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Tobramycin Disposition in the Lung Following Airway Administration

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TOBRAMYCIN DISPOSITION IN THE LUNG FOLLOWING AIRWAY ADMINISTRATION

A Dissertation submitted in partially fulfillment of the requirements for the degree of **Doctor of Philosophy** at Virginia Commonwealth University

By

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^{99m}Tc-DTPA	technetium-99 m-labelled diethylene triamine pentaacetic acid
A	the absorbable amount
abs%	percent of total lung dose absorbed at time *t* following inhalation
AGT	artificial glass thorax
ALF	alveolar lining fluid
ANOVA	analysis of variance
A_t/A_0	the fraction of administered dose remaining in the sac
AUC	area under drug serum concentration versus time curve
AUC_∞	area under drug serum concentration versus time curve from time zero to infinity
AUC_{inh}	AUC_{inh} is the total area under the drug blood concentration versus time curve following inhalation
AUC_{0-t}^{sputum}	area under concentration versus time curve for tobramycin in sputum
B	the amount of drug bound to tissue
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
C_{max}	the peak level of drug concentration in the airways

C_{peak}^{sputum}	the "peak" sputum concentration
C_{trough}^{sputum}	the "trough" sputum concentration
CF	cystic fibrosis
CFC	chlorofluorocarbon
CFTR	cystic fibrosis transmembrane conductance regulator
CL	total body clearance
COD	coefficient of determination (or r^2)
D	the administered dose
ELF	epithelial lining fluid
EMIT	enzyme-multiplied immunoassay
F	the fraction of the lung dose that was absorbed following inhalation at time infinity
F_a	the absorbable fraction of each administered dose reaching perfusate following airway administration to the IPRL
F-Na,	sodium fluorescein
F_p	the fraction of each administered dose transferred to the perfusate
g	practical osmotic coefficient of tobramycin
GI	gastrointestinal
HPLC-MS	high performance liquid chromatography–mass spectrometry
IACUC	Institutional Animal Care and Use Committee
IPRL	Isolated Perfused Rat Lung
IV	intravenous
k_{12}(k_{12}')	the association rate constants for binding

$k_{21}(k_{21}')$	the dissociation rate constants for binding
K4	Krebs-Henseleit solution (KHS) with 4% (w/v) BSA
k_a	the apparent first-order rate constant for absorption
$k_{cp}(k_{pc})$	the apparent first-order inter-compartmental transfer rate constants
k_e	the apparent first-order rate constant for dialysis
k_{el}	the apparent first-order elimination rate constant from the central compartment
KHS	Krebs-Henseleit solution
k_{nal}	the first-order rate constant responsible for non-absorptive loss
LLOQ	lower limit of quantification
LOD	limit of detection
LogD (pH 7.4)	logarithm of the distribution coefficient between octanol and water at pH7.4
LogP	logarithm of the partition coefficient between octanol and water for the neutral form
MDI	metered dose inhaler
MIC	minimum inhibitory concentration
MIC_{90}	MIC required to inhibit the growth of 90% of organisms
MSC	model selection criterion
MW	molecule weight
ND	not determined
NE	not estimated
NR	not reported

NS	normal saline
OAT	organic anion transporters
OCT	organic cation transporters
P	the amount of solutes absorbed into the perfusate in the IPRL
P-gp	P-glycoprotein
PK	pharmacokinetic
R	the amount of solutes released into the receiver solution from the dialysis sac
SD	standard deviation
$t_{1/2}$	the half life of the terminal phase of drug serum concentration time profiles or absorption from the lung
TLD	total lung deposited dose
U	the untransferable (unabsorbable) amount of each dose in the IPRL
USP-NF	United States Pharmacopeia and National Formulary
V_c	the apparent volume of the central compartment
V_d	volume of distribution
V_p	the apparent volume of the peripheral compartment
V_β	the apparent volume of distribution
β	the terminal first-order, whole body elimination rate constant (Chapter III); the same term is used as a first-order rate constant in the analysis of IPRL absorption data and/or dynamic data in Chapter IV to represent absorption and/or dialysis of tobramycin after the drug has apparently equilibrated between compartments.

Abstract

TOBRAMYCIN DISPOSITION IN THE LUNG FOLLOWING AIRWAY ADMINISTRATION

By Min Li, M.S.

A dissertation submitted in partially fulfillment of the requirements for the degree of **Doctor of Philosophy** at Virginia Commonwealth University

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Tobramycin disposition following airway administration was evaluated by meta-analysis of human data in the literature and, experimentally, using a realistic ex vivo model, the isolated perfused rat lung preparation (IPRL). Pulmonary bioavailability of inhaled tobramycin in published studies was re-evaluated separately for CF and healthy adults, with the drug's intrinsic pharmacokinetic (PK) parameters obtained from intravenous (IV) studies in the literature. While

large variations in tobramycin's clearance precluded accurate assessment of its bioavailability, the results were indicative of substantial pulmonary absorption, in spite of its hydrophilic and poly cationic properties. To explore its disposition kinetics and mechanisms following airway administration, tobramycin absorption was investigated as a function of dose in the IPRL. The cumulative fraction of the administered tobramycin dose reaching the perfusate versus time, was bi-exponential and dose-dependent, unlike that of the marker solutes fluorescein and mannitol, both of which showed first-order and dose-independent kinetics. A kinetic model that incorporated lung tissue binding (or sequestration) alongside passive absorption was employed successfully to describe the aminoglycoside's disposition in the IPRL following airway administration. Tobramycin's absorption was fast with the first-order absorption rate constants (0.065-0.070 min^{-1}) close to those seen with fluorescein (0.076 min^{-1}), but a dose-, and concentration-dependent slow onset tissue binding prolonged its presence in the rat lung. Binding was confirmed by independent dynamic dialysis experiments using sliced lung prepared from the intact IPRL, immediately following airway administration using an identical technique as that used in tobramycin absorption studies. Dosing solution osmolality and pH had negligible effects on the drug's disposition in the IPRL, when these were investigated over experimental ranges that could be used clinically. While tobramycin itself was found to accelerate mannitol's absorption, and thus affect airway epithelial integrity when administered at high doses, the effect was undetectable at a dose level in rat lungs that was believed to produce airway concentrations corresponding to those in human patients using TOBI®. These findings may partly explain the apparent success of inhaled tobramycin therapy in the treatment of pulmonary infections.

Chapter I
BACKGROUND AND SIGNIFICANCE

Tobramycin administration direct to the airways via nebulizer has brought great improvements to the health and wellbeing of cystic fibrosis (CF) patients and others suffering from chronic pulmonary infections with pathogens such as *Pseudomonas aeruginosa* (Chuchalin et al., 2009). The topical administration of this class of antibiotics by inhalation offers an attractive alternative, delivering high concentrations of drug directly to the site of infection while minimizing the risk of toxicities associated with high systemic exposure (Geller et al., 2002; Touw et al., 1995). Although the efficacy and safety of inhaled tobramycin is well-recognized in clinical circles, its absorption and disposition following airway administration has not been thoroughly studied in humans and animals, and it remains possible that its pulmonary disposition influences its efficacy.

Historically, tobramycin has been administered parenterally for treatment of lung infections with limited success; serum to lung drug concentration ratios of 10:1 are typical (Moriarty et al., 2007), meaning that high serum concentrations are required to get sufficient drug into the airways and these often caused toxicity (Selimoglu, 2007; Todd and Hottendorf, 1995). These observations, taken with tobramycin's extremely poor oral bioavailability (Jaresko and Alexander, 1995; Phillips and Shannon, 1997), all appear to be associated with the low permeability of epithelia to this hydrophilic drug. Tobramycin's biopharmaceutical properties may be further complicated by the drug's tendency to accumulate in or on certain epithelial cells, e.g. in the kidney or inner ear

(Mingeot-Leclercq and Tulkens, 1999; Selimoglu, 2007). Clearly, topical administration of tobramycin by nebulizer has the advantage that high concentrations may be targeted to the airways where its antimicrobial effect is believed to be concentration-dependent and its efficacy is related to the magnitude of the airway C_{max} (the peak level of drug concentration in the airways) to MIC (minimum inhibitory concentration) ratio (MacArthur et al., 1984). However, the ideal duration over which concentration should be maintained in the airways of infected patients is unknown.

In general, tobramycin bioavailability from the lung following inhalation is therefore presumed to be slow and incomplete, in favor of sustaining its local antimicrobial activities in the airways, due to its hydrophilicity and polycationic properties at physiologic pH (Pai and Nahata, 2001; Poli et al., 2007). However, tobramycin studies in the lung following inhalation are quite limited, and there is insufficient published scientific evidence to support this theory. In the study of Cooney et al., mean absolute bioavailability (±SD) in 6 CF adults was estimated to be 9.1 (±3.8) %, based on the ratios of AUC (area under drug serum concentration versus time curve) normalized by dose (Cooney et al., 1994). In this case, the data was normalized based on the "emitted dose" from the nebulizer (Microstate Ultra Nebulizer was used for inhalation) compared to the intravenous (IV) dose as the reference; notably, however, the true lung dose was not determined. Tobramycin pulmonary bioavailability was also assessed using a population pharmacokinetic approach for multiple doses (twice daily for 28 days) of TOBI® (300 mg in 5 mL 0.25 normal saline; Chiron Corporation; Seattle, WA) administered via a jet nebulizer (PARI LC; Pari Respiratory; Richmond, VA) in a phase III clinical trial in which 258 CF patients were enrolled (Geller et al., 2002). In that study, the bioavailability of inhaled tobramycin was reported as 11.7 %, calculated using the "population estimate of the apparent clearance" (CL/F= 49.6 L/h) with an average value for

systemic clearance, CL of 5.79 L/h from 3 published IV studies (Geller et al., 2002). Geller's result was comparable with that from Touw et al. (1997), given the large inter-patient variations in both studies; Touw et al. estimated bioavailability at 17.5% based on the urinary output of 6 CF adults after a single dose of 600 mg tobramycin via an ultrasonic nebulizer (WISTO SENIOR; Wisto, Woerden, Netherlands) (Touw et al., 1997).

Notably, the fractions of the administered doses depositing in the lung immediately following tobramycin inhalation were determined in none of the studies described above. Therefore, values for pulmonary bioavailability were calculated based on the nominal doses loaded in the nebulizer or the emitted doses believed to be delivered from them. Due to the generally inefficient lung delivery of nebulizer devices, large drug losses are expected during drug administration and only a small dose fraction can reach the capillary-perfused regions for absorption into the systemic circulation (Coates et al., 2000; Touw et al., 1995). The fraction of each administered dose eventually deposited in the lung depends on the device, formulation and the airways geometry and breath patterns of users; the average percentage of loaded dose is expected to range between 5 and 15% for the ultrasonic and jet nebulizers used in these studies (Ilowite et al., 1987; Lenney et al., 2011; Touw et al., 1997). In this scenario therefore, although tobramycin pulmonary bioavailability was stated to be low (Cooney et al., 1994; Geller et al., 2002), the results indicate that the systemically available fractions of the total lung doses (mass deposited in the lung region) delivered by nebulizer may actually approach 1, in spite of the drug's ionization and hydrophilicity.

In the study of Cooney et al., the absorption rate was also assessed after calculating the cumulative fraction of drug absorbed as function of time using the Loo-Riegelman method

(Cooney et al., 1994). The mean absorption time estimated for the 6 subjects ranged from 0.25 to 1.5 hours. Due to the small number of subjects and large inter-subject variability seen in this study, the reliability of this result is debatable. In the study of Touw et al., the first-order absorption rate constant (k_a) for tobramycin after inhalation was estimated using compartmental pharmacokinetic (PK) analysis; values were reported in the range of 0.56-4.16 hr^{-1} with wide inter-subject variations (Touw et al., 1997), while the terminal $t_{1/2}$, which referred to the half life of the terminal phase of drug serum concentration time profiles, ranged from 8.9 to 19.3 hours (Touw et al., 1997). In this study, this prolonged terminal $t_{1/2}$, compared to those observed typically in IV studies in the range of 1.5 to 3 hours (Levy et al., 1984; Touw et al., 1993; Town et al., 1996), was interpreted to be due to the phenomenon of "flip-flop" that implied a delayed absorption from a depot of tobramycin in the lung. Similarly, the prolonged terminal $t_{1/2}$ (about 8 hours) following inhalation was also observed in another study conducted by Conte et al. in healthy volunteers (Conte et al., 1993). While these prolonged serum half-lives have been observed, the values are not consistent with those for absorption from Cooney et al.(1994); nor has the phenomenon been seen in other studies with similar designs (Geller et al., 2002; Newhouse et al., 2003; Pilcer et al., 2008; Poli et al., 2007). The discrepancies may be attributed to differences in study designs, sampling duration and frequencies, PK analysis methods, accuracy, precision and LLOQ (lower limit of quantification) of assay methods, as well as variations between subjects, studies, and dosing regimens. Overall however, the reports emphasize our poor understanding of pulmonary absorption and disposition.

Despite this controversy, tobramycin is believed to exit the lung slowly following inhalation. This theory may be derived from the persistence of high tobramycin concentration in airway secretions following inhalation in animals and humans (Eisenberg et al., 1997; Gibson et al., 2003;

Lenoir et al., 2007; Valcke and Pauwels, 1991). Evidence of high alveolar lining fluid (ALF) concentrations that persisted for as long as 6 hours and remained far in excess of the MIC (minimum inhibitory concentration) of susceptible respiratory pathogens was shown in rats following endotracheal administration (Valcke and Pauwels, 1991). In humans, potential tobramycin accumulation in respiratory secretions with repeated dosing TOBI (300 mg b.i.d.) in young CF patients (≤6 years old) was also observed (Gibson et al., 2003), although a short half life of drug disappearance from sputum was generally found after single dose administration (Geller et al., 2003; Weber et al., 1994). Tobramycin persistence in ALF of pneumonia patients collected by bronchoalveolar lavage (BAL) was also shown in a clinical trial after IV administration, in which a slower rate of drug disappearance occurred from ALF than from serum (Carcas et al., 1999) was thought to indicate that the ALF constituted a deep compartment for tobramycin. Moreover, in vitro studies also found that tobramycin bound to sputum and that binding appeared saturable (Mendelman et al., 1985). In spite of these results, however, tobramycin binding or sequestration in the lung and the extent to which binding actually affects the drug's disposition following inhalation has not been studied.

While the sites and mechanisms of drug binding or sequestration in lung tissue remain to be investigated, the drug's multiple positive charges and the presence of "ubiquitous negative charges on the surface of cell membranes" has been mentioned as one type of possible interaction in the airways (Patton et al., 2004). The hypothesis was inferred partly from results of the studies of aminoglycosides' nephrotoxicity, in which the drugs were believed to initially bind to acidic phospholipids in the brush-border membrane of proximal tubule cells and then cause accumulation of this class of drugs in the epithelial cells of renal proximal tubules (Nagai and Takano, 2004).

Notably, aminoglycosides' binding to proximal tubule and inner ear cells has been investigated for the studies of aminoglycoside nephrotoxicity and ototoxicity. Since there are the similarities in composition and structure (phospholipid bilayers) among epithelial membranes, the binding of aminoglycosides to other epithelia, especially to the lung, appears to merit further investigation.

In summary, the extent and rate of tobramycin absorption, the existence of tissue binding and/or lung retention remains uncertain, especially from the kinetic and mechanistic aspects, in part because of the difficulties of studying pulmonary disposition in animals and humans. Since a solute's physicochemical properties affect its penetration through the pulmonary barrier (Patton et al., 2004), an understanding of tobramycin's molecular characteristics is important. Tobramycin is an aminoglycoside with a relatively large molecular weight (MW=467.5 Da). The structure of this compound (Figure I.1) consists of an aminocyclitol connected to two amino sugars by glycosidic bonds. There are 5 amino groups in the structure and the pKas shown in Figure I.1 are as reported by Szilagj *et al.*(1993); these amino groups will be predominately ionized and the drug should carry about 2.5-3 positive charges based on the reported pKa values under physiological conditions (pH7.4). The predicted LogP (logarithm of the partition coefficient between octanol and water for the neutral form) and LogD (pH 7.4) (logarithm of the distribution coefficient between octanol and water at pH7.4) were reported to be -4.224 and -9.54, respectively, indicating the drug's extreme hydrophilicity and how this is enhanced by protonation of the amino groups (LogP and LogD prediction were performed by ACD/PhysChem Suite (Advanced Chemistry Development, Inc., Toronto, Canada), a software used for prediction of physicochemical properties).

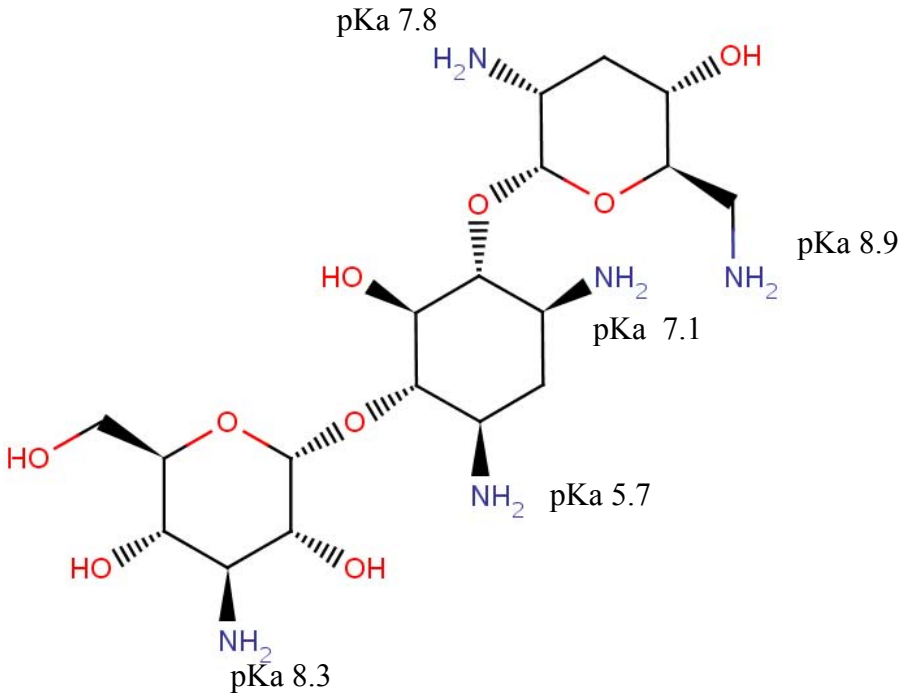


Figure I.1 Tobramycin chemical structure and pKa values

Solute absorption from the airways of the lung is generally believed to occur via diffusion and/or partitioning (Brown and Schanker, 1983). For hydrophilic solutes, passive diffusion is expected via paracellular tight junctions between epithelial cells as the primary pathway through pulmonary barrier, due to the difficulties that such solutes experience in penetrating into the phospholipid bilayers in epithelial cell membranes (Brown and Schanker, 1983; Effros and Mason, 1983; Schanker and Less, 1977; Schneeberger, 1991). The absorption of lipophilic solutes, to the contrary, occurs by transcellular diffusion/partition, and correlates with molecular partition coefficients between octanol and water (Brown and Schanker, 1983). The absorption of lipophilic compounds is generally faster than that of hydrophilic ones, given a similar range of molecular size (Brown and Schanker, 1983), since the surface area of the epithelial cell membranes in the

lung, available for transcellular diffusion, is much larger than that for intercellular or paracellular diffusion via tight junctions between cells.

Tight junctions are known as a complex belt-like network of strands and fibrils which are polymers of interacting transmembrane proteins and lipids (Su and Sheppard, 2008); they are believed to represent relatively impermeable structures in which "aqueous pores" reside (Sakagami, 2000). These pores may alternate between open and closed configurations as channels for solute transport (Claude, 1978; Reuss et al., 1991). These hypothetical "pores" with various sizes in the pulmonary epithelium were demonstrated in several studies by Schanker and coworkers (Enna and Schanker, 1972; Lin and Schanker, 1983; Schanker and Hemberger, 1983). In those studies, the in vivo disappearance rates of miscellaneous hydrophilic compounds from the lungs were determined by quantifying the dose remaining in the lungs at various time points following intra-tracheal instillation or aerosol inhalation in anesthetized animals (Brown and Schanker, 1983; Enna and Schanker, 1972). Notably, the absorption of a series of non-metabolized and lipid-insoluble saccharides, with similarities to aminoglycosides in structure and metabolic properties (non-metabolized in lung), were also investigated by Schanker's group (Enna and Schanker, 1972). The absorption of these saccharides appeared to be dose-independent with the first-order absorption rate constants, k_a, ranging from 0.025-10.4 h^{-1} and where k_a values were inversely related to molecular weight. The mean absorption half life of mannitol (MW=182 Da), sucrose (MW=342 Da) and inulin (MW=5,250 Da) in rat lungs were reported to be 65, 87, and 225 minutes respectively. In their study, passive paracellular diffusion (via tight junctions) was concluded as the pathway for these saccharides' absorption from the airways into the blood, because both the

extent and rate constants for absorption were dose- and concentration-independent (Enna and Schanker, 1972).

Clearly, mechanisms other than diffusion and partitioning, such as transporter-mediated absorption or vesicle-mediated endocytosis and transcytosis, may be involved in solute absorption from the airways (Patton et al., 2004). In a recent review of drug transporters in the lung, the expression and potential impact of transporters (such as P-glycoprotein, P-gp, and organic cation and organic anion transporters, OCT and OAT, etc.) were described in detail (Bosquillon, 2010). At this time however, there is no strong evidence to indicate the involvement of transporters in the absorption of drugs delivered to the lung (Bosquillon, 2010). Similarly, our knowledge of pulmonary absorption by vesicle mediated endocytosis and transcytosis is limited and mostly associated with macromolecules (Patton, 1996).

In general, solute disposition in the lung following inhalation is a complex function of regional deposition, mucociliary clearance, active and/or passive absorption, tissue sequestration and metabolism (Byron et al., 1994; Niven et al., 1990). Unfortunately, the kinetics and mechanisms describing the behavior of individual solutes remain largely theoretical, due to the lack of appropriate experimental systems for characterization of these processes. In vivo studies in animals, combined with intratracheal instillation or aerosol administration, have been applied to characterize solute absorption rate and extent following airway administration in Schanker and others' laboratories (Brown and Schanker, 1983; Sakagami et al., 2003). While such methods enable the direct assessment of drug pharmacokinetics in vivo, reproducible airway dosing and considerable animal sacrifice are necessary (large numbers are required) to reduce the inter-animal

variations in whole body clearance that make characterization of absorption and disposition possible (Sakagami, 2006).

More recently, reconstructed lung epithelium, cultured from human lung adenocarcinoma cell lines or primary cultured alveolar epithelial cells, have been used to clarify the kinetics and mechanisms of solute transepithelial transport in vitro (Sakagami, 2006). The convenience and low cost of cell culture are potentially great advantages, when these methods are compared to the use of whole animals. Unfortunately, the variables associated with lung cell isolation and culture and a lack of standardization create uncertainties so that results between laboratories from similarly cultured cells are rarely comparable (Sakagami, 2006). Moreover, the relevance of using these reconstructed barriers to represent complex intact lung is still debatable. For these reasons, the ex vivo model of the isolated perfused lung appears to resolve some deficiencies in both these in vivo and in vitro methods; it also offers a great alternative for the study of lung-specific pharmacokinetics, by virtue of its well-maintained structural and functional integrity and the absence of the whole body complication introduced by solute distribution and elimination in vivo (Sakagami, 2006). The ex vivo method provides advantages, such as enabling relatively accurate and reproducible drug administration to the airways, convenient sampling of perfusate and lavage fluids, and easy determination of mass balance (Tronde et al., 2003). Hence, this model has found use for investigations of absorption, tissue sequestration, metabolism and mucociliary clearance. The isolated perfused rat lung (IPRL) preparation described by Byron and Niven, that combined with a highly reproducible "forced intratracheal instillation" technique for solute administration into the airways, brought great improvements to the accurate and precise determination of absorption rate and extent (Byron and Niven, 1988; Byron et al., 1986). In part because the IPRL

preparation is studied in a physiological normal orientation (horizontally suspended) with a horizontally exteriorized trachea cannula, the preparation has found its way into text books as a way of studying aerosol deposition (Byron et al., 1986; Sinko, 2006). The method has also been employed successfully in many studies to address issues regarding the kinetics and mechanisms of solute absorption and disposition following airway administration in a number of different academic groups (Byron et al., 1994; Niven and Byron, 1990; Niven et al., 1990; Pang et al., 2005; Sakagami et al., 2002).

The use of the IPRL preparation for the study of solute binding and/or sequestration after airway administration is yet to be described. The affinity and capacity of solute tissue binding has largely been investigated in vitro using equilibrium dialysis with tissue homogenates or slices (Pacifici and Viani, 1992; Wilkinson et al., 1986*)*. Clearly, structurally modified tissues are unlikely to behave like intact organs, and therefore results from in vitro equilibrium dialysis experiments fail to provide usable predictive information for solute tissue binding in vivo. In this thesis, an attempt has been made to improve the bio-relevance of an in vitro binding assay and to marry the results to those from the intact organ. The dialysis of sliced isolated lung tissues was performed immediately after drug administration to the airways, to gain more insight to the in vivo lung tissue binding of tobramycin by mimicking the environment in which the drug was administered. Dynamic dialysis under sink conditions was first described by Meyer and Guttman (Meyer and Guttman, 1968; 1970). The technique was modified in this dissertation to support the use of the IPRL preparation for the combined characterization of tobramycin's lung tissue binding behavior and absorption. This method, initially applied for protein binding studies, has been used successfully by other groups to study solute-ligand binding, by virtue of its providing more kinetic

information than equilibrium dialysis alone (Farrell et al., 1971; Hashimoto et al., 1984; Leo et al., 1999; Meyer and Guttman, 1968; Pedersen et al., 1977).

This dissertation aims to enhance the understanding of pulmonary absorption and retention of tobramycin in the airways following inhalation by targeting and describing experiments designed to study the kinetics and mechanisms of absorption and disposition in the lung. In an attempt to elucidate the kinetics and extent of pulmonary absorption in humans, the systemic bioavailability of inhaled tobramycin in adults was re-evaluated by performing a meta-analysis of PK studies of aerosolized tobramycin using the available data in the literature. To explore the kinetics and mechanisms responsible for aerosolized tobramycin's pulmonary disposition, the absorption and binding behavior of tobramycin was then assessed in the most realistic ex vivo model of the rat lung, the IPRL, as a function of dose and local concentration, using a previously described method to administer the drug reproducibly (Byron and Niven, 1988). The effect of pH and osmolality of dosing solutions was also investigated to eliminate possible secondary factors that may impact the drug's absorption and disposition. By combining the IPRL preparation with dynamic dialysis, a disposition model for tobramycin binding and/or sequestration was developed and validated after drug administration to the airways. Based on the results from these binding experiments, a kinetic model for tobramycin absorption and disposition following airway administration in the IPRL was used to interpret and discuss the data from the IPRL. The findings in this study are discussed in the light of the literature concerning the lung's interactions with inhaled compounds, perhaps to aid in the future development of drugs designed to target lung tissue.

Chapter II
HYPOTHESES AND SPECIFIC AIMS

The efficacy and safety brought about by administration of tobramycin via inhalation is well-recognized for the treatment of lung infections in clinical circles (Chuchalin et al., 2009). However, it remains unclear whether the antibiotic's physicochemical properties are such as to lead to slow absorption, tissue binding and/or lung retention to sustain the drug's presence and antimicrobial properties for periods between dosing. This dissertation was aimed to enhance the understanding of tobramycin's absorption from, and retention in, the airways and lung tissues, by targeting experiments designed to study the kinetics and mechanisms responsible for the drug's disposition following airway administration. The central hypothesis was that tobramycin was slowly absorbed from airways to the blood following local delivery to the lung, primarily by passive diffusion via the paracellular pathway, due to its hydrophilic and polycationic properties at physiological pH (Szilagyi et al., 1993). Tobramycin's bioavailability following airway administration, was presumed to be low, based on the reportedly poor permeability of aminoglycosides across gastrointestinal barriers seen following oral administration (Jaresko and Alexander, 1995; Phillips and Shannon, 1997). Because aminoglycosides and tobramycin have been shown to bind to tissues in kidney and inner ear to form "deep compartments" (French et al., 1981; Mingeot-Leclercq and Tulkens, 1999; Nagai and Takano, 2004; Schentag, 1978; Schentag et al., 1978; Stepanyan et al., 2011), we also hypothesized that the drug could bind to lung tissue and that the degree of this binding could be dose- and/or concentration-dependent. Because of slow absorption, any retention

in the airways was thought likely to prolong the drug's antimicrobial efficacy following local delivery. Finally, because these characteristics were believed to be due primarily to the drug's physicochemical properties, we hypothesized that the drug's formulation in dosing solutions with different osmolalities and small changes in pH were not likely to significantly affect its pulmonary disposition.

The following **Specific Aims** were used to test these hypotheses:

1. **To estimate tobramycin's pulmonary bioavailability following inhalation in humans using pharmacokinetic (PK) meta-analysis.** Selected tobramycin blood level and sputum concentration data from CF and healthy adults were taken from the clinical literature and re-analyzed using non-compartmental and compartmental approaches to assess tobramycin's pulmonary bioavailability. Both the rate and extent of absorption of the lung deposited dose was assessed. The available sputum PK data following inhalation were also systematically reviewed and discussed in the light of the earlier meta-analysis of blood level data. Specific Aim 1 is described in detail in this thesis in Chapter III.

