

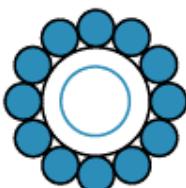
IN VITRO TOXICOLOGY AND HOLLOW FIBER BIOREACTORS

BY JOHN J.S. CADWELL

The recent introduction of hollow fiber bioreactor cartridges represents an important advancement in the field of *in vitro* toxicology. Most toxicologists believe *in vitro* testing methods are a useful, time and cost effective tool for drug discovery but it is generally accepted that many of the available tests are not effective for examining both time and concentration, and do not closely mimic human kinetics. This is because they do not properly take into account pharmacodynamic actions (what a drug does to the body) and pharmacokinetic actions (what a body does to the drug). Recently, with the use of hollow fiber bioreactor cartridges, this has changed. Hollow fiber bioreactor cartridges have surpassed standard *in vitro* toxicology methods by mimicking changes in drug concentration over time as they would occur in humans. The numerous advantages of the hollow fiber bioreactors applied to *in vitro* toxicology are discussed more fully below as they relate to antibiotics and other drugs.



Pharmacokinetic-pharmacodynamic (PK-PD) principles for antibiotics were initially identified in the 1940s and 1950s by Dr. Harry Eagle. Using animal models he identified the time dependence of penicillin bactericidal activity (ref), the concentration dependence of streptomycin and bacitracin activity and the mixed pattern for tetracyclines. These data were applied to the clinic where it was found that continuous infusion of penicillin resulted in the most rapid cures while avoiding drug related toxicities. The most effective dosage regimens for concentration dependant antibiotics like streptomycin were those that resulted in the highest initial peak concentrations. Out of this work were derived the first principles of antibiotic action, that both time and concentration play an important role in antibiotic effectiveness.



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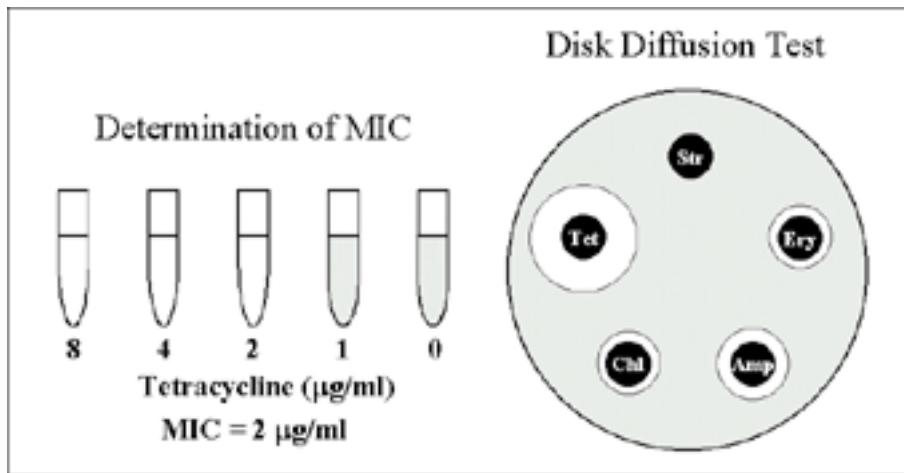
In the case of antibiotic development, it is critical to define both the time and concentration interactions of the drug with the target organisms as early as possible. The drug's spectrum of activity and potency against different strains of bacteria also needs to be determined. Traditional assays for susceptibility include the disc test, the E-test and broth dilution assay. These provide a quantitative measurement of the drug's potency and are defined as the minimum inhibitory concentration or MIC. The MIC is the lowest concentration of the drug that prevents a bacterial inoculum from growing to visibly detectable levels. The 3 most common PK-PD measures derived from the MIC are the duration of time a drug concentration remains above the MIC, the ratio of the maximal drug concentration to the MIC and the ratio of the area under the concentration time curve at 24 hours to the MIC. MIC is the first and most important specification for an antibiotic that helps to define its therapeutic potential. However the MIC tells us nothing about:

- 1) Whether an antibiotic is bacteriostatic or bactericidal,
- 2) Whether this activity is time dependant or dosage dependant
- 3) The rate of bacterial killing
- 4) Whether the drug exhibits a post-antibiotic effect when it falls below the MIC
- 5) What parameters of drug exposure most influence efficacy
- 6) The most effective pharmacodynamic targets for optimal dosing and
- 7) Which dosage profiles either prevent or facilitate the development of resistance.

