Pharmacoanalytical Assays of *Erwinia* Asparaginase (Erwinase) and Pharmacokinetic Results in High-risk Acute Lymphoblastic Leukemia (HR ALL) Patients: Simulations of Erwinase Population PK-PD Models

VASSILIOS I. AVRAMIS[1,2], SAGRARIO MARTÍN-ARAGÓN[1,3], EARL V. AVRAMIS[1,4] and BARBARA L. ASSELIN[5]

[1]*Division of Hematology/Oncology, Department of Pediatrics, Childrens Hospital Los Angeles, USC Keck School of Medicine, Los Angeles, CA 90027;*
[2]*University of Southern California Keck School of Medicine, Los Angeles, CA 90033, U.S.A.;*
[3]*Pharmacology Department, School of Pharmacy, Complutense University of Madrid, 28040 Madrid, Spain;*
[4]*UC Davis, Davis, College of Environmental Toxicology, Davis, CA 95616;*
[5]*Pediatric Hematology/Oncology, University of Rochester Medical Center, Rochester, NY 14642, U.S.A.*

Abstract. *Background: Asparaginases are the cornerstone therapy of many successful combination regimens for the treatment of acute lymphoblastic leukemia (ALL), the most common malignancy in children and adolescents. Currently, two asparaginase formulations are available in the US, native Escherichia coli asparaginase (ASNase) and pegaspargase. A third formulation native Erwinia asparaginase (Erwinase, ERW) has recently been made available under a licensing exception for personal use. We report here the development and validation process of ERW pharmacoanalytical assays and the results in a few patients. Materials and Methods: We developed and systematically validated the ERW enzyme activity and ERW concentration, anti-ERW antibody and related assays. Pharmacokinetic and pharmacodynamic (PK-PD) studies were performed in a limited number of patients who received 6,000 IU/m^2 x 3 per week x 2 courses, and 4 patients who received 25,000 IU/m^2 x 3 per week x 2 courses of ERW. Results: The linearity and range of the Erwinase calibration lines for the pharmacoanalytical assays were excellent. The accuracy and precision were better than the FDA limit allows for oncology biological products (<30%) coefficient of variation (%CV) and related parameters in the quantification of ERW concentration. The validation of these parameters was equal to or better than during the assay development. PK-PD analyses of ERW in a few patients yielded an average half-life of elimination of 15.8±1.64 hours. There was an excellent PD response post ERW administration resulting in an ERW concentration-dependent asparagine (ASN, <0.5 μM) and glutamine (GLN, <50 μM) deamination. Pharmacodynamic correlations demonstrated that 0.1 to 0.2 IU/ml of ERW in serum were sufficient for 90% GLN and/or ASN deamination for up to 2 weeks. No anti-ERW antibody [Ab(+)] was seen among those few patients. None of the other 5 patients had an adverse event. Based on these post hoc results, simulations on various doses and schedules of this drug have been made. Conclusion: The pharmacoanalytical assays were excellent tools to evaluate the PK and PD data of ERW in pediatric patients with HR ALL. However, this initial PK-PD evidence needs further validation in future clinical trials. Insights into the PD contributions of ERW in anti-E. coli ASNase Ab(+) patients will guide us in optimal design and use of ERW as part of combination chemotherapy regimens in future clinical trials.*

Correspondence to: Dr. Vassilios I. Avramis, Childrens Hospital of Los Angeles, Hematology/Oncology, 4650 Sunset Blvd, Mail 57, Los Angeles, CA 90026, U.S.A. e-mail: vavramis@chla.edu

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The discovery that asparaginase (*Escherichia coli* L-asparagine amidohydrolase, ASNase) possesses anti-cancer properties began in the 1950's in the United Kingdom (1-6). Asparaginases represent a key drug, which is used in treatment of haematopoietic malignancies, in particular in acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma, and act by depriving tumor cells of the amino acid asparagine (ASN) (7, 8). In most cases, repeated administration of ASNase is required for effective therapy, but this can be restricted by undesirable effects caused by immune responses and anti-asparaginase antibody [Ab(+)] production. Despite several recent insightful reviews

detailing the history and mechanisms of action of ASNases against ALL, important questions still remain (9-15). The primary mechanism of action of ASNase is to deaminate ASN and, to a lesser extent, GLN, in serum.

Rapid depletion of ASN and GLN in the patients' serum ensures optimal leukemic blast-kill. In contrast, a gradual ASN or GLN depletion may allow the leukemic blasts to adapt and survive (16-18). Repeated or prolonged treatment with ASNases can lead to an Ab(+) response and this may neutralize the enzyme activity and prevent the protein from remaining in the circulation (10-12, 19-20). Ultimately, this can lead to a diminished therapeutic effect with the risk of infusion reactions (12, 16). More insidiously, there is a danger that antibodies will not only neutralize the foreign enzyme, but will also cross-react with a vital component in normal tissues with subsequent host reactions. Immediate (Type I) and delayed (Type II) immune hypersensitivity reactions are further immunological concerns (12). Therefore, the aim for the use of biological oncology products (enzymes) in cancer therapy is to allow repeated treatments without compromising their efficacy or the safety of patients. Potentially this can be achieved by understanding and controlling the immune response and by selecting the appropriate ASNase enzyme accordingly (16). However, a significant proportion of patients treated with ASNase develop allergic reactions or undergo neutralization of the enzyme so they could require treatment cessation or alteration of the formulation and the dosage, respectively. Thus, there is a need for another structurally unrelated ASNase to manage patients sensitized to *E. coli* ASNase. Many investigators have determined the adverse effects that anti-ASNase Ab(+) have had on treatment outcomes in ALL patients (10, 12, 20). In the presence of Ab(+), the ASNase enzymatic activity and the percentage of ASN and GLN depletion become non-existent (12, 16). Once Ab(+) appears, it neutralizes the antigen with loss of its specific enzymatic activity and the subsequent rebounding of ASN and GLN to control concentrations in serum.

The toxicities of ASNases are serious. Liver and pancreas dysfunction are presented as coagulopathies and pancreatitis, with CNS dysfunction seen less frequently (12, 21). Hypersensitivity is the most common dose-limiting toxicity seen with the *E. coli* ASNase formulations, which share immunological cross-reactivity (16). The severity of clinical symptoms of allergy, ranging from localized erythema at the injection site to systemic anaphylaxis, are reported in up to two thirds of patients receiving intensive schedules of the native form (5, 7-10, 16, 21). Patients with allergic reactions usually have neutralizing IgG Ab and other subtypes of immunoglobulins in their serum. In the majority of cases (85%-90%) these Ab(+) interfere with the therapeutic effect of ASNase by neutralizing enzymatic activity and/or increasing the rate of enzyme clearance (10, 19-21, 22). Evaluations of Ab(+) in relapsed pediatric patients suggest that development of a high anti-ASNase Ab(+) titer is associated with inferior treatment response(s) (16, 19-20). In CCG-1961, occurrence of ASNase allergy was not associated with inferior outcome, while the presence of "silent hypersensitivity" as defined by the presence of anti-ASNase Ab(+) without clinical history of allergy symptoms was identified in a subset of patients with poor outcome (16).