2. **To characterize the kinetics and mechanisms of tobramycin's disposition in the rat lung following administration to the airways and to build a kinetic model to describe the results.**

a) ***To assess tobramycin disposition in rat lungs using a realistic ex vivo model, the isolated perfused rat lung preparation or IPRL.*** Tobramycin disposition in the rat lung following

airway administration was studied as a function of dose and local concentration using a previously described method (Byron and Niven, 1988). To explore the kinetics and mechanisms of the drug's absorption from the airways, the mean cumulative absorbed fraction of each administered dose absorbed into the perfusate, F_p, was compared to that of the absorption markers, fluorescein (known for its rapid and complete absorption) and mannitol (known for its paracellular absorption), as a function of time following administration. To support the development of a disposition model with the presence of drug binding and/or sequestration to the lung tissues, dynamic dialysis of sliced IPRL tissues was studied immediately after drug administration to the airways, to assess the binding or tissue sequestration properties of tobramycin. These experiments and the resultant kinetic model for tobramycin absorption and disposition in the IPRL are described in detail in this thesis in Chapter IV.

b) ***To investigate the effects of osmolality and pH of dosing solutions on tobramycin's absorption from the airways of the IPRL***. The potential airways' anatomical and functional alteration due to the administration of non-isotonic and non-physiologic pH solutions to the airways has been reported to alter solute transport through the airways (Beasley et al., 1988; Eschenbacher et al., 1984; Glauser et al., 1979; Schanker and Less, 1977). In the literature, the osmolality of tobramycin dosing solutions appears to be theoretically unpredictable (Law, 2001) in that measurements have indicated much smaller osmolality values than those based on theoretical calculations. Therefore, in some experiments, in which absorption from the airways of the IPRL was studied, the osmolality of dosing solutions was measured experimentally while in others, the effects of varying the

concentration of NaCl (0.225-0.9% w/v) and/or the pH of the dosing solutions (tobramycin concentration held constant) was assessed.

c) ***To evaluate the effect of tobramycin on airway epithelial integrity in the IPRL***. Because aminoglycosides and other polycations in certain concentration ranges have been reported to affect epithelial integrity and/or alter solute permeation through epithelia in vitro and in vivo (Dong et al., 2011; Todd et al., 1994), the effect of tobramycin on mannitol absorption from the airways of the IPRL was investigated following co-administration of mannitol with tobramycin at different concentrations to the airways. Because mannitol is accepted as a marker solute for paracellular absorption through different epithelia, the approach was used to elucidate whether the tobramycin itself could increase its own permeability at high drug concentrations by affecting the airway epithelial integrity.

In summary, the work described in thesis Chapters III and IV focus on the evaluation of tobramycin's absorption and retention following airway administration in humans and rats, respectively. These culminate in Chapter IV, where a disposition model is described that invokes dose and concentration-dependent drug binding and/or sequestration to lung tissues to explain the kinetic handling of tobramycin by the rat lung. Chapter V is aimed to validate and place constraints on that disposition model by describing the results of experiments designed to eliminate the possibilities that perturbations other than tissue binding or sequestration may have been responsible for some of the effects predicted at concentrations likely to be seen following inhalation.

Chapter III *

PHARMACOKINETIC META-ANALYSIS TO ESTIMATE TOBRAMYCIN'S PULMONARY BIOAVAILABILITY FOLLOWING INHALATION IN HUMANS

III.a INTRODUCTION

Inhaled tobramycin is recommended for the maintenance treatment of lung infections associated with *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients, as an alternative to intravenous (IV) administration, in order to deliver high antibiotic concentrations direct to the site of infection while minimizing the drug's systemic toxicity (Cheer et al., 2003; Geller et al., 2002; Touw et al., 1995). Although its efficacy and safety are well-recognized, tobramycin's pulmonary biopharmaceutics are not well understood in humans, partly due to the general technical difficulties involved with (a) characterizing the true dose administered to the lungs by different pulmonary delivery systems in the clinic and (b) the analytical challenges associated with determining the low blood levels of tobramycin resulting from inhalation (McGlinchey et al., 2008; Pechere and Dugal, 1979).

Notably, there are no published studies in which tobramycin's pulmonary bioavailable fraction, F, of the total lung deposited dose (TLD) has been determined and none in which an IV reference experiment has been performed in the same subjects. In CF patients, absorption has been estimated

*Some of this chapter has been published in Li and Byron (2012)

with reference either to nominal loaded doses placed in the delivery devices (e.g. ampoule label claims added to nebulizer reservoirs) or the in vitro emitted doses from the chosen delivery system (e.g. apparent output from a powder inhaler after the use by subjects) (Cooney et al., 1994; Geller et al., 2002; Touw et al., 1997). Not surprisingly, pulmonary bioavailability estimates with no consideration of the true doses delivered to the lung, show large variations across the literature, due to the high variability in delivery efficiency of inhalation devices for different formulations, as well as inter-individual variations associated with breathing patterns, respiratory physiology and function, and the drug's variant pharmacokinetics that are seen in CF patients.

In humans, aminoglycosides are neither plasma protein bound nor metabolized, and are mainly distributed through the body's extracellular compartments; excretion of the unchanged drug in urine occurs via glomerular filtration with a small percentage of the filtered fraction reabsorbed by the renal proximal tubules (Pechere and Dugal, 1979). Systemic bioavailability of this class of drugs from the GI tract is very low, since the drugs' blood levels concentration was reported to be extremely low following oral administration (Jaresko and Alexander, 1995; Phillips and Shannon, 1997; Weber et al., 1995). Drug accumulation or tissue sequestration has been documented in the labyrinth of the inner ear and the renal cortex of the kidney after IV administration; this is believed to cause the ototoxicity and nephrotoxicity associated with most aminoglycosides (Selimoglu, 2007; Todd and Hottendorf, 1995). To deal with the toxicity of aminoglycosides, in pharmacokinetic studies, a 3-compartment model has often been used that contains a so-called "deep compartment", responsible for drug accumulation or tissue sequestration (French et al., 1981; Schentag, 1978; Schentag et al., 1978). However, the slow phase of elimination (γ), that is associated with this deep compartment, was generally not easy to characterize, as it appeared about

24 hours following IV administration with a half live of a few days, and there were analytical difficulties assaying extremely low drug concentrations in these circumstances. In clinical practice, after a single dose, the 2-compartment open body model, in which the rapid distribution phase corresponds to fast distribution through fluid compartments and elimination occurs via renal excretion, is generally sufficient to describe tobramycin disposition after IV administration (Aminimanizani et al., 2002; Inclan et al., 2005).

In CF patients, aminoglycosides are generally believed to show different intrinsic pharmacokinetic behavior to that seen in non-CF humans, with an increased total body clearance (CL) and/or a larger volume of distribution (Vd) (Horrevorts et al., 1988; Levy et al., 1984). Larger inter-subject variability in PK parameters, was also often seen in CF in comparison to non-CF patients, probably because of the disease physiological and pathological complex (Mauro et al., 1995; Town et al., 1996). From the point of view of aerosol delivery moreover, CF patients are generally known to have mucus build-up in the airways and decreased mucociliary clearance, because of the abnormal chloride ion secretion in the respiratory tract caused by reduced or absent function of the cystic fibrosis transmembrane conductance regulator (CFTR) (Donaldson et al., 2007). Overall, these factors in CF may contribute to further variability in tobramycin pulmonary bioavailability following inhalation.

In this chapter, a meta-analysis is described that was performed using literature data to assess tobramycin's pulmonary bioavailability (the rate and extent of absorption of the lung deposited dose) separately in CF and healthy adults. This meta-analysis combined the inhalation PK data in which lung dose determinations were performed with tobramycin's IV data from the literature.

These PK data were re-evaluated by non-compartmental and compartmental approaches to assess the pulmonary bioavailable fraction (F) and the apparent first-order absorption rate constant following inhalation. In the effort to evaluate the absorption kinetics, the time-course of tobramycin blood concentration profiles following inhalation were re-analyzed by deconvolution, using the Loo-Riegelman method (Gibaldi and Perrier, 1975). This method is known as a very useful and rigorous method for the evaluation of absorption kinetics that applies generally to linear multi-compartment PK models (Gibaldi and Perrier, 1975) and is one that has been used successfully in inhalation work when dealing with experimental data (Clark and Byron, 1986).

Following aerosol delivery and deposition in the lung, in the case that drug biological transformation and degradation are absent in the lung, the converse of absorption is, of course, drug retention in the airways. It is notable therefore, that tobramycin lung retention following inhalation, should favor its antipseudomonal efficacy, and be a subject of interest to those studying its clinical pharmacology and aerosol development (Patton et al., 2004; Ruddy et al., 2013). It is hardly surprising therefore, that the sputum concentration of tobramycin following inhalation in CF patients has been extensively investigated in many studies to assess the drug's in vivo local antibacterial activity, the apparent delivery efficiency of various inhalation devices, and even tobramycin's potential accumulation in the lung with repeated administration (Eisenberg et al., 1997; Geller et al., 2003; Poli et al., 2007; Ruddy et al., 2013). The topic is complicated by the difficulty in obtaining sputum samples for analysis that are in some way "representative" of the drug's "non-absorption" following delivery to different sites on an enormous epithelial surface. In general therefore, there is high variability seen in tobramycin sputum concentrations following inhalation, and it makes the results difficult to interpret. These variations are attributed to the

different delivery efficiencies of devices, the time and methods to collect airway secretion samples, and the human subject's disease status and airway anatomy (Gibson et al., 2003). Nevertheless, to gain more insight into drug retention in the airways, the available sputum PK data following inhalation are systematically reviewed and discussed, in an attempt to add this information to the meta-analysis. These data are not available for healthy humans in the literature and therefore, only studies in CF patients were included in this analysis.

III.b METHODS

Pulmonary bioavailability

Tobramycin PK studies after IV and inhalation administration were systematically retrieved by subject searching for "tobramycin pharmacokinetics" in PubMed (1968-2013 July) and manually adding articles retrieved from the reference sections of key articles obtained from that PubMed search. Studies were selected for analysis if they met the following *Eligibility criteria*:

1. Tobramycin PK data from adult CF or adult healthy humans with ages between 17 and 65 years old were included if the drug was administered either intravenously or by inhalation and drug blood concentrations were determined for at least 5 time points after administration (for IV studies, the sampling duration covered both distribution and elimination phases, while for inhalation studies the sampling duration spanned both absorption and elimination phases).
2. Impaired renal functions or critical illness were excluded to avoid unexpected changes in clearance under these conditions (Leroy et al., 1978; Pechere and Dugal, 1976). Abnormal

renal function was defined as creatinine clearance out of the normal range 80-160 ml/min/1.73 m^2.

3. Research studies involving inhalation were selected only if the lung dose determination could be assessed with confidence from the article in question.
4. Articles which failed to indicate that blood concentrations were greater than the reported assay lowest limit of quantitation (LLOQ) were rejected as were those in which blood level data were reported with sparse sampling schedules, such as those from routine therapeutic drug monitoring using peak and trough levels.

The eligible data were categorized into CF and healthy groups, and analyzed separately by three different methods to assess the rate and extent of tobramycin absorption following inhalation.

Method 1: This was a non-compartmental approach. The value of the fraction of the lung dose that was absorbed at time infinity, F, was estimated from:

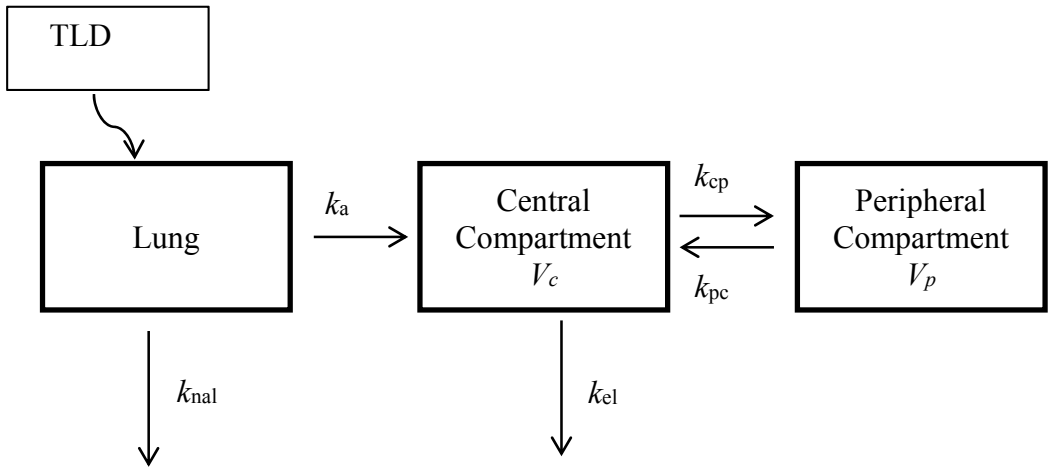
$$F = CL/(TLD/AUC_{inh}) \qquad \text{eq. III.1}$$

where AUC_{inh} is the total area under the drug blood concentration versus time curve following inhalation, CL is total body clearance and TLD is total lung dose; values for AUC_{inh} were used either as reported in the literature, or they were calculated trapezoidally. AUC values from the ultimate concentration value (C_{last}) through time infinity were estimated from C_{last}/k where k was the negative log-linear slope of the concentration versus time data collected during the elimination phase in the inhaled drug study. The values of CL, for CF and healthy subjects, were obtained separately by averaging the pooled data from the eligible IV studies in each population across the literature, by taking the sum of the reported mean values for CL multiplied by the number of subjects in each study and finally dividing by the total number of subjects across all studies. The

mean (±SD where available) values of CL in each IV study was used as reported or in the event that it was not reported, calculated from CL=β*V_β or dose/AUC_∞ and expressed per kg body weight; β, V_β and AUC_∞ refer to the terminal first-order, whole body elimination rate constant, the apparent volume of distribution and the total area under the concentration versus time curve following IV administration from time zero to infinity, respectively; the values of the PK parameters such as β, V_β and AUC_∞ , were used as reported.

Method 2: This was based on curve fitting of the inhalation data to equations representing the compartmental model shown in Scheme III.1. In this case, F with respect to the known value for TLD was given by $k_a/(k_a+k_{nal})$, the ratio of the first-order absorption rate constant (k_a) to the total first-order rate constant for removal of drug from the lung following inhalation (equal to the sum of k_a and k_{nal}, where k_{nal} is the first-order rate constant responsible for non-absorptive loss). A 2-compartment PK model was chosen to analyze both the IV and inhalation data, because that was reported to provide the best description for tobramycin disposition following systemic exposure in clinical trials (Aminimanizani et al., 2002; Inclan et al., 2005). The PK parameters k_{cp} and k_{pc} are the apparent first-order intercompartmental transfer rate constants, k_{el} is the apparent first-order elimination rate constant from the central compartment, and V_c is the apparent volume of the central compartment. The values of k_{cp}, k_{pc}, k_{el} and V_c for CF and healthy subjects were derived separately from the IV data for each population. In short, the blood level versus time curves from these IV studies were digitally copied and the concentration data at each time point extracted using DataThief II (*http://datathief.org*), a graph reading java software and dose- and body weight-normalized where necessary. Curve fitting of the IV data was then performed in accord with a 2-compartment PK model using non-linear regression and SCIENTIST® 3.0 (MicroMath, Saint

Louis, MO), to provide best estimates for k_{cp}, k_{pc}, k_{el} and V_c. Similarly, non-linear regression was then employed to analyze the inhalation data in accord with Scheme III.1, where the values of k_{cp}, k_{pc}, k_{el} and V_c were held constant at the best estimates obtained from the IV data. TLD was fixed at its reported value while k_a and k_{nal} were allowed to float to produce the best curve fits of the inhaled blood level versus time data, plus best estimates for k_a and k_{nal} and F.



Scheme III.1 Pharmacokinetic model describing pulmonary clearance, absorption, distribution and elimination of the inhaled tobramycin total lung dose, TLD. First-order rate constants k_a, k_{nal}, k_{pc}, k_{cp}, and k_{el} represent absorption into the central sampled compartment with apparent volume Vc, non-absorptive loss from the lung, distribution between the central and peripheral compartment with apparent volume Vp, and elimination, respectively.

Method 3: The Loo-Riegelman method (Gibaldi and Perrier, 1975) was used to assess the absorption properties of tobramycin following inhalation; the intrinsic PK parameters of this two-compartment open body model drug were determined in CF and healthy subjects as described above (Method 2). The percent of TLD absorbed at time t, $abs\%$, was then calculated by eq. III. 2-5 calculated (Gibaldi and Perrier, 1975):

$$abs\% = \frac{A_{(t)}}{A_\infty} \times 100\% \qquad \text{eq. III.2}$$

where $A_{(t)} = V_C C_P + A_{P(t)} + V_C k_{10} AUC_{0-t}$ eq. III.3

$$A_{P(t)} = A_{P(t_1)} e^{-k_{21}\Delta t} + \frac{k_{12} A_{C\,(t_1)} \left(1 - e^{k_{21}\Delta t}\right)}{k_{21}} + k_{12} V_C \left(\frac{\Delta C_p}{\Delta t}\right)\left(e^{-k_{21}\Delta t} + k_{21}\Delta t - 1\right)/k_{21}^2 \quad \text{eq. III.4}$$

and $A_\infty = V_c k_{10} AUC_\infty$ eq. III.5

where $A_{(t)}$ and A_∞ are the amount of drug absorbed systemically at time *t*, and the amount of drug that is ultimately absorbed, respectively; $A_{P(t_1)}$and $A_{P(t)}$, are the amounts of drug in the peripheral compartment at the time of the first of any two consecutive sampling periods (i.e. $t = t_1$) and at time *t*, while $A_{C\,(t_1)}$ is the amount of drug in the central compartment at the time of the first of the sampling period; AUC_{0-t} and AUC_∞ are the area under the plasma concentration versus time curve from time zero to time *t*, and from time zero to infinity, respectively; C_P is the drug plasma concentration at time *t* and ΔC_P is the change of C_P in each period of time, $\Delta t = t - t_1$.

The percentage remaining unabsorbed (100 - *abs%*) versus time plots were generated and used to predict the absorption rate constant for tobramycin after administration to the airways, assuming that absorption occurred only via the lung.

Sputum pharmacokinetics

Tobramycin concentrations in sputum sampled from adults and children (Ages ≥6 years) with CF after single or repeated inhalation doses were obtained from the systematic literature review described above. The subset of articles that were used for a review of sputum concentrations included those by Griese et al., 2013, etc. shown in Table III.4. To evaluate the variability associated with different study designs and methods, the drug formulations and delivery devices for inhalation, and the dosing and sampling regimens in these studies were tabulated and summarized. The values of C_{peak}^{sputum}, the "peak" sputum concentration, generally determined from samples taken 10-60 minutes post administration, and C_{trough}^{sputum}, the pre-dosing level, as well as AUC_{0-t}^{sputum}, area under the tobramycin sputum concentration time curve, immediately after dosing to the last sample time point (8 or 12 h), were compared across these studies to evaluate the

evidence for potential lung retention and/or accumulation of tobramycin in sputum after repeated inhalations.

III.c RESULTS AND DISCUSSION

Pulmonary bioavailability

A total of 633 papers were obtained from the keyword search in PubMed. Of these, only 18 studies satisfied the *Eligibility criteria* for meta-analysis to determine tobramycin's pulmonary bioavailability. The eligible studies included 15 IV PK studies in adult humans. IV studies were categorized into 2 groups: CF (6 studies) and Healthy (9 studies). For the inhalation PK studies, two studies that met the Eligibility criteria were included: Pilcer et al. 2008 (in CF adults) and Newhouse et al. 2003 (in healthy adult volunteers), while a similar study by Lenney et al. was excluded from the meta-analysis because of the data insufficiency in this paper for the estimation of F or the absorption rate constants by Method 1, 2 and 3 (Lenney et al., 2011). In the inhalation studies, the lung doses were estimated using γ-scintigraphic imaging to characterize the deposited dose in the lung, after labeling formulations with ^{99m}Tc before administration (Newhouse et al., 2003; Pilcer et al., 2008)

Method 1

The weighted mean value for tobramycin total body clearance (CL) was 1.88 ml/min/kg in CF adults, obtained from a total of 50 subjects in six IV PK studies, and 1.23 ml/min/kg in healthy adults, from a total of 80 subjects in nine IV studies. The individual reports and values are summarized in Table III.1. These clearance values are believed to be typical in these populations

and are consistent with excretion that occurs primarily via glomerular filtration. Notably, in the IV studies, values of CL for CF patients were generally larger than those for healthy subjects, although substantial variations were associated with both populations. The relatively larger values seen in CF were consistent with the findings of many other studies, in which an increased volume of distribution and CL were seen in CF (de Groot and Smith, 1987; Levy et al., 1984). The reasons for this PK change in CF patients remain unclear; enhanced renal clearance, diminished tubular reabsorption or an additional alternative route of excretion such as respiratory or gastrointestinal elimination in CF have all been mentioned (Horrevorts et al., 1985; Levy et al., 1984).

For CF patients in Pilcer's study (2008), using the values of CL obtained from IV studies, the average estimates of F for 3 formulations administered in different doses, were 1.03 (lipid-coated dry powder), 1.09 (uncoated dry powder), and 1.70 (nebulized TOBI®) (Table III.2). The unrealistically high absorbed fraction, $F >> 1$ for the aqueous TOBI® formulation, delivered by a hand-held PARI LC Star nebulizer (PARI GmbII; Starnberg, Germany), could have been due to either the choice of an inappropriate CL value for Pilcer's patients (Table III.1) or an experimental underestimated TLD assessment by Pilcer et al. (2008). The inaccuracy in the case of TOBI® may also indicate that values of F from Pilcer's powder formulations, delivered by the aerolizer® (Novartis, Switzerland), are also unreliable (Table III.2).

Table III.1 Total body clearance values for tobramycin reported or calculated from IV PK studies in CF and normal human adults

CF			Healthy		
Ref	n	CL* (ml/min/kg)	Ref	n	CL* (ml/min/kg)
Butterfield 2013	9	1.2±0.2	Champoux 1996	12	1.3
Moriarty 2007	14	1.6	Mauro 1995	8	1.2±0.1
Aminimanizani 2002	6	2.0	Woodworth 1994	6	1.2±0.1
Bates 1997	8	1.4±0.4	Pleasants 1988	15	1.4
Cooney 1994	6	2.5±0.3	Guglielmo 1987	8	1.3±0.2
Horrevorts 1985	7	3.1±0.9	Winslade 1987	11	1.2±0.4
			Pcherere 1976	4	0.8
			Lode 1975	12	0.9
			Regamey 1973	4	1.8±0.1
Total	50	1.88**	Total	80	1.23**

* mean (±SD where available) as quoted or calculated ($CL=\beta*V_\beta$ or dose/AUC_∞) and normalized for reported body weight; ** weighted mean by sample size from each study.

Method 2

The compartmental curve fitting approach, advocated by Sakagami (Sakagami, 2004) to determine the absorption of drugs following inhalation, also relied on the accurate assessment of intrinsic PK parameters determined from IV data in a different subject cohort; however values of F >>1 cannot be obtained using this method (Scheme III.1). In this case, two of the previously selected IV studies: Aminimanizani et al. (2002, for CF adults) and Pleasants et al. (1988, for healthy adults) were selected for analysis, mainly because (a) those two studies showed most frequent sampling over a sufficient length of time (more than 8 hours) following IV administration

and (b) the assay methods were believed to be reliable (Aminimanizani et al., 2002; Pleasants et al., 1988). The assay methods, fluorescence polarization immunoassay (TDx; Abbott Laboratories, Irving, TX), used in those two studies, were judged to be sensitive, accurate, precise and reproducible, compared to other analytical methods, such as enzyme-multiplied immunoassay (EMIT), radioenzymatic assays and microbiological assays used by others (Cheng et al., 1987; Touw et al., 1996). Best estimates of V_c, k_{cp}, k_{pc}, and k_{el} for the IV data in Aminimanzani et al. and Pleasants et al. obtained using non-linear regression in accord with 2-compartment open model are shown in Table III.3 alongside values for the model selection criterion, MSC, and coefficient of determination, COD, both of which indicated excellent fits to the data.

Table III.2 Values for F from analysis of inhalation PK data for tobramycin by Methods 1 and 2

	Lung deposited dose(mg)/% of nominal	F	
		Method 1	Method 2[a]
Pilcer 2008 (CF)			
Lipid-coated powder from DPI	12.6/53.0%	1.03	0.99
Uncoated powder from DPI	8.5/34.1%	1.09	NE
TOBI® (PARI LC Star)	22.8/7.6%	1.70	NE
Newhouse 2003 (Normal)			
DPI	27/34.3%	0.83	0.85
TOBI® (PARI LC PLUS)	15/5.0%	0.78	0.76

[a] Values shown for curve fits in Figure III.2;
NE = not estimated (data fit not possible with fixed PK; Table III.3)

Table III.3: Pharmacokinetic parameters estimated by curve fitting IV tobramycin blood level data to a 2-compartment open body model [a]

Data Source (ref)	V_c (L/kg)	k_{cp} (hr^{-1})	k_{pc} (hr^{-1})	k_{el} (hr^{-1})	MSC [b]	[c] r^2
Aminimanizani 2002	0.178 (0.033-0.208) [d]	0.92 (0.24-1.94) [d]	1.00 (0.53-3.16) [d]	0.64 (0.43-2.43) [d]	4.1-6.5	0.996-0.999
Pleasants 1988	0.125 (0.112-0.137) [e]	1.28 (0.75-1.91) [e]	1.66 (0.99-2.33) [e]	0.65 (0.55-0.75) [e]	4.8	0.998

[a] Gibaldi and Perrier, 1975; [b] Model Selection Criterion; [c] coefficient of determination; [d] median value (range) of the best estimates from 6 individual blood drug concentration versus time curves; [e] best estimate (95% CI) from curve fitting the mean blood drug concentration versus time curve.

In the study of Newhouse et al. in healthy volunteers, the PK data for a tobramycin dry powder (PulmoSphere™ formulation; PStob; Inhale Therapeutic Systems; San Carlos, CA) delivered from a passive dry powder inhaler, was also compared to those from a commercial nebulized tobramycin product TOBI® delivered by PARI LC Plus nebulizer (Newhouse et al., 2003). The values of F were 0.83 for the powder formulation and 0.78 for nebulized TOBI® (Table III.2). Despite these apparently "reasonable" estimates for F, the wide variation seen around the average value of CL (1.23; reported range 0.8 - 1.8 ml/min/kg) for normal subjects that were used to analyze these data (50 adults, Table III.1) did not inspire confidence in this assessment of tobramycin's pulmonary bioavailability. Overall, the results of Method 1 were inconclusive with respect to F, largely because of uncertainty in values for CL. Even so, the data were indicative of substantial pulmonary absorption, in spite of the known poly-cationic state of this drug at physiologic pH (Szilagyi et al., 1993). As expected (Weber et al., 1995), there was also little evidence seen for any contribution of GI absorption to tobramycin blood levels following pulmonary administration.

In the inhalation study for healthy subjects, the Newhouse data (Newhouse et al., 2003) showed good agreement with the PK model described in Scheme III.1, as shown in Figure III.1. The calculated F values are shown in the final column of Table III.2. The values of F in normal subjects for nebulized TOBI® and dry powder were 0.76 and 0.84, respectively, consistent with the values calculated by Method 1. The estimates for k_a ranged 0.30 – 0.41 h^{-1} in normals, corresponding to absorption half lives 1.7-2.3 hours, close to that of technetium-99 m-labelled diethylene triamine pentaacetic acid (^{99m}Tc-DTPA), a hydrophilic solute with a similar molecule size as tobramycin and recognized as a paracellular absorption marker following aerosol administration in human (Bondesson et al., 2007; Coates and O'Brodovich, 1986). For CF patients in the study of Pilcer et al. (2008), however, the method failed to provide good fits to the inhalation data for the treatment groups using both nebulized TOBI® and the uncoated powder; plasma concentrations in Pilcer, that apparently resulted from the reported TLD, could not be reached in simulations with this model, even when very large k_a values were employed. It is possible that underestimation of TLD and/or variations in the drug's intrinsic PK between patient cohorts were responsible for this apparent "failure" in curve fitting, but this also means that the estimate of F from Method 2 (= 0.99, Table III.2) for Pilcer's CF patients must also be viewed with suspicion, alongside the value for k_a (= 1.0 h^{-1}, Figure III.1). Notably, the possible underestimation of TLD in Pilcer's study, discussed for Method 1, above, could also be the explanation for the results seen here, even though two quite different approaches were used for calculation.

