidate in rodents is usually performed. Drug candidates possessing an adequate half-life for therapeutic effects to occur can enter later phases of development. Drug candidates possessing an insufficient half-life may need to be chemically altered and retested before entering later phases of development, or discarded altogether.

Numerous techniques exist for determining the concentrations and half-life of drugs. High performance liquid chromatography (HPLC) has been used to separate and characterize numerous small-molecule drugs based on molecular weights^{11,12}. Fluorescence assays can track a drug based on innate fluorescence or that of an attached fluorophore^{13,14}. Radioisotopes (typically ¹⁴C, but also ³²P, ³⁴S, etc.) incorporated into drug molecules allow them to be systemically tracked via scintillation counting¹⁵. Radioimmunoassays are competitive inhibition techniques where an unlabelled drug competes for antibody binding with its radioactive counterpart (typically labeled with ³H, ⁷⁵Se, ¹²⁵I, etc.)¹⁶⁻¹⁸. Labeling a drug with a biologically rare element (such as Se) and tracking it using elemental mass spectrometry is also possible¹⁹. However, all these methods are labor-intensive,

require sophisticated instruments, involve complicated chemical syntheses, or require handling radioisotopes. Such techniques are therefore impractical for the high-throughput screening of half-lives (100+ drugs at a time).

Here, we describe TOX_{HL} : a simple method for the determination of the half-lives of drugs based on survival outcomes alone. TOX_{HL} involves 3 experiments: an acute toxicity experiment, a cumulative toxicity experiment, and a multi-dose experiment at different dosing intervals. Data obtained from these 3 experiments was sufficient to calculate the peritoneal half-life of an antimicrobial peptide ($\Omega 76$ ¹⁹) in BALB/c mice. The half-life of $\Omega 76$ calculated here was in good agreement with previous pharmacodynamic data obtained using mass spectrometry.

2 Results

2.1 Defining and determining (non)cumulative toxicity

Here, we define and contrast the terms *acute toxicity*, *maximum nonlethal dose*, *cumulative toxicity*, and *noncumulative toxicity*. We use two antimicrobial peptides: $\Omega 76$ ¹⁹ and Pexiganan^{20,21}, to demonstrate these concepts. $\Omega 76$ is a designed antimicrobial peptide effective against systemic infections of resistant *A. baumannii*. Here, $\Omega 76$ is used as an example drug that displays noncumulative toxicity and is compatible with TOX_{HL} . Pexiganan is a broad-spectrum peptide that is effective at treating infected wounds. Here, Pexiganan is used as an example drug that displays cumulative toxicity and is incompatible with TOX_{HL} . It should be noted that the toxicity results for Pexiganan described here should not be used to assess its suitability as a topical agent, a role where systemic toxicity is unimportant.

Acute toxicity comprises the lethal effects of a single dose of a drug, rather than the combined lethal effects of multiple doses of a drug. BALB/c mice (6-8 weeks, 20 g) were injected intraperitoneally with varying doses of $\Omega 76$ and Pexiganan, at 2-fold concentration increments. 5 mice per concentration cohort were used. All mice were observed for 5 days for mortality. Mice treated with both $\Omega 76$ and Pexiganan display mortality that increases in a dose-dependent manner. These results are depicted in Figure 1A. The LD_{50} values for $\Omega 76$ and Pexiganan were determined to be 170 mg/kg and 60 mg/kg respectively¹⁹, using linear (non-logarithmic) interpolation. However, for TOX_{HL} , determining the *maximum nonlethal dose* (MND) rather than the LD_{50} is more important. We define the MND as the highest concentration of a drug, administered as a single intraperitoneal dose, that does not cause any mortality in mice observed for 5 days. Here, the MND values for $\Omega 76$ and Pexiganan are 32 mg/kg and 64 mg/kg respectively (Figure 1A, shown in arrows). These results are consistent with our previously reported values, which were performed on smaller cohorts¹⁹.