Erwinase® (ERW, crisantaspase) (EC 3.5.1.1.) is an L-asparaginase isolated from *Erwinia chrysanthemi* which does not cross-react with the Ab(+) against the *E. coli* ASNase formulations. ERW is available in the U.S. for personal use from OPi SA (Lyon, France). Clinical studies need to be conducted in order to determine the optimal dose and schedule of the ERW formulation, its population pharmacology, toxicities and possible impact on event-free survival (EFS) in HR ALL pediatric patients treated with this ASNase on a personal use. In patients with obvious clinical allergy symptoms who were also Ab(+) to *E. coli* formulations (anamnestic response), ERW doses were used equally substituting native *E. coli* ASNase dosing in a limited number of patients (16, 23). Hence, ERW was safely and successfully administered to these few patients on a compassionate basis (16). The assays for quantifying ERW were developed in order to evaluate these patients. We present here their development and quality control with the pertinent accuracy and precision and in-study validation. More recently, 5 other patients received ERW in a more intensive dosing schedule upon clinical allergy to *E. coli* formulations. Interesting and useful PK-PD data for ERW are available from these patients (22). Based on these limited clinical studies, pharmacokinetic (PK) and pharmacodynamic (PD) population modeling (NONMEM) has been used to provide new insights into the optimal dose and formulation of ERW that may be used in future clinical trials.

Patients and Methods

Patients and treatment. Between May 2006 to the present, patients with newly diagnosed ALL and HR features were enrolled on CCG studies receiving *E. coli* pegaspargase after the appropriate COG IRB review and the individual informed consent. Patients who developed clinical allergy to native ASNase or pegaspargase treatment, if symptoms persisted, were switched to ERW treatment available since May 2006 under a special licensing exception for personal use. ERW was administered at a dose of 6,000 IU/m^2 and then increased to 10,000 IU/m^2 and was substituted for each dose of *E. coli*-ASNase in patients with clinical allergy with CHLA IRB approval (16, 23). More recently, and based on the compensation for the shorter half-life of this ASNase, high doses and schedules, 4 patients received ERW at 25,000 IU/m^2 x 3 doses per week x 2 weeks per course of treatment (150,000 IU/m^2/ treatment).

ASNase formulations. All preparations of ASNase were given by intramuscular (*i.m.*) injection. Both native *E. coli* and ERW formulations are lyophilized crystalline powders. Doses were 6,000 IU/m^2 for each dose of native *E. coli*-ASNase (ELSPAR, Merck & Co., Inc. West Point, PA, USA).

Assay development

HPLC amino acid determinations. The same serum specimen was used for all three ERW assays and the amino acid quantifications by HPLC. Amino acid levels for ASN, GLN, ASP, and GLU were determined using a pre-column derivatization and reversed-phase high-performance (C18, μC18 reverse phase column in line with a pre-column mini-column filter) liquid chromatography (HPLC) method as reported previously (10, 16, 21). Briefly, the serum specimens were derivatized in batches of 24 specimens as described elsewhere (10, 24) in the presence of an external standard using a UV detector. The elution schema consisted of a complex isocratic elution for 10 minutes followed by a gradient elution from 0% methanol into 50% methanol in sodium acetate, pH 6.50, controlled by a computer-specific program which collected the UV changes and produced a peak and area under the curve (AUC) analysis for each amino acid (Waters, Inc., Bedford, Mass). The calibration lines of the peak ratio or AUC ratio of each amino acid of interest (ASN, GLN, ASP and GLU) to the external standard were linear (R^2=0.99), with all mean data points being included within the 95% confidence interval (95% CI) range (10, 16). When the peak ratio for any amino acid of interest varied more than the ±5% assay error from its own AUC ratio (sample HPLC assay) for any given serum sample, a repeat HPLC quantification assay was performed with each serum specimen derivatized individually (validation assay) (10). This validation procedure corrected the error and thus the precision of the assay. Derivatized standard amino acid solutions were made and used over the period of 12-24 months (the stability of the derivatized amino acid solutions was within ±5% of the original quantification over this time period). A single standard solution of the known concentrations of the amino acid mixture was run every day after a background HPLC run to obtain a flat base-line and before any unknown/experimental samples were assayed manually. We found that computer controlled sample auto-injection did not assist with the accuracy nor the precision of this assay. Similarly, quality controls were performed of the total calibration line every few months and the linearity and parallelism of these lines were excellent (10, 16). The lowest linear point of quantification (LOQ) of low amino acid concentrations in serum was 0.01 μM and the highest varied from 50 to 1000 μM for each of those amino acids, depending on their physiological range in human serum specimens. However, the lowest quantification concentration or the level defined as being below the limit of quantification (BLQ) within the ±5% error (lower sigmoidal point of the curve) was 0.001 μM (10). This range of a $\log_{10}$ between BLQ and LOQ gives great accuracy for the determination of the low, but significant, amino acid concentrations and any values below, which are very low concentrations, hence, they are rarely used for PD analyses.

ERW enzymatic activity assay. Quantification assays have been developed with quality assurance and quality control for ERW enzymatic activity, anti-ERW antibody, and Erwinase protein quantification in human sera in a similar manner to native *E. coli* of PEG-ASNase (10, 16, 23). Briefly, serum ERW activity is measured using an enzymatic reaction that converts L-ASN to L-aspartate and ammonia in the presence of ASNase. Pre-ASNase (control) and post-treatment-ASNase serum specimens from ALL patients were collected during various phases of therapy and placed immediately in an ice-water bath (0˚C) to prevent *ex vivo* amino acid deamination. Serum samples were shipped on dry-ice and stored at –80˚C until analysis. Any samples not shipped per protocol requirements were strictly forbidden from these assays.

The linearity of the calibration lines, the lower LOQ, the inter- and intra-batch accuracy and precision of these assays will be reported. The results from these assays have been used for the evaluation of Erwinase PK-PD and are much better than the US FDA requirements for Biological Oncology Products.

The calibration line for quantification of ERW from 0.0125 to 0.60 IU/ml was generated by regression of the mean +/– SDEV data by logistic function of a standard line equation: $y = a + bx$ or its appropriate semi-log equivalent line equation. The linear portion of the sigmoidal relationship between ERW concentration and optical density (OD 405 nm) was determined as being from 0.025 to 0.6 IU/ml (23). For this purpose serial dilutions of the biological oncology product were made from the stock solution – pharmaceutical preparation that the patients received.

The intra-assay precision (intra-batch error) expressed as percent of coefficient of variation (%CV - or relative error) and variance ($SDEV^2$ or σ^2) were calculated from the relative response values of the various drug concentrations in these assays. A summary of the %CV and/or σ^2 is shown below. Other statistical evaluation derivatives of SDEV of each ERW concentration have been evaluated (Fisher information =1/variance =1/σ^2), but due to their rarity they have not been used here. Because of the low value of SDEV, both %CV and variance were relatively low. More importantly, when variance was plotted *vs.* the concentrations of the ERW assayed, a near horizontal line (very low slope) was obtained signifying the great accuracy and precision of these pharmacoanalytical assays. The in-study validations for the ERW activity assay were also monitored by "spiked" standards and calibration lines every time these experiments were used to quantify ERW in human serum for many assays. Recently, we had the opportunity to redevelop these assays for the new ERW formulation in the U.S. The results of these new assays are superimposable on the "old" ones so that the sample assays can be compared with a high accuracy and precision.