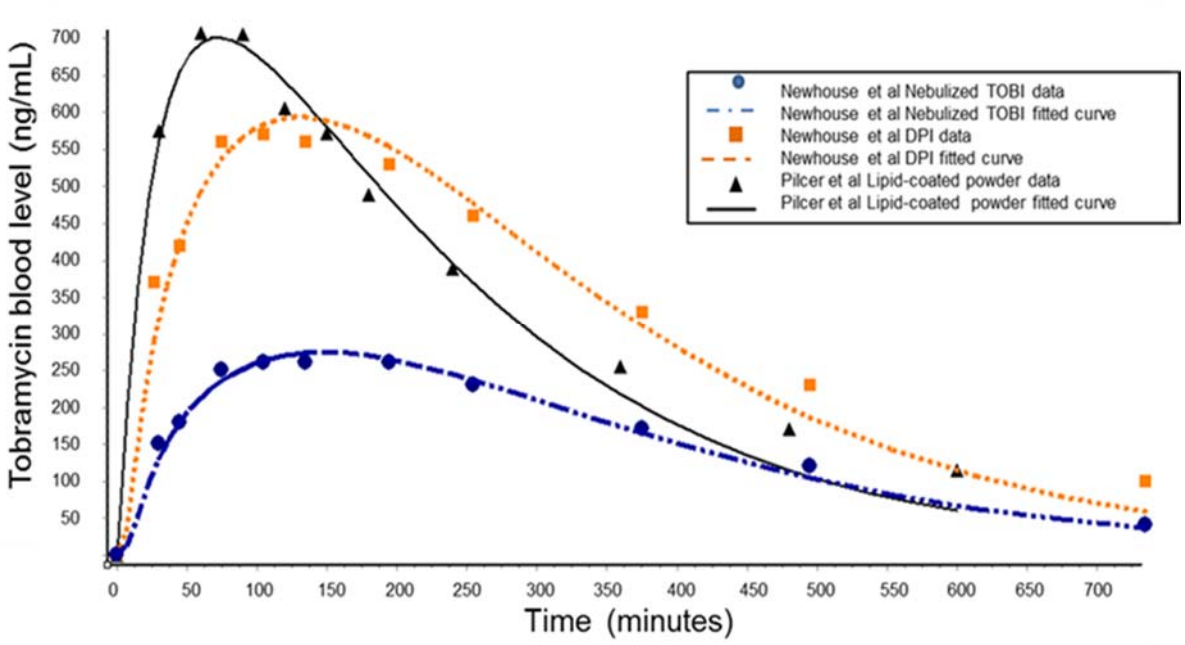


Figure III.1 Mean tobramycin blood level versus time curves following inhalation; data from Pilcer et al.(2008) and Newhouse et al.(2003). Best fits using nonlinear regression analysis to the PK model in Scheme III.1 according to Method 2 are shown as curves in which all rate constants other than k_a and k_{nal} were held constant at the values shown in Table III.3. Pilcer data for formulations other than the lipid coated powder could not be fitted with the fixed rate constants shown in Table III.2 (NE in Table III.2). Estimates for the absorption rate constant k_a were 1.00, 0.41 and 0.30 h^{-1} for Pilcer (TOBI®), Newhouse (DPI) and Newhouse (TOBI®), respectively.

Method 3

When the Loo-Riegelman method was used to analyze the data in the study of Newhouse et al. (2003) , the amount of bioavailable drug calculated for the nebulizer formulation TOBI® and the dry powder formulation was 12.2 mg (TLD=15 mg) and 26.1 mg (TLD=27 mg), corresponding to values for F = 0.82 and 0.97, respectively. These results were consistent with those from Methods 1 and 2, indicating tobramycin's pulmonary bioavailability as close to 100%. The percentages of drug remaining unabsorbed (100 - *abs%*) versus time plots (Figure III.2) in log-linear plots for both formulations indicated that tobramycin absorption from the lung appeared to be first-order. For Method 3 however, the absorption half lives fell in the range from 2.7-3.4 hours. Pilcer's data from CF patients for the nebulizer TOBI® could also be analyzed using this technique yielding the values for the bioavailable dose equal to 48.2 mg, more than two fold higher than the experimental determined lung deposited dose (22.8 mg) stated in the paper. For the lipid-coated and the uncoated drug powder formulation, the bioavailable doses were estimated at 15.3 mg and 11.4 mg, corresponding to 21 and 35% higher than the reported TLD for these two formulations, respectively. Clearly, these values for TLD, and the apparently non-linear absorption profiles in Figure III.2, for CF patients, were influenced significantly by the chosen estimates for the drug's intrinsic PK parameters; those were derived entirely from the data of Aminimanizani et al. (2002), and they may well be unrepresentative of the behavior of tobramycin in Pilcer et al.'s patients. Notably however, given intrinsic PK parameters from selected IV experiments that are representative of a drug's behavior following extravascular absorption, this approach (Method 3) may be an acceptable deconvolution method capable of determining independently, a value for the bioavailable dose.

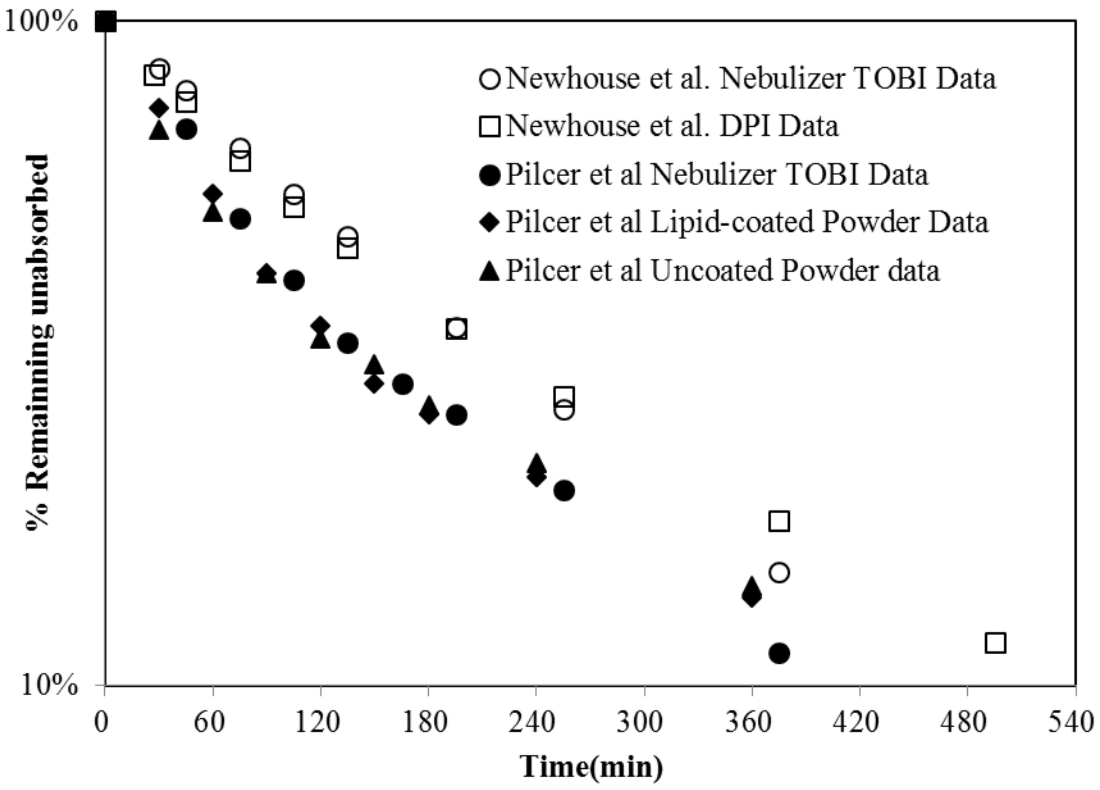


Figure III.2 Semi-Log plot of percentages of bioavailable tobramycin remaining to be absorbed versus time following inhalation. Percentages of bioavailable tobramycin remaining to be absorbed were calculated for the inhalation formulations in the studies of Pilcer et al.(2008) and Newhouse et al.(2003) by the Loo-Riegelman method (Gibaldi and Perrier, 1975). The apparent absorption half lives were 3.4 hr (nebulizer TOBI) and 2.7 hr (the DPI) for the data from healthy adults in the study of Newhouse et al.(2003), and 3.2, 3.3 and 3.4 hrs for the nebulizer TOBI, lipid-coated and uncoated DPI, respectively, in the study of Pilcer et al.(2008) on CF patients.

Notably, tobramycin absorption data (the percentages of drug remaining unabsorbed (100 - abs%) versus time) from the CF patients in the study of Pilcer et al. (2008) showed a slight deviation from mono-exponential absorption kinetics, showing a suspected biphasic absorption

behavior. One explanation for this apparent biphasic absorption behavior may possibly be decreased mucociliary clearance caused by mucus build-up, often associated with CF patients (Donaldson et al., 2007). Alternatively, drug binding and/or sequestration in lung tissues, that tends to prolong the drug's absorption after airway administration, may also be responsible for this biphasic absorption.

Sputum pharmacokinetics

Ten studies in CF patients were analyzed in an attempt to evaluate tobramycin concentration data in sputum following repetitive dosing by inhalation. Table III.4 shows the summary of sputum PK studies that were considered. After inhalation of tobramycin solution (either 300 mg in 4 mL of ½ normal saline (1/2NS) or 300mg in 5 mL ¼ NS were delivered by nebulizer, the drug sputum concentration could exceed by more than 100 times the reported tobramycin minimum inhibitory concentrations (MICs) for *Pseudomonas aeruginosa*; MICs have been shown to vary between 0.5 and 32 mg/L for isolates obtained from patients with cystic fibrosis treated with once- or thrice-daily intravenous tobramycin while the MIC_{90} (MIC required to inhibit the growth of 90% of organisms) after inhalation treatment was reported as 16 mg/L (Burkhardt et al., 2006; LiPuma, 2001). These high drug levels in sputum have been used in the literature to imply antimicrobial efficacy after local drug delivery to the lung.

Despite the large variations between subjects, the mean or median C_{peak}^{sputum} and AUC_{0-t}^{sputum}, were comparable between studies, given comparable formulations and delivery systems, e.g. TOBI® (300 mg/5mL in ¼ NS and pH 6.0) delivered by PARI LC PLUS® (Table III.4). The eFlow® nebulizer was reported to achieve a higher C_{peak}^{sputum} than the PARI LC PLUS® in one study

(Hubert et al., 2009), while this conclusion was not reached by others (Govoni et al., 2013; Griese et al., 2013), possibly due to large inter-subject variations. The values of tobramycin sputum concentrations determined in the study of Griese et al.(Griese et al., 2013) were relatively higher than those of other studies using the same formulation and delivery system for unknown reasons, although the high variability seen in sputum concentrations appear to be due mostly to variable inhalation and deposition patterns, airway obstruction and anatomy differences seen with age and disease, mucus plugging, individual sputum production rates and the use of different assay methods for sputum samples (Hubert et al., 2009).

Tobramycin retention and accumulation in the lung following inhalation were generally assessed by evaluation of the drug's persistence after a single dose and the changes in "peak" and "trough" sputum concentrations, as well as AUC_{0-t}^{sputum} values after repeated dosing. Theoretically, the drug peak concentrations in sputum may behave most likely as a surrogate of initial deposition in the airways and could be very variable (Okubo and Iyobe, 2002). Drug trough concentrations in sputum determined pre-dosing (approximately 12 h following last inhalation) can probably better reflect the true concentrations in the lung, since drug deposited in the upper airways such as laryngopharynx and trachea can be removed by clearance and swallowing within a couple of hours.

In general, tobramycin concentrations in sputum rapidly decreased 1-2 h after administration of TOBI® by PARI LC PLUS®, and then became undetectable 3 to 12 h after a single dose by inhalation, indicating rapid removal of this drug from the lung, by absorption into the systemic circulation, mucociliary clearance and swallowing. However, the values of trough tobramycin concentrations at steady state (pre-dose drug concentration in sputum at day 15 or day 28) after

twice daily dosing were reported to be 148±354 μg/g and 135±93 μg/g, by Hubert et al. in children and Govoni et al. in adults, respectively, implying that some drug accumulation occurred in the airways after repeated dosing, and not all of the administered dose is absorbed 12 hours after inhalation. Consistent with that data for escalated trough levels, tobramycin was found to accumulate 3.7-fold in the sputum with eFlow® and 2.1-fold with PARI LC PLUS®, shown by the ratio of sputum $AUC_{0\text{-}8h}$ between Day 15 and Day 1 in the study of Hubert et al. In contrast however, the study of Govoni, et al. reported tobramycin accumulation in sputum after 28 days' dosing (twice daily) as "low", because the ratios of C_{peak}^{sputum} and AUC_{0-8h}^{sputum} (geometric mean at 1.06 for C_{peak}^{sputum} and 1.61 for AUC_{0-8h}^{sputum}) between Day 28 and Day 1 differed insignificantly from unity (Govoni et al., 2013). In Govoni et al.'s study however, the large inter-subject variations seen in sputum concentrations likely disguised the possible change in C_{peak}^{sputum} and AUC_{0-8h}^{sputum}, caused by repeated dosing (Govoni et al., 2013).

In another study, tobramycin accumulation in the lung after 28 days' repeated dosing (twice daily) by inhalation in CF patients was evaluated in children less than 6 years old, using tobramycin concentrations in epithelial lining fluid (ELF) collected by bronchoalveolar lavage (BAL) (Gibson et al., 2003). The drug concentrations in ELF are believed to be more representative of levels in the deep lungs, compared to drug concentration in the sputum sample (Tayman et al., 2011), although ELF is more difficult to collect and concentration data more difficult to interpret. Nevertheless, tobramycin was detectable in ELF an average 12 h after the last dose at Day 28, with a mean (±SD) concentration of 74 (±50) μg/mL (Gibson et al., 2003). Compared with the mean (±SD) drug concentrations of only 90 (±54) μg/mL in ELF at the "peak" level (45 min after inhalation) and generally undetectable concentrations after 12 h after a single or first dose (Gibson

et al., 2003; Rosenfeld et al., 2001), these results clearly indicated drug accumulation in the lung after repeated doses. High trough drug levels in the ELF of young children at steady state, combined with the sputum results in CF patients in the studies of Govoni, et al. (2013) and Hubert et al.(2009), with ages ≥6 yrs, all support a finding of tobramycin retention and accumulation in respiratory secretions when repeated inhalation occurs in CF patients.

Table III.4 Summary of sputum pharmacokinetics for tobramycin following inhalation in CF patients.

Ref	N[1]	Age of subjects yrs	Sputum sampling times(post -dose)	Device/ Formulation	First day		Last day	
					[2] C_{peak}^{sputum} (mean±SD) ug/g	[3] AUC_{0-t}^{sputum} (mean±SD) µg*hr/g	$^{2}C_{peak}^{sputum}$ (mean±SD) µg/g	[3] AUC_{0-t}^{sputum} (mean±SD) µg*hr/g
Govoni 2013	27	≥18	0.5, 1.5, 3 and 8 h on day 1 and day 28	PARI eFlow® (300mg/4mL)	540.8 ±676.5	529.4±714.4	518.6±639.7	529.4±714.5
				PARI LC PLUS® (300mg/4mL)	491.3 ±690.1	573.1±706.6	819.9±1611.1	758.8±1470.4
Griese 2013	76	average 20	10 min at day 7	PARI eFlow® (150mg/1.5mL)	NR	ND	[4,8]2589 (1967,3211)	ND
				PARI LC PLUS® (300mg/5mL)	NR	ND	[4,8]2272 (1659,2885)	ND
Ruddy 2013	10	≥10	0.5-0.6 h on day 2 - 3 and day 26-28	PARI LC PLUS® (300mg/5mL)	[5] 961.0 (681.5,1.536)	ND	[5]1323 (520,1730)	ND
Hubert 2009	25	≥6	0, 0.5,1.5, 2 and 8 h on day 1 and day 15	PARI eFlow® (300mg/5mL)	981±1191	1688±2046	1572±2182	3731±7235
				PARI LC PLUS® (300mg/5mL)	754±927	1511±1744	769±823	1941±2301
Lenoir 2007	21	≥6	0.5 h on day 1 and day 28	PARI LC PLUS® (300mg/4mL)	[9] 695.6±817.0	ND	[9] 716.9±799	ND
Poli 2007	11	18-37	0.5, 3, 6,12,24 h	PARI LC PLUS® (300mg/4mL)	1289±851	NR	ND	ND
				PARI LC PLUS® (300mg/5mL)	816±681	NR	ND	ND
Geller 2007	84	≥6	0.5, 1, 2, 4, 8 and 12 h	PARI LC PLUS® (300mg/5mL)	737±1028	974±1143	ND	ND
				T-326 DPI (4*28mg)	1048±1080	1275±1359	ND	ND
Geller 2003	49	15-36	0.2,1, 2,4 and 8 h	AeroDose® 5.5 RP (90 mg/1.5 mL)	959±952	1307±978	ND	ND
				PARI LC PLUS® (300mg/5mL)	985.7±839.3	1471±1278	ND	ND
Eisenberg 1997	60	11-41	10 min, 1 and 2 h	UltraNeb (600mg/20mL)	[6]1359 (44-7782)	ND	ND	ND
				Sidestream (300mg/5mL)	[6]393 (57-1998)	ND	ND	ND
				PARI LC® (300mg/5mL)	[6]452 (47-3724)	ND	ND	ND
Weber 1994	7	≥7	0.05, 1, 2, 3, 4 h	Pulmo-Aid (80mg/4mL)	[7]325 (59-629)	NR	ND	ND

NR: not reported; ND: not determined; [1] number of subjects; [2] the "peak" sputum concentration, generally determined from samples taken 10-60 minutes post administration; [3] area under concentration versus time curve for tobramycin in sputum determined trapezoidally in each study; t = the last time points for sputum determination; [4] mean (90%CI); [5] median (25th, 75th percentiles); [6] median (range); [7] mean (range); [8] sputum concentrations determined on day 7; [9] concentration unit in µg/mL.

III. d CONCLUSIONS

In summary, the absorption of tobramycin from the lung appears to occur slowly but to be more complete than is commonly assumed based on the drug's theoretical behavior as an aminoglycoside poly-cation at physiologic pH (Szilagyi et al., 1993). The large variance seen in published studies of tobramycin's intrinsic pharmacokinetics (IV) precluded accurate assessment of its pulmonary bioavailability. A cross-over study, where drug plasma level versus time profiles are compared following IV and inhalation administration to the same subject cohort, is needed to eliminate the uncertainties associated with the evaluation of its fractional pulmonary absorption (F) in humans. In CF patients, tobramycin absorption from the lung following inhalation appeared to occur more rapidly than in normals but this could not be stated with certainty from the drug's pharmacokinetic data. The results from the sputum PK meta-analysis however, indicated that drug retention and accumulation in the lung following inhalation appeared to occur after repeated dosing, at least in CF patients. It was possible therefore, that some mechanism for tobramycin binding and/or sequestration was involved in extending the drug's retention at the site of its action in humans. Further investigation into the kinetics and mechanisms of tobramycin disposition following airway administration in animals could thus be an alternative way to explore the drug's binding and/or sequestration in lung tissues.

Chapter IV*

TOBRAMYCIN DISPOSITION IN THE RAT LUNG FOLLOWING AIRWAY ADMINISTRATION

IV.a INTRODUCTION AND THEORY

Administration of tobramycin directly to the airways via nebulizer has brought great improvements to the health and wellbeing of cystic fibrosis (CF) patients and others suffering from chronic pulmonary infections with pathogens such as *Pseudomonas aeruginosa* (Chuchalin et al., 2009).The efficacy and safety brought about by topical use of this drug is well-recognized in clinical circles, although it remains unclear whether the antibiotic's physicochemical properties lead to slow absorption, tissue binding and/or lung retention to sustain the drug's anti-infective properties for periods between dosing (Li and Byron, 2012; Patton et al., 2004). Notably, tobramycin is believed to cross epithelial barriers poorly and it is therefore marketed primarily as an injectable (Jaresko and Alexander, 1995; Phillips and Shannon, 1997). Our recent meta-analysis of pharmacokinetic data in humans following IV and inhalation administration found that it was not possible to come to a statistically sound conclusion about its bioavailability and possible binding to, or sequestration in, lung tissue following inhalation administration due largely to the variance associated with the available data (Li and Byron, 2012), even though tobramycin and

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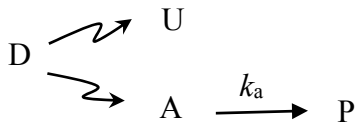
other aminoglycoside antibiotics are known to manifest some of their toxicity through tissue binding or sequestration and the creation of "deep compartments" (Mingeot-Leclercq and Tulkens, 1999; Nagai and Takano, 2004; Stepanyan et al., 2011). Notably, and probably because of the physiologic existence of these drugs as polycations, transfer into cells is slow and intracellular sequestration can be persistent. For these reasons we have used the isolated perfused rat lung (IPRL) to systematically investigate the disposition of tobramycin in the lung following its administration directly to the airways. The IPRL has been used similarly before (Byron et al., 1986; Niven and Byron, 1988; Niven et al., 1990; Sakagami et al., 2002). In the most simple cases, when solutes like fluorescein are administered in solution to the airways of this preparation, they behave in accord with Scheme IV.1. Absorption of fluorescein then occurs in a dose-independent, first-order fashion with no evidence of binding or metabolism. However, the nominal dose, or dose loaded into a dosing cartridge (Figure IV.1) usually results in a smaller administered dose (D*)* (Scheme IV.1), only part of which, the absorbable amount, *A* (*A* = D - U), where U is the transferable (unabsorbable) amount of each dose, can be transferred to the perfusate (P*)*. This is because the bronchial circulation in the IPRL is severed and only solute that deposits proximal to the actively perfused pulmonary circulation can be absorbed into the perfusate (Byron et al., 1986; Niven and Byron, 1988). In such a case, solute in perfusate will increase mono-exponentially toward an asymptotic value equal to the initial condition, A_0. The fraction of the administered dose (D) transferred to the perfusate (F_p) for a solute described by Scheme IV.1, is given as a function of time, *t*:

$$F_P = F_a \times (1 - e^{-k_a t}) \qquad \text{eq. IV.1}$$

where F_a is the absorbable fraction (= A_0/D) and k_a is the apparent first-order rate constant for absorption.



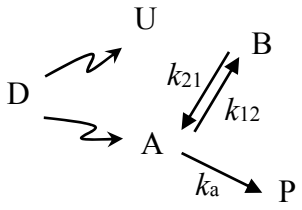
Scheme IV.1 Kinetic models describing solute (fluorescein) dosing and absorption from the airways of the IPRL in the absence of tissue binding or sequestration. Compartmental abbreviations show the administered dose (D), the untransferable (unabsorbable) amount of each dose (U), and the absorbable amount, A, (D=U+A), where P is the amount absorbed into the perfusate at time t. The apparent first-order rate constant for absorption from the airways is designated as *k*a.

In the case of other non-metabolized drugs that are absorbed by apparent first-order kinetics, binding or sequestration to lung tissue can be modeled in accord with Scheme IV.2. This Scheme differs from Scheme IV.1 because of the addition of a "bound drug" compartment (B) and the association and dissociation rate constants (k_{12} and k_{21}). In Scheme IV.2, which is analogous to a two-compartment pharmacokinetic model, the drug in A and B should behave in a similar fashion to drug in plasma and drug in tissue, respectively, whereas drug in P should behave in the same way as cumulative elimination. Thus, the fraction in the perfusate, F_p, for Scheme IV.2 is given by a rearranged form of eq. 216 from Gibaldi and Perrier(Gibaldi and Perrier, 1975) and the data for drug in perfusate should conform to

$$Fp = Fa * (1 - \frac{k_a - \beta}{\alpha - \beta} e^{-\alpha t} - \frac{\alpha - k_a}{\alpha - \beta} e^{-\beta t}) \qquad \text{eq. IV.2}$$

where $\alpha = \left(k_a + k_{12} + k_{21} + \sqrt{(k_a + k_{12} + k_{21})^2 - 4k_{21}k_a}\right)/2$

and $\beta = \left(k_a + k_{12} + k_{21} - \sqrt{(k_a + k_{12} + k_{21})^2 - 4k_{21}k_a}\right)/2.$



Scheme IV.2 Kinetic models describing solute (tobramycin) dosing and absorption from the airways of the IPRL in the presence of tissue binding or sequestration. Compartmental abbreviations show the administered dose (D), the untransferable (unabsorbable) amount of each dose (U), and the absorbable amount, A, (D=U+A), whereas P and B are the amount of drug absorbed into the perfusate and the amount of drug bound to, or sequestration in, tissue at time t, respectively. The apparent first-order rate constant for absorption from the airways is designated as ka, whereas k_{12} and k_{21} are first-order association and dissociation rate constants for binding or sequestration.

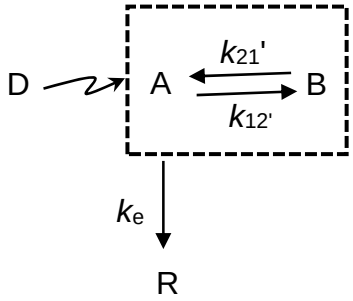
In this current study, the dynamic dialysis techniques, first described by Meyer and Guttman and used subsequently by other groups to study solute-ligand binding (Bottari et al., 1975; Hashimoto et al., 1984; Hiji et al., 1978; Meyer and Guttman, 1968; 1970; Pedersen et al., 1977; Sparrow et al., 1982), were first used to support the use of the IPRL binding and sequestration model described in Scheme IV.2. When dynamic dialysis under sink conditions is used to extract solutes from sliced IPRL tissue following initial airway dosing to that tissue, a model analogous to Scheme IV.2, Scheme IV.3 applies (Meyer and Guttman, 1968). In contrast to Scheme IV.2, Scheme IV.3 lacks an untransferable component, i.e. U = 0, because all of D that is administered to the airways becomes available for dialysis, so the amount released into the receiver solution, R, becomes equal to D as t tends to infinity. Furthermore, the binding or sequestration rate constants, k_{12}' and k_{21}', differ from those in Scheme IV.2 because sliced tissue and drug release from the dialysis sac differ from the intact IPRL. The

first-order rate constant for solute release, k_e, from a dialysis sac into R depends largely on the diffusive properties of the solute and the conditions of dialysis. However, the fraction of administered dose remaining in the sac (A_t/A_0), at time t, should be a reflection of the solute's tissue binding, and should theoretically conform to eq. 218 from Gibaldi and Perrier (Gibaldi and Perrier, 1975) , where

$$A_t/A_0 = \frac{k_e-\beta}{\alpha-\beta}e^{-\alpha t} + \frac{\alpha-k_e}{\alpha-\beta}e^{-\beta t} \qquad \text{eq. IV.3}$$

in which $\alpha = \left(k_e + k_{12}' + k_{21}' + \sqrt{(k_e + k_{12}' + k_{21}')^2 - 4k_{21}' k_e}\right)/2$

and $\beta = \left(k_e + k_{12}' + k_{21}' - \sqrt{(k_e + k_{12}' + k_{21}')^2 - 4k_{21}' k_e}\right)/2$



Scheme IV.3: Kinetic model describing solute dosing and dialysis from the IPRL in the presence of tissue binding or sequestration; the dialysis sac is shown diagrammatically as a dashed line. Compartmental abbreviations show the administered dose (D) and the dialyzable amount, A, (A=D), while R and B are the amount released into the receiver solution and the amount of drug bound to or sequestered in tissue at time t, respectively. The apparent first-order rate constant for dialysis is designated as *k*e, while k_{12}' and k_{21}' are the association and dissociation rate constants for binding/sequestration to the sliced tissue.

IV.b MATERIALS AND METHODS

Experimental Design. Tobramycin, mannitol and fluorescein were administered in different aqueous solution formulations, by forced intratracheal instillation, to the airways of the

isolated perfused rat lung preparation (Figure IV.1) using a previously described method (Byron and Niven, 1988). Using this technique, studies were performed to explore the kinetics and mechanisms responsible for tobramycin's pulmonary disposition. **IPRL Absorption Studies** were performed to elucidate the effects of concentration on airway-to-perfusate transfer (absorption). Prior to the analysis of data from those studies, **IPRL Binding Studies** were performed using dynamic dialysis (Meyer and Guttman, 1968). Those binding experiments employed identical surgical preparation, perfusion and airway dosing as those for **IPRL Absorption Studies**, after which the extent and rate of tobramycin and/or mannitol dialysis from lung tissue was determined.

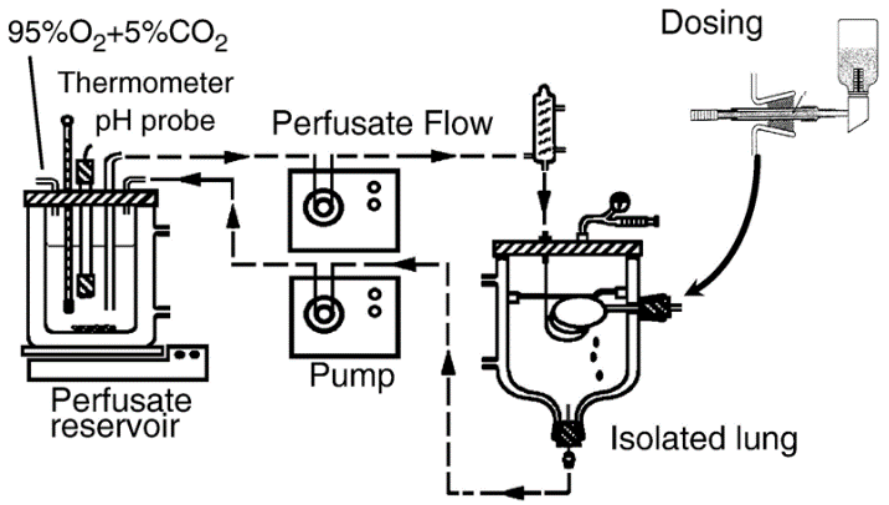


Figure IV.1 The IPRL preparation showing perfusate circulation. The lung was suspended horizontally in the artificial glass thorax (AGT) after cannulation of the pulmonary artery and trachea (Sakagami, 2006).