Cumulative toxicity comprises the lethal effects of multiple doses of drug when injected intraperitoneally at MND concentrations. 10 BALB/c mice (6-8 weeks, 20 g) were treated with 11 doses of Pexiganan, administered at the MND (32 mg/kg) at 12 hour intervals, over the course of 5 days. Despite Pexiganan never exceeding the MND concentration, 100% mortality was observed. This indicates that a single dose of Pexiganan causes a small amount of damage in mice, which is insufficient to cause mortality. However, this damage is not reversed within the dosing interval timespan (12 hours), and additional doses only serve to incrementally increase the total damage caused to mice, until a threshold is reached and death occurs. Autopsies indicate that the mechanism of cumulative toxicity for Pexiganan may involve cumulative damage to peritoneal organs culminating in intestinal inflammation (Figure 2B), which is absent in mice treated with $\Omega 76$ (Figure 2A).

Noncumulative toxicity can be defined in contrast to cumulative toxicity. For a drug to display noncumulative toxicity, multiple doses injected intraperitoneally at MND concentrations should cause no mortality. 10 BALB/c mice (6-8 weeks, 20 g) were treated with 11 doses of $\Omega 76$, administered at the MND (64 mg/kg) at 12 hour intervals, over the course of 5 days. $\Omega 76$ never exceeded the MND concentration, and consequently neither intestinal inflammation nor mortality was observed (2A, Figure 1B). Our previous work¹⁹ further confirmed that $\Omega 76$ does not cause nephrotoxicity or hepatotoxicity in similarly treated mice. TOX_{HL} can only be used for drugs displaying noncumulative toxicity, which makes performing the acute and (non)cumulative toxicity experiments depicted in Figure 1A,B essential.

2.2 Experiments and input terms required for TOX_{HL}

Once noncumulative toxicity for a given drug is established (Figure 1B), TOX_{HL} relies on the results of 2 experiments: acute toxicity experiments (Figure 1A) and multi-dose toxicity experiments (Figure 1C).

Multi-dose toxicity experiments are required to determine Δt_M : the dosing interval that causes mortality, and $S_{\Delta t_M}$: the

Figure 1. All experiments required to determine the elimination half-life using TOX_{HL} . BALB/c mice (6-8 weeks, 20 g) were used for all experiments. **(A)** Acute toxicity experiments to determine survival vs. concentration of drug injected intraperitoneally. Determining the maximum nonlethal dose (MND), which is the highest concentration of a drug that causes no mortality, is essential for later noncumulative toxicity experiments. Here, the MND for Pexiganan is 32 mg/kg (blue arrow), and the MND for $\Omega 76$ is 64 mg/kg (red arrow). **(B)** Multi-dose (non)cumulative toxicity determination experiments. Here, mice were intraperitoneally injected with 11 doses of drug (at the MND concentration), at 12 hour intervals over the course of 5 days. All mice treated with Pexiganan died, indicating cumulative toxicity. All mice treated with $\Omega 76$ survived, indicating noncumulative toxicity. The difference between the survival outcomes of the $\Omega 76$ and Pexiganan cohorts are statistically significant ($p=7e-3$, Fisher's test). **(C)** After $\Omega 76$ displayed noncumulative toxicity, we determined the dosing interval (Δt) between MNDs at which mortality is first observed. 5 doses at the MND were injected intraperitoneally at Δt of 15, 7.5 and 3 minutes. 67% survival (33% mortality) was observed a $\Delta t = 3$ minutes (Δt_M , highlighted in yellow). The expected concentration of $\Omega 76$ responsible for 67% survival was calculated from panel A using linear interpolation (non-logarithmic), and found to be 135.11 mg/kg. Note that a separate set of similar experiments was described in our previous work¹⁹, but these experiments were only used to assess the toxicity of $\Omega 76$.

Figure 2. Peritonea of BALB/c mice (6-8 weeks, 20 g) treated with Ω 76 and Pexiganan for (non)cumulative toxicity experiments. **(A)** Peritoneum of a mouse treated with 11 doses of 64 mg/kg Ω 76 administered at 12 hour intervals over 5 days, and euthanized immediately after the last dose. All peritoneal organs appear intact. **(B)** Peritoneum of a mouse treated with 32 mg/kg Pexiganan administered at 12 hour intervals until mortality occurred. Here, intestinal inflammation is clearly visible (yellow arrow) and may be the cause of cumulative toxicity.