Anti-Erwinase antibody ELISA assay. Anti-Erwinase antibody titers were measured using a direct antibody-capture enzyme-linked immuno-sorbent assay (ELISA), which is a multi-step serum assay in a similar manner to that of native *E. coli* of PEG-ASNase (9) with minor modifications for the accommodation of the new computerized equipment (10, 16, 23). This assay uses the VECTASTAIN ABC kit, which was obtained from Vector Laboratories, Burlingame, CA, USA. Briefly, anti-ERW Ab titers were measured using an antibody-capture enzyme-linked immunosorbent assay (ELISA). In the case of ERW, a rabbit anti-ERW Ab(+) was developed and it was used for the initial anti-ERW assay development. Eventually a human anti-ERW Ab became available and it was used for these assays (16, 23). The linearity of the calibration lines of the anti-ERW Ab(+) assay was applied as described above. Pre-ASNase (control) and post-treatment-ASNase serum specimens from ALL patients were collected during various phases of therapy and placed immediately

in an ice-water bath (0˚C) and shipped on dry-ice and stored at –80˚C prior to ELISA assay. The linear portion of the sigmoidal relationship between ERW concentration and optical density (OD490 nm) was determined from 3.125% (LOQ) to 100% of 1:100 dilution of anti-ERW Ab(+) and up to 200% by using the 1:50 anti-ERW Ab(+) dilution.

The intra-assay precision (intra-batch error) expressed as percent of coefficient of variation (%CV - or relative error) and variance ($SDEV^2$ or σ^2) were calculated from the relative response values of the various drug concentrations in these assays. A summary of the %CV and/or σ^2 is shown below. More importantly, when variance was plotted *vs.* the dilutions of anti-ERW Ab(+) assayed a line with a very low slope was obtained, signifying its accuracy and precision throughout the range of Ab(+) determination. Similarly, Ab(+) assay validation was monitored by Ab(+) standards and calibration lines every time these experiments were used to assay ERW in human patient serum for many assays. Recently, we had the opportunity to redevelop these assays for the new ERW formulation in the U.S. The results of these new assays are superimposable on the "old" ones so that the sample assays can be compared with high accuracy and precision.

Erwinase protein assay (VECTASTAIN). The protein assay for either ERW or pegaspargase antigen (protein) was based on the reported method by Asselin *et al.*, (9) with minor modifications for the currently used equipment (16, 23). This assay uses an indirect antibody-capture enzyme-linked immuno-sorbent assay (ELISA). In this assay, the antibody against the specific antigen (protein) is plated first and then the antibody–antigen reactions take place in a multi-step serum assay in a similar manner to native *E. coli* of PEG-ASNase based on the Vectastain ABC kit (Vector Laboratories). The linearity of the calibration lines of the anti-ERW Ab(+) assay was as described above (16, 23). Pre-ERW (control) and post-treatment-ERW serum specimens from ALL patients were collected during various phases of therapy and placed immediately in an ice-water bath (0˚C) and shipped on dry-ice and stored at –80˚C prior to ELISA assay. In reality, the same serum specimen is used for all three Erwinase assays and the amino acid quantifications by HPLC.

Pharmacokinetics and pharmacodynamics of Erwinase in pediatric patients with obvious clinical allergy to E. coli formulations. There are no published detailed PK or PD studies of ERW in pediatric ALL patients in the US. We present here the PK and PD data from 5 pediatric ALL patients. Although these are a limited number of patients, the results obtained are superimposable on the ones obtained from the CCG-1961 trial (23). Hence, due to their strong clinical significance after a much higher ERW dose, we believe that these evaluations should be reported here so that they can be performed in a new clinical study.

Statistical analyses. Population pharmacokinetic parameters were determined using the limited sampling technique after multiple doses of ERW in a similar manner to the one reported previously (10, 12, 16). A one-compartment, open-model population pharmacokinetic analysis was performed on serum ERW activity values after each dosing using the nonlinear mixed effects model (NONMEM) computer program, designed to fit general statistical regression-type models, and allowing for estimation of average population values of PK parameters, as well as estimation of inter- and intra-individual variabilities, as described previously (10, 12, 25). Percentage of ASN and GLN deamination was expressed as (amino acid pretreatment level – amino acid post-treatment level)/(amino acid pretreatment level) x100%. Statistical comparisons of PD parameters used non-paired *t*-tests. Finally, correlations between ERW activity and ASN and GLN deamination were sought, expressed as % of pre-treatment control. The population maximum input of ASN (Imax) was estimated.

Results

The %CV for the ASNase assays were 10.28% for the Ab(+) ELISA assay, 4.72% for the ERW activity, and 8.45 % for the HPLC assay. These parameters for ERW are very similar to the ones obtained for pegaspargase, which have been reviewed by the FDA who licensed it (Oncaspar™) as front-line in both pediatric and adult ALL patients (July 2006).

Erwinase activity assay. The standard curve of ERW, and hence, the range of ERW quantification, from 0.025-0.6 IU/ml were found to be linearly correlated with the optical density (OD 405 nm) ranging from 0.2 to 1.5 with an R^2=0.999 (Figure 1). The negative control human serum OD 405 nm averaged 0.002 OD units or it was 2 orders of magnitude lower than the lowest linear OD value of ERW (0.025 IU/ml). This major difference allows us to clearly determine the lowest possible EWR concentration quantification and bifurcate them from the "true" negative control.

Validation at a similar (OD 450 nm) wavelength showed an equal linearity and characteristics, but at lower OD units. The intra-batch and inter-batch precision of the ERW activity assay in the range of the drug concentrations from the calibration curve are shown in Table I. The variance is mode-dependent on the SDEV of each ERW drug concentration determination, so it varies more than the %CV, which is a relationship between SDEV and the mean of each ERW drug concentration. The excellent accuracy of the ERW assay is shown in Table II.

Significant sigmoidicity was seen in calibration curves when 9 different ERW activity concentrations were used, 2 at lower plus one at higher ERW concentrations (0.0075-1.2 IU/ml, data not shown). However, the linear portion of the calibration line ranged between the ERW concentrations shown in Figure 1. The relative error (SDEV) was >12.5% in the lowest ERW concentration (0.025 IU/ml) and with %CV for the higher ERW concentrations in the range of 3%-5.7%. The QC samples of intra-day and inter-day accuracy for those ERW and native *E. coli* ASNase concentrations (performed the same days as the ERW assays) are shown in Table II. The % relative errors are similar for both ERW and native ASNase activity determination. Additional calibration lines representing many assays on separate dates (days-

Table I. *Intrabatch and interbatch precision of Erwinase activity assay x 3 experiments in triplicate each drug concentration.*

ERW standard X3 batches	0.025 IU/ml	0.05 IU/ml	0.1 IU/ml	0.2 IU/ml	0.4 IU/ml	0.6 IU/ml	Intrabatch %CV* Mean of means
	% CV*						n=3 x 3
Interbatch %CV*							
Mean of means	12.456	5.676	4.01	1.92	4.54	3.06	5.483
±SDEV	7.23	3.286	2.442	5.61	2.255	1.91	2.06
±SE	4.173	1.9	1.41	1.98	1.30	1.10	1.19

*Mean of means from 3 separate experiments in triplicate determination each drug concentration.