Animals. Specific pathogen-free, 300–400g, male Sprague-Dawley rats (Hilltop Lab Animals Inc., Scottsdale, PA) were used throughout. The animals were housed with access to water and food at 30-70% relative humidity (RH) and 18-26˚C, and a 12-hr light/dark cycle

for ≥ 2 days before sacrifice. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University.

Chemicals and Materials. Tobramycin (≥98% free base) and bovine serum albumin, BSA, ≥98% (agarose gel electrophoresis) lyophilized powder (Sigma-Aldrich, St Louis, MO) were stored according to USP-NF 2007 (United States Pharmacopeia and National Formulary, 2007) . Krebs-Henseleit solution (KHS) containing 4% (w/v) BSA (K4) was freshly prepared and used as perfusate in the isolated perfused rat lung preparation. [^{3}H]tobramycin and [^{3}H]mannitol solutions (0.8 Ci/mmol, 1 mCi/mL; 10-30 Ci/mmol, 1 mCi/mL, respectively) were purchased from Moravek Biochemicals (Brea, CA) and PerkinElmer Life and Analytical Sciences (Waltham, MA), and sodium fluorescein (F-Na) was obtained from Arocos Organics (Geel, Belgium). Ecoscint XR scintillation cocktail (National Diagnostics, Atlanta, GA) was used for liquid scintillation counting. Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Dialysis Tubing (Snakeskin®, 10K molecular weight cut-off, MWCO; Thermo Scientific, Rockford, IL) was washed with water and immersed in aqueous buffer solutions for ≥30mins prior to use.

Dosing Solutions. Aqueous radiolabeled tobramycin solutions were prepared in different concentrations (100, 20, 2, 0.2, 0.04 and 0.02 mg/mL) containing a nominal radiolabel content of 18 μCi/mL in 0.45% w/v NaCl and adjusted to pH 7.4 with H_2SO_4. Radiolabeled mannitol was prepared with different concentrations at 12 μCi/mL, while sodium fluorescein (unlabeled) solutions were prepared in pH 7.4 phosphate buffer and used at a concentration of 0.2 mg/mL. While the majority of the experiments involving tobramycin were performed with dosing

solutions containing 0.45% w/v NaCl (1/2NS), osmolality was varied in some investigations by using different concentrations of sodium chloride.

IPRL Binding Studies. A dynamic dialysis method (Meyer and Guttman, 1968) was modified to investigate solute binding or sequestration to lung tissue. Tobramycin binding was investigated across doses (0.002, 0.004, 0.2 and 2 mg) by comparing its interaction with lung tissue to that of mannitol (a non-binding solute). In binding studies, the IPRL preparation was used and dosed in exactly the same way as described later for **IPRL Absorption Studies**. After dosing, the isolated lung preparation was taken out of the artificial glass thorax (AGT), sliced into 60-90 rectangular pieces (maximum linear dimension = 4mm) and transferred to a dialysis sac (dry length: 15 cm, washed and immersed in KHS solution prior to use) with 10 mL of K4. To determine the intrinsic diffusive properties of tobramycin and mannitol in this system, solute dialysis was also studied in the absence of lung tissue and BSA, from sacs with identical total volumes of KHS. Sacs were closed, with minimal headspace, by ligating with cotton thread, after which they were immersed at time zero in the perfusate reservoir containing 200 mL magnetically-stirred receiver solution (KHS or K4) maintained at 37˚C and pH 7.4. The solute mass enclosed in the sac at time zero (A_0) was determined by subtracting the solute lost during lung slicing (by assay of rinse solutions used in the procedure) from the administered dose, D (determined as described in the **IPRL Absorption Studies**). One mL samples were removed for assay from the receiver solution at 5, 10, and 20 minutes after which a 100 mL samples was withdrawn at each time point of 30, 60, 90, 120, 180 minutes and hourly thereafter. An equal volume of solute-free receiver solution (37^{o}C) was added to replace each sampling aliquot and restore the receiver volume to 200 mL. The

amount of solute remaining in the sac as a function of time (A_t) was calculated assuming mass balance.

IPRL Absorption Studies. Use of the IPRL preparation (Figure IV.1) to study solute absorption was carefully controlled as described previously (Byron and Niven, 1988; Sun et al., 1999). Briefly, a rat lung was surgically removed and housed in an artificial glass thorax (AGT) maintained at 37°C. Krebs-Henseleit solution (KHS) with 4% (w/v) BSA was used as perfusate (K4; 200 mL) and recirculated through the pulmonary circulation via the pulmonary artery at a constant flow rate of 15 mL/min. A metal dosing cartridge containing 0.1 mL dosing solution was inserted into the trachea via a tracheal cannula. A metered dose inhaler (MDI, 25 μL drug-free CFC propellants per actuation) was connected to the dosing cartridge and actuated once. The dosing solution was propelled into the lung as a coarse spray, and the lung was inflated simultaneously to ~6 mL. The dosing cartridge was removed and the lung allowed to deflate (Byron and Niven, 1988). The administered dose (to the airways of the IPRL) was determined from the initial weight of the primed dosing cartridge, solution density and concentration after subtracting the mass remaining in the dosing cartridge after administration (determined by assay). The perfusate samples were taken from the well-mixed reservoir at time zero (blank sample, immediately prior to dosing) and subsequently at 1, 3, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min following dosing. Solute concentrations were determined as described under "Assay Methods". Sufficient IPRL studies were performed to yield no less than four fully viable preparations for each dosing solution, as evidenced by the absence of any signs of edema onset over 120 min (preparations that were "nonviable" at times $\leq$ 120min were discarded). We have shown previously, and observe

consistently with this preparation, that "signs of edema onset" occur when the (blood-free) lungs change in outside color and texture from smooth white to a grey and/or patchy appearance; this appearance change is an early indicator of the preparation's declining viability, shortly after which values for the wet lung/dry lung weight ratio, epithelial permeability to solutes and other effects change markedly (Byron et al., 1986; Niven et al., 1990). At the end of each IPRL absorption study, the lung tissues were collected, homogenized and assayed for solute remaining in both the airways and the lung tissue.

Assay Methods. [^{3}H]Tobramycin and [^{3}H]mannitol concentrations were determined by scintillation counting relative to standards prepared freshly for each experiment (Liquid Scintillation Analyzer, Tri-Carb 2800-TR, PerkinElmer Life and Analytical Sciences) after first validating the radioactivity assay for tobramycin with high performance liquid chromatography–mass spectrometry (HPLC-MS) to ensure that chemical degradation and/or metabolism did not occur in lung tissue or perfusate over the duration of a typical experiment (see Appendix III and IV). In brief, perfusate samples containing radiolabeled solutes were used neat or diluted in K4. One milliliter aliquots were added to 5 mL scintillation cocktail (Ecoscint XR; National Diagnostics) in 7-mL polypropylene scintillation vials and well mixed. Total radioactivity was expressed as disintegrations per minute. Disintegrations per minute from accurately prepared [^{3}H]tobramycin or [^{3}H]mannitol standard solutions in K4 were measured independently for each assay series in order to calculate solute concentrations in unknown test solutions. Fluorescein concentrations in perfusate were assayed by spectrofluorophotometer (RF-5301 PC, Shimazu Corporation, Kyoto, Japan; λ_{ex} and λ_{em} = 490

and 520 nm, respectively), as described earlier (Byron and Niven, 1988; Byron et al., 1986; Sakagami et al., 2002).

Data analysis. Kinetic Analysis was performed according to the theory described above and Schemes IV.1-3. The data from IPRL binding studies using dynamic dialysis were expressed in accord with Scheme IV.3, as the fraction of the solute dose (At/A_0) remaining in the sac as a function of time. The k_e value for tobramycin was first determined by studying its dialysis kinetics in the absence of IPRL components (tissue and protein). In this case, linear regression analysis of the first-order data for ln $[A_t/A_0]$ versus t was performed to determine its value (Meyer and Guttman, 1968). The binding rate constants, in the presence of lung tissue, k_{12}' and k_{21}', were then determined by fitting data for $[A_t/A_0]$ versus t to eq. IV.3 by least mean square non-linear regression analysis. The value for k_e was fixed, whereas k_{12}' and k_{21}' were allowed to float. Goodness-of-fit was assessed using the calculated r^2 and model selection criterion (MSC) by Scientist 3.0 (MicroMath Scientific Software, Salt Lake City, UT). Data from IPRL Absorption Studies were grouped in accord with the nominal dose initially added to the dosing cartridge. Each nominal dose resulted in a mean value for D (Scheme IV.1), due to solution retention in the cartridge. Fluorescein absorption into perfusate was expressed as the mean fraction of each administered dose reaching the perfusate, F_p, versus time. Best estimates for fluorescein's mean absorbable fraction, F_a (=A_0/D; Scheme IV.1) and its apparent first-order rate constant for absorption, k_a, were obtained by fitting the unweighted data for F_p versus time to eq. IV.1 using Scientist 3.0. Because fluorescein that penetrates the airways proximal to the circulating perfusate is known to be completely absorbed, this provided a mean value for the solute's eventually absorbable fraction, F_a that could be used

across solutes. Tobramycin data was analyzed according to Scheme IV.2, where best estimates for the rate constants describing binding and absorption (k_{12}, k_{21}, k_a) at different nominal doses were determined by curve-fitting tobramycin F_p (fraction of administered dose in perfusate) versus time data to eq. IV.2.

IV.c RESULTS

IPRL Binding Studies. Tobramycin dialysis from the sac is shown as mean A_t/A_0 (fraction of administered dose remaining in the sac) versus time in Figure IV.2A. Biphasic (biexponential) profiles resulted from experiments in which drug release from sliced lung tissue occurred after airway dosing to the IPRL at five different dose levels; smaller doses showed greater values for tissue retention with time. In the absence of lung tissue, control experiments showed mono-exponential (apparent first-order) release of tobramycin from the dialysis sac under sink conditions (Meyer and Guttman, 1968). Following the data analysis, the mean dialysis rate constant, k_e (Scheme IV.3), for tobramycin was found to be dose-independent ($0.0107\pm0.0021 min^{-1}$; Figure IV.2A). Mannitol, a nonbinding solute, produced monoexponential and dose-independent A_t/A_0 profiles in the presence and absence of IPRL tissue (Figure IV.2B). Tobramycin dialysis data from IPRL tissue (Figure. IV.2A) was fitted to eq. IV.3 to produce the best estimates of the rate constants in Scheme IV.3. These are shown in Table IV.1 for each dose; dashed curves in Figure IV.2A were produced by simulation using the rate constant values shown in the table. Tobramycin's dissociation rate constant (k_{21}') was effectively dose-independent, whereas k_{12}' appeared saturable and decreased with ascending dose.

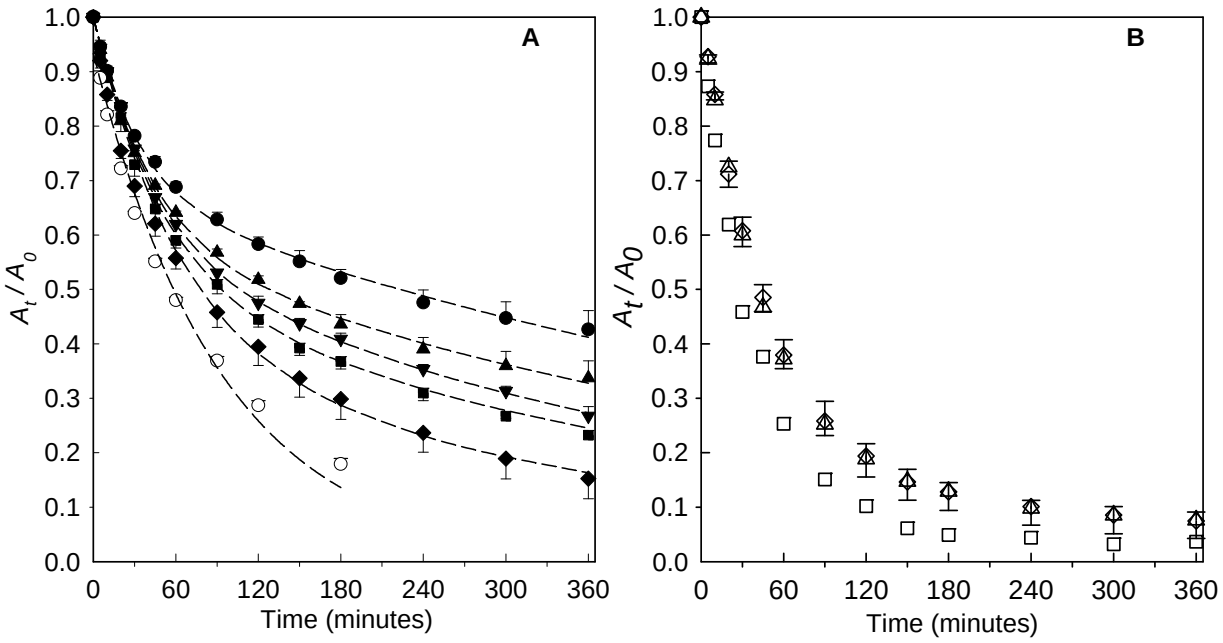


Figure IV.2. Mean fraction of solute remaining in the dialysis sac, A_t/A_0, versus time. (A) Tobramycin at different nominal doses. Dashed lines show the best fits to eq. IV.3. Error bars are sample SDs (n≥3). ●, 0.002 mg; ▲, 0.004 mg; ▼, 0.2 mg; ■, 2 mg; ◆,10 mg; ○, control (tobramycin 2 mg and 0.002 mg in absence of IPRL). (B) Mannitol at two nominal doses. △, 0.02 mg; ◇, 2 mg; □, control, (0.02 mg of mannitol in absence of IPRL).

Table IV.1: Best estimates (95% CI) of tissue binding/sequestration rate constants in Scheme IV.3 (eq. IV.3) from dynamic dialysis alongside the coefficient of determination, r^2, and "model selection criterion" (MSC).

The value of k_e was fixed at 0.0107 min^{-1}, its value in the absence of lung tissue.

Dose mg	k_{12}' min^{-1}	k_{21}' min^{-1}	k_{12}'/k_{21}'	r^2	MSC
0.002	0.0174 (0.0160-0.0189)	0.0041 (0.0033-0.0048)	4.29	0.9999	5.74
0.004	0.0112 (0.0099-0.0124)	0.0037 (0.0027-0.0046)	3.02	0.9998	5.70
0.2	0.0094 (0.0087-0.0100)	0.0043 (0.0037-0.0050)	2.17	0.9999	7.03
2	0.0070 (0.0060-0.0080)	0.0038 (0.0025-0.0051)	1.83	0.9997	5.89
10	0.0052 (0.0031-0.0074)	0.0039 (0.0033-0.0045)	1.3.	0.9982	4.29

IPRL Absorption Studies. F_p versus time data for fluorescein (Figure IV.3) was fitted to eq. IV.1 to obtain best estimates of the absorbable fraction (F_a) and the first-order rate constant for absorption (k_a) for this nonbinding solute; the values were 0.75 and 0.076 min^{-1}, respectively (Table IV.2). Although mannitol absorption was too slow to reach an asymptote within the IPRL's viable lifetime (Sun et al, 1999), its k_a values shown in Table IV.2 were derived by fitting mannitol data to eq. IV.1 assuming that the absorbable fraction, F_a, was the same as that of fluorescein (F_a = 0.75). While that assumption may not be completely accurate, the approach was supported by the absence of a statistical difference between mannitol's F_p (fraction of administered dose in perfusate) versus time profiles at different nominal doses (0.02 and 2 mg; t-test, $p < 0.05$; data not shown) and the agreement of k_a values (Table IV.2) with reports in the literature for mannitol in the *in situ* rat lung where absorption half lives were derived by measuring solute disappearance from the lung (Brown and Schanker, 1983). In contrast to fluorescein and mannitol, tobramycin's absorption was clearly dose-dependent, the rate and extent of its pulmonary absorption increasing with the magnitude of the administered dose (Figure IV.3). At 120 minutes following tobramycin administration, the values for mean F_p (± SD; nominal dose) were 0.449 (± 0.024; 0.002 mg); 0.505 (± 0.021; 0.02 mg); 0.587 (± 0.028; 0.2 mg) and 0.612 (±0.050; 2 mg). Tobramycin's F_p versus time data showed good agreement with Scheme IV.2 and eq. IV.2, as indicated by the continuous curves shown in Figure IV.3. Best estimates of the rate constants used to generate these curves are reported in Table IV.2 alongside the values for the coefficient of determination, r^2, and the model selection criterion (MSC). The rate constant for absorption appeared to be dose-independent (range from 0.065 to 0.070 min^{-1}) and close to that of fluorescein (0.076 min^{-1}). The rate constant for dissociation from intact IPRL tissue (k_{21}) was also relatively constant (0.018-0.022 min^{-1}), while that for association (k_{12}) decreased from 0.164 to 0.072 min^{-1} with increasing

airway dose from 0.002 to 2 mg. As a result, the ratio of k_{12}/ k_{21} decreased from 8.9 to 3.4 when the nominal dose was increased from 0.002 to 2 mg.

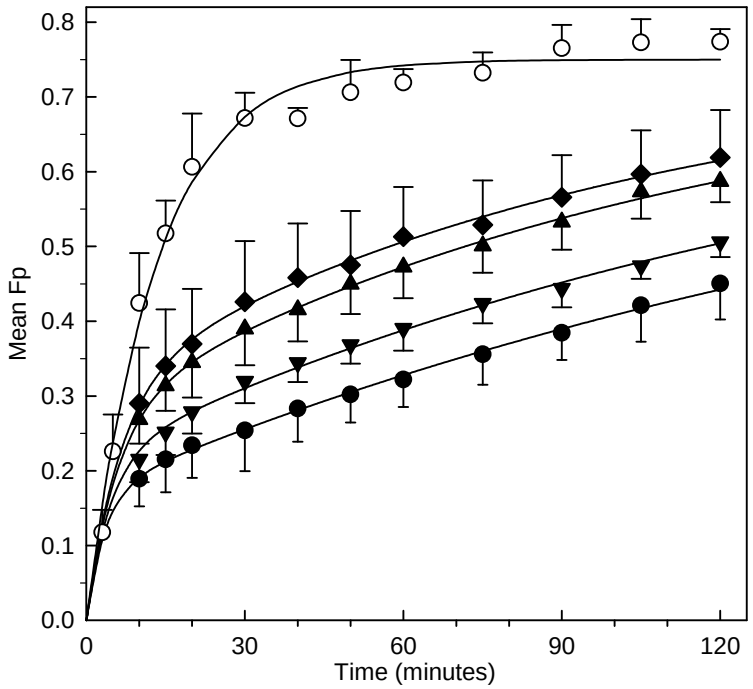


Figure IV.3. Mean F_p = mean (±SD) fraction of administered dose (D) transferred to the perfusate versus time for fluorescein (nominal dose: 0.02 mg; open symbols) and tobramycin (closed symbols) in the IPRL. Nominal doses were 0.002 (●; n=6); 0.02mg (▼; n=5); 0.2mg (▲; n=5) and 2mg (◆; n=4). Solid curves are the best fits for all profiles to eq. IV.1 (fluorescein) and eq. IV.2 (tobramycin) based on Schemes IV.1 and 2, respectively. F_a, the absorbable fraction of the administered dose, was fixed at 0.75, whereas k_a, k_{12}, and k_{21} were allowed to float to produce the best estimates shown in Table IV.2.

Table IV.2. Estimated parameters (95% CI) and goodness-of-fit for different solutes in accord with Scheme IV.1 (fluorescein and mannitol) and Scheme IV.2 (tobramycin) in the IPRL Absorption Studies with $F_a = 0.75$.

Blank cells for k_{12} and k_{21} indicate data consistent with Scheme IV.1.

	Dose mg	k_a min^{-1}	k_{12} min^{-1}	k_{21} min^{-1}	k_{12}/k_{21}	r^2	MSC
Tobramycin	0.002	0.0651 (0.0150-0.0852)	0.1639 (0.0970-0.2308)	0.0184 (0.0161-0.0207)	8.91	0.9998	6.04
	0.02	0.066 (0.0572-0.0748)	0.1307 (0.1226-0.1388)	0.0205 (0.0184-0.0225)	6.38	0.9998	6.06
	0.2	0.0695 (0.0610-0.0781)	0.0905 (0.0714-0.1097)	0.0223 (0.0199-0.0246)	4.06	0.9999	6.89
	2	0.07 (0.0626-0.0774)	0.0721 (0.0575-0.0867)	0.0214 (0.0189-0.0239)	3.37	0.9999	6.82
Mannitol	0.02	0.0184 (0.0154-0.0213)				0.9896	2.25
	2	0.0198 (0.0180-0.0216)				0.995	3.45
Fluorescein	0.02	0.0761 (0.0694-0.0828)				0.9988	4.8

IV.d Discussion

Binding of tobramycin and other aminoglycosides to various tissues has been used to explain the formation of deep compartments in pharmacokinetic studies " (Mingeot-Leclercq and Tulkens, 1999; Nagai and Takano, 2004; Stepanyan et al., 2011), some epithelial cells of which (e.g. kidney proximal tubule and inner ear hair cells) are associated with nephro- and ototoxicity (Hiel et al., 1993; Just and Habermann, 1977; Nagai and Takano, 2004; Todd and Hottendorf, 1995). It is noteworthy, and probably because of the physiologic existence of

these drugs as polycations, transfer into cells is slow and intracellular sequestration can be persistent. In the hope of elucidating the drug's binding to and/or intracellular sequestration by lung tissue, dynamic dialysis was performed both in the presence and absence of tissue, and from the sliced IPRL, after dosing the airways using the same technique as that used for *IPRL* absorption studies. The open circles in Figure IV.2A show the apparent first-order, dose-independent dialysis of tobramycin in the absence of lung tissue in which there was no evidence of drug binding. The comparator data shown in Figure IV.2B, for the nonbinding solute mannitol, was monoexponential and dose-independent (r^2 >0.98) both in the presence and absence of tissue, indicating only that the presence of sliced lung tissue slightly reduced the value of k_e for diffusive release from the sac (Scheme IV.3; k_e for mannitol = 0.025 and 0.019 min^{-1} in the absence and presence of sliced IPRL tissue, respectively) by hindering diffusion. Most notably, the tobramycin data for A_t /A_0 (fraction of administered dose remaining in the sac) versus time at different doses in the presence of lung tissue were described well by eq. IV.3 and Scheme IV.3 (r^2>0.998) showing clear evidence of dose-dependent lung tissue binding or sequestration, as summarized by the decreasing values for the forward binding rate constant, k_{12}' and the ratio k_{12}'/k_{21}' as doses were increased (Scheme IV.3, Table IV.1). While the model described in Scheme IV.3 assumes that tissue binding is zero at t=0 (and in practice, some binding must have occurred during the short period used for tissue slicing and dialysis set-up), these values for k_{12}' and k_{21}' implied that tissue binding occurred relatively slowly after airway administration and that all three rate constants in Scheme IV.3 had a similar order of magnitude. Efforts to determine whether significant binding/sequestration was due to tissue or the constituents of the IPRL airway lining fluid implied that lung tissues themselves were largely responsible for antibiotic sequestration.

Bronchoalveolar lavage samples; diluted with KHS containing low tobramycin concentrations (consistent with doses of approximately 0.002 mg) showed no difference in drug dialysis kinetics from the IPRL tissue-free control data for A_t/A_0 in Figure IV.2A.

This "slow-on" and "slow-off" binding behavior seen during dynamic dialysis supported the kinetic analysis of F_p versus time data in IPRL absorption studies. When the ex vivo IPRL was used, the values of the aminoglycoside's binding constants in Scheme IV.2 (Table IV.2) were quantitatively different to those from the dialysis experiments (association and dissociation occurred faster under conditions involving absorption into perfusate), although the overall trend in the rate constant values was the same as that seen during dialysis. Neither fluorescein nor mannitol showed dose-dependent binding behavior and both showed monoexponential absorption properties (k_a and F_a values) consistent with reports in the literature (Brown and Schanker, 1983; Byron and Niven, 1988). Tobramycin, however, showed decreasing rates of fractional absorption (transfer from airways to perfusate) as a function of decreasing dose and increasing binding/sequestration. Notably, dosing solution osmolality appeared to have no effect on F_p versus time data. For example, F_p at 120 min for a tobramycin dose at 0.2 mg in 0.9% w/v NaCl showed no statistical difference to that from 0.2 mg in 0.45% w/v NaCl (*t* test, $P < 0.05$). However, the data for F_p versus time at different doses were described well by eq. IV.2 and Scheme IV.2 (r^2>0.998) with clear evidence of dose-dependent and possibly saturable lung tissue binding summarized by the decreasing values for the forward binding rate constant, k_{12} and the ratio k_{12}/k_{21} as doses were increased (Table IV.2). The significant difference between the retention of this solute in lung tissue was related to tobramycin's slow binding or sequestration following its administration. Values for

k_{21} appeared unrelated to dose, but values of k_{12} decreased as the dose was increased, but without evidence of capacity limitation. While the exposure of different tissue sites was clearly possible for absorption and dialysis experiments (IPRL was sliced immediately following dosing), the trend in the data for the lung tissue binding or sequestration constant, k_{12} (increases with decreasing dose) and k_{21} (effectively dose-independent), was consistent with the rate constant data for the sliced IPRL.

To the best of our knowledge, this is the first work to investigate tobramycin binding to and/or sequestration by lung tissue and explore its effects on the drug's pulmonary disposition following airway administration. Dynamic dialysis, initially used for protein binding studies, was successfully employed to study tissue binding and sequestration, and the results were consistent with those from the realistic ex vivo IPRL model. Based on the results, it was possible to calculate the apparent elimination half-life from the IPRL (equal to 0.693/β; eq. IV.2) as 2.3, 1.8, 1.3 and 1.2 hours for nominal doses of 0.002, 0.02, 0.2 and 2 mg, respectively. Notably, if binding did not occur, absorption into the perfusate (and thus, elimination from the IPRL) should occur with a half-life of a ~10 minutes ($0.693/k_a$; Table IV.2), similar to that for fluorescein anions (Niven and Byron, 1988; Table IV.2). The 1.8-hour half-life at a nominal dose of 0.02 mg is consistent with the in vivo lung elimination half-life of 2.1 hours reported by Valcke and Pauwels (1991) in rat alveolar lining fluid following aerosol administration of a similar dose .

In clinical treatment of humans, tobramycin inhalation solution (TOBI®; Novartis, Basel, Switzerland), the commercial nebulizer formulation used for cystic fibrosis patients, is

prescribed as an intermittent (28 days on/28 days off) treatment with 300 mg/5 mL nebulized and inhaled twice daily regardless of age (≥6 yrs) or body weight. Since the percentage of each nebulizer dose reaching the lung via the recommended PARI LC PLUS nebulizer has been reported to be ~15% (Lenney et al., 2011), a lung dose of ~45 mg could be expected in a 70-kg human. Based on weight scaling, this corresponds approximately to a nominal dose of approximately 0.2 mg to the airways of the rat lungs used in the IPRL studies described here, where the k_{12}/k_{21} ratio was 4.06 (Table IV.2). Although species differences may well exist, similar cell constituents and concordance with alveolar surface area, lung volume, capillary volume and body weight have been reported across many species, including rats and humans (Crapo et al., 1983; Cryan et al., 2007; Plopper, 1983). Therefore, in conclusion, these studies in rat lung appear to support the existence of tobramycin retention in lung tissue following airway administration at doses likely to produce airway concentrations seen in humans. As a result of this slow-on and slow-off tissue binding or sequestration, the antibiotic's longevity in the lung is extended. It is possible that this may account, at least in part, for the apparent success of tobramycin inhalation therapy seen in clinical practice.

Chapter V

THE EFFECTS OF OSMOLALITY, pH AND TOBRAMYCIN CONCENTRATION OF DOSING SOLUTIONS ON AIRWAY EPITHELIAL INTEGRITY IN THE RAT LUNG FOLLOWING AIRWAY ADMINISTRATION

V.a INTRODUCTION

Pharmaceutical formulators often aim to make solutions for inhalation isotonic with blood and neutral with respect to pH. In practice, they frequently compromise these "targets", but need to observe certain limits for osmolality and pH deviations to avoid issues associated with airway irritation and potential pulmonary epithelial damage (Weber et al., 1997). Administration of non-isotonic and non-physiologic pH solutions to the airways of sensitive individuals has been reported to cause cough, bronchoconstriction, airway hyperactivity, or bronchospasm (Eschenbacher et al., 1984; Godden et al., 1986) and the occurrence of such airway reactions has sometimes been associated with alterations in pulmonary epithelial integrity, evidenced by an increase of intercellular space and the opening of tight junctions (Ohashi et al., 1992); this can lead to increases in airway epithelial permeation to solutes that diffuse via paracellular pathways, such as mannitol and ^{99m}Tc-DTPA (technetium-99 m-labelled diethylene triamine pentaacetic acid) (Wan et al., 2000; Wells et al., 1994).