Figure 3 can also be described mathematically, using Equation-set 1:

$$\begin{aligned}
 N_0 &= N_k & N_1 &= N_0 e^{-\lambda \Delta t_M} \\
 N_2 &= N_1 + N_k & N_3 &= N_2 e^{-\lambda \Delta t_M} \\
 N_4 &= N_3 + N_k & N_5 &= N_4 e^{-\lambda \Delta t_M} \\
 N_6 &= N_5 + N_k & N_7 &= N_6 e^{-\lambda \Delta t_M} \\
 & & N_8 &= N_7 + N_k \\
 \text{Note: } T_{1/2} &= \frac{\log_e 2}{\lambda}
 \end{aligned} \tag{1}$$

Equation-set 1 can be simplified as described in Equation-set 2.

$$\begin{aligned}
 N_8 &= N_7 + N_k \\
 N_8 &= N_6 e^{-\lambda \Delta t_M} + N_k \\
 N_8 &= (N_5 + N_k) e^{-\lambda \Delta t_M} + N_k \\
 &\vdots (\text{expanding \& simplifying}) \\
 \frac{N_8}{N_k} &= e^{-4\lambda \Delta t_M} + e^{-3\lambda \Delta t_M} + e^{-2\lambda \Delta t_M} + e^{-\lambda \Delta t_M} + 1 \\
 &\text{Let } e^{-\lambda \Delta t_M} = x : \\
 \frac{N_8}{N_k} &= x^4 + x^3 + x^2 + x + 1 \\
 &\vdots (\text{simplifying geometric series}) \\
 \frac{N_8}{N_k} &= \frac{1 - x^5}{1 - x}
 \end{aligned} \tag{2}$$

$S_{\Delta t_M}$ (67%) mice survive at concentration N_8 (Figure 1C, highlighted). Using linear (non-logarithmic) interpolation, $S_{\Delta t_M}$ survival occurs at a single-dose $\Omega 76$ concentration of 135.11 mg/kg (Figure 1A, highlighted). Once the value of N_8/N_k is known, Equation-set 2 can be solved for x . $T_{1/2}$ can be calculated from x using Equation-set 3.

$$\begin{aligned}
 e^{-\lambda \Delta t_M} &= x \\
 \therefore \lambda &= -\frac{\log_e(x)}{\Delta t_M} \\
 T_{1/2} &= \frac{\log_e(2)}{\lambda} \\
 \therefore T_{1/2} &= -\log_e(2) \times \frac{\Delta t_M}{\log_e(x)}
 \end{aligned} \tag{3}$$

For $\Omega 76$, $N_8/N_k = 135.11/64 = 2.11$, $x = 0.55$, and $T_{1/2} = 3.48$ minutes.

Note that the description of TOX_{HL} provided above describes equations for a 5-dose cumulative toxicity experiment (Figure 1C). The solution provided in Equation-set 2 can easily be modified for an n -dose cumulative toxicity experiment (Equation 4, where $n \geq 2$).

$$\frac{N_8}{N_k} = \frac{1 - x^n}{1 - x} \tag{4}$$

All experiments and calculations required to calculate $T_{1/2}$ using for TOX_{HL} are provided in Figure 4. A python script that calculates $T_{1/2}$ using TOX_{HL} is provided on GitHub (https://github.com/preetham-v/TOX_HL) and is made available as a webserver (<http://proline.biochem.iisc.ernet.in/toxhl/>).

Figure 4. All experiments and calculations used by TOX_{HL} to calculate $T_{1/2}$ are provided in order. (1): Perform the acute toxicity experiment (Figure 1A). (2): obtain N_k value from acute toxicity experiment. (3): Use N_k to guide the dosing concentration for the multi-dose toxicity experiment (Figure 1B). (4): Obtain values for Δt_M and $S_{\Delta t_M}$ from the multi-dose toxicity experiment. (5): Calculate N_8 concentration by looking at the mortality corresponding to $S_{\Delta t_M}$ in the acute toxicity graph. (6): Calculate x using the values of N_k and N_8 , using Equation-set 2. (7,8): Use x to calculate λ and $T_{1/2}$ using Equation-set 3.