Current assays for the determination of antibiotic efficacy are not able to examine the potential for the development of resistance since antibiotic resistance develops over time. A time related assay capable of examining the effects of antibiotic pharmacokinetics on a population of organisms large enough to reveal the emergence of resistance is clearly required.

No variables (Time and Concentration are fixed)

Current methods for performing *in vitro* static assays for toxicological results and efficacy are relatively historic in nature. They consist of discs of paper soaked in differing concentrations of the drug in question placed onto petri dishes containing agar and the target organism. The size of the ring of inhibition of bacteria growth can be used to calculate the minimum inhibitory concentration. The E-test is a refinement of this assay where a plastic strip is coated with a gradient of the drug and then placed onto agar containing the target bacteria. There are two types of dilution assays that can be used to determine the MIC of a particular antibiotic. They are the broth macrodilution assay and the broth microdilution assay. The difference between these two assays is the volume of broth used and how the assay can be quantified. For the macrodilution assay 15 ml test tubes are used and the antibiotic is serially diluted over a wide range. The MIC is determined by the visual detection of bacterial growth inhibition. The microdilution assay is performed in a similar manner but a microtiter plate is used instead of test tubes and the volumes are typically at least 100x smaller. The use of microtiter plates allows quantitation to be easily performed in an automatic plate reader.



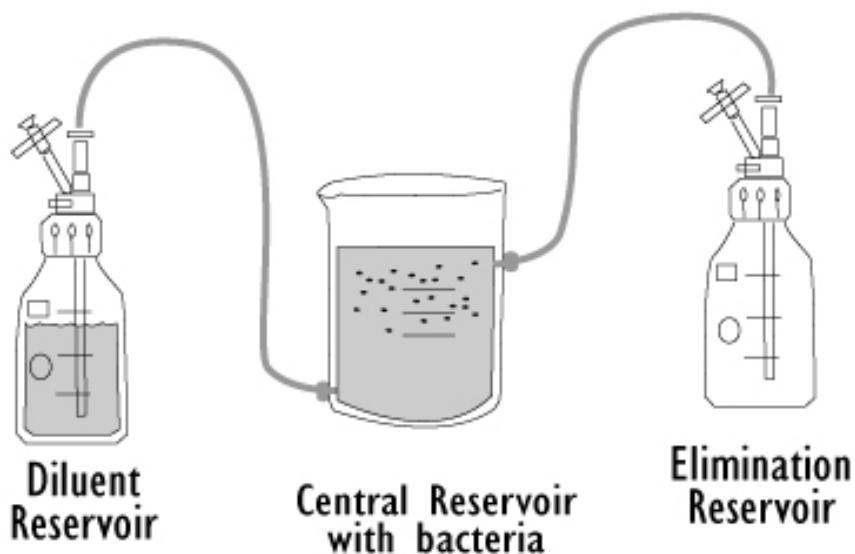
One variable (Time is variable, Concentration is fixed)

Advancement over the MIC assay is the static time kill assay. In this assay cultures of bacteria are exposed to static concentrations of an antibacterial agent over a defined period of time. Unlike the MIC assay, samples are taken at intervals of time and actual counts of viability are taken. The static kill time assay does provide some information about the rate of efficacy but the static concentrations of antibacterial agents are still lacking in clinical relevancy.

Two variables (Time and Concentration are both variables)

Animal models of infection

In vitro pharmacodynamic models, MIC and static time-kill assays provide good methods for examining pharmacodynamics. They lack the complex interactions between host and drug that is present in animal and eventually human clinical models. Some of the advantages of animal models include 1) the ability to evaluate clinical efficacy in addition to antibacterial pharmacodynamics 2) examination of the interaction between drug therapy and host immune response and 3) ability to examine the interaction of drug with serum proteins. However, there are also problems associated with animal models. It is expensive to maintain animal colonies and time consuming to perform the experiments. Many infections simply cannot be reproduced in animals. Experimental design can be subject to regulatory approval and the experiments need to be carried out in a humane fashion. Drug kinetics may be very different than that found in humans. The total bacterial load is generally small so development of resistance may not be revealed. The kinetics of different routes of administration cannot be easily compared. Depending upon the organism and species this approach can have varying validity when compared to the human response. There is a requirement for a method that can span the gap between placement of paper discs onto agar and the infusion of drug into an animal that can precisely mimic the human pharmacodynamic and pharmacodynamic profile. The capability to control both time and concentration of drug exposure to an organism is required.



