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DHOP Nº 078/2021

San Lorenzo, 16 de Junio de 2021

Lic. Neris Aguilar, Administrador
Hospital de Clínicas
Facultad de Ciencias Medicas - UNA
De mi mayor consideración

Me dirijo a Ud., a fin de dar respuesta a la Nota DCP 1171/2021 en relación al llamado de LPN 09/2021 ADQUISICION DE MEDICAMENTOS E INSUMOS MEDICOS PARA EL DPTO. DE HEMATOLOGIA PEDIATRICA, recibida en fecha 15/06/2021.

ITEM 31

Solicitamos la inclusión de esas certificaciones a fin de precautelar la calidad del producto tratándose de una enzima biológica. La L-Asparginasa es un biológico de vital importancia en el tratamiento de las leucemias. En los últimos años se ha comercializado en el país, esta droga, sin certificado de buenas prácticas en el país de origen, ni registro de estudios clínicos que certifiquen su eficacia.

La Dra. Silvia Brandalise de Brasil, referente mundial en las leucemias pediátricas ha realizado un estudio sobre la composición y seguridad de estas drogas encontrando que muchas de ellas que se comercializan en Brasil contienen muchas impurezas y bajas disponibilidad de la droga. Muchas de esas marcas se comercializan también en nuestro mercado. Es por eso que insistimos en la presentación de documentaciones que certifiquen las buenas prácticas.

Sin otro particular, hallo propicia la ocasión para reiterarle mis más cordiales saludos.

Dr. Diego D. Figueredo
Oncología Pediátrica
Reg. Prof. N° 6778

Mg. Prof. Dra. **ANGÉLICA JIMÉNEZ de SAMUDIO,** Jefa
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D.P.C.P
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ADMINISTRADOR
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Research Paper

Low Bioavailability and High Immunogenicity of a New Brand of *E. coli* L-Asparaginase with Active Host Contaminating Proteins

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ABSTRACT

The drug L-asparaginase is a cornerstone in the treatment of acute lymphoblastic leukemia (ALL). The native *E. coli* L-asparaginase used in Brazil until recently has been manufactured by Medac/Kyowa. Then a decision was taken by the Ministry of Health in 2017 to supply the National Health System with a cheaper alternative L-asparaginase manufactured by Beijing SL Pharmaceutical, called Leuginase®. As opposed to Medac, the asparaginase that has been in use in Brazil under the trade name of Aginasa®, it was not possible to find a single entry with the terms Leuginase in the Pubmed repository. The apparent lack of clinical studies and the scarcity of safety information provided to the hospitals by the drug distributor created a debate among Brazilian pediatric oncologists about issues of safety and efficacy that culminated eventually in a court decision to halt the distribution of the new drug all over the country. Boldrini Children's Center, a non-profit pediatric oncohematology hospital, has conducted its own evaluation of Leuginase®. Mass spectrometry analyses found at least 12 different contaminating host-cell proteins (HCP) in Leuginase®. The presence of two HCP (beta-lactamase and malate dehydrogenase) was confirmed by orthogonal methodologies. The relative number of HCP peptides ranged from 19 to 37% of the total peptides identified by mass spectrometry. *In vivo* studies in mice injected with Leuginase® revealed a 3 times lower plasma bioavailability and the development of higher antibody titres against L-asparaginase in comparison to Aginasa®-injected animals. The decision to buy a new drug based on its price alone is not safe. Developing countries are especially vulnerable to cheaper alternatives that lack solid quality assurance.

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1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer in childhood. According to the last Brazilian cancer incidence data, approximately 3000 children (up to 19 years of age) are diagnosed with ALL annually (Estimativa 2018, 2017). Brazilian children enrolled in prospective cooperative clinical trials achieve survival rates comparable to those reported by centres in Western Europe and North America (Ribeiro et al., 2007; Möricke et al., 2010; Silverman et al., 2010; Hunger et al., 2012; Brandalise et al., 2010, 2016). ALL treatment is based fundamentally on combination chemotherapy. One essential drug is L-asparaginase, an enzyme produced in *Escherichia coli* that catalyzes the hydrolysis of asparagine into ammonia and aspartic acid (Pieters et al., 2011). The clinical effectiveness of this drug is based on asparagine depletion and the selective vulnerability of lymphoblasts whose survival is dependent on extracellular sources of asparagine (Müller and Boos, 1998).