2.4 Validating TOX_{HL} using data from a standard pharmacokinetic assay

Our previous work involved the pharmacokinetic characterization of $\Omega 76$ in mice¹⁹. 70 mg/kg N selenomethionine-labeled $\Omega 76$ (Nselmet- $\Omega 76$, the molar equivalent of 64 mg/kg unlabeled $\Omega 76$) was injected intraperitoneally into BALB/c mice (6-8 weeks, 20 g), and withdrawn at different time intervals using cardiac punctures. We observed that Nselmet- $\Omega 76$ reached a serum concentration maxima (C_{max}) at 4.59 minutes post-injection. This indicates that the peritoneal elimination half-life of $\Omega 76$ is within the range of $0 \rightarrow 4.59$ minutes, and is in good agreement with the TOX_{HL} value of 3.48 minutes.

3 Discussion

In this work, we described TOX_{HL} , using which the half-life of a drug can be determined based on the survival outcomes of 3 simple experiments performed on mice. TOX_{HL} is cost-effective, easy to perform, and requires no instruments or special reagents. We described and validated TOX_{HL} using an example antimicrobial peptide ($\Omega 76$). Using TOX_{HL} , the peritoneal half-life of $\Omega 76$ in mice was found to be 3.48 minutes. This value was in good agreement with the peritoneal half-life range of $0 \rightarrow 4.59$ minutes, calculated using a conventional ICP-MS technique that tracked selenium. A python script (https://github.com/preetham-v/TOX_HL) and webserver (<http://proline.biochem.iisc.ernet.in/toxhl/>) implementing TOX_{HL} have been made available to the community.

TOX_{HL} can be modified in many ways to meet a user's requirements. We have used 5 intraperitoneal doses (n) to determine M and S_M (Figure 1C). However, any number of doses ($2 \leq n < \infty$) may be used. S_M will increase with n , which is advantageous as it eliminates time-errors when dosing at a small n . However, the amount of drug used will increase with n , necessitating the synthesis/purchase of greater quantities. The total volume of drug injected intraperitoneally will also increase. Extreme values of n (for example, 20 doses of 200 μ L each), would cause abdominal distention in mice, altering the elimination kinetics. Larger n should therefore be accompanied by smaller injection volumes. TOX_{HL} models first order elimination kinetics (3). For exceptional drugs following non-exponential kinetics, a user can replace the exponential equations in Equation-set 1 with decay equations of their choosing. TOX_{HL} can also be used to calculate the bloodstream half-life of drugs, simply by performing intravenous rather than intraperitoneal injections in mice.

It should be noted that TOX_{HL} was only validated using a single molecule ($\Omega 76$). Additional experiments using more molecules would help further validate our approach. TOX_{HL} can only be used for drugs possessing noncumulative toxicity, which has to

be tested prior to half-life calculation (Figure 1B). TOX_{HL} requires a larger number of mice in comparison to conventional methods. Consequently, a larger amount of drug is required for these tests.

Nevertheless, we expect TOX_{HL} to be especially useful for the half-life determination of a library of compounds, created from a parent compound displaying noncumulative toxicity. For example, a 100-1000 member peptide library, created using saturation mutagenesis of a parent antimicrobial peptide, could quickly and easily be assayed using TOX_{HL}.

4 Methods

For all experiments described in this work, BALB/c mice (6-8 weeks, 20 g) were housed at the Central Animal Facility (CAF, IISc) and fed *ad libitum* with pellet feed and water. All animal experiments were approved by the Institutional Animal Ethics Committee, IISc (Project No. CAF/Ethics/550/2017). A detailed description of all methods used is provided in the results section. However, it should be noted that 20 mg/mL stocks for both Ω 76 and Pexiganan were prepared in physiological saline (0.8% NaCl) and stored at -80 °C. Both peptides display poorer solubility in phosphate buffered saline (PBS). These stocks were diluted in saline to obtain the concentration needed for the specific experiment. All intraperitoneal injections were 200 μ L in volume, as larger volumes could alter peritoneal elimination kinetics.

