

## Extended Stability of Reconstituted Lyophilized Erwinia L-asparaginase in Vials

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**Abstract.** *Background/Aim:* L-Asparaginase (L-ASNase) is used as a tumor-inhibitory drug on paediatric acute lymphoblastic leukemia (ALL). ERW-ASNase is commercialised as a lyophilized powder stable only for 8 hours once reconstituted and, consequently, the leftover is usually discarded. The aim of this study will be to analyse the stability of the reconstituted lyophilised ERW-ASNase. *Materials and Methods:* In the present study, we analysed the enzymatic stability of reconstituted ERW-ASNase after conservation in three different temperature conditions for 2 and 5 days. *Results:* Our results show that ERW-ASNase is stable at 4°C, -20°C and -80°C for up to 5 days, retaining 95% of the initial enzymatic activity in all three storage temperatures tested. *Conclusion:* It is feasible to reuse the remaining content of ERW-ASNase vial after reconstitution, which allows the optimization of the content of ERW-ASNase vials use and reduces the cost of this formulation usage, making it more accessible.

The search for approaches that selectively target tumour cells is one of the challenges for developing drugs designed for the treatment of neoplastic disease (1). In this context, L-asparaginase (L-ASNase) has found its therapeutic niche by

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significantly reducing the levels of asparagine in the plasma of the patients (2). Asparagine is an essential amino acid involved in the development of blast cells generated during lymphoproliferative processes, such as in acute lymphoblastic leukemia (ALL); therefore, a limitation in asparagine levels prevents the blasts from synthesizing proteins and promotes their apoptosis (3, 4). The mechanism of action of L-ASNase has proven to be so effective that its therapeutic use is considered one of the greatest advances in the hematology field during the last 50 years, changing the natural course of treatment for ALL patients (5). Specifically, it has allowed response rates of over 70% (6) and has increased the 5 years-survival rate in more than 90% of patients (7).

Currently, L-ASNase is available in three different forms: two of them produced in the microorganism *Escherichia coli* (*E. coli*) as engineered proteins, i) the first one is the native form (ASNase, L-asparaginase or Kidrolase®) and ii) the second one is in a pegylated form (PEG-ASNase, pegaspargase or Oncaspar®); iii) the third formulation is produced in *Erwinia chrysanthemi* (ERW-ASNase, asparaginase Erwinia or Erwinase®) and its major difference compared to the *E. coli* counterparts is its reduced immune response (8). Therefore, ERW-ASNase is extensively used in those patients where an immune hypersensitivity to some of the forms derived from *E. coli* occurs or a silent inactivation has developed (9). ERW-ASNase is commercially available as a lyophilised product containing 10,000 IU per vial for its reconstitution in an injectable solution. After administration, ERW-ASNase average half-life is 16 hours (10), thus, the patient requires 2-3 weekly administrations. The recommended dose of ERW-ASNase for the paediatric population, assuming a child's surface area is between 0.7 and 1.2 m<sup>2</sup>, is 20,000 IU/m<sup>2</sup> (8), however, according to the manufacturer instructions, it shows 8 hours

stability once reconstituted (11). Reconstituted protein preservation for subsequent administrations (at 48 and 72 h) is not possible, resulting in loss of economic resources. We hypothesised that reconstituted ERW-ASNase stability is in the range of days, aiming to enable the optimization of the vials content usage for consecutive administrations, thus, reducing the costs associated with the drug disposal in the treatment of ALL. The present study aimed to analyze the stability of lyophilised ERW-ASNase (Erwinase®) formulation under different preservation conditions.

## Materials and Methods

**ERW-ASNase reconstitution and stability assay.** ERW-ASNase was obtained as a commercially available lyophilized powder in vials from Erwinase® (Jazz Pharmaceuticals plc, Dublin, Ireland). One single Erwinase® powder vial was reconstituted under sterile conditions following the technical data sheet guidelines. Briefly, this vial was reconstituted in 2.0 ml of sterile distilled water to obtain a final solution concentration of approximately 5,000 IU/ml, which was aliquoted into several vials. To evaluate the enzymatic activity of this ASNase preparation, each aliquot was diluted 10,000-fold in a saline solution of NaCl 0.9% in order to obtain a concentration of the drug within the linear detectable range of the assay. The activity of the stock solution at zero time point (t0) was established as the reference activity (100%). Subsequently, several aliquots of the stock solution (5,000 IU/ml) were stored in sterile Eppendorf tubes under three different conservation temperatures: i) 4°C, ii) -20°C and iii) -80°C. ERW-ASNase activity was measured after 2 (t2) and 5 (t5) days. Each sample was analysed in duplicate, and a third measurement was performed when the standard error was higher than 5%. The enzymatic activity loss after storage was calculated as a percentage by setting the t0 activity as the reference value.