Table II. *Accuracy of Erwinase and Native E. coli ASNase activity assay x 3 experiments (3 batches) in triplicate each drug concentration (1999).*

ERW Conc.*, IU/ml	Determined ERW Conc., IU/ml	% Error ERW Conc.	*E. coli* ASNase Conc.,* IU/ml	Determined *E. coli* ASNase Conc., IU/ml	% Error *E. coli* ASNase Conc.
0.025	0.024974	–0.104%	0.025	0.022	–12.00%
0.05	0.04988	–0.24%	0.05	0.0472	–5.60%
0.1	0.09961	–0.39%	0.1	0.095	–5.34%
0.2	0.19814	–0.93%	0.2	0.193	–3.60%
0.4	0.3922	–1.95%	0.4	0.392	–2.09%
0.6	0.5752	–4.13%	0.6	0.629	4.83%
Mean±SDEV		–1.291% 1.55%	Mean±SDEV		–4.18% 5.86%

*Mean of means from 3 separate experiments in triplicate determination each drug concentration.

months) were conducted. The parallelism of the validation of the ERW calibration lines was excellent and is shown in Figure 1. Parallelism is conceptually similar to the pre-study assay validation. It should be noted that parallelism is not typically evaluated during a method development, but rather is used as a validation tool over an extended period of time or throughout the duration of the pharmacoanalytical assays of many months (for the duration of a clinical study).

The lower limit of quantification (LOQ) for ERW in human serum was 0.025 IU/ml. At this low concentration(s) there was relative small SDEV and low %CV (relative Error) and hence, variance. Although the LOQ was slightly higher in ERW than PEG-ASNase in human specimens this method yielded similar LOQ values to those of native ASNase, which is a similar Biologic Oncology Product ASNase formulation with 8-hour stability post-rehydration from the lyophilized powder.

Anti-Erwinase antibody [Ab(+)] assay. The standard line for the determination of anti-ERW Ab(+) was established from 1:1000 dilution (100%) to as low as 1:32000 dilution (3.125%) from 100 U/ml rabbit or human anti-ERW Ab(+). The linearity of the diluted anti-ERW Ab(+) and hence, the range of anti-ERW Ab(+) quantification, were found to be linear with the optical density (OD) ranging from 0.4 to 0.9 at 490 nm and with an $R^2 \geq 0.999$ by a direct ELISA assay determination (Figure 2). More recently, and on the recommendation by the FDA, lower Anti-ERW Ab(+) dilutions were used, *i.e.*, 1:100 and 1:50. Any lower Ab(+) dilution yielded many uncertainties of non-specific binding to serum proteins and hence, the possibility of false positives.

The negative control human serum at 1:100 dilution OD 490 nm averaged 0.001 OD units or it was greater than 2 orders of magnitude lower than the lowest linear OD value of ERW Ab(+). This major difference between an Ab(+) and Ab(–) sera determinations allows us to clearly determine the lowest possible EWR Ab(+) quantification and bifurcate them from the "true" negative control.

The intrabatch and interbatch precision of the anti-ERW Ab(+) assay in the range of the calibration curve drug concentrations are shown in Table III (Mean of means of

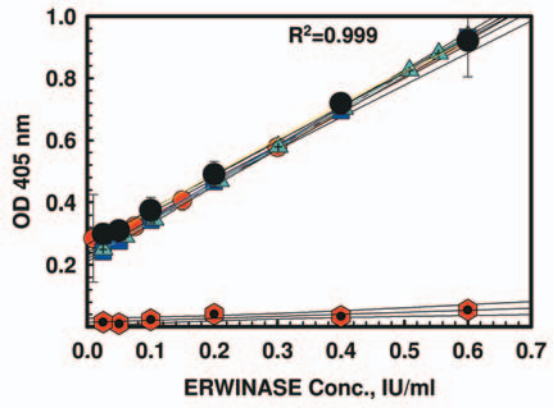


Figure 1. *Calibration line showing the relationship between the Erwinase concentration average (black circles as "mean of means", and individual n=3 determinations) ±SDEV ±95% confidence intervals (%CI) diluted in human serum and optical density OD 405 nm response for Erwinase standard solutions. There is a remarkably low variability as the SDEV is contained within the size of each symbol. The n=3 individual calibration lines and their mean demonstrate the parallelism and superimposability of these calibration lines of Erwinase. All symbols are the mean of n=3 ±SDEV ±95% CI. Parallelism is typically evaluated during in-study validation and it represents the variability (relative error) of these assays that are generated during a study. The negative control OD 405 nm is also shown as being extremely low and highly statistically different from the lowest linear limit of quantification (LOQ) of Erwinase. This along with the excellent parallelism shown here allows us to be very confident in that, when a patient's serum sample was assayed has an extremely high accuracy with low error of a false positive.*

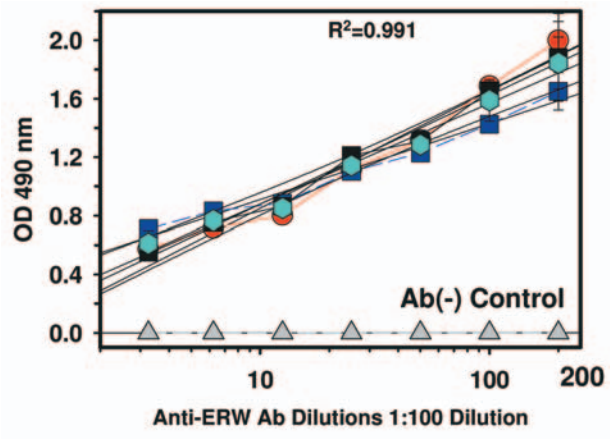


Figure 2. *Calibration line showing the relationship between the average human anti-Erwinase antibody [Ab(+)] at 1:100 (1:50 dilutions for 200%) dilutions in human serum (n=3 in triplicate or n=9) ±SDEV ±95% confidence intervals (%CI) and optical density OD 490 nm response with the new Erwinase antigen standard solutions. The Negative control OD 490 nm is also shown as being extremely low and highly statistically different from the lowest linear limit of quantification (LOQ) of anti-ERW Ab(+). This along with the excellent parallelism shown here allows us to be very confident in that, when a patient's serum sample was assayed has an extremely high accuracy with low error of a false Ab(+) positive.*