The diagram above shows the one-compartment model.

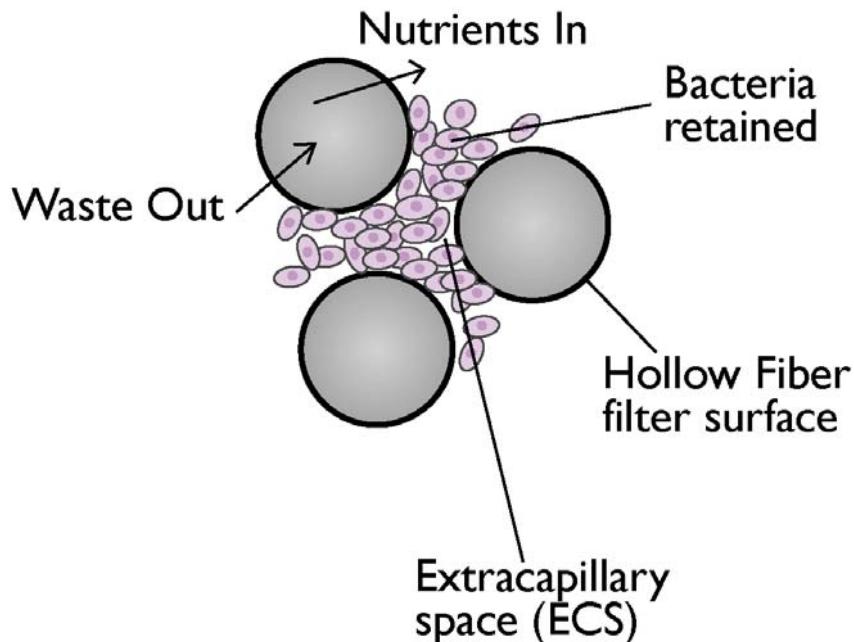
One-Compartment Module

To address some of these issues and present the test organism with a dynamic concentration of drug that more closely mimics *in vivo* pharmacokinetics the one-compartment model was developed. This model consists of a central reservoir containing the organisms, a diluent reservoir and a waste reservoir. Peak concentrations of drug are added to the central reservoir and the elimination profile is mimicked by the addition of drug free diluent to the central reservoir and removal of an equal volume of drug (and organism) containing medium into the waste reservoir. The versatility of this model allows for the modeling of nearly any desired elimination half-life within the limits presented by the somewhat large volume of the central reservoir and the peristaltic pumps used to add and remove diluent. One disadvantage of the one-compartment model is the loss of organism during the dilution of the drug since the main parameter measured is the number of viable organisms. Another disadvantage is that only the elimination profile can be modeled, not the absorption profile. The size of the central reservoir precludes extremely rapid changes in drug concentration due to diffusional limitations. Another disadvantage is the biohazard presented while working with antibiotic resistant and pathogenic organisms in such an open system.

Hollow Fiber Cartridge Two-Compartment Models

To address these shortcomings the two-compartment *in vitro* pharmacokinetic model utilizing hollow fiber bioreactors was developed. Hollow fiber bioreactors are modules containing thousand of hollow fibers; small tubular filters 200 microns in diameter. The fibers are sealed at each end so that liquid entering the ends of the cartridge will necessarily go through the insides of the fibers. The pore size of the fibers is selected to retain the organisms while allowing drugs and other small molecule to freely cross the fiber. Bacteria or cells are inoculated on the outside of the fibers, trapped in the space called the extra-capillary space, or ECS. The ECS is defined by

the space outside the fibers but within the cartridge housing. Medium from the central reservoir continuously recirculates through the inside of the fibers providing oxygenation and nutrition support. Small molecules such as drugs, glucose and metabolic waste products can easily cross the fiber while larger bacteria, cells and viruses cannot cross the fiber.



The diagram above shows the cross-section of a hollow fiber bioreactor cartridge.

The design of the two-compartment model is quite similar to the one-compartment model except that the organism to be tested is confined within the small volume of the ECS (20mls), physically separated from the central reservoir by the semi-permeable membrane. The concentration of the drug in the central reservoir equilibrates rapidly with the medium in the ECS containing the organisms, which is relatively small in volume. The volume of the central reservoir can be adjusted to permit rapid changes in drug concentration.