**Asparaginase activity assay.** The asparaginase enzymatic activity was measured by a spectrophotometric method using the *medac* Asparaginase-Aktivitäts-Test MAAT kit (*medac* GmbH, Wedel, Germany), a validated protocol compared to the reference method *L-aspartic acid β-hydroxamate* (AHA) (12). Each aliquot of ERW-ASNase stock solution (5,000 IU/ml) was diluted 10000-fold by two consecutive dilutions: i) a first dilution 1/100=10 µl of stock solution + 990 µl of saline solution and ii) a second dilution 1/100=10 µl of previous dilution + 990 µl of saline solution. According to the instructions, 20 µl of the last dilution was then mixed in 180 µl of the dilution buffer supplied in the MAAT kit. The resulting solutions were incubated with an asparagine analogous substrate capable of releasing a green chromatic complex upon reaction. The degradation product was determined spectrophotometrically at 690 nm wavelength using the Thermo Scientific™ Multiskan™ GO spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the asparagine enzymatic activity was calculated within a linear range from 30 to 600 IU/l.

## Results

**ERW-ASNase stability after a 2-5 days storage at low temperatures.** We performed a total of 14 ERW-ASNase activity measurements. All samples were measured in

Table I. ERW-ASNase activity and conservation. Direct absorbance of enzymatic activity (at 690 nm) was transformed to IU/l, according to the kit specifications. Activity values were normalised to day 0 activity, and absolute values were calculated as a percentage of activity (shown in brackets). The data shown correspond to the average value of the measurements.

Conservation	Activity IU/l		
	Day 0 t0	48 h t2	120 h t5
	328		
4°C	---	325 (99.1%)	324 (98.7%)
-20°C	---	303 (92.4%)	306 (93.3%)
-80°C	---	302 (92.0%)	320 (97.6%)

duplicate for the reference time point (t0), t2 and t5 time points at the storage temperature conditions of 4°C, -20°C and -80°C. The reference enzymatic activity at t0 was measured as 328 IU/l (standard deviation lower than 5%). The resulting ERW-ASNase enzymatic activity oscillated between 302 and 325 IU/l, independently of the storage time and temperature (Table I). The enzyme retained over 92% of its activity in all cases, while storage at 4°C showed the best preservation results with values over 98.7% of its original activity. In summary, the loss of ERW-ASNase activity detected was less than 8% (standard deviation lower than 5%) in all tested storage times and conditions.

## Discussion

The incorporation of L-ASNase in the treatment of patients with ALL has led to a paradigm change for this disease. Indeed, circulating asparagine elimination has allowed dramatically improving the survival of these patients (13). Currently *E. chrysanthemi* (Erwinase®) is used in those patients who are intolerant to *E. coli* derived asparaginase, both the native and the pegylated form (9). Even though ALL is not a disease with a high incidence, ERW-ASNase is considered a drug with a high budgetary impact, so it is essential to propose strategies that help optimize treatment costs. In our study, we sought to analyze the stability of the L-ASNase activity contained in the Erwinase® vials and evaluate whether this presentation would allow subsequent administrations in the time frame of the treatment, which is every 48-72 h. Our results show that the ERW-ASNase solution obtained from the reconstituted Erwinase® lyophilized powder retains more than 92% of its activity after 5 days when preserved in all three low-temperature tested conditions. The storage condition at 4°C was the best of all tested, probably due to the enzyme activity loss during the freeze-thaw process occurring at -20 and -80°C

temperatures. The microbiological stability was not analysed in this study, so it is important to highlight that, in case of applying this strategy for the optimization of the treatment with ERW-ASNase, it will be essential to work in aseptic conditions that ensure not only chemical stability but also the correct microbiological conservation.

In conclusion, the activity of ERW-ASNase (Erwinase<sup>®</sup>) remains stable at 4°C, -20°C and -80°C for at least 5 days after reconstitution. This allows the vials to be used in subsequent administrations covering the whole period of the patient's treatment, with obvious saving implications in the cost of paediatric treatment of ALL.

### Conflicts of Interest

The Authors declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

### Authors' Contributions

VRMM: Wrote most of the manuscript. RDR: Designed and implementation of the experimental study. IMP: Helped with experiments and provided data of table. HGM: Hematologist responsible for writing the paper's introduction. MGC: Pediatrician that contributed to introduction. GJN: Pharmacist responsible for the discussion and who chose the asparaginase activity kit. JFMA: Pharmacist responsible for section of materials and methods. DVJ: Contributed to discussion of results and editing of the paper. GNF: Director of the group and coordinator of the work and paper's writing.

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