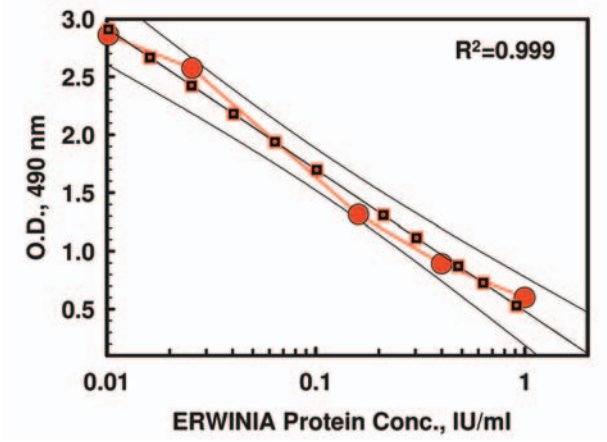


Figure 3. *Calibration line showing the relationship between the average Erwinase Protein Vectastain Assay (n=3 in triplicate or n=9) ±SDEV ±95% confidence intervals (%CI) diluted in human serum and optical density OD 490 nm response for the Erwinase protein standard solutions.*

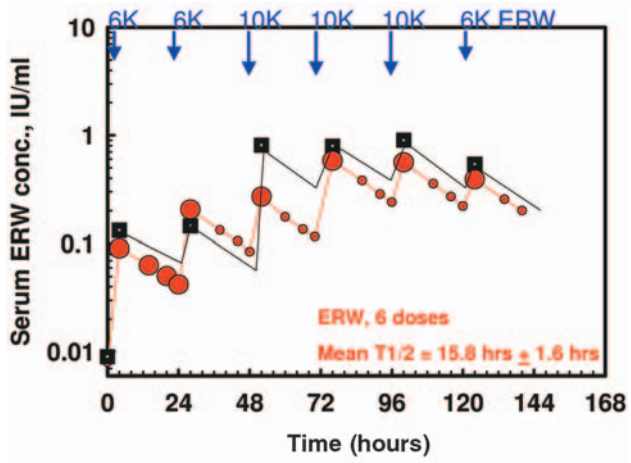


Figure 4. *Pharmacokinetic (PK) profile of Erwinase in one pediatric patient with relapsed ALL after 6 ERW doses; Doses #1 and #2: 6,000 IU/m²; Doses #3, #4, and #5: 10,000 IU/m²; Dose #6: 6,000 IU/m² (circles: ERW activity; squares: ERW protein). The half-life after the 1st EWR dose was 18.3 hours and the half-life average of ERW after 6 doses was 15.8±1.64 hours. There was an accumulation of ERW activity after the 2nd dose of ERW with a subsequent increase nearly doubling the peak ERW serum activity concentration to 0.75 IU/ml, when ERW dose increased from 6,000 to 10,000 IU/ m2. The higher serum peak ERW activity was proportionally reduced after the 6th ERW dose reduction.*

the %CV from 3 separate experiments in triplicate determination for each ERW concentration (±SDEV and ±SE of %CV). The Relative Error (%CV) was >12.5% in the lowest range of anti-ERW Ab(+) dilutions. The QC samples of intra-day and inter-day accuracy for those anti-ERW and anti-native *E. coli* ASNase Ab(+) dilutions (performed on the same days as the ERW assays) are shown

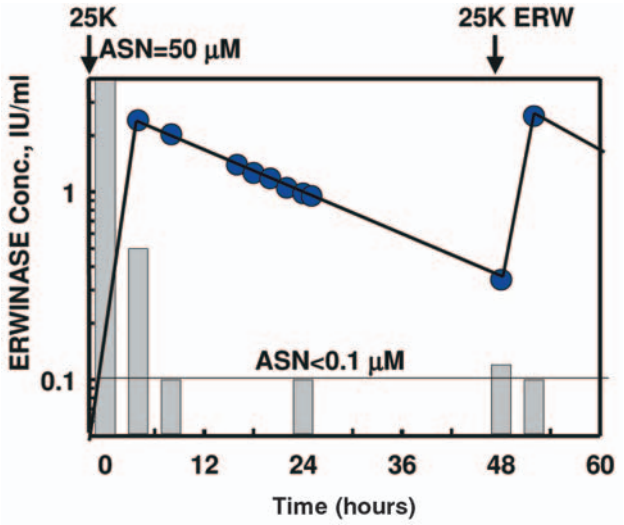
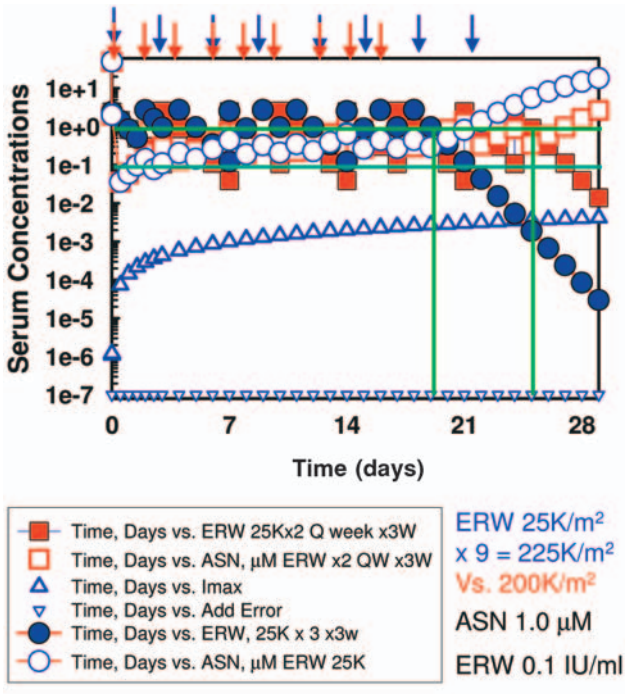


Figure 5. *Pharmacokinetic (PK) profile of Erwinase in 4 pediatric patients with ALL front line who had a clinical reaction to E. coli Pegaspargase after 2 ERW doses of 25, 000 IU/m^2; These doses continued x 3 per week x 2 weeks. The serum ERW concentration vs. time data have been examined as if they were from ONE patient with NONMEM analyses yielding a higher peak of 2.4 IU/ml and with a POST-HOC analysis for a half-life after the 1st EWR dose of 15.61 hours in n = 4 patients. There is an excellent concordance between the half-life in these patients after the 25 K/m^2 doses and the average of ERW after 6 doses of 15.8±1.64 hours (Figure 4). There was no apparent peak accumulation of ERW activity after the 2nd dose. The higher serum peak ERW activity was proportionally reduced. The bars indicate the pharmacodynamic ERW effect on ASN at the assayed time points of 0, 4, 8, 24, 48 and 52 hours. All post-ERW administration ASN concentrations were well below 1 μM and most of them were below 0.1 μM. The Imax for ASN was 1E-10 nmoles/min/ml. The GLN deamination at the same time points was well below 70 μM.*