Public health services are provided freely to almost 75% of the Brazilian population by the Government (Montekio et al., 2011). In the beginning of 2017 the Brazilian Ministry of Health acquired a new L-asparaginase produced by Beijing SL Pharmaceutical (China) with the trade name of Leuginase®. Due to eminent risk of shortage, the Ministry of Health purchased this new drug based on price and under less stringent import conditions, *i.e.* solely on registration in the country of origin and Good Manufacturing Practices certificate, without comparability studies. The virtual absence of clinical studies with the drug prompted our institution to investigate the purity, bioavailability and immunogenicity of Leuginase®, provided to the hospitals directly by the Government, in comparison to Medac L-asparaginase – the drug in prior use in the Country under the trade name of Aginasa® (Medac/Kyowa).

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2. Methods

2.1. Reagents

Leuginase® (Beijing SL Pharmaceutical) vials used in this study were from batch number 2016100101, validation date 10/23/2018. Aginasa® (Medac/Kyowa) vials were from batch number G140371A, validation date 07/31/2017. Antibodies used in ELISA were: anti-L-asparaginase (Abcam, ab55824), HRP-conjugated goat anti-mouse IgG (KPL, 074-1806), and HRP-conjugated goat anti-rabbit IgG (KPL, 04-15-16).

2.2. Protein Quantification

Protein mass quantifications of the L-asparaginase preparations were done using a fluorescence assay (Qubit Protein Assay, ThermoFisher Scientific), according to the manufacturer recommendations.

2.3. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Assay

The LC-MS/MS method adopted for the analysis conducted at LNBio (Campinas, Brazil) is described here. The corresponding method used at MS Bioworks (Ann Arbor, MI, USA) can be provided on request. L-asparaginase samples were reconstituted in saline (0.9% NaCl), 30 μg of protein were aliquoted and 10 μL Ureia 8 M and 0.4 μL 250 mM DTT were added. This mix was then incubated at 56 °C for 25 min, followed by addition of 0.57 μL 500 mM iodoacetamide and incubation for 30 min at room temperature in the dark. After alkylation, 0.4 μL 250 mM DTT was added again and incubated for 15 min. After these steps of reduction and alkylation, samples were digested by addition of 53.25 μL 50 mM ammonium bicarbonate, 0.74 μL 100 mM $CaCl_2$ and 1 μg of trypsin or chymotrypsin (Sequence Grade Modified, Sigma Aldrich) and incubated at 37 °C for 13 h. The reaction was stopped by addition of trifluoroacetic acid to a final concentration of 1%. Samples were then de-salted by the method of Stage Tips (Rappsilber et al., 2007). The samples were dried in a vacuum concentrator and reconstituted in 135 μL of 0.1% of formic acid. Two μL containing 0.44 μg of the resulting peptide mixture was analyzed on an ETD enabled LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with LC-MS/MS by an EASY-nLC system (Proxeon Biosystem) through a Proxeon nanoelectrospray ion source.

Peptides were separated by a 2–30% acetonitrile gradient in 0.1% formic acid using a C18 PicoFrit Column (20 cm × ID75 μm, 5-μm particle size; New Objective) and an EASY-nLC at a flow rate of 300 nL/min over 30 min. The nanoelectrospray voltage was set to 2.2 kV, and the source temperature was 275 °C. The scan MS spectra (m/z 300–1600) were acquired in the Orbitrap analyzer after accumulation to a target value of 1×10^6 (Brandalise et al., 2010). Resolution in the Orbitrap was set to $r = 60{,}000$ (m/z 400). Peptide ions were sequentially isolated to a target value of 80,000 and fragmented in the HCD (high collisional dissociation) energy (normalized collision energy of 40%). The signal threshold for triggering an MS/MS event was set to 7500 counts. An activation time of 0.1 ms was used.

The raw files were processed using Proteome Discoverer 1.4 (Thermo Scientific), and the MS/MS spectra were searched using the Sequest software against the Uniprot SwissProt *E. coli* database (Release: March 31