Aminoglycosides and other polycations in certain concentration ranges have also been reported to affect the integrity of various epithelia, including the airways, as manifested by increases in epithelial permeation and damage caused by the cytotoxicity of these compounds in vivo and in vitro (Dong et al., 2011; Florea et al., 2006; Karasawa and Steyger, 2011; Nagai and Takano, 2004; Schipper et al., 2000; Seki et al., 2008). As to aminoglycosides specifically, this class of drugs in certain concentration ranges has been reported to affect the permeation of vestibular epithelia and proximal tubular epithelia, and also could cause cell apoptosis and necrosis due to high local concentrations seen in studies of drug-induced oto- and nephro-toxicity (Karasawa and Steyger, 2011; Kim et al., 2005; Todd et al., 1994).

As all these factors may also perturb tobramycin's disposition following airway administration, the IPRL studies in this chapter were aimed to validate tobramycin's disposition model in the IPRL described in Chapter IV, or at least to define the boundaries of its applicability; this by planning and executing a series of experiments designed to eliminate alternate perturbation possibilities that could interfere with the binding models described earlier.

V.b MATERIALS AND METHODS

IPRL Absorption Studies were performed using materials and general methods identical to those described in detail in Chapter IV of this thesis. Only the dosing solutions that were used for administration to the airways differed with respect to their formulation. Two separate series of **IPRL Absorption Studies** were performed in which the effects of realistic, but minor, variations in (i) dosing solution osmolality and (ii) dosing solution pH were studied. In a separate

experimental arm (iii) the effects of tobramycin concentration on mannitol's absorption were investigated. For each dosing solution, no less than four fully viable preparations were studied and, as before, the absorption results from each group ($n \geq 4$) were expressed as mean cumulative fraction of the administered dose absorbed into the perfusate, F_p, versus time.

(i) [^{3}H] tobramycin dosing solutions at 2 mg/mL were prepared in 0.225 % or 0.9 % w/v NaCl and adjusted to pH 7.4 with H_2SO_4. The osmolality of these tobramycin dosing solutions and their 2 mg/mL tobramycin counterparts described in Chapter IV (in which the vehicle was 0.45% w/v NaCl) were determined using the freezing point depression method using an Advanced® Model 3320 Micro-Osmometer (Advanced Instruments, Inc., Norwood, MA), to measure and report the apparent osmolality of these solutions. Tobramycin absorption data obtained from these dosing solutions in 0.225 % and 0.9 % w/v NaCl were compared to the control data shown in Chapter IV, in which the same dose of tobramycin was administered in 0.45% w/v NaCl at pH 7.4.

(ii) To elucidate the effects of pH of the dosing solution on tobramycin absorption, [^{3}H] tobramycin dosing solutions at 2 mg/mL were prepared in 0.45% w/v NaCl and adjusted to pH 4.6 or 8.6 with H_2SO_4 when necessary (pH of 2 mg/mL solutions of the base was $\geq$ 8.6). The absorption of tobramycin from these solutions was determined and compared to the control data at the same nominal tobramycin lung dose and pH 7.4 reported in Chapter IV.

(iii) The effects of tobramycin concentration on mannitol absorption were investigated by studying the absorption of [^{3}H] mannitol from dosing solutions containing 0.2 mg/mL mannitol in 0.45% w/v NaCl and pH 7.4 to which tobramycin (non-labeled) was added at 2, 20 and 60 mg/mL. Mannitol's absorption after its co-administration with these different tobramycin doses was investigated and compared to that of mannitol in the absence of tobramycin .

Statistical analysis A repeated measures analysis of variance (ANOVA) was used to model mean tobramycin absorption as a function of time separately for each dosing solution (e.g. with different values of osmolality and pH), and compare the drug's absorption within different dosing solutions for each evaluation as described in Methods. The model included fixed effects for time, dosing solution, interaction between dosing solution and time, and a random effect by subject or IPRL preparation. A Bonferroni multiple comparison procedure was used to adjust the overall significance level with $\alpha = 0.05$.

V.c RESULTS AND DISCUSSION

As described in the conclusion to Chapter IV, and because the percentage of nebulizer doses likely to reach the lung from the recommended PARI LC PLUS nebulizer is ~15% (Lenney et al., 2011), a lung dose of ~45 mg would be expected in a 70-kg human inhaling TOBI, in accord with the manufacturer's instructions, twice daily (TOBI is formulated at 60mg/mL in 1/4 Normal Saline (NS) or 0.225% w/v NaCl). The experiments in this thesis using the IPRL were designed to bracket the likely doses (and thereby airway surface concentrations) seen in humans, based on weight scaling, where a lung dose of 45mg corresponds approximately to 0.2 mg in the airways of the rat lungs used here. The justification for this approach was based on the literature in which similar cell constituents and concordance with alveolar surface area, lung volume, capillary volume and body weight have been reported across species, including rats and humans (Crapo et al., 1983; Cryan et al., 2007; Plopper, 1983). The experiments described in this chapter in which the effects of pH and osmolality were evaluated were focused therefore on a single nominal dose of 0.2 mg

delivered to the airways by intratracheal administration of 2 mg/mL tobramycin in a 0.1 mL coarse spray of solution in different vehicles.

Effects of dosing solution osmolality on tobramycin absorption

Table V.1 shows the experimentally determined osmolality values of tobramycin solutions prepared in 0.225, 0.45 or 0.9 %w/v NaCl. The absorption data for tobramycin at a nominal 0.2 mg dose are shown, expressed as F_p as a function of time, from either highly hypotonic (80 mOsmol/kg) through isotonic (298 mOsmol/kg) dosing solutions in Figure V.1.

Table V.1 Osmolality of tobramycin solutions at different concentrations at pH 7.4 (±0.2) prepared in 0.9% (normal saline, NS), 0.45% (1/2 NS) or 0.225% w/v NaCl (1/4 NS) solution.

Tobramycin concentration			
mg/mL	mmol/L	Vehicle	mOsmol/kg
0	0	1/2NS	150
0.02	0.04	1/2NS	151
0.2	0.4	1/2NS	150
2	4.3	1/2NS	157
2	4.3	NS	298
2	4.3	1/4 NS	80
20	42.8	1/2NS	195
60	128.5	1/2NS	257
100	214.1	1/2NS	330

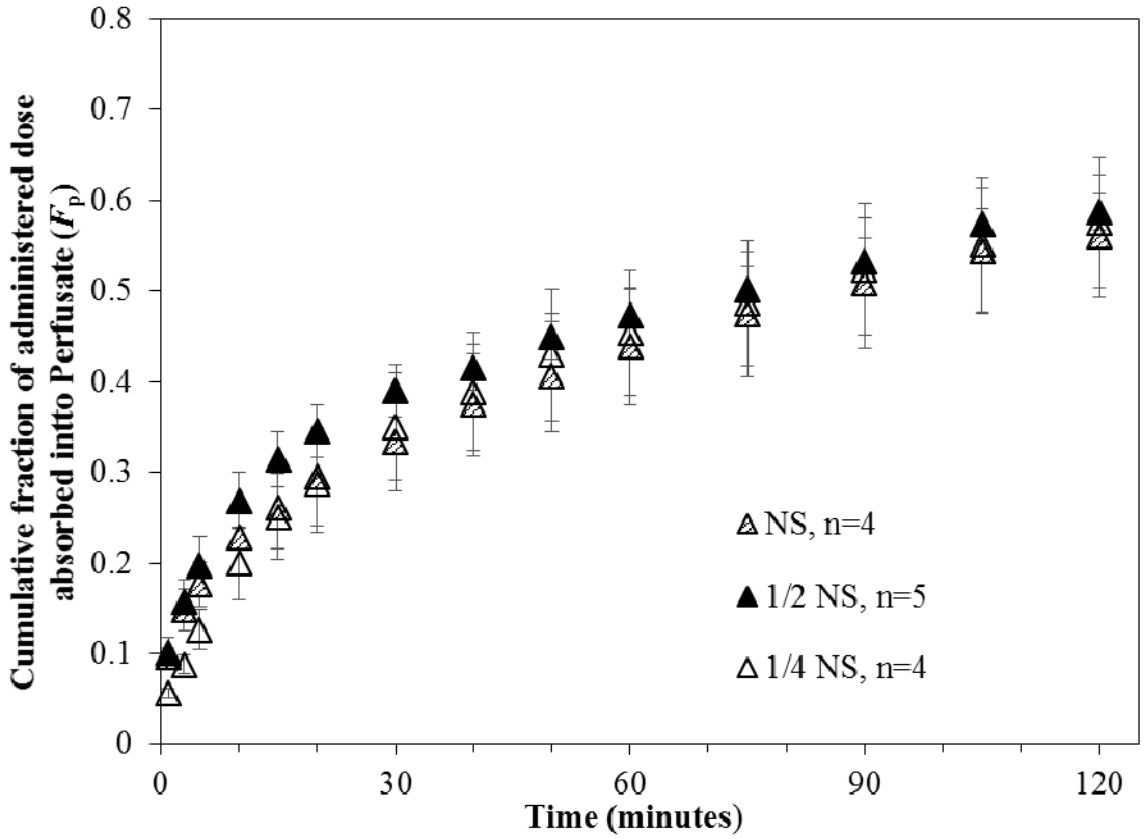


Figure V.1 Mean cumulative fraction of administered dose versus time following administration of tobramycin in vehicles with different osmolality. Tobramycin dosing solutions used in these experiments contained 2 mg/mL tobramycin at pH 7.4 in 0.9 % (normal saline, NS), 0.45% (1/2 NS), and 0.225% w/v NaCl (1/4 NS). Error bars are sample standard deviation (n=4 or 5).

The osmolality of the dosing solutions that were studied appear to be representative of the range used in clinical practice. The absorption data show clearly that no statistical difference in absorption kinetics was detectable in the IPRL throughout the 2 h period of an IPRL experiment (p value > 0.0035) due to changing osmolality. Accordingly, the results imply that these changes in osmolality do not affect the modeling and interpretation of the drug's disposition that are discussed in detail in Chapter IV. Furthermore, the results also indicate that for even these significant volumetric additions of solution to the epithelial surfaces of the lung (0.1 mL in a 300

g rat lung) failed to disturb the epithelial barrier sufficiently to change the absorption of the aminoglycoside.

The values for osmolality that were measured are worthy of some further explanation however. The theoretical osmolality of 0.9%w/v NaCl (Normal saline or NS) is close to 300 mOsmol/kg (Sinko, 2006). The value seen in Table V.1 for the 2mg/mL tobramycin solution in NS is consistent with this value and shows the minimal contribution of the aminoglycoside to the overall osmolality at the concentrations used for the absorption studies (in which the contribution of tobramycin (≤2 mg/mL or 4.3 mmol/L) was small). It is not until tobramycin concentrations reached and exceeded 20mg/mL in 0.45% w/v NaCl, that a significant increase of osmolality was seen due to the drug itself; drug contributions of 45, 107, and 180 mOsmol/kg were calculated for concentrations at 20, 60 and 100 mg/mL, respectively, by subtracting the osmolality contributed by the vehicle (150 for 0.45% w/v NaCl) from the total osmolality of these solutions shown in Table V.1.

Theoretically, at pH 7.4, tobramycin is expected to have around 2.5-3 positive charges based on the reported pKas for its amino groups and according to acid-base equilibrium theory (Szilagyi et al., 1993). If the number of charges per tobramycin molecule at pH 7.4 is equal to 2.5, there should also be an increase in osmolality due to the addition of sulfate ion concentration needed to bring about the pH adjustment to a value of 7.4. Theoretically therefore, ignoring the contributions of NaCl and assuming complete dissociation of tobramycin and sulfate ions in solution, the osmolality of part-neutralized tobramycin at 20, 60, and 100 mg/mL, should be close to 95, 289 and 482 Osmol/kg, respectively, assuming that the density of these solutions was not significantly changed from that of water. The measured smaller osmolalities shown in Table V.1, due to the

tobramycin and sulfuric acid in the higher concentration pH 7.4 solutions, imply significant non-ideality, perhaps due to ionic interactions, in the solutions containing 20, 60 and 100mg/mL (the theoretical osmolality of a 100mg/mL tobramycin solution in ½ NS at pH 7.4 is expected to be ~ 632 mOsm/kg in the event of complete ionic dissociation). Notably however, these non-ideal results shown in Table V.1 for high concentration tobramycin solutions are consistent with the reported osmolalities for Tobramycin Solution for Inhalation (60mg/mL) in the monograph in United States Pharmacopoeia (USP), where the drug solution in ¼ NS should lie in the range 150-200 mOsm/kg (USP29-NF24: http://www.pharmacopeia.cn/v29240/usp29nf24s0_m83766.html) and the experimentally determined osmolality values for tobramycin solutions at 40 and 80 mg/mL in sterile water, ½ NS or NS at neutral pH according to Law (Law, 2001).

These large differences in osmolality that are seen between the experimental measurements and the theoretical calculations for tobramycin imply the existence of strong interionic interactions in solution. In an attempt to quantitatively assess this phenomenon, the practical osmotic coefficient of tobramycin, *g*, as a measurement of the drug's dissociation, was estimated to be 0.47, 0.37 and 0.37 for tobramycin concentration at 20, 60, and 100 mg/mL at pH 7.4, respectively, using the equation eq. V.1 (Sinko, 2006):

$$g = \frac{mOsmol_{apparent}}{v*m} \qquad \text{eq. V.1}$$

where $mOsmol_{apparent}$ refers to the contribution of osmolality by tobramycin as an electrolyte; *v* is the number of ions into which an electrolyte dissociates (a value of 2.25 for tobramycin sulfate was used, corresponding to 2.5 charges associated with tobramycin molecules in pH 7.4); *m* refers to the electrolyte molality and is assumed to be equal to the value of molarity, assuming the density of these solutions was not effectively changed from that of water.

Values of $g \leq 0.5$ imply that tobramycin sulfate behaves as less than half of the total ions into which tobramycin sulfate theoretically dissociates in solution. Although it is difficult to understand this atypical electrolyte behavior and the mechanisms responsible for it, this uncommon colligative characteristic of tobramycin clearly shows the uniqueness of this compound as a polyvalent electrolyte.

In the literature, it has been suggested that a solution for inhalation should have an osmolality between 150 and 550 mOsm/kg (Weber et al., 1997). Others have reported that administration of non-isotonic solutions to the lungs via inhalation in the clinic are acceptable, even though such administrations may produce bronchial reactions manifested by cough, bronchoconstriction, airway hyperreactivity, or bronchospasm in some individuals (Eschenbacher et al., 1984; Weber et al., 1997). The effect of hypotonic solutions on the airway permeability of ^{99m}Tc-DTPA was evaluated by Wells et al. (Wells et al., 1994) who were able to show that enhanced airway permeation could be detected across isolated sheep trachea when the tracheal lumen was filled with hyposmolar fluid (128 mOsmol/kg). Based on the results shown in Figure V.1, Wells' findings do not appear to be relevant to the absorption of tobramycin from the airways of the rat lung.

Effects of dosing solution pH on tobramycin absorption

Mean cumulative fraction of administered dose absorbed into the perfusate, F_p, from dosing solutions of different pH are shown as function of time in Figure V.2. Tobramycin dosing solutions were prepared at the same drug concentration, 2 mg/mL, but adjusted to three different pH values with the addition of sulfuric acid: values of pH = 4.6, 7.4 and 8.6, believed to be representative of

the range used in clinical practice. Statistically, administration of tobramycin under these conditions showed no alteration in the drug's absorption kinetics when compared to the control (p value > 0.0035). It was noteworthy that administration of these basic or acidic dosing solutions to the IPRL airways appeared to have no effect on the isolated rat lung's viability (no edema was observed during the typical 2 hours' experiment period), and thus, it was probable that these pH values and the addition of solutions as coarse sprays failed to disrupt the epithelial integrity of the airways of the IPRL. Because the pH values that were chosen should affect the degree of ionization of the aminoglycoside, the results also implied that the modified ionization status of the amino groups in each tobramycin dosing solution had no effect on the drug's absorption, binding or sequestration at this (realistic) dose and airway surface concentration. This could of course, simply be an indication that the buffering power of the organ and its circulating perfusate simply overwhelms the addition of a pH-modified solution bolus containing 2mg/mL tobramycin.

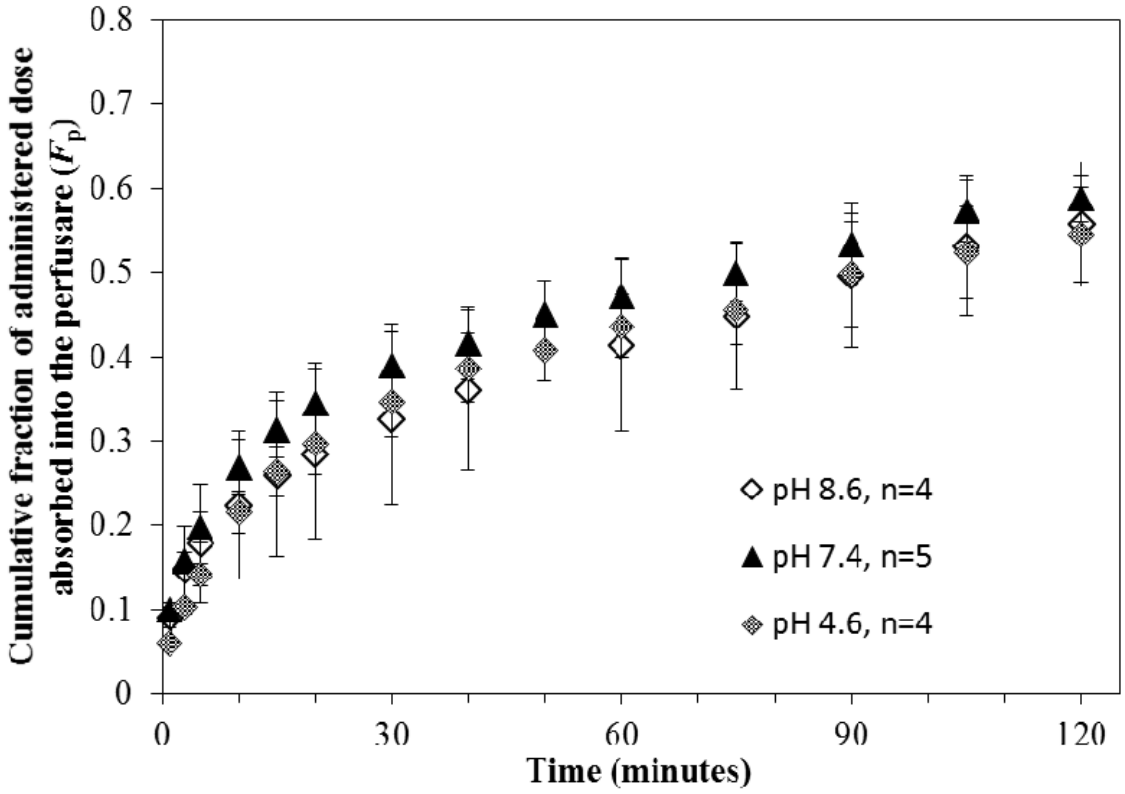


Figure V.2 Mean cumulative fraction of administered dose versus time, F_p, for tobramycin dosed at 0.2mg using 0.1mL of 2mg/mL solutions in 0.45% w/v NaCl at either pH 8.6, 7.4 or 4.6. Error bars are sample standard deviations (n=4 or 5).

Effect of tobramycin concentration on mannitol absorption

The absorption of the hydrophilic monosaccharide mannitol and/or its permeation rate through epithelial membranes is believed to be a measure of simple diffusion through hydrophilic "pores" or passive paracellular transport. In short, the solute is believed to be a marker for the integrity of tight junctions and the "leakiness" of epithelial monolayers such as those seen in the airways of the lung (Wan et al., 2000). Mannitol absorption data, therefore, should be usable as a surrogate measure of epithelial integrity in a preparation such as the IPRL; factors that accelerate its absorption, perhaps alter the barrier separating (in the IPRL) the airways from the perfusate. The results of IPRL absorption studies following airway administration of mannitol alone or its co-

administration with unlabeled tobramycin in different concentrations, are shown in Figure V.3. The nominal lung dose of mannitol in all these IPRL studies was held constant at 0.02 mg/rat lung (0.2 mg/mL in a fixed solution volume of 0.1 mL). Quite clearly, co-administration 0.2 mg tobramycin with mannitol, failed to bring about alterations in mannitol absorption profiles (mean F_p versus time) when these were compared to the tobramycin-free control data (p value > 0.0035; Figure V.3). At the high dose extremes however, where tobramycin concentrations were 60 mg/mL or greater (nominal doses of 6mg or more), the disruptive effect of tobramycin on airway epithelial integrity could be seen clearly as a major accelerant for mannitol absorption in the IPRL (p value < 0.0035; Figure V.3). Notably, also at this dose, among a total of 7 rat lungs that were used for this experimental condition, only 4/7 showed a lack of edema over the 120 minute study; three rat lungs became edematous within one hour of dosing. This decrease in viability appeared to be due to the presence of high tobramycin concentration in the airway lumen, a conclusion that was supported by an attempt to investigate tobramycin's absorption at a dose of 10 mg where edema was observed in all IPRL preparations within one hour of dosing. These observations implied that the values for F_p versus time at the 6 mg dose were actually an underestimate of the true values, due to the obligatory discard of non-viable preparations.

The tobramycin dose effect on mannitol absorption can also be seen, both in Figure V.3 and Table V.2, where values for F_p were compared to the tobramycin-free control at identical sample times and show that the effect begins to be manifested even at doses as low as 2 mg (0.1 mL, tobramycin concentration = 20 mg/mL), when both mannitol and tobramycin were administered in the same solution (p value < 0.0035). Although there was no observation this co-administration affected the isolated rat lungs' viability as seen from a higher tobramycin dose of 2 mg, it is

possible that this dose level of tobramycin in the dosing solution could cause a certain degree of membrane disruption such as the cleavage of tight junctions and accelerated mannitol's absorption.

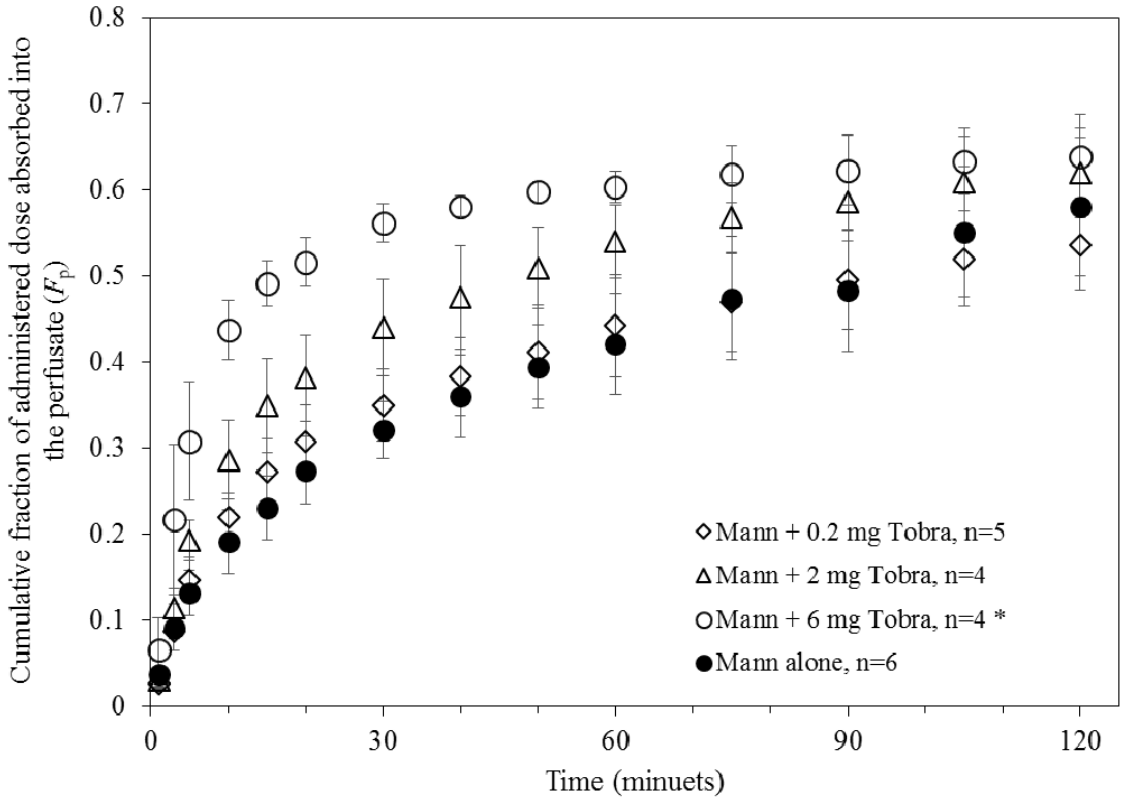


Figure V.3 Mean cumulative fraction of administered mannitol dose versus time. The closed circles refers to the mean F_p data from the administration of 0.02 mg mannitol alone, while the open symbols refer to the data from the co-administration of 0.02 mg mannitol with tobramycin at different doses. * indicates that the mean values of F_p were calculated from 4 out of 7 rat lungs, by discarding the results from the isolated rat lungs in which edema occurred during the 2 hr's experiment period.

Table V.2 The increase in mean F_p for mannitol with time seen due to co-administration with 2 or 6 mg tobramycin to the airways of the IPRL.

	ΔF_p $(F_{p,coadministration} - F_{p,control})^{\#}$	
Time (min)	2 mg	6 mg
1	-0.007	0.028
3	0.023*	0.125*
5	0.061*	0.176*
10	0.094*	0.245*
15	0.118*	0.260*
20	0.107*	0.242*
30	0.119*	0.241*
40	0.115*	0.221*
50	0.115*	0.203*
60	0.119*	0.183*
75	0.093*	0.144*
90	0.103*	0.139*
105	0.058	0.082
120	0.040	0.059

control data for F_p was taken from the results of administration of mannitol alone at a lung dose of 0.02 mg. Statistical significance is shown as * (p value <0.0035).

V.d CONCLUSIONS

Osmolality and pH variations in airway dosing solutions in the range 80 through 298 mOsmol/kg and 4.6 to 8.6, respectively, failed to alter the rate or extent of tobramycin transfer to perfusate following airway administration to the IPRL in doses designed to produce airway concentrations typical of those used clinically. These experiments, that were referenced in the text of Chapter IV, are thought to be useful adjuncts to the data showing that aminoglycoside disposition following inhalation at clinically relevant doses is unlikely to be influenced significantly by small changes in formulation variables such as NaCl content and pH; there was no evidence that these changes affected the epithelial integrity of the IPRL. Tobramycin itself

however, was implicated as an agent capable of epithelial damage, when its effects were studied by co-administration with mannitol. To our knowledge, this is the first report of the effect of aminoglycosides on airway epithelial integrity. Based on the results however, drug induced epithelial changes are expected to be minimal at lung doses seen for the clinical treatment of humans.

Tobramycin's membrane damaging effects were clearly dose and concentration dependent however, and may begin to be seen at IPRL doses as low as 2 mg. While these experiments differed from those described in Chapter IV and involved mannitol administration in the same solution as the aminoglycoside, the slight elevation in the reported values for tobramycin's k_a with dose (Table IV.2) may have been due in part to the drug's membrane toxicity. It is impossible to say whether this latter observation has any clinical significance, especially given the fact that the drug is mostly used to treat individuals with CF who have abnormal airway epithelia in the first place and the treatment of their infections is a clinical imperative. Overall, these findings appear to eliminate the possibilities that significant airway perturbations other than tissue binding and sequestration are responsible for the drug's pulmonary pharmacokinetics, and that it is those phenomena that bring about the drug's prolonged presence in the lung following airway administration.