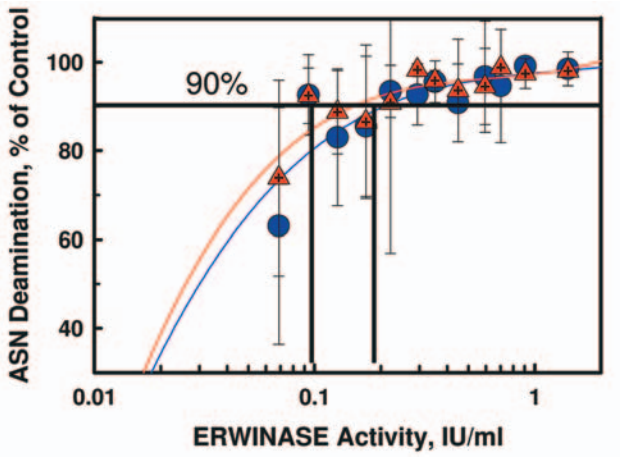


Figure 6. *The pharmacodynamic relationship between Erwinase serum enzymatic activity and its substrates, ASN and GLN deamination post-ERW administrations in sera from the 5 patients treated with the higher doses. Serum ASN declined from a population concentration mean of 42±9 μM to less than 1 μM for post-administration of Q2 days pediatric patients with HR ALL. There is a casual dose-serum peak- ASN deamination in these few patients (Figures 4 and 5). Similarly, GLN was deaminated from a population concentration mean of 320 μM to less than 50 μM and it attempted to rebound to less than 75 μM. The results are shown as a sigmoid pharmacodynamic relationship (parabolic) between the population mean of deaminated ASN and GLN levels expressed as % of pre-treatment controls versus log10 of ASNase enzymatic activity. The sigmoid relationship is of the 3rd order of regression and it is the upper portion of the sigmoid fit according to Michaelis-Menten population PK-PD modeling. The highest GLN % deamination achieved was 98%±1.1% and for ASN 94%±4%. These PD results may indicate that GLN may have to be deaminated to a greater degree in serum by ERW so that it cannot be used as a substrate for the de novo biosynthesis of ASN in the tissues with the catalytic aid of asparagine synthetase. Of importance is that the "time" does not appear in these relationships. However, "time" is embedded in the concentrations of ERW enzymatic activity, which was obtained from data presented in Figures 4 and 5. Minimal enzymatic activity of 0.1 to 0.2 IU/ml was needed for optimal ASN deamination of approximately 90% compared to baseline.*

Figure 7. *Simulations of the ERW 25K x 3 doses per week x 3 weeks (9 ERW doses = 225K/m^2) and the ERW 25K x2doses per week x 3 weeks (8 ERW doses = 200K/m^2) under NONMEM population PK-PD modeling. Under intermediate Imax of ASN representing the vast majority of high risk pediatric ALL patients showed that the ERW 25K x2 doses per week x 3 weeks regimen is much more beneficial in its ability to deaminate ASN <1 μM up to Day 28, whereas the ERW 25K x3 doses per week x 3 weeks deaminated ASN to <1 μM only up to day 21-22.*

Table III. *Intrabatch and interbatch precision of anti-Erwinase antibody (Ab) assay x 3 experiments (3 Ab batches) in triplicate each drug concentration.*

Anti-ERW Ab* X3 batches	3.125%	6.25%	12.5%	25%	50%	100%l	Intrabatch %CV* Mean
	% CV						(n=3x3)
Interbatch %CV*							
Mean of means	4.38	12.15	5.44	7.48	5.41	5.46	6.72
±SDEV	3.73	2.82	1.29	1.29	3.03	1.51	0.511
±SE	2.15	1.63	0.95	0.74	1.75	0.87	0.29

*Mean of means from 3 separate experiments in triplicate determination each drug concentration.

in Table IV. The % relative errors are similar for either ERW or Native ASNase Ab(+) accuracy determination. The parallelism of the validation of the anti-ERW Ab(+) calibration lines was not done due to the limited patient-specimens, but it was excellent for native Ab(+).

Erwinase protein (Vestastain) assay. The standard curve for the determination of ERW protein and hence, the range of ERW protein concentration quantification, was established from 0.0102 to 1.0 IU/ml and was found to be inversely linear with the optical density (OD 490 nm) ranging from 2.8 to 0.5 with an R^2=0.999 by an indirect ELISA assay determination (Figure 3). The intrabatch and interbatch precision of the ERW activity assay in the range of the calibration curve drug concentrations are shown in Table V (Mean of means of the %CV from 3 separate experiments in triplicate determination for each ERW concentration (±SDEV and ±SE of %CV). The relative Error (SDEV) was remarkably among the lowest even in the low ERW concentration (0.0102 IU/ml).

The % relative errors were similar for either pegaspargase or Native ASNase protein accuracy determination and were also excellent, however these assays were not used in the CCG clinical trials after the first handful of patients due to the lack of adding interesting data and the excess assay cost. The parallelism of the validation of the *E. coli* ASNase antigen protein lines was excellent.

Pharmacokinetic profile of Erwinase. Erwinase was administered in relapsed ALL pediatric patients as a daily single intramuscular dose of 6, 000 IU/m^2 on day 3x2 doses; the 3rd ERW dose was increased to daily 10, 000 IU/m^2 x 3 doses and finally a 6th ERW dose of 6, 000 IU/m^2 x 1 of re-induction. There was no apparent statistical difference between ERW protein (antigen) and its enzymatic activity, and no anti-ERW Ab(+) was detected. Frequently obtained serum specimens after the 1st ERW dose were analyzed and following our previous publications, the ERW enzymatic activity set of data was used for the PK analyses. The apparent peak serum concentrations of ERW activity increased with multiple ERW doses and nearly doubled with the increase from 6K to 10K daily administrations (Figure 4). More recently, 4 patients received ERW 25,000 IU/m^2 x 3 doses per week, x2 weeks after clinical reaction to Pegaspargase and verification of anti-*E. coli* Ab(+) (Figure 5). Based on PK modeling of ERW and the subsequent fit of the peak - trough concentrations, the PK model-projected $T_{1/2}$ of ERW activity averaged 15.8±1.64 hours (n=6 evaluations ± SDEV).

In all PK evaluations, ERW obeyed a one-compartment open model analyzed by the NONMEM population program. The population POSTHOC (best fit) analysis showed a serum peak concentration average of ERW enzymatic activity in this ALL patient population of 0.7 IU/ml and 2.4 IU/ml after the low and after the high doses of the ERW, respectively. The $T_{1/2}$ of elimination was 16 and 15.8 hours for the low and high ERW doses of the drug, respectively. The volume of distribution averaged 1.62 L/m^2 equivalent value to the serum volume (aqueous phase) and there were no differences in the two sets of ERW doses.