Hollow fiber cartridges were first used by Zinner (1) and Blaser (2) for bacterial testing in the 1980's and by Billelo et al (3) for anti-HIV drugs in the 1990's. Hollow fiber cartridges offer the advantages of having an extremely high surface area to volume ratio, in excess of 150 cm² per milliliter of volume, providing rapid and uniform distribution of the drug within the ECS. Several different types of hollow fiber polymers are commercially available to allow for compatibility with drugs of different chemistries.

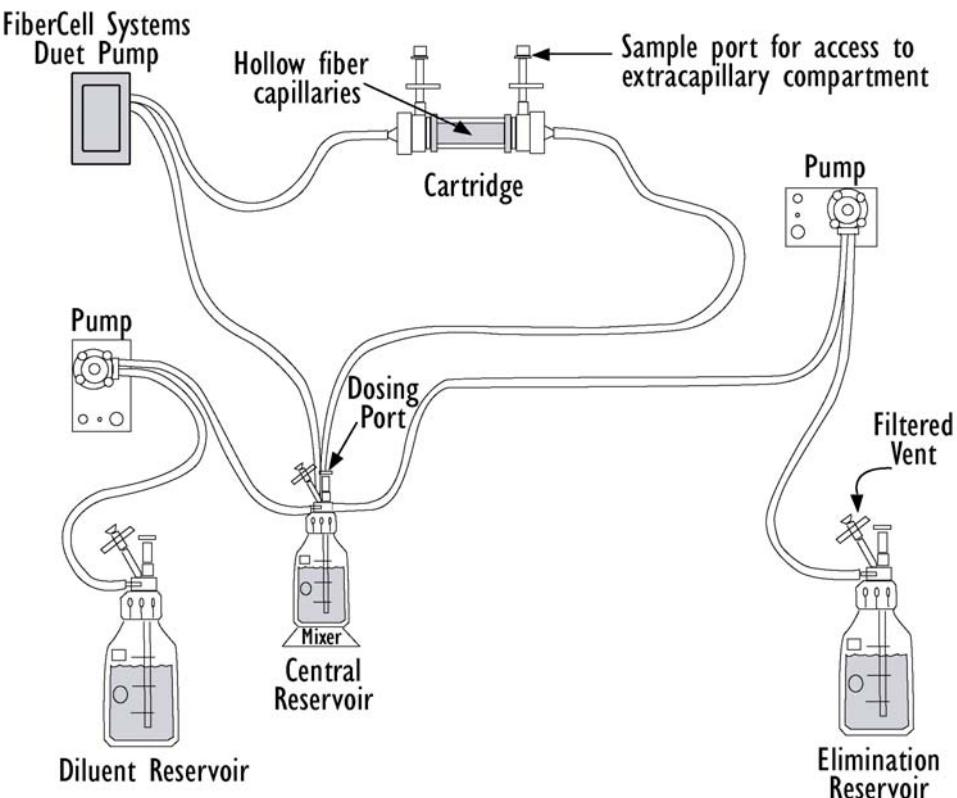


Diagram above shows the two-compartment hollow fiber model.

The advantages of the two-compartment hollow fiber system are numerous. The target bacteria are contained within a very small volume, 10-20 mls, so they are at a similar concentration to *in vivo* infections and the drug can equilibrate rapidly within the compartment. Representative samples can be taken easily without significantly affecting the bacteria population. Drug resistant, highly pathogenic and highly biohazardous organisms are safely contained in a sealed environment. Large numbers of organisms can be tested in one experiment so the emergence of drug resistance is easily quantified. Both absorption and elimination kinetics of the drug being tested can be precisely and independently controlled. The kinetics of multiple drugs can also be controlled so drug/drug interactions and combination therapies can readily be examined. The system is compact enough that multiple cartridges can be conveniently manipulated in a relatively small space.