Chapter VI
SUMMARY AND CONCLUSIONS

Nebulized tobramycin therapy has improved the health and well-being of many cystic fibrosis (CF) patients in spite of its pulmonary disposition being poorly understood. In this dissertation, a meta-analysis of the pharmacokinetic (PK) data in humans is described as a precursor to systematic series of experimental investigations using the isolated perfused rat lung preparation (IPRL). In an attempt to elucidate both the kinetics and extent of pulmonary absorption in humans, the bioavailability of inhaled tobramycin in healthy and CF adults was re-evaluated in Chapter III using both non-compartmental and compartmental approaches partnered with the drug's intrinsic PK parameters obtained from intravenous (IV) studies in the literature. This work revealed that in both CF and healthy adults, tobramycin absorption from the lung following inhalation was more complete than is commonly assumed based on the drug's theoretical behavior as an aminoglycoside poly-cation at physiologic pH (Szilagyi et al., 1993). However, due to the large variance seen in published studies of tobramycin's intrinsic PK after IV administration, and the fact that patients, in which the drug's inhalation blood level versus time curves were reported, differed from those used to determine tobramycin's intrinsic PK, the overall results from meta-analysis did not inspire much confidence in the accuracy with which the drug's pulmonary bioavailable fraction in humans could be determined. Likewise, in Chapter III, the rate of the drug's absorption from the lung following inhalation, measured using different modeling techniques was found to produce apparent half lives for pulmonary absorption that ranged from 0.7 through 2.3 hours. Data analysis difficulties were also noted for modeling with some of data

to determine absorption rate constants (see legend, Figure III.1), all of which assumed irreversible absorption from the airways into the systemic circulation. It was only following a review of the sputum concentration data in patients who had experienced repetitive aerosol dosing, that the existence of drug binding or sequestration in the lung became a possibility (see Table III.4)

To enhance the understanding of the inhaled drug's disposition kinetics and mechanisms, tobramycin's absorption following airway administration was investigated systematically as a function of dose and local concentration, using a realistic ex vivo model, the IPRL, as described by Byron and Niven (1988). Tobramycin's absorption, from airways to perfusate, expressed as cumulative fraction of the administered dose absorbed into the perfusate versus time, was seen to be bi-exponential and dose-dependent, unlike that seen for the marker solutes fluorescein and mannitol that both showed first-order and dose-independent kinetics (Chapter IV). HPLC-MS assay techniques were developed with a sufficiently small limit of quantitation (LOQ) to study the absorption of this chromophore-free aminoglycoside at some of the higher doses studied in the IPRL (Appendix I). Those assay techniques, when compared with the radiolabel assay (Appendix III) used for most studies reported in Chapter III produced statistically comparable absorption profiles (Appendix IV), showing that radiolabel degradation was not significant in the period of study. Furthermore, the dose-dependent disposition kinetics seen with tobramycin in the IPRL did not appear to be associated with metabolic degradation, since this class of drugs fails to undergo metabolism in the body and is excreted unchanged almost entirely by the kidney after IV administration (Israel et al., 1976; Schentag et al., 1978).

In this scenario, a kinetic model for tobramycin that incorporated lung tissue binding and/or sequestration alongside passive absorption was proposed and employed successfully to describe the aminoglycoside's disposition in the IPRL following airway administration. Based on the rate constant data obtained from the kinetic model in the IPRL, tobramycin's absorption occurred fast with first-order absorption rate constants (k_a = 0.065-0.070 min^{-1}) close to those seen with fluorescein (0.076 min^{-1}), while a dose-, and concentration-dependent lung tissue binding mechanism, appeared able to prolong the drug's presence in the rat lung. This dose-dependent tissue binding or sequestration appeared to potentially saturable, indicated by the fact in which forward binding rate constants (k_{12}) decreased as doses (and administered concentrations) were increased, while backward rate constants (k_{21}) appeared unrelated to dose (see Table IV.2).

This kinetic model was validated using dynamic dialysis experiments involving elution studies from the whole sliced IPRL preparation. The dynamic dialysis techniques, advocated by Meyer and Guttman and used subsequently by other groups to study solute-ligand binding (Bottari et al., 1975; Hashimoto et al., 1984; Hiji et al., 1978; Meyer and Guttman, 1968; 1970; Pedersen et al., 1977; Sparrow et al., 1982), were modified and used with sliced IPRL tissues in this dissertation to study the lung tissue binding of tobramycin by directly mimicking the environment in which the drug was administered to the airways of the preparation. Those studies confirmed that tissue binding occurred relatively slowly after airway administration and that all three rate constants (for dialysis, association and dissociation) had a similar order of magnitude (see Scheme IV.3, Table IV.1). Although the binding sites remained unclear, lung tissues were shown to be clearly responsible for the antibiotic's sequestration; this because the constituents of the IPRL airway lining fluid collected by bronchoalveolar lavage showed no significant binding to tobramycin in control dialysis studies. While such disposition models appeared to be consistent with observations

of prolonged sputum concentrations following repeated dosing in the clinic(Gibson et al., 2003; Govoni et al., 2013; Hubert et al., 2009), the human PK data for this drug were too variable to discern the magnitude of such effects (Chapetr III). Moreover, it is not known whether prolongation of tobramycin's concentrations in the lung due to such binding enable better killing or whether the antimicrobial effects would be enhanced simply by maximizing the airway Cmax/MIC ratio for a short duration as discussed by MacArthur et al.(1984).

It was unexpected that tobramycin could be absorbed from the airways of the IPRL faster than mannitol (k_a = 0.018-0.019 min^{-1}), a paracellular (hydrophilic) absorption marker with a much smaller molecular size (MW 182 Da) than tobramycin (MW 467.5 Da). While the mechanisms responsible for the drug's apparent rapid absorption in the IPRL were not studied in this dissertation, it was noteworthy that tobramycin itself was found to affect airway epithelial integrity when co-administered at doses as large as 2 and 6 mg to the IPRL airways with mannitol. In this situation, mannitol's absorption was accelerated when co-administered with tobramycin and the viability of the isolated lung preparation appeared to decrease when increasing tobramycin's airway dose to the IPRL. The drug's disruptive effects on airway epithelia however, were undetectable at a lung dose level of 0.2 mg in rats that was believed to produce similar airway concentrations to those seen in human patients using TOBI. Based on this reasoning, tobramycin induced epithelial changes are expected to be minimal at lung doses seen in the clinical treatment of humans by inhalation. To our knowledge, this is the first report of the effect of aminoglycosides on airway epithelial integrity even though this class of drugs in some concentrations have been reported to affect the permeation of vestibular and proximal tubular epithelia, and also cause cell apoptosis and necrosis due to high local concentrations (see studies of drug-induced oto- and

nephrotoxicity; Karasawa and Steyger, 2011; Kim et al., 2005; Todd et al., 1994). Whether this observation has any clinical significance, especially given the fact that the drug is mostly used to treat lung-infected individuals with CF, who have abnormal airway epithelia in the first place, is unknown. Nevertheless, these findings may add value to those dealing with formulation development and dosing strategy designs for other inhaled aminoglycosides.

Overall, by eliminating the variability associated with systemic clearance, and performing a series of systematic investigations in an ex vivo lung preparation, this dissertation increased our knowledge of tobramycin disposition following airway administration. As a polycation at physiological pH (Szilagyi et al., 1993), the aminoglycoside showed a unique ability to bind or sequester in the lung that appeared to prolong the drug's local presence and, thereby, extend its anti-infective effects following inhalation. The drug's tissue binding properties may account, at least in part, for the apparent success of tobramycin inhalation therapy seen in clinical practice. The findings in this dissertation may also assist with development of future research in this important therapeutic area of antibiotic therapies for pulmonary administration, given that the models developed in Chapter IV enable kinetic interpretation of the clinical studies in humans in which repetitive dosing showed, albeit inconclusively, accumulation and prolonged antibiotic durations following topical delivery. Further exploration of the mechanisms responsible for aminoglycosides' lung sequestration with other aminoglycosides, may enable future design and development of drugs for pulmonary administration with prolonged lung retention.

Appendices

Appendix I

HPLC MASS SPECTROMETRIC ANALYSIS OF TOBRAMYCIN IN PERFUSATE

AI.a Introduction

In this appendix, the development and validation of an HPLC-MS method suitable for tobramycin assay in perfusate in the IPRL is described. The method overcomes the adsorption problems of aminoglycosides on glass and other surfaces (Josephson et al., 1979) and employs solid phase extraction followed by reverse phase HPLC with trifluoroacetic acid (TFA) as an ion-pairing agent. Mass detection using a single quadrupole mass spectrometer in positive detection mode with electrospray ionization (ESI) confers sensitivity and specificity.

AI.b Methods and materials

Chemicals and materials

Tobramycin was purchased from Sigma-Aldrich (MO, USA), acetonitrile (CAN; HPLC-grade), methanol (HPLC-grade), buffer salts (KCl, KH_2PO_4, $CaCl_2$, $MgSO_4$, $NaHCO_3$) and dextrose were supplied by Fisher Scientific (Pittsburgh, PA). Bovine serum albumin (BSA), trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were purchased from Sigma-Aldrich (St. Louis, MO). Bond-ELUT-C18 cartridges (100mg, 1 mL) used for solid phase extraction (SPE) were obtained from Varian Inc. (Walnut Creek, CA). Pipette tips (200uL and 1000uL) and centrifuge tubes (2mL) were made of polypropylene (PPE) and purchased from Fisher Scientific. Small volume HPLC

vial inserts (250uL, PPE) were obtained from MicroSolv (Eatontown, NJ). The perfusate (K4) is the modified Krebs-Henseleit solution(KHS) with 4% bovine serum albumin (BSA) (w/v) containing 4.7mM KCl, 143.3mM NaCl, 1.2mM $MgSO_4$, 2.52mM $CaCl_2$, 1.2mM KH_2PO_4, 5mM glucose, prepared as described in Chapter IV. The perfusate pH was adjusted to 7.4 at room temperature; solutions were stored at 4°C and used within 48 hours.

HPLC-MS method development

Chromatographic and mass spectrometric conditions. Alliance 2695 system (Waters, Milford, MA) was used for chromatographic separation by a Hypersil Gold C_{18} column (150×4.6mm, 3μm; Thermo Scientific, Waltham, MA). Isocratic elution at a flow rate of 0.3 mL/min was used with the mobile phase consisted of acetonitrile (ACN): 0.1% v/v TFA water solution (volume fraction ratio, 30:70), where TFA functioned as an ion pairing agent to increase tobramycin's retention on the column. Chromatographic performance (peak shape and symmetry) using mobile phase containing different TFA concentrations was assessed to determine the appropriate TFA concentration in the mobile phase for the analysis. The injection volume was hold at 10 μL. The mass spectrometry detector was a ZMD 4000 (Waters Corporation, Milford, MA) equipped with an electrospray interface in positive ionization mode. The parameters were set as: capillary voltage 3.78 kV; cone voltage 20 V; extractor 6 V; Rf Lens 0.1 V; source block temp 130 °C; desolvation temp 350 °C; nitrogen desolvation flow 600 L/min. Tobramycin was determined by single ion monitoring (SIM) for the molecular ion $[M+H]^+$ at *m/z* 468.61. Masslynx NT 3.5 (Waters) was used for the HPLC-MS system control and data processing.

Calibration standards and quality controls. The stock standard solution of tobramycin at 1 mg/mL was prepared in water and further diluted with ACN –H2O (80:20) containing 0.4% TFA, to obtain the standard solutions at concentrations of 50, 20, 10, 5, 2, 1, and 0.5 µg/mL. These standard solutions were then diluted by 10 fold with blank perfusate to obtain perfusate calibration standards at concentrations of 5, 2, 1, 0.5, 0.2, 0.1, 0.05 µg/mL. Quality control samples in perfusate at the lowest limit of quantification (LLOQ) and low, medium and high concentration levels were prepared in triplicate in the same way as these calibration standards.

Solid phase extraction and sample pretreatment. Tobramycin perfusate samples, calibration standards in perfusate and quality control samples were pretreated with 5% v/v HFBA water solution in a volume ratio of 1:1 followed by centrifugation at 14,000 rpm for 10 minutes at 4 °C (Eppendorf, Centrifuge 5804R, Hamburg, Germany) to enable protein precipitation and simultaneously, ion pairing. A volume of 1 mL supernatant solution was then taken from each sample and solid phase extraction was performed for sample clean-up with Bond-ELUT-C_{18} cartridges (100mg, 1 mL) using the following procedure:

Conditioning: 1 mL methanol and 1ml 5 mM HFBA water solution;

Loading: 1 mL supernatant solution prepared above;

Washing: 1 mL 5 mM HFBA water solution (×2);

Eluting: 1 mL ACN: H_2O 90:10 (containing 1% v/v TFA).

Eluent solutions collected in polypropylene (PPE) centrifuge tubes were evaporated to dryness at 40 °C under vacuum and reconstituted with the dilution solvent. These reconstituted solutions were transferred to 250 µL polypropylene (PPE) inserts and aliquots of 10 µL were injected onto the HPLC column.

The adsorption of tobramycin onto the surfaces of glass HPLC vials and polypropylene inserts were assessed using tobramycin standard solutions prepared in different dilution solvents. The dilution solvent was selected from H_2O, ACN –H_2O (80:20), ACN –H_2O (30:70) containing 0.1% or 0.4% v/v TFA, and ACN –H_2O(80:20) containing 0.1% or 0.4% v/v TFA, based on the results of screening experiments designed to assess peak shape, injection reproducibility and adsorption losses. The storage and transfer containers used in sample pretreatment were all made of polypropylene in order to avoid surface adsorption losses onto glass that have previously been reported with aminoglycosides (Josephson et al., 1979).

Validation Procedures.

Calibration. Perfusate calibration standards after solid phase extraction were analyzed by HPLC-MS. Tobramycin peak areas from the corresponding chromatograms versus nominal concentrations were fitted using a Masslynx regression method based on a best-fit procedure (Figure AI.3). Deviation of the calculated concentration of each calibrator from the nominal one, expressed as the percentage of the nominal concentration (% Dev), should be within ±15% except at the LLOQ level in which a deviation of ±20% was permitted.

Accuracy, precision and extraction recovery. Accuracy was defined as the ratio, expressed as a percentage, of the experimentally determined concentration to the spiked drug concentration in each quality control sample. Precision was expressed by coefficient of variation, CV%, of the experimental determined concentrations of each quality control sample injected in 3 separate runs within a day (= SD / Mean× 100%, where "Mean" is the mean of the experimentally determined concentrations from 3 separate runs and SD is the standard

deviation of the "Mean"). The efficiency and reproducibility of solid phase extraction was evaluated by extraction recovery rate, expressed as percentages, calculated by eq. AI.1:

$$\text{Extraction rate} = {Area_{extracted}}\Big/{Area_{reference}} \times 100\% \qquad \text{eq. AI.1}$$

where $Area_{extracted}$ refers to the peak area obtained from each extracted quality control, $Area_{reference}$ is the average peak area of tobramycin obtained from the standard solutions prepared in duplicate with the dilution solvent at the same nominal concentration as that in the quality control sample.

Matrix effects.

Matrix effects, defined as the impact of the perfusate components and endogenous metabolic byproducts derived from the dying IPRL tissue during recirculation through the rat lung vasculature on the assay's accuracy**,** were assessed for "IPRL-free" blank perfusate and 4 batches of IPRL control perfuste, in which two batches of IPRL control perfusate (Batch 1 and 2) were obtained by circulating purfusate in the IPRL vasculature for 0.5 hour using two different rat lungs, and another two batches of control perfusate (Batch 3 and 4) were collected from the re-circulated perfusate using a third rat lung after 0.5 hour (Batch 3) and 2 hours (Batch 4). These four batches of IPRL control perfusate as well as "IPRL-free" blank perfusate sample were pretreated by solid phase extraction, and reconstituted with the dilution solvent. Matrix effects were evaluated using both a post-column infusion and a post-extraction addition method. In the post-column infusion method, tobramycin standard solution at 1000 ng/mL was infused into the MS detector by a syringe pump at a flow rate of 5 μL/min, while the mobile phase was also pumped to the MS detector at a flow rate of 0.3 mL/min operated by a Tee-valve device. Aliquots of 10 μL of reconstituted solutions of these perfusate extracts were injected onto the column when performing post-column

infusion. The chromatograms were collected based on single ion monitoring of m/z 468.61; a negative peak was expected to indicate signal suppression while a positive peak was expected show signal enhancement due to the components in the matrices. For the post-extraction addition method, only the batch of perfusate which was recirculated in IPRL for 2 hours and the "IPRL-free" blank perfusate, were used for this purpose. These perfusate batches were sampled and extracted by solid phase extraction; after evaporating to dryness, a volume of 50 µL standard solutions in dilution solvent at the concentrations of 50, 10, 1 µg/mL were added to the dried 2ml centrifuge tubes containing perfusate residues and diluted by 450 µL dilution solvent to produce tobramycin solutions at concentrations at 5000 (high), 1000 (medium), and 100 (low) ng/ml (two replicates at each concentration for each batch of perfusate). The corresponding standard solutions at these concentrations were prepared similarly in duplicate but using 2 ml clean centrifuge tubes, as reference solutions. Matrix effects were evaluated by % deviation (% dev.) of mean peak area obtained from the injections of those solutions prepared with perfusate matrices $\overline{Area_{post-extration}}$, from that in the reference solutions, $\overline{Area_{reference}}$, calculated by:

$$\%\ \text{dev.} = \frac{\overline{Area_{post-extration}} - \overline{Area_{reference}}}{\overline{Area_{reference}}} \times 100\% \qquad \text{eq. AI.2}$$

Adsorption test on the IPRL apparatus.

Since the IPRL apparatus included glassware, tobramycin adsorption onto the complete IPRL apparatus was assessed for perfusate samples at low concentration (100 ng/mL) in three replicates to evaluate the applicability of the IPRL standard apparatus for studies of tobramycin disposition. Briefly, 200 mL of blank perfusate was circulating in the IPRL apparatus at 37 ˚C simulating the real experimental condition except the absence of an isolated rat lung. An aliquot of 0.1 mL

tobramycin solution in water at 200 μg/mL was added the perfusate, and sampling of perfusate from the IPRL system was begun at 5 minutes after adding the tobramycin solution and followed at 30, 60, 90 and 120 minutes. The experiment was repeated three times and the profiles of tobramycin concentration versus time were plotted and the adsorption of tobramycin onto the IPRL apparatus in the presence of perfusate was evaluated as a function of time by comparing tobramycin concentration at each sample time point with the spiked tobramycin concentration of 100 ng/mL, using mixed-effects repeated-measures ANOVA with *p* value 0.05 by JMP 8.0, to assess whether there was statistically significant drug loss during circulation of this tobramycin perfusate solution through the IPRL apparatus system for 2 hours.

AI.c Results and discussion

Chromatograms and mass spectrums

Volatile ion pairing agent TFA was employed in this study to achieve analyte retention in the C_{18} column. The retention time of tobramycin was increased and peak shape was improved by increasing the concentration of TFA in the mobile phase as shown in Figure AI.1. A mobile phase consisting ACN-0.1% v/v TFA (30:70) was chosen because of the improved chromatographic performance that resulted from this condition in which the analyte showed moderate retention at 5.4 min and a symmetric peak shape

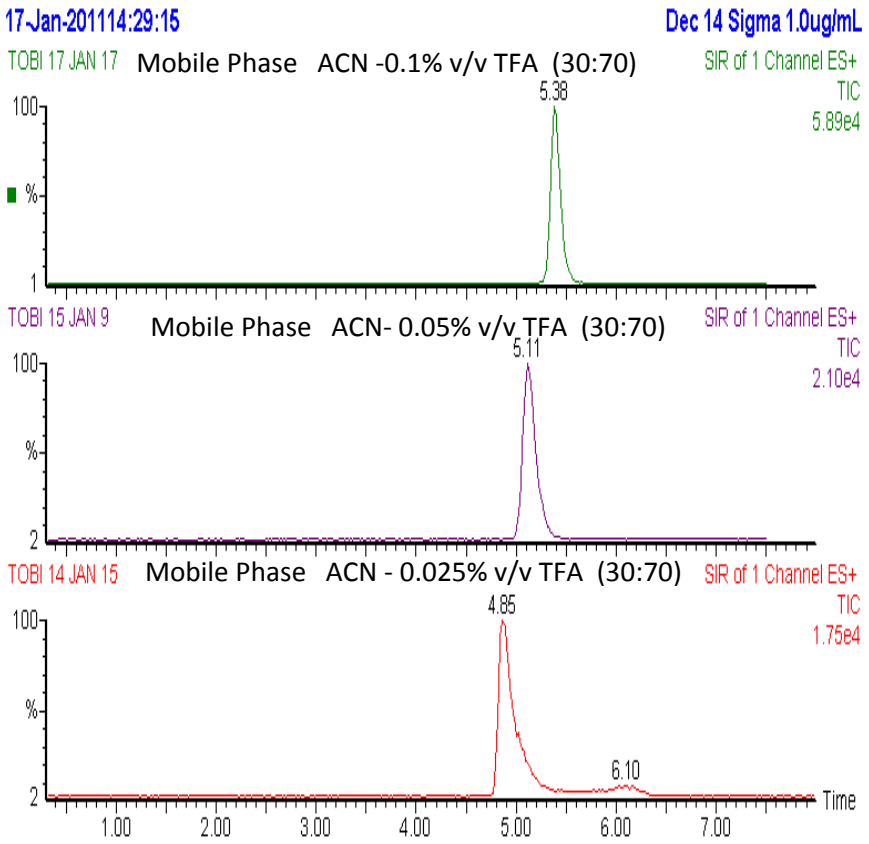


Figure AI.1 Effects of TFA concentration in mobile phase on chromatography.

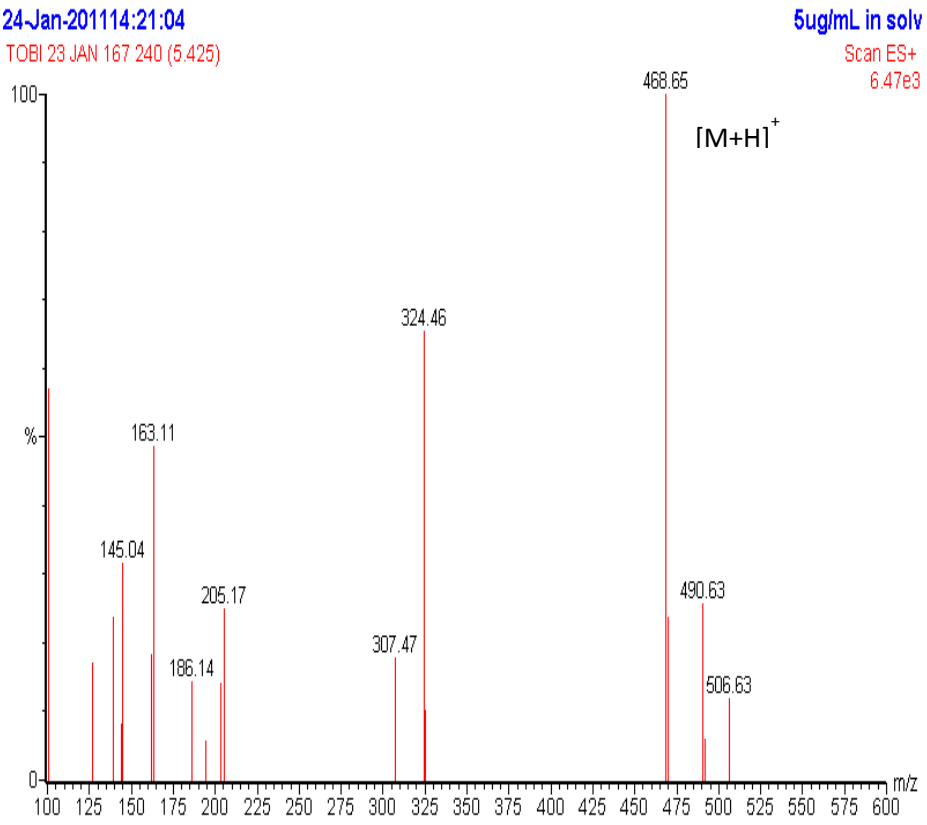


Figure AI.2 Mass spectrum of tobramycin

The mass spectrum of tobramycin is shown in Figure AI.2 in which peaks corresponding to the molecular ions $[M+H]^+$ at m/z 468.65 was observed in addition to the daughter ions at m/z 324.46, and 163.11, resulting from cleavage of glycosidic bonds and the subsequent loss of the aminosugar rings (Li et al., 2009). The molecular ion $[M+H]^+$ at m/z 468.65 was chosen for single ion monitoring in this method for its greatest abundance and specificity.

Solid phase extraction and sample pretreatment.

The perfusate matrix is the modified Krebs-Henseleit buffer containing 4% (w/v) bovine serum (BSA). The large amount of buffer salts and albumin in the perfusate made direct injections of samples onto the LC column followed by mass spectrometric detection impossible. Therefore, solid phase extraction was employed for the sample pretreatment after a deproteination procedure

with 5% v/v HFBA, before loading to C_{18} SPE cartridges. With these sample preparation procedures, a complete extraction was achieved with good reproducibility (see *Accuracy, precision and extraction recovery* in Table AI.3). Since the extraction recovery appeared to be complete and reproducible, the use of an internal standard seemed not to be necessary. Therefore, the quantification was based on the external standard method and validated as suitable for the assay of tobramycin in perfusate.

Although aminoglycosides' adsorption on container surfaces has been known and reported (Josephson et al., 1979), very few published bioanalytical methods addressed the problem. For tobramycin solutions in solvents such as water, water/acetonitrile mixture, or with mobile phase modifier agents such as TFA, there are no specific studies regarding aminoglycosides adsorption from solution on container surfaces. In this study, tobramycin adsorption onto the surfaces of 2 mL glass HPLC vials versus 250 µL polypropylene (PPE) inserts was evaluated when tobramycin standard solutions were prepared in different vehicles such as water, ACN-H_20, ACN-H_20 containing TFA. As for the storage in 2mL HPLC glass vials, the detected concentrations of tobramycin standard solutions in water or ACN–H_2O (80:20) at concentration of 1000 ng/mL were observed to decrease 44.3% after 2 hours and the peak of tobramycin was hardly detected after 9 hours, while 1000 ng/mL tobramycin in ACN –H_2O (80:20) containing 0.4% v/v TFA showed no change of the detectable concentration (data not shown). While ACN –H_2O (80:20) containing 0.4% v/v TFA as dilution solvent appeared to prevent tobramycin adsorption, the tobramycin peak for 100 ng/mL tobramycin standards prepared with this solvent was split in the tail or showed a shoulder when stored in glass vials. Interestingly, tobramycin peak was symmetric and absent of tail or peak shoulder for the same solution when a PPE insert was used for HPLC injections. Moreover, PPE containers could effectively reduce the drug's adsorption onto surfaces. However,

when tobramycin standards were stored in PPE inserts, the detected concentrations of 100 ng/mL decreased by 69.9 and 74.6 ng/mL after 9 hours in water and ACN–H_2O (80:20), respectively. Using ACN–H_2O (80:20) containing 0.4% v/v TFA as the solvent, the detectable concentrations of these standards at 100, 500 and 1000 ng/mL showed no decrease after 9 hours (see Table AI.1). In conclusion, PPE inserts used with a dilution solvent containing 0.4% TFA removed the adsorption problem. Furthermore, assay precision was also improved when this dilution solvent was employed (Table AI.2).

Table AI.1 Detectable concentrations of tobramycin standard solutions prepared in ACN–H_2O (80:20) containing 0.4% v/v TFA at 100, 500 and 1000 ng/mL stored in PPE inserts for 9 h at room temperature before injection.

Nominal Conc.(ng/ml)	After 9 h storage in PPE inserts (Mean Conc.±SD)
1000	1003.9±91.5
500	491.3±22.4
100	104.9±6.9

Table AI.2 Precision of repeated injection of 100 ng/mL tobramycin standard prepared in different dilution solvents.

Dilution solvent	5 runs Precision (CV%)
ACN-H20 (30:70) 0.1% TFA	14.3%
ACN-H20 (30:70) 0.4% TFA	19.0%
ACN-H20 (80:20) 0.1% TFA	13.1%
ACN-H20 (80:20) 0.4% TFA	5.3%

Validation

Calibration Figure AI.3 shows the calibration curve for tobramycin standards in perfusate (7 concentration levels, 50-5000 ng/mL) using an external standard. The peak area of tobramycin

versus the nominal concentrations in the standards was best fitted with a quadratic equation (software: Masslynx NT 3.5) to provide the solid curve with correlation coefficient equal to 0.995. The % deviation of the determined concentrations for each calibrator to the nominal ones were all within 11% as shown in Figure AI.3.

Nominal Conc (ng/mL)	%Dev
5000	-3.2
2000	10.5
1000	4.5
500	-1.5
200	-14
100	-2.4
50	4.4

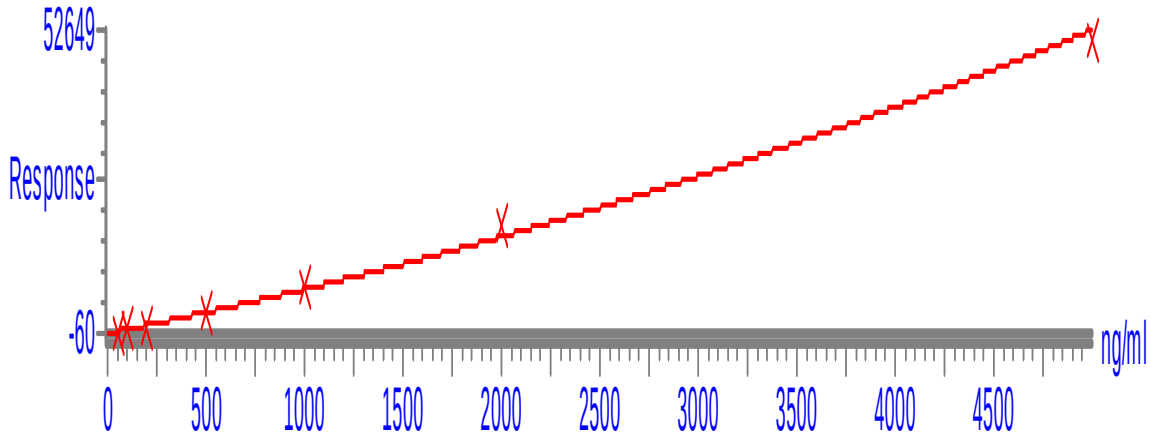


Figure AI.3 Calibration curve of tobramycin standards in perfusate

Accuracy, precision and extraction recovery

Accuracy, precision and extraction recovery of solid phase extraction for quality controls of tobramycin in perfusate are presented in Table AI.3. Accuracies ranged from 89.9 to 102.3%

and precision, expressed as CV%, were within 5.5% for tobramycin quality control standards at LLOQ, low, medium and high concentration levels. The extraction recovery rates of solid phase extraction for quality controls at low, medium and high concentrations were 101.6%, 103.7% and 97.5%, respectively. LLOQ was set to 50 ng/mL, according to the FDA guidance for Bioanalytical Method Validation that required accuracy at this concentration level ranging from 80-120% of nominal and precision (CV%) of repeated measurements less than 20% (http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf).