Pharmacodynamic (PD) correlations between Erwinase and ASN and GLN deamination. The serum ASN concentration mean remained statistically lower than the baseline (Pre-ERW Tx) up to day 7, well below 1 μM after the 25K ERW dose x 3 doses per week (Figure 5). Although the treatment continued for two 2 weeks no long-term samples were drawn. The equivalent ASN deamination after the lower ERW doses was <3 μM at trough levels. Deamination of ASN was complete in this patient receiving daily ERW low doses from a few hours to day 6. Similarly, GLN deamination was well below 20-30 μM by day 7 and below

Table IV. *Accuracy of anti-Erwinase (6% Ab dilutions) and in-study accuracy of anti-Erwinase antibody (Ab) assay (7 Ab% dilutions) x 3 experiments (3 Ab batches) in triplicate each drug concentration.*

Anti-ERW Ab, % dilution	Determined Anti-ERW Ab, % dilution	% Error ERW Conc.	Anti-*E. coli* Ab, % Dilution	Determined *E. coli* Ab, % Dilution	% Error *E. coli* Ab, % Dilution
		1.5625%	1.43%	–8.48%	
3.125%	3.75%	–0.0001%	3.125%	2.87%	–8.12%
6.25%	6.23%	–0.32%	6.25%	5.92%	–5.04%
12.5%	12.44%	–0.48%	12.5%	12.08%	–3.39%
25%	24.68%	–1.28%	25%	24.61%	–1.57%
50%	48.83%	–2.34%	50%	49.74%	–0.517%
100%	95.62%	–4.38%	100%	100.14%	0.14%
Mean ±SDEV		–1.291% ±1.55%	Mean ±SDEV		–3.89% ±3.52

Table V. *Accuracy of Erwinase protein assay x 3 experiments (3 batches) in triplicate each drug concentration.*

ERW Conc., IU/ml	Determined ERW Protein Conc., IU/ml	% Error ERW Protein Conc.
0.0102	0.010195	–0.001%
0.256	0.026	0.173%
0.16	0.1603	0.212%
0.4	0.401	0.113%
1.0	0.989	-2.85%
Mean±SDEV		–0.535% 1.29%

Table VI. *Post-hoc analyses of ERW 25K/m^2 dosing x 2 or 3 doses per week x 3 weeks in induction and the reciprocal ASN deaminated serum levels. The 4th column indicates the Imax of ASN input into the circulation as it increases based on the additive error (per minute) (12, 25).*

Time Day	ERW 25Kx2/w x3Weeks ERW, IU/ml	ASN, μM 1.00E-06	Imax INPUT ASN 1.00E-07	ERW 25Kx3/w x3Weeks ERW, IU/ml	ASN, μM
0.16667	2.018	0.06	0.0000251	2.018	0.06
2	0.3	0.15	0.0002891	2.7	0.14
7	2.439	0.71	0.0010091	2.521	0.44
14	2.439	1.02	0.0010091	2.521	0.63
21	2.439	1.39	0.0030241	0.122	0.82
25	0.889	0.34	0.0036007	0.002	5.54
30	0.005	4.28	0.0043211	1.04783E-05	21.66

55 μM after the two ERW doses. Due to the limited number of patients no graphs have been attached.

However, based on these results and on the experience with the *E. coli* ASNase formulations, a population PD model was constructed which took into consideration the serum enzymatic ERW activity and serum deaminated ASN. Based on the Michaelis-Menten equation the model predicted at "steady-state" that serum asparagine could be determined up to Day 28 post- 3 weeks of ERW administration. It should be emphasized that despite the number of observations, neither Native *E. coli* nor Pegaspargase achieved as low population GLN deamination as post-ERW. This may indicate that the presence of either lower GLN in serum of these few Ab(+) to *E. coli* ASNase HR ALL patients may validate the greater glutaminase affinity that the ERW enzyme is known to possess, hence its greater PD effect on GLN, which in turn, may deaminate ASN more efficiently.

More importantly, the population average ERW enzymatic activity was correlated with the serum ASN or GLN deamination, expressed as percentage of pre-treatment control, yielding the upper portion of a sigmoid curve per Michaelis-Menten relationship (Figure 6). The highest GLN % deamination achieved was 98%±1.1% and for ASN 93%±4% at 1 IU/ml ERW concentration. At the higher ERW doses with their reciprocal higher ERW peak and trough levels these % deamination values became even better. This apparent pharmacodynamic correlation between the serum deamination and ERW activity showed that a minimal enzymatic activity of 0.1 to 0.2 IU/ml was needed for optimal ASN deamination of >90%-95% compared to Pre-Tx baseline. As seen in Figure 5, the majority of patients had an enzymatic activity of ≥0.2 IU/ml up to day 2 after a single ERW drug administration and 0.1 IU/ml up to day 3 after a single ERW dose, thus providing a minimum optimal ERW concentration when the drug was administered Q48 hours x 3 doses per week. The collective population Imax of ASN was evaluated by NONMEM in these patients at 1E-10 nmoles/min/ml, with apparent K_m estimations of 14 and 456 μM for ASN and GLN, respectively.

No emergence of anti-ERW Ab(+) appeared nor was there any association with immediate disappearance of ERW enzymatic activity and rebound of serum ASN and multiple doses of ERW were not associated with any clinical manifestations of hypersensitivity reactions.

Simulations of Erwinase. Based on the similarity of the PK parameters from 3 different ERW doses we decided to use the population Post-hoc (average) PK-PD values to construct various simulated (SIMs) ERW dose and schedule regimens. These regimens were examined under various input (maximum Input - Imax) (25) for ASN values ranging from 1E-8 to 1E-4 nmoles/min/ml and with increasing

Additive Errors. The ERW 6K x 3 doses per week x 3 weeks (like native *E. coli* dosing) was not very effective in achieving trough levels at or above 0.1 IU/ml of activity with a reciprocal ASN trough level well above 6 μM. The ERW 10K x 3 doses per week x 3 weeks was much better the previous dose regimen by achieving ASN less than 1 μM for up to 21-22 days. Then, the ERW 25K x 3 doses per week x 3 weeks (9 ERW doses = 225K/m^2) and the ERW 25K x2doses per week x 3 weeks (8 ERW doses = 200 K/m^2) were evaluated. A remarkable result was produced under low, intermediate, or high Imax of ASN. SIMs showed that the ERW 25K x2 doses per week x 3 weeks regimen is much more beneficial in its ability to deaminate ASN <1 μM up to Day 28, whereas the ERW 25K x3 doses per week x 3 weeks deaminated ASN to <1 μM only up to day 21-22 (Figure 7).

Discussion

Asparaginases are included in most treatment protocols for both pediatric and adult patients with newly diagnosed ALL (1-12). For the first time, this report provides the pharmacokinetic and pharmacodynamic data from the intramuscular administration of ERW ASNase in pediatric ALL, who were reactive to native *E. coli* and pegaspargase in the clinic. These elemental PK data are similar to those which have been reported earlier (16, 23). It should also be emphasized that in our limited study ERW was given only on a compassionate basis, and only during re-induction therapy.