The hollow fiber bioreactor based two-compartment model gained widespread acceptance as a result of the work by Drusano et al with mammalian cells infected with HIV. (Antimicrobial Agents 1994) The testing of anti-viral agents is especially problematic as in most cases, such as HIV, there are no appropriate animal models and it can be difficult to culture sufficient cells to support viral growth. At the time of this work hollow fiber bioreactors were in common use for the production of monoclonal antibodies and recombinant proteins and had also been used for the production and growth of HIV. The two-compartment model utilizing hollow fiber bioreactor cartridges has been used for antibiotic testing against many organisms including Klebsiella

pneumoniae, MRSA Staph aureus, pseudomonas aeruginosa and tuberculosis. Another area of great interest is in the determination of optimal antibiotic dosages for potentially weaponized organisms. In the event of a bioterrorist incident using modified organisms the hollow fiber method could rapidly determine the most effective antibiotics and their optimum dosage profiles. Lister and Wolter demonstrated the power of the model for looking at antibiotic combination therapies. In their 2005 paper they demonstrated that the combination of levofloxacin and imipenem prevented the emergence of drug resistance from clinical isolates of Pseudomonas aeruginosa even when subpopulations resistant to both drugs are present. Drusano et al (2007) determined the relationship between garenoxacin exposure and emergence of quinalone resistant subpopulations and found that different targets for the area under the concentration-time curve over 24h/MIC ratio were required for different bacteria. The data from the hollow fiber two-compartment model can be critical for determining dosage profiles and drug combinations that can prevent the emergence of resistant strains. In the case of tuberculosis data have been generated showing that ciprofloxacin and Isoniazid's antibiotic activity ceases not because of depletion of organisms in log growth phase but because of emerging resistance. These results could not have been found through any other type of study.



Example of the hollow fiber bioreactor cartridge setup. Note the large volume of diluent on the outside of the incubator and peristaltic pumps for controlled addition of diluent.

The two-compartment hollow fiber bioreactor system has also demonstrated distinct advantages when compared to previously used static and one-compartment models and can be used to determine optimum dosing schedules as well as revealing the mechanisms of resistance development. Historical *in vitro* methods for evaluating efficacy of antibiotics suffer from two

fundamental shortcomings. The first is that antibiotic concentrations remain static; they are not varied in a dynamic fashion, as they would be when administered *in vivo*. Flux in concentration of antibiotic should reflect the adsorption rate, bioavailability, volume of distribution and excretion rate. None of these parameters can be controlled using current methods. The second shortcoming is the number of organisms exposed to the drug is necessarily limited so mechanisms of resistance cannot be studied effectively. Hollow fiber bioreactors offer a more *in vivo* like way to model physiological processes. Bacteria can be grown at densities and numbers that reflect *in vivo* infection potential. The smaller volume provides more rapid equilibration of drug concentrations and a more uniform growth environment. This high-density culture also supports cell pathogen interactions such as virus infections and parasitic growth that more accurately reflect disease states. Therapeutic modalities utilizing antibiotic, anticancer and anti-parasitic agents depend not only upon a maximum tolerated dosage but the time course of administration, usually of multiple dosages. These can be easily modeled in a hollow fiber system

Hollow fiber technology offers higher levels of reproducible control of both concentration and time of drug exposure in complex growth, infection, treatment, and sampling regimens. This system permits more realistic simulation of *in vivo* drug effects in a dynamically controlled system providing data that more accurately reflects biological responses. They are fully disposable and provide a biosafe environment for the potential testing of drug resistant, weaponized, or genetically modified organisms. The two-compartment model can be a cost effective supplement to the evaluation of clinical efficacy both for existing antibiotics and in the development process for new antibiotics as part of the submission process for FDA approval.

Advantages of Hollow Fiber Bioreactor Cartridges vs. **Static Methods**

- Dosage profiles can be controlled over time
- Mechanisms of resistance can be revealed
- Data is more clinically relevant

Advantages of Hollow Fiber Bioreactor Cartridges vs. the **One-compartment Model**

- Bacterial load remains constant
- Biohazardous organisms safely contained
- Both absorption and elimination curves can be modeled.
- Rapid half lifes can be modeled

Advantages of Hollow Fiber Bioreactor Cartridges vs. **Animal Models**

- High bacterial and viral loads can be tested, dosage profiles that result in resistance will more likely be revealed.
- Absorption, excretion and metabolic profiles can be more closely modeled on the human half-life.

- Not all animals can be infected with the organism of interest.
- Extreme doses can be modeled
- Combination therapies can be easily controlled and tested
- No animal testing protocols, restrictions.
- Less expensive
- Closed, bio-safe system for pathogens
- Easier to test multiple pathogens with the same drug
- Larger N per experiment (cartridges in series)
- Bacteria, viruses, cancer and parasites can be tested.
- Dosage and metabolic profiles can be more precisely controlled.

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