Table AI.3 Accuracy, precision and extraction recovery

Nominal Conc. (ng/mL)	Accuracy (%) 3 replicates		Precision 3 replicates	Recovery (%) 3 replicates	
	Mean	SD	CV%	Mean	SD
5000	93.9	3.0	4.0	101.6	4.3
1000	102.3	4.1	3.8	103.7	4.6
100	89.9	3.8	5.5	97.5	4.5
50	101.8	2.8	2.6		

Matrix effects

Reconstituted solutions of "drug-free" perfusate residues were injected to the HPLC column as described in the post-column infusion method. The chromatograms are shown in Figure AI.4. Ion suppression occurred at a retention time of 4.5 and 5.7 min, while ion enhancement occurred at 6.4 min for all the injections of these "drug-free" perfusate samples (Figure AI.4). These effects did not complicate tobramycin determinations (retention time = 5.4 min, Figure AI.1). The chromatogram of blank perfuate showed no obvious difference from those from the 4 batches of IPRL control perfusate (See Figure AI.4). Likewise, there was no detectable difference in the

chromatograms when the perfusate was recirculated in the pulmonary vasculature of the IPRL for either 0.5 or 2 h (Figure AI.4) and also no detectable difference for the IPRL control perfusate obtained from different rats (data not shown). Although the peak of tobramycin (retention time at 5.4 min) could be resolved even with matrix effects, further confirmation was performed using the post-extraction addition method to assess whether the residuals (after solid phase exaction) of perfusate components and endogenous metabolic byproducts from the dying tissue during recirculation in the rat lung vasculature affected the assay's accuracy. Table AI.4 shows % deviation (% dev.) of mean peak area obtained from the injections of tobramycin standards prepared with perfusate matrix $\overline{Area_{post-extration}}$ from that in the reference solutions (the standard solution prepared in the absence of perfusate matrix), $\overline{Area_{reference}}$. In these results, the values of % dev. for low (100 ng/mL), medium (100 ng/mL) and high (5000 ng/mL) concentrations were within 15%, indicating that the matrix components in the blank perfusate and IPRL control perfusate re-circulating in the isolated rat lung vasculature did not change the assay's accuracy for spiked standards prepared in the presence of extracted perfusate. These results also implied that this solid phase extraction technique could effectively remove interfering matrix components other than the analyte.

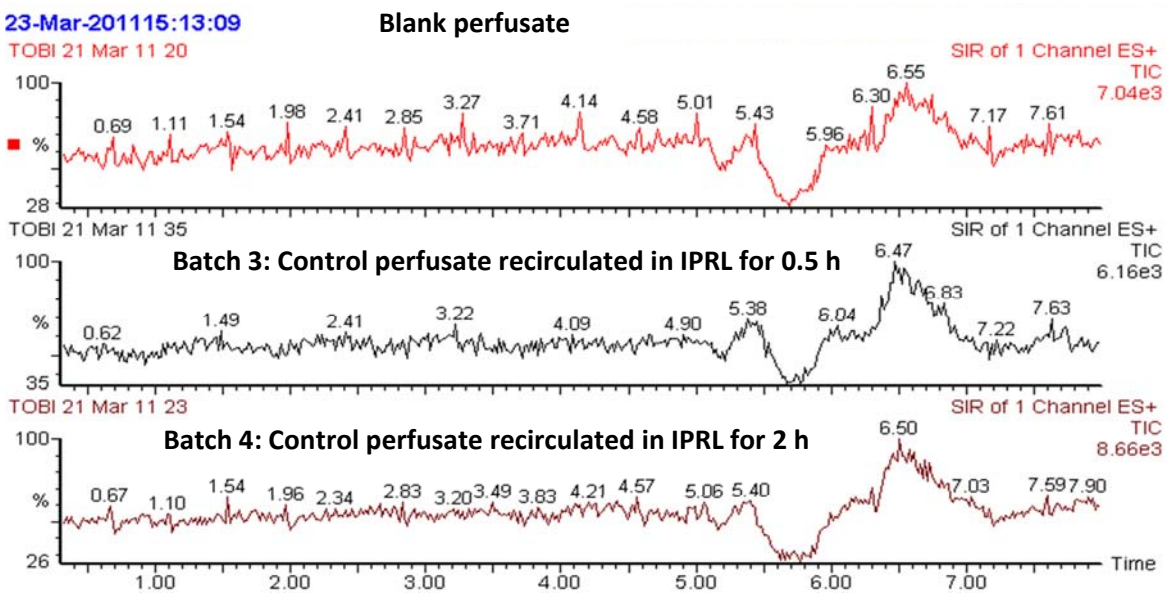


Figure AI.4 Chromatograms of perfusate with post-column infusion of 1 µg/mL standard solution

Table AI.4 % dev. of mean peak area of tobramycin in perfusate matrices (extracted) from that in the reference solutions (standard solutions prepared in the absence of perfusate matrices)

Nominal Conc. (ng/mL)	for "IPRL-free" blank perfusate	for IPRL control perfusate that recirculated in the pulmonary vasculature of the rat lung for 2h
	% dev.	% dev.
5000	2.1	-2.7
1000	5.7	5.7
500	7.1	-6.5
100	13.8	8.3

Adsorption tests in the IPRL apparatus

Circulating tobramycin perfusate solution in the IPRL apparatus in the absence of rat lungs at 37°C for 2 hours failed to show a concentration decrease due to adsorption loss to the apparatus. The determined concentrations at each sampling time showed no significant changes from the

nominal concentration of 100 ng/mL over 2 hours using mixed-effects repeated-measures ANOVA (p value > 0.05; Figure AI.5). It is possible that the high content of BSA in the perfusate effectively prevented the drug's adsorptive losses onto the apparatus in this case.

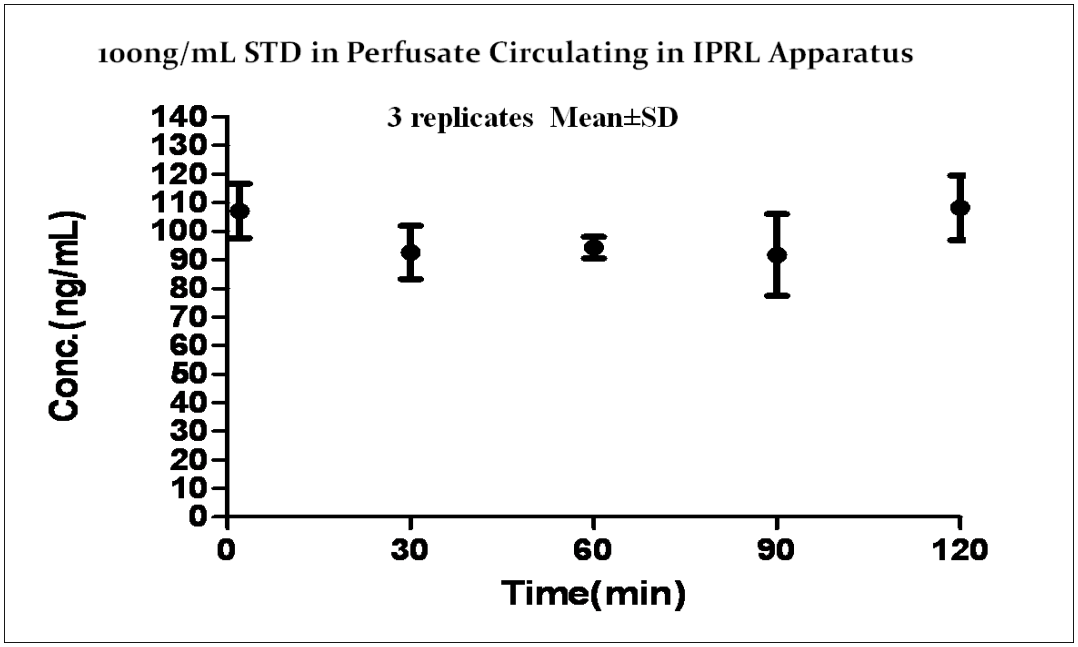


Figure AI.5 Adsorption test of tobramycin on the IPRL apparatus

Appendix II

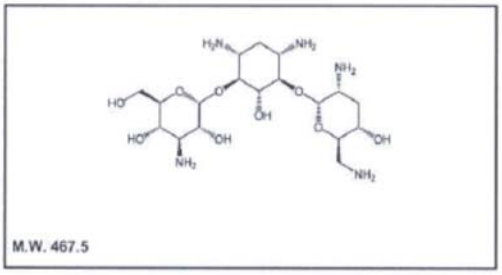
RADIOACTIVE [^{3}H]TOBRAMYCIN SPECIFICATIONS

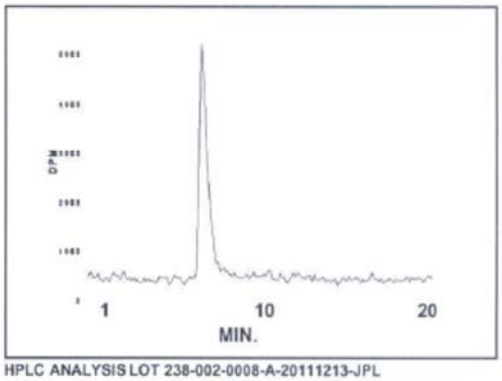
This shows a copy of certificate of analysis and product data sheet with analytical specifications provided by Moravek Biochemicals, Inc. Brea, CA. The claimed radiochemical purity of the tritiated drug was 98.5% on Dec. 14, 2011.

Product Data Sheet

MT-1001074

Tobramycin, [^{3}H]-





HPLC ANALYSIS LOT 238-002-0008-A-20111213-JPL
File Name: intc4434 Date and Time: 12/14/2011 9:19:09 A
Unit 12 Radio

Peak #	Area %	Time	Area
1	98.50	6.56670	15707.58768
2	1.50	7.86330	239.13053
Totals	100.00		15946.71821

Lot #: 238-002-0008-A-20111213-JPL

Specific Activity: 0.8 Ci/mmol

Concentration: 1.0 mCi/ml; 584.4 µg/ml

Packaged in: Water solution

Date of Analysis: December 14, 2011

Radiochemical Purity: 98.5%

Column: Supelco Discovery C18 4.6 x 250mm

Flow Rate: 1 ml/min.

Mobile Phase: Water : acetonitrile : heptafluorobutyric acid
(70 : 30 : 0.1)

Storage Recommendation: Store at -20°C.

Product Warranty: Stated on the reverse side of this Product Data Sheet.

Caution: Not For Use In Humans Or Clinical Diagnosis. This product is intended for investigational or manufacturing use only. It is pharmaceutically unrefined and is not intended for use in humans. Responsibility for its use in humans, as a diagnostic reagent, and compliance with federal laws rests solely with the purchaser.

Moravek Biochemicals, Inc. 577 Mercury Lane, Brea, California 92821 www.moravek.com info@moravek.com Phone (714) 990-2018 Fax (714) 990-1824

MT-1001074

Tobramycin, [^{3}H]-

238-002-0008-A-20111213-JPL

A) All chromatograms were run using the HPLC method described on the Product Data Sheet.

Concentrations and volumes:

Standard solution concentration was 2 mg/ml.

Tobramycin, [^{3}H]- concentration was 100 µCi/ml.

Volume of standard alone injection was 10 µl.

Volume of **Tobramycin, [^{3}H]-** alone injection was 1 µl.

Co-injection solution consisted of 1 µl **Tobramycin, [^{3}H]-** + 10 µl standard.

Volume of co-injection was 11 µl.

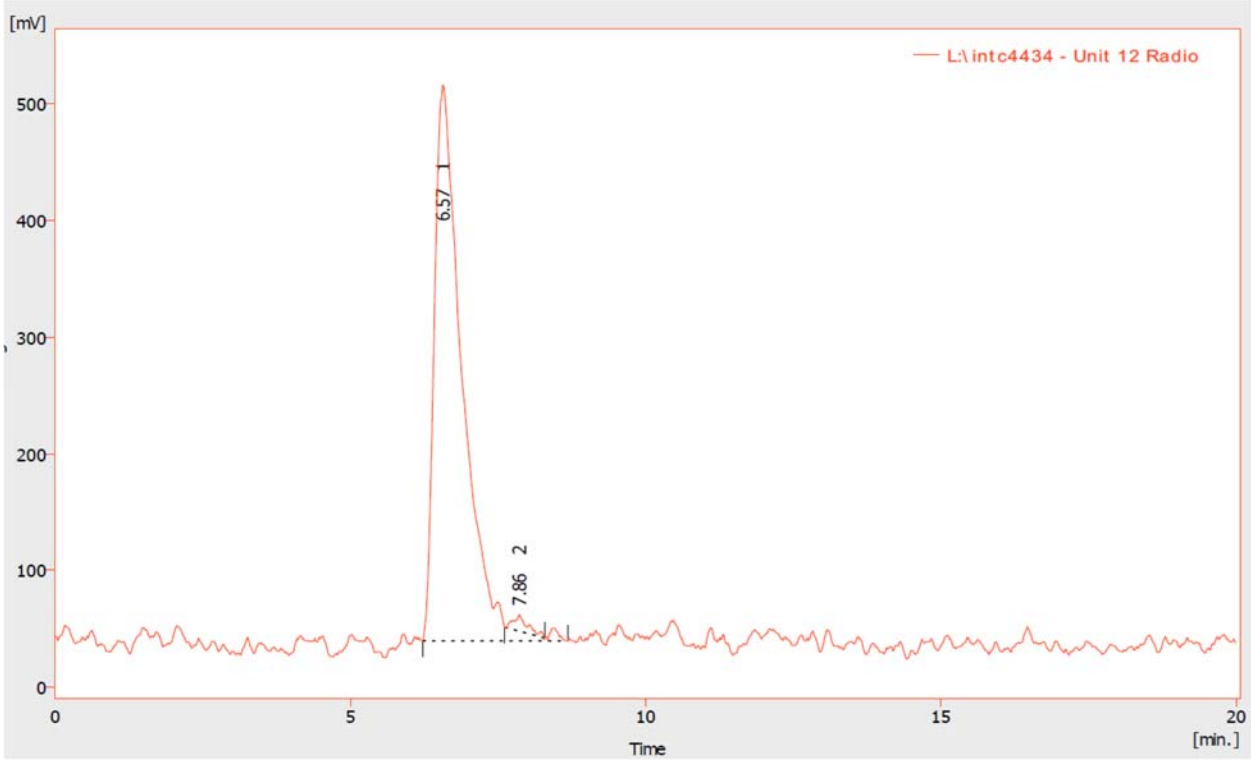
Volume of blank injection was 1 µl.

B) Mass spectrometry – positive mode

MT-1001074
Tobramycin, [3H]-
Lot 238-002-0008-A-20111213-JPL

Chromatogram Info:

File Name	: L:\intc4434	File Created	: 12/14/2011 9:19:26 AM
Origin	: Acquired	Acquired Date	: 12/14/2011 9:19:09 AM
Project	: Test	By	: Administrator
Method	: Unit12_20_min_run	By	: Administrator
Description	: Radiochemical trace of 3H material alone		
Created	: 8/8/2007 9:24 AM	Modified	: 12/14/2011 1:09 PM
Column	:	Detection	: Radiochemical
Mobile Phase	:	Temperature	:
Flow Rate	:	Pressure	:
Note	:		



12/14/2011 1:10 PM Chromatogram L:\intc4434.PRM

Result Table (Uncal - L:\intc4434 - Unit 12 Radio)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	6.567	15707.588	475.544	98.50	97.1	0.47
2	7.863	239.131	14.429	1.50	2.9	0.25
	Total	15946.718	489.974	100.00	100.0	

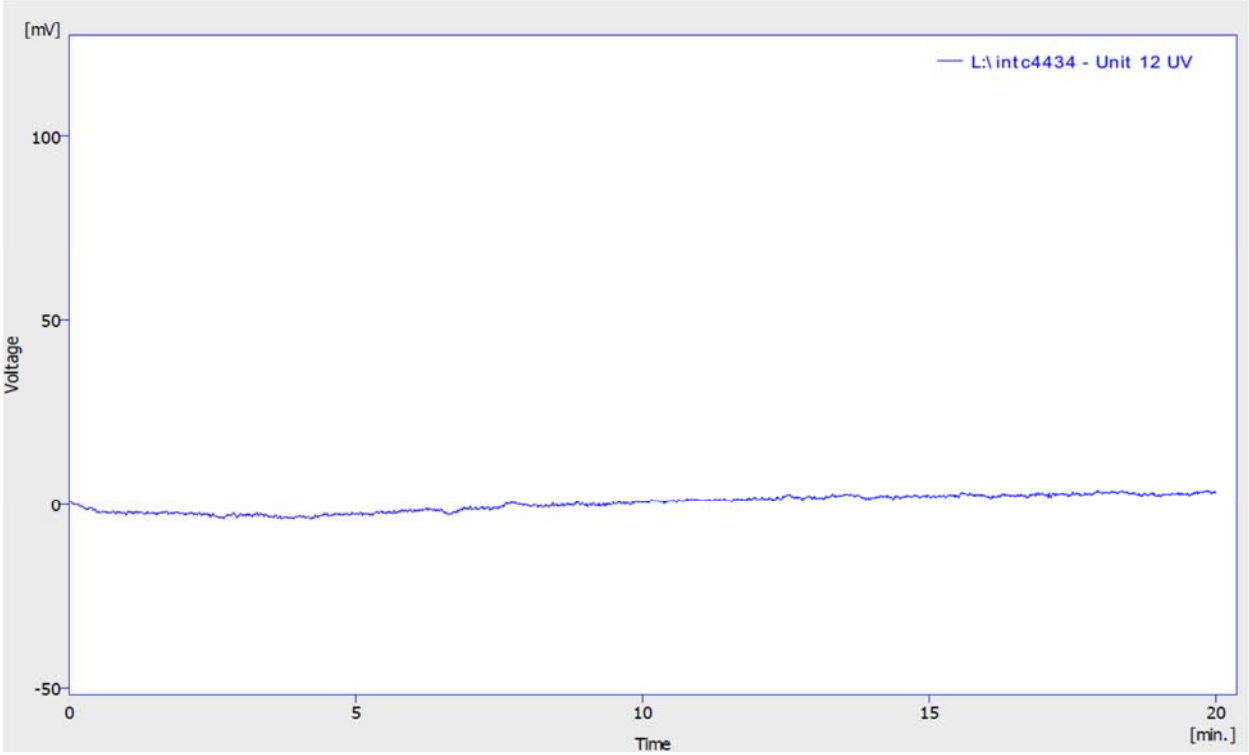
12/14/2011 1:17 PM

Chromatogram L:\intc4434.PRM

MT-1001074
Tobramycin, [3H]-
Lot 238-002-0008-A-20111213-JPL

Chromatogram Info:

File Name	: L:\intc4434	File Created	: 12/14/2011 9:19:26 AM
Origin	: Acquired	Acquired Date	: 12/14/2011 9:19:09 AM
Project	: Test	By	: Administrator
Method	: Unit12_20_min_run	By	: Administrator
Description	: UV trace of 3H material alone		
Created	: 8/8/2007 9:24 AM	Modified	: 12/14/2011 1:17 PM
Column	:	Detection	: UV 190nm
Mobile Phase	:	Temperature	:
Flow Rate	:	Pressure	:
Note	:		



Chromatogram L:\intc4434.PRM

Result Table (Uncal - L:\intc4434 - Unit 12 UV)

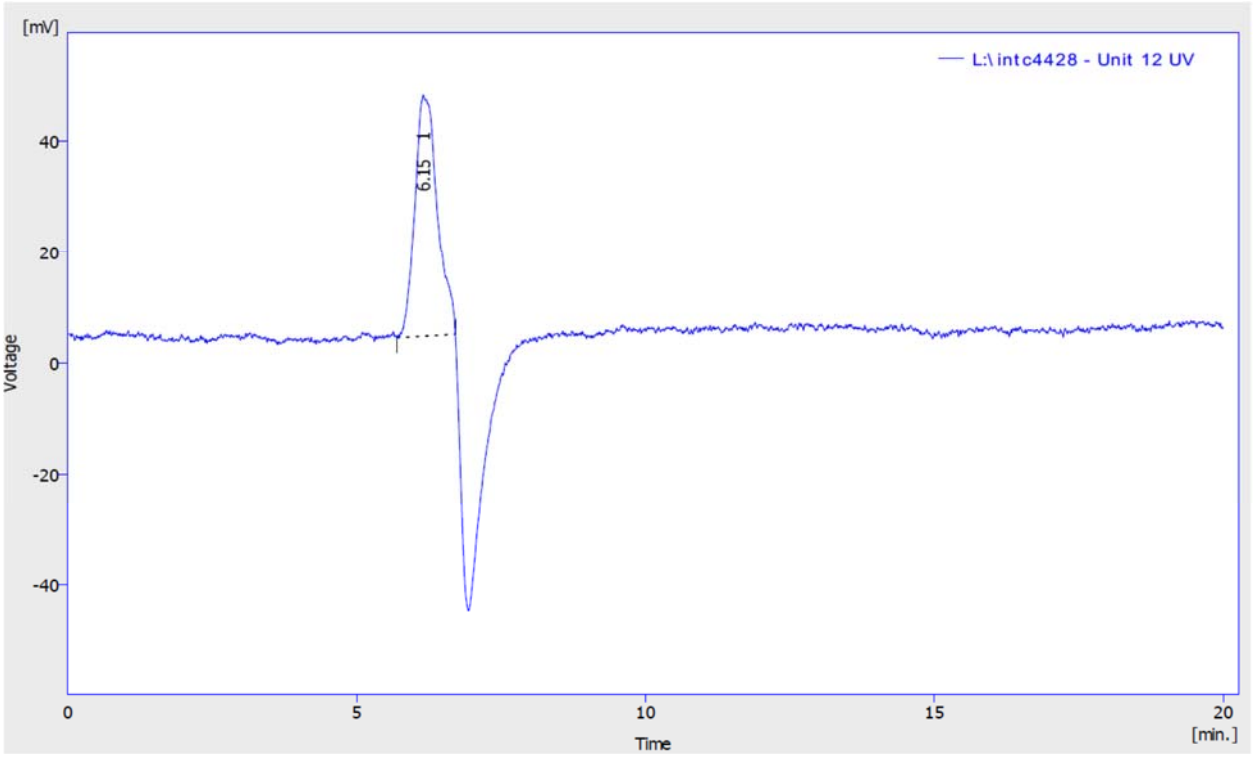
Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
No peak to report					

12/14/2011 1:19 PM Chromatogram L:\intc4428.prm

MT-1001074
Tobramycin, [3H]-
Lot 238-002-0008-A-20111213-JPL

Chromatogram Info:

File Name	: L:\intc4428	File Created	: 12/13/2011 2:20:30 PM
Origin	: Acquired	Acquired Date	: 12/13/2011 2:20:14 PM
Project	: Test	By	: Administrator
Method	: Unit12_20_min_run	By	: Administrator
Description	: UV trace of standard alone		
Created	: 8/8/2007 9:24 AM	Modified	: 12/14/2011 1:19 PM
Column	:	Detection	: UV 190nm
Mobile Phase	:	Temperature	:
Flow Rate	:	Pressure	:
Note	:		



Result Table (Uncal - L:\intc4428 - Unit 12 UV)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	6.150	1181.132	43.463	100.00	100.0	0.41
	Total	1181.132	43.463	100.00	100.0	

MT-1001074
Tobramycin, [3H]-
Lot 238-002-0008-A-20111213-JPL

Chromatogram Info:

File Name	: L:\intc4435	File Created	: 12/14/2011 9:39:39 AM
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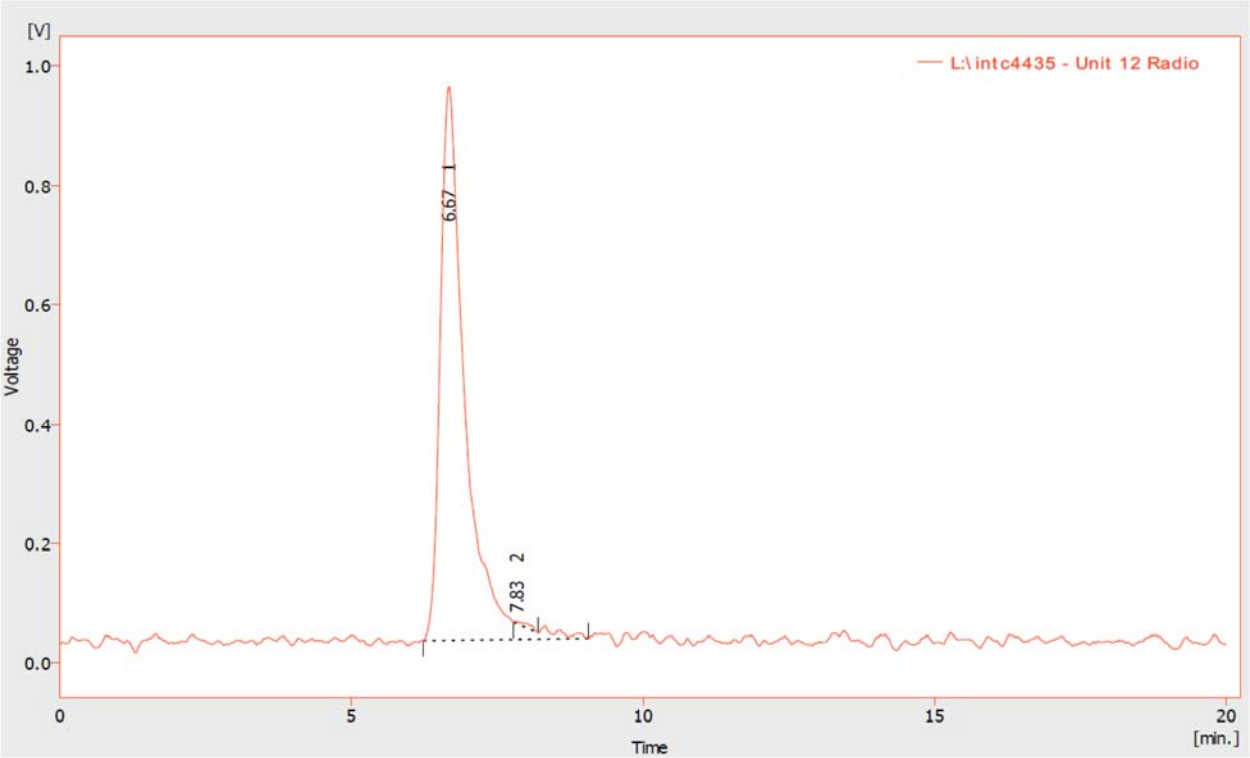
Project	: Test	By	: Administrator
Method	: Unit12_20_min_run	By	: Administrator
Description	: Radiochemical trace of 3H material co-injected with standard		
Created	: 8/8/2007 9:24 AM	Modified	: 12/14/2011 1:22 PM
Column	:	Detection	: Radiochemical
Mobile Phase	:	Temperature	:
Flow Rate	:	Pressure	:
Note	:		



Result Table (Uncal - L:\intc4435 - Unit 12 Radio)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	6.670	27546.206	926.459	99.58	99.6	0.40
2	7.833	116.598	3.642	0.42	0.4	0.08
	Total	27662.804	930.101	100.00	100.0	

12/14/2011 1:22 PM Chromatogram L:\intc4435.prm

MT-1001074
Tobramycin, [3H]-
Lot 238-002-0008-A-20111213-JPL

Chromatogram Info:

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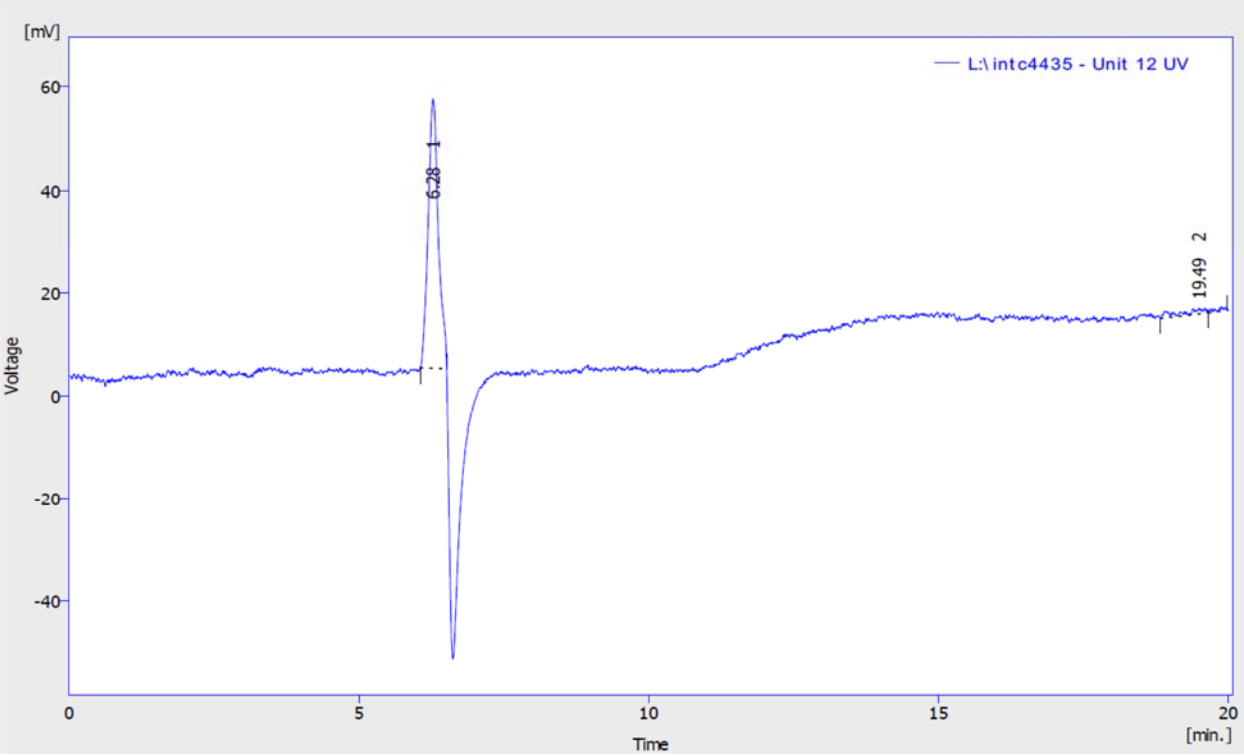
Method	: Unit12_20_min_run	By	: Administrator
Description	: UV trace of 3H material co-injected with standard		
Created	: 8/8/2007 9:24 AM	Modified	: 12/14/2011 1:22 PM
Column	:	Detection	: UV 190nm
Mobile Phase	:	Temperature	:
Flow Rate	:	Pressure	:



Result Table (Uncal - L:\intc4435 - Unit 12 UV)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	6.280	639.626	52.316	94.34	96.3	0.18
2	19.493	30.473	1.172	4.49	2.2	0.09
3	19.883	7.927	0.863	1.17	1.6	0.04
	Total	678.026	54.351	100.00	100.0	

12/14/2011 1:18 PM

Chromatogram L:\intc4427.prm

MT-1001074
Tobramycin, [3H]-
Lot 238-002-0008-A-20111213-JPL

Chromatogram Info:

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Description	: UV trace of blank injection		
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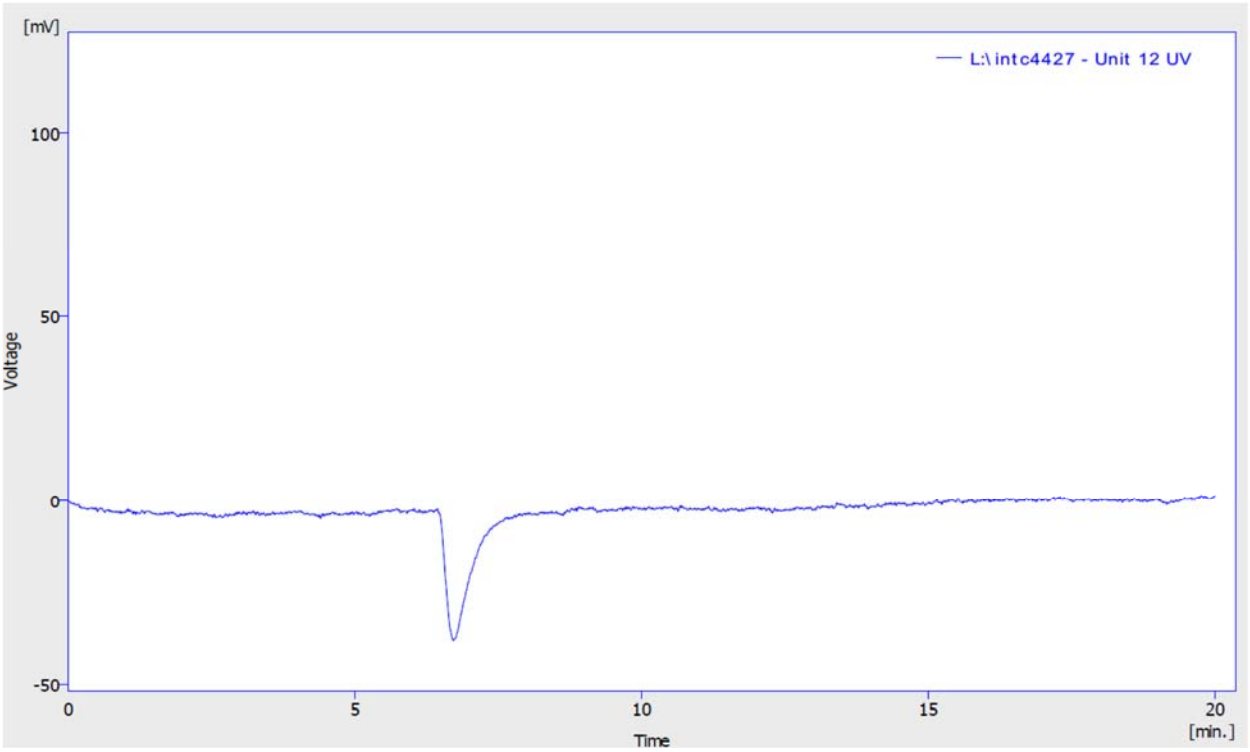
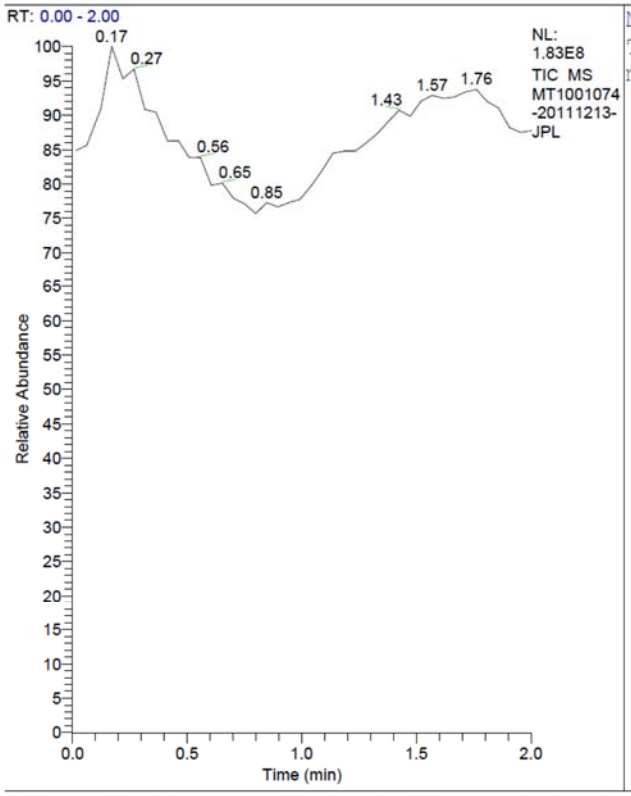
Flow Rate	:	Pressure	:
Note	:		



Result Table (Uncal - L:\intc4427 - Unit 12 UV)

Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
No peak to report					

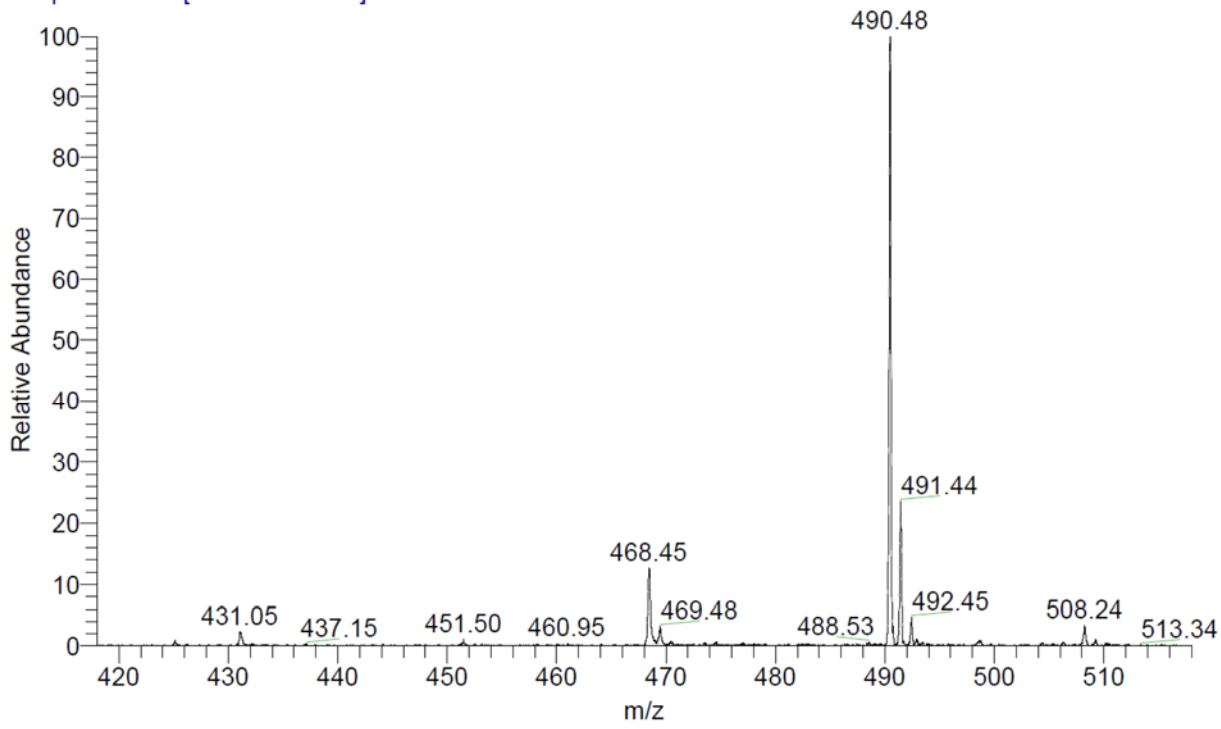
C:\Xcalibur\data\MT1001074-20111213-JPL 12/13/2011 4:07:49 PM MT1001074-20111213-JPL



MT1001074-20111213-JPL#1-42 RT: 0.01-2.00
T: + p ESI Z ms [418.00-518.00]
m/z= 463.55-512.17

m/z	Intensity	Relative
468.48	15019378.0	19.60
469.43	4168921.0	5.44
470.44	1130001.9	1.47
473.47	738280.9	0.96
474.53	864304.3	1.13
477.04	702914.3	0.92
478.21	605942.0	0.79
483.12	645945.3	0.84
488.55	734538.8	0.96
489.41	584120.6	0.76
490.47	76635544.0	100.00
491.43	18759380.0	24.48
492.43	4068593.8	5.31
493.04	775581.0	1.01
498.57	1630105.9	2.13
504.33	670741.7	0.88
506.31	738534.7	0.96
508.23	3441736.5	4.49
509.22	1004399.5	1.31
510.29	659529.7	0.86

MT1001074-20111213-JPL #1-42 RT: 0.01-2.00 AV: 42 NL: 1.98E6
T: + p ESI Z ms [418.00-518.00]



	A	B	C	D	E	F
1	Number of tritium atoms	Specific Activity (Ci/mmol)	Relative Abundance	Number of Ci	Number of mmoles	
2						
3	0	0	100.00%	0	1	
4	1	28.8	3.11%	0.89568	0.0311	
5	2	57.6	0.00%	0	0	
6	3	86.4	0.00%	0	0	
7	4	115.2	0.00%	0	0	
8	5	144	0.00%	0	0	
9	6	172.8	0.00%	0	0	
10						
11				0.89568	1.0311	
12					0.969838037	
13		**Overall Specific Activity:**	0.868864533			
14						
15						
16	Column A is the number of Tritium atoms in a molecular species					
17						
18	Column B is the specific activity of a molecular species when it contains the number of Tritium atoms in column A					
19						
20	Column C is the relative abundances of each molecular species from the mass spectrometer.					
21	In a mass spectrum there is always a 100% reference peak.					
22						
23	Column D is the number of mCi present in the mixture with a specific activity of column B (B x C)					
24						
25	Column E is the relative percentage of mMoles present in the mixture based on the 100% reference value being 1 mMole (1 x C)					
26						
27	Cell D11 is the total number of Ci. Cell E11 is the total number of mmoles.					
28						
29	Dividing the total number of Ci by the total number of mmoles gives the specific activity in cell C12 (D11 / E11)					

Appendix III

RADIOACTIVE [^{3}H]TOBRAMYCIN ASSAY IN PERFUSATE

Perfusate samples containing [^{3}H]tobramycin obtained from IPRL experiments were assayed in a Liquid Scintillation Analyzer (LSA) (Tri-Carb 2800-TR, PerkinElmer) directly or after appropriate dilution in blank perfusate; the counts were corrected by subtracting the background due to blank perfusate. Tobramycin concentrations in perfusate samples for each IPRL experiment were calculated based on the comparison of radioactive content between perfusate samples and the dosing solution used in the same IPRL study. For each IPRL experiment, the dosing solution prepared with trace amount of [^{3}H]tobramycin was diluted 2000 fold by blank perfusate to prepare the control solution for the assay. The total radioactivity in samples, expressed as DPMs (disintegrations per minute), in 1 mL of each solution was determined by LSA, after mixing with 5 mL of scintillation cocktail (Ecoscint XR, National Diagnostics, Atlanta, GA) followed by 4 hours of storage in the dark at room temperature prior to analysis. Control solutions were prepared in duplicate. Corrected DPMs of perfusate samples were used for calculation of tobramycin concentration, C_{sample}, according to the formula:

$$C_{\text{sample}} = DPM_{\text{sample}} / \text{mean } DPM_{\text{control}} \times C_{control}^{nominal} \qquad \text{eq. AIII.1}$$

where DPM_{sample} and mean DPM_{control} are the corrected radioactive counts from samples and duplicate controls, respectively, while $C_{control}^{nominal}$ refers to the nominal concentration of each control solution (0.5‰ of solute concentration in dosing solution).

A comparison of the HPLC-MS and radioactive analysis methods is shown for tobramycin absorption in Appendix IV. Notably, such a comparison could only be performed at a relatively high dose (2mg) that produced perfusate concentrations enabling accurate assay by HPLC-MS. Radioactive methods were needed for lower doses.

Appendix IV

COMPARISON OF IPRL ABSORPTION DATA FOR TOBRAMYCIN: RADIOACTIVITY ASSAY VERSUS HPLC-MS

AIV.a Introduction

The HPLC-MS method described in Appendix I successfully assayed tobramycin perfusate samples at concentrations no less than 50 ng/mL. Nevertheless, the detection sensitivity was not sufficient for the studies of tobramycin absorption in the IPRL at doses lower than 0.2 mg, in which tobramycin concentrations in the re-circulating perfusate in most cases were < 50 ng/mL if sample time less than 10 minutes following administration. In order to study tobramycin's disposition kinetics for a wide range of airway doses (from 0.002 to 2 mg) in the IPRL therefore, a radioactivity assay using liquid scintillation counting (described in Appendix III) was employed. This appendix describes a comparison of the cumulative fraction of administered dose absorbed into the perfusate, F_p, as a function of time, from two parallel groups of IPRL absorption experiments in which the surgery, establishment and maintenance of the IPRL, and the dosing technique and perfusate sampling, were all fixed except perfusate samples were assayed either by HPLC-MS or radioactivity. The Appendix also describes the general methods used to calculate F_p from the concentration data that was collected in each experiment.

AIV.b Methods

IPRL absorption studies using either tritium-labeled tobramycin or unlabeled tobramycin were performed according to Chapter IV. For six separate IPRL absorption experiments using tobramycin dosing solution at 20 mg/mL containing 18μCi/mL [^{3}H]tobramycin, perfusate samples were analyzed by radioactivity assay as described in Appendix III. For 4 separate IPRL absorption experiments using unlabeled tobramycin solutions at 20 mg/mL, perfusate samples were analyzed using the HPLC-MS method described in Appendix I. For each IPRL experiment, tobramycin concentrations in the re-circulating perfusate at each sampling time, $C_t^{perfusate}$, were determined to calculate the cumulative absorbed amount from airway to the perfusate, and the cumulative fraction of administered dose absorbed into the perfusate (F_P) by the equation:

$$F_P = \frac{C_t^{perfusate} \times V_t + \sum(C^{sample} \times V^{sample})}{Administered\ dose} \qquad \text{eq. AIV. 1}$$

where $C_t^{perfusate}$ and V_t are the drug concentration in the circulated perfusate and the total volume of the re-circulating perfusate in the IPRL system at time t, respectively; C^{sample} is the drug concentration in each perfuate sample, and V^{sample} is the volume of that perfusate sample withdrawn at each sampling time (=1mL); $\sum(C^{sample} \times V^{sample})$ is the total amount of drug in perfuate samples taken before time t. Notably, no blank perfusate was added to replace perfusate after each sample was withdrawn in IPRL absorption experiments. After the last time point, the recirculating perfusate was collected and measured, to determine V_{last} and used to calculate V_t. The values of F_P at each sampling time were compared statistically between the two assay methods using multiple t tests and a p value 0.05.

AIV.c Results and discussion

For each IPRL experiment, sampling time, $C_t^{perfusate}$, V_t , cumulative amount of dose absorbed into perfusate from the airway, and F_P are tabulated in Table AIV.1 (using the radioactivity assay)

and Table AIV.2 (using the HPLC-MS method). The mean±SD of F_p values at each sampling time determined by the HPLC-MS method and the radioactivity assay are shown in Fig. AIV.1. For the IPRL experiments using the HPLC-MS method, there were not sufficient data at 1, 105 and 120 minutes to perform a statistical comparison. The comparison of Fp therefore, was performed at 3, 5, 10, 20, 30, 40, 50, 60, 75, 90 minutes for the two parallel groups of IPRL absorption experiments. There was no statistical difference of the values F_p between the two parallel groups at any of these sampling times (t-test, p value >0.5), indicating that the IPRL absorption results were consistent when the two different assay techniques were employed. The results implied that assaying perfusate samples based on radioactivity content by LSA were accurate and specific for tobramycin since the IPRL absorption data determined by this technique were comparable with those determined by the HPLC-MS method in which tobramycin was quantitated using single ion monitoring of tobramycin's molecular ion $[M+1]^+$.

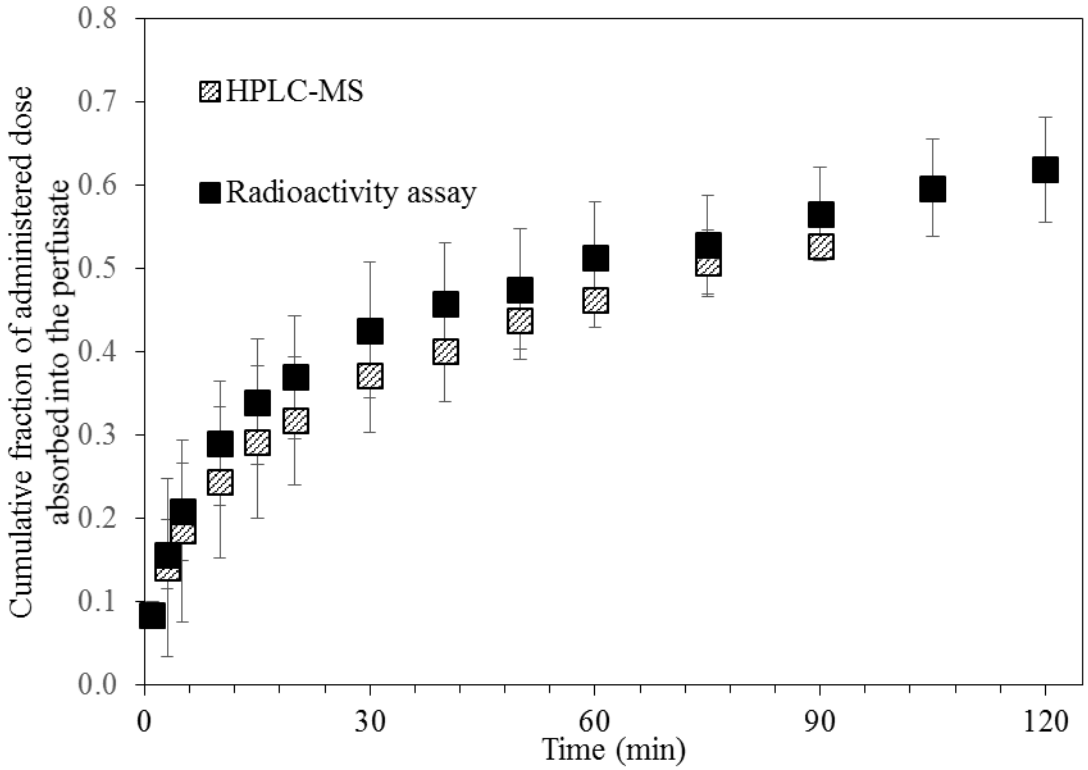


Figure AIV.1 Mean cumulative fraction of administered tobramycin dose absorbed into perfusate versus time determined by HPLC-MS or the radioactivity assay (nominal doses are 2 mg)

Table AIV.1 IPRL absorption data of tobramycin determined by radioactivity assay (6 IPRL preparations)

Rat Lung #1 [^{3}H]tobramycin

Administered dose 2027.5 μg

Sampling time (min)	$C_t^{perfusate}$ (μg/mL)	V_t (mL)	Cumulative absorbed amount (μg)	F_p
1	0.632	200	126.44	0.062
3	1.559	199	310.93	0.153
5	2.316	198	460.82	0.227
10	2.980	197	591.54	0.292
15	3.490	196	691.52	0.341
20	3.608	195	714.58	0.352
30	4.443	194	876.62	0.432

40	4.609	193	908.66	0.448
50	4.640	192	914.60	0.451
60	5.204	191	1022.20	0.504
75	5.705	190	1117.42	0.551
90	5.895	189	1153.33	0.569
105	6.338	188	1236.66	0.610
120	6.632	187	1291.67	0.637

Rat Lung # 2 [^{3}H]tobramycin

Administered dose 2289.4 µg

Sampling time (min)	$C_t^{perfusate}$ (µg/mL)	V_t (mL)	Cumulative absorbed amount (µg)	F_p
1	0.765	213	162.95	0.071
3	1.108	212	235.61	0.103
5	1.409	211	299.24	0.131
10	2.029	210	429.43	0.188
15	2.420	209	511.02	0.223
20	2.953	208	621.86	0.272
30	3.351	207	704.30	0.308
40	3.865	206	810.32	0.354
50	4.069	205	852.03	0.372
60	4.647	204	970.02	0.424
80	4.998	203	1041.27	0.455
90	5.384	202	1119.19	0.489
105	5.881	201	1219.18	0.533
120	6.153	200	1273.47	0.556

Rat Lung # 3 [^{3}H]tobramycin

Administered dose 2255.2 µg

Sampling time (min)	$C_t^{perfusate}$ (µg/mL)	V_t (mL)	Cumulative absorbed amount (µg)	F_p
1	1.033	213	219.93	0.098
3	1.598	212	339.88	0.151
5	2.106	211	446.95	0.198
10	3.326	210	703.21	0.312
15	4.026	209	849.58	0.377
20	4.549	208	958.19	0.425

30	5.152	207	1083.12	0.480
40	5.036	206	1174.48	0.521
50	5.359	205	1247.49	0.553
60	5.972	204	1385.87	0.615
75	6.682	203	1394.54	0.618
90	6.926	202	1443.89	0.640
105	7.236	201	1506.28	0.668
120	6.325	200	1640.36	0.727

Rat Lung # 4 [^{3}H]tobramycin

Administered dose 1800.7 µg

Sampling time (min)	$C_t^{perfusate}$ (µg/mL)	V_t (mL)	Cumulative absorbed amount (µg)	F_p
1	0.708	204	144.35	0.080
3	1.441	203	293.20	0.163
5	1.920	202	389.95	0.217
10	2.733	201	553.31	0.307
15	3.236	200	653.94	0.363
20	3.460	199	698.65	0.388
30	3.931	198	791.84	0.440
40	4.205	197	845.77	0.470
50	4.456	196	895.05	0.497
60	4.682	195	939.00	0.521
75	4.428	194	889.74	0.494
90	5.150	193	1029.23	0.572
120	5.243	192	1046.92	0.581

Rat Lung # 5 [^{3}H]tobramycin

Administered dose 1953.22 µg

Sampling time (min)	$C_t^{perfusate}$ (µg/mL)	V_t (mL)	Cumulative absorbed amount (µg)	F_p
1	0.885	203	179.69	0.092
3	1.343	202	272.14	0.139
5	1.665	201	336.84	0.172
10	2.276	200	459.12	0.235
15	2.844	199	572.26	0.293

20	3.008	198	604.74	0.310
30	3.524	197	706.46	0.362
40	3.964	196	792.58	0.406
50	4.168	195	832.60	0.426
60	4.549	194	906.52	0.464
75	4.831	193	961.14	0.492
90	5.076	192	1008.27	0.516
105	5.329	191	1056.71	0.541
120	5.648	190	1117.61	0.572

Rat Lung # 6 [^{3}H]tobramycin

Administered dose 1926.2 μg

Sampling time (min)	$C_t^{perfusate}$ (μg/mL)	V_t (mL)	Cumulative absorbed amount (μg)	F_p
1	0.977	202	197.36	0.102
3	2.197	201	442.56	0.230
5	2.918	200	586.72	0.305
10	3.906	199	783.47	0.407
15	4.267	198	854.93	0.444
20	4.531	197	906.82	0.471
30	5.152	196	1028.52	0.534
40	5.319	195	1061.15	0.551
50	5.328	194	1062.85	0.552
60	5.310	193	1059.40	0.550
75	5.438	192	1084.06	0.563
90	5.914	191	1174.89	0.610
105	6.139	190	1217.67	0.632
120	6.218	189	1232.62	0.640

Table AIV.2 IPRL absorption data of tobramycin determined by HPLC-MS assay (4 IPRL preparations)

Rat Lung #7 unlabeled tobramycin
Administered dose 1791.5 μg

Sampling time (min)	$C_t^{perfusate}$ (μg/mL)	V_t (mL)	Cumulative absorbed amount (μg)	F_p
3	1.908	204	389.25	0.217
5	2.456	203	500.52	0.279
10	3.032	202	616.87	0.344
15	3.271	201	664.84	0.371
20	3.525	200	715.62	0.399
30	3.828	199	775.94	0.433
40	4.023	198	814.59	0.455
50	4.448	197	898.26	0.501
60	4.489	196	906.24	0.506
75	4.721	195	951.52	0.531
90	4.702	194	947.98	0.529

Rat Lung #8 unlabeled tobramycin
Administered dose 1814.1 μg

Sampling time (min)	$C_t^{perfusate}$ (μg/mL)	V_t (mL)	Cumulative absorbed amount (μg)	F_p
1	1.118	200	223.66	0.123
3	1.854	199	370.04	0.204
5	2.271	198	452.60	0.249
10	2.696	197	536.37	0.296
15	3.057	196	607.06	0.335
20	3.195	195	633.96	0.349
30	3.524	194	697.79	0.385
40	3.477	193	688.77	0.380
50	3.750	192	741.22	0.409
60	3.850	191	760.31	0.419
75	4.340	190	853.43	0.470
90	4.342	189	853.78	0.471

Rat Lung # 9 unlabeled tobramycin

Administered dose 1799.2 μg

Sampling time (min)	$C_t^{perfusate}$ (μg/mL)	V_t (mL)	Cumulative absorbed amount (μg)	F_p
1	0.452	200	90.49	0.050
3	1.066	199	213.23	0.119
5	1.534	198	306.28	0.170
10	2.167	197	431.76	0.240
15	2.710	196	537.47	0.299
20	3.057	195	604.14	0.336
30	3.726	194	732.36	0.407
40	4.211	193	823.71	0.458
54	4.392	192	855.60	0.476
60	4.646	191	900.54	0.501
75	5.033	190	970.36	0.539
90	5.299	189	1016.43	0.565

Rat Lung # 10 unlabeled tobramycin

Administered dose 1834.7 μg

Sampling time (min)	$C_t^{perfusate}$ (μg/mL)	V_t (mL)	Cumulative absorbed amount (μg)	F_p
3	0.209	203	42.44	0.023
5	0.365	202	73.84	0.040
10	0.852	201	171.79	0.094
15	1.492	200	299.83	0.163
20	1.699	199	341.00	0.186
30	2.385	198	476.87	0.260
40	2.888	197	575.97	0.314
50	3.396	196	675.51	0.368
60	3.939	195	781.42	0.426
75	4.502	194	890.52	0.485
90	5.047	193	995.86	0.543
105	5.238	192	1032.54	0.563
120	5.491	191	1080.73	0.589

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Vita

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