The primary focus of this manuscript is the development and validation of the immunoassays necessary to support PK studies with accuracy and precision. By thoroughly defining and examining each part of these pharmacoanalytical assays, they become the first step in the resulting validation (10, 24). These assay quality controls should be concise and the validation process straightforward. In order to evaluate the parameters of precision and accuracy prior to conducting limited ERW PK and PD studies, we developed and systematically validated the ERW pharmaco-analytical assays. These assays were produced in a handful of ERW batches and their results were near-identical to those for the *E. coli* both native and Pegaspargase (10, 16, 23). The standard curve (line) of Erwinase enzymatic activity and hence, the range of ERW quantification, from 0.025 - 0.6 IU/ml were found to be linear with the optical density (OD 405 nm) ranging from 0.2 to 1.5 with an R^2=0.99. The actual lowest limit of ERW activity based on the extrapolation of this calibration line is 0.01 IU/ml (Figure 1). However, this low level will most likely not be used in our clinical trial for ERW drug concentration calculation. When the new ERW calibration lines were added, Mean of Means from 3 batches, 2 sent by OPi and one from a patient's ERW dose to the "old" calibration Mean of Means line, again, excellent linearity and parallelism were determined. All means are included within the 95% Confidence Intervals (95% CI) (Tables I and II).

Moreover, the robustness of an assay is based on its variability per drug concentration determined. To this effect we have examined the % coefficient of variation (%CV) and its Variance (σ^2) per drug concentration and through out the ERW calibration range. Since some older theory states that variance is a better measurement of the variability of an assay than %CV this parameter was also estimated for the "old" and the new ERW calibration assays. There are near-horizontal lines produced of either %CV or σ^2 *vs.* the ERW concentrations (data not shown) indicating that this assay has a great accuracy and precision thought out its calibration line range. Both accuracy and precision are excellent for ERW enzymatic activity (Tables I and II). Thus, in answering the practical question - How credible is the assay? We can reply that % CV is good, but Variance (σ^2) as estimated per ERW Drug concentration is much better, especially when it does not change very much (No statistically significant difference) with each increasing ERW drug concentration determination.

As described above, a typical assay development and validation will include at least 3-6 precision and accuracy determinations in multiple drug concentrations (4-6 or more per standard line) to define the overall consistency and QC of the biologic assay. QC includes (but are not required by the FDA regulations) stability of the drug during the assay, specificity, selectivity, lower limit of linearity and range and lower limit of quantification (LOQ), not to mention, parallelism and In-Study validation once the clinical trial is in progress for these assays. Each calibration line should evaluate the negative control with non-zero points and for all drug concentrations, so that the accuracy of the drug can be assessed. The validation standards should re-define the range of the assay and no values below the LOQ may be seen or used. Overall the imunoassays that we have developed and presented here (Figures 1-3 and Tables I-V), are highly sensitive and selective that have been used to quantify ERW protein, its activity and its antibody in a biologic matrix of assays for the early PK-PD analyses in pediatric patients. It should be emphasized that the ERW protein assay although useful it provides very little additional information of significance and based on its cost, it is not yielding a high return of data information for its cost (Figure 4).

Asselin *et al.*, reported the first clinical PK evaluation of ERW in pediatric ALL patients in the early 1990's. These studies reported a half-life for ERW of 16 hours (9). However, after multiple IM injections of ERW to 24 patients of 6,000 or 10,000 IU/m^2 treated per CCG-1961 protocol in newly diagnosed HR ALL children who had an

obvious clinical reaction to *E. coli* ASNase formulations, the half-life was estimated to be approximately 18 hours (23). In these evaluations, the ERW treatment achieved a prolonged duration of ASN (3-4 weeks) and GLN (2 weeks) deamination activity, determined in serum amino acid depletion by a validated HPLC assay (10, 16). This was the most direct parameter of ERW ASNase pharmacodynamic activity in these patients.

More importantly, we also demonstrated that for optimal ASN or GLN deamination and depletion, the minimal therapeutic level of ERW enzymatic activity should be 0.1-0.2 IU/ml, which is similar to what was found recently in our patients receiving the higher ERW dose (23). The range of minimum therapeutic ERW concentration is lower than that suggested for PEG-ASNase in other pediatric (10, 16, 17) and adult studies (18, 21). This unexpected PD phenomenon may indicate that GLN may have to be deaminated to a greater degree in serum by ERW thus, it cannot be used for the *de novo* biosynthesis of ASN in the tissues (mesenchymal cells, liver, *etc.*) under the catalysis of asparagine synthetase (ASNS). This is supported by the lower K_m for ASN and GLN after ERW at "steady-state" conditions than after Pegaspargase (10). These results indicate that, at least for these few pediatric HR ALL patients, the minimal interval of IM administration of multiple ERW doses should be approximately 2 days or x 3 doses per week, and lasting for at least 3 weeks, which is similar to the time interval of IM dosing for native *E. coli* ASNase used in the CCG-1962 study (10, 12). ERW is non-cross-reacting with the anti-*E. coli* ASNase antibodies and hence, despite its lower half-life, possesses an ability to produce sufficient serum ERW activity levels which produce optimal ASN and GLN deamination in these HR ALL patients (16, 23). In this pilot study with high dose ERW, none of these HR ALL patients developed anti-ERW Ab with or without "silent hypersensitivity" hence, no patient resulted in immediate disappearance of the ERW enzymatic activity and normalization of serum ASN or GLN levels in serum.

Limited response rate data exists when anti-*E. coli* Ab(+) is determined after the administration of a chemotherapy regimen containing native or Pegaspargase in pediatric ALL patients in front line or in relapse with low or high Ab ratios over negative controls (12, 16). Further, an interim analysis of the pediatric CCG 1961 protocol showed that the presence of antibodies without clinical hypersensitivity was correlated with an adverse outcome compared to patients who had no antibodies and no clinical allergy, or to patients who had clinical allergy and were switched to *Erwinia* ASNase (16, 23).

The role of ERW administration with or without dose intensification and prolonged duration of ASN and GLN deamination has not been studied extensively in ALL patients. Our assay development and validation and preliminary PK results allow us to conduct a future clinical study with ERW to examine its PK-PD effect on outcome of ALL patients.

Based on the development of population PK-PD models for *E. coli* native and Pegaspargase formulations (10, 12, 25) the post-hoc results from the ERW PK-PD model fitting (Figures 5 and 6) were used to develop the necessary tools for the PK-PD model prediction of ERW peak and trough concentrations and ASN deamination after various administration schedules and time intervals of the drug. Due to space limitations, many figures with interesting data did not make it into this manuscript, but suffice it to say that the most important simulated PK-PD data is shown in Figure 7. Based on these "artificial" simulations, which will need validation in a future clinical trial, a high dose ERW under either time interval should be considered to provide excellent peak and sufficient trough levels of ERW enzymatic activity, hence, equivalence among non-inferiority ASNase formulations (12, 16, 25). Lastly, ERW which has a lower KM for GLN, provides excellent ASN deamination of equivalence (Table VI and Figure 7) with a therapeutic dose regimen of Pegaspargase (12, 25). Taking all this information together, we are currently planning to conduct a clinical trial to determine the toxicity and rate of anti-ERW antibody formation after multiple doses of post-remission with PEG-ASNase in HR pediatric patients with ALL.

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