References

- McNaught, A. D. & McNaught, A. D. *Compendium of chemical terminology*, vol. 1669 (Blackwell Science Oxford, 1997).
- Taft, D. R. Drug excretion. In *Pharmacology*, 175–199 (Elsevier, 2009).
- Rollins, D. E. & Klaassen, C. D. Biliary excretion of drugs in man. *Clin. pharmacokinetics* **4**, 368–379 (1979).
- Mathur, D. *et al.* Peplife: a repository of the half-life of peptides. *Sci. reports* **6**, 36617 (2016).
- Gorodischer, R. & Koren, G. Salivary excretion of drugs in children: theoretical and practical issues in therapeutic drug monitoring. *Dev. pharmacology therapeutics* **19**, 161–177 (1992).
- Ito, S. & Lee, A. Drug excretion into breast milk—overview. *Adv. drug delivery reviews* **55**, 617–627 (2003).
- Crandall, D. C. & Leopold, I. H. The influence of systemic drugs on tear constituents. *Ophthalmology* **86**, 115–125 (1979).
- Chandrasekaran, S., Bayne, W. & Shaw, J. E. Pharmacokinetics of drug permeation through human skin. *J. pharmaceutical sciences* **67**, 1370–1374 (1978).
- Cone, E. J. Mechanisms of drug incorporation into hair. *Ther. drug monitoring* **18**, 438–443 (1996).
- Andrew, B. & Shargel, L. *Applied Biopharmaceutics & Pharmacokinetics* (Mcgraw-hill Education-Europe, 2015).
- Kauffman, R. E. *et al.* Pharmacokinetics of chloramphenicol and chloramphenicol succinate in infants and children. *The J. pediatrics* **98**, 315–320 (1981).
- Li, J. *et al.* Pharmacokinetics of colistin methanesulphonate and colistin in rats following an intravenous dose of colistin methanesulphonate. *J. Antimicrob. Chemother.* **53**, 837–840 (2004).
- Formelli, F., Pollini, C., Casazza, A. M., di Marco, A. & Mariani, A. Fluorescence assays and pharmacokinetic studies of 4-deoxydoxorubicin and doxorubicin in organs of mice bearing solid tumors. *Cancer chemotherapy pharmacology* **5**, 139–144 (1981).
- Alberts, D. S., Bachur, N. R. & Holtzman, J. L. The pharmacokinetics of daunomycin in man. *Clin. Pharmacol. & Ther.* **12**, 96–104 (1971).
- Heeg, J. F., Hiser, M. F., Satonin, D. K. & Rose, J. Q. Pharmacokinetics of probucol in male rats. *J. pharmaceutical sciences* **73**, 1758–1763 (1984).
- Paxton, J., Rowell, F. & Cree, G. Comparison of three radioligands, selenium-75, Iodine-125, and tritium, in the radioimmunoassay of methotrexate. *Clin. chemistry* **24**, 1534–1538 (1978).
- Back, D. *et al.* The effects of ampicillin oral contraceptive steroids in women. *Br. journal clinical pharmacology* **14**, 43–48 (1982).
- Michiels, M., Hendriks, R. & Heykants, J. A sensitive radioimmunoassay for fentanyl. *Eur. J. Clin. Pharmacol.* **12**, 153–158 (1977).

19. Nagarajan, D. *et al.* ω 76: A designed antimicrobial peptide to combat carbapenem- and tigecycline-resistant *Acinetobacter baumannii*. *Sci. advances* **5**, eaax1946 (2019).
20. Ge, Y. *et al.* In vitro antibacterial properties of pexiganan, an analog of magainin. *Antimicrob. agents chemotherapy* **43**, 782–788 (1999).
21. Lamb, H. M. & Wiseman, L. R. Pexiganan acetate. *Drugs* **56**, 1047–1052 (1998).

Author contributions statement

D.N. conceived and designed TOX_{HL}, and performed all experiments. P.V. created a python script and webserver implementing TOX_{HL}. C.T. and A.D. co-performed mouse experiments. D.C. and N.C. coordinated the study, planned experiments, and provided resources. All authors reviewed the final draft of the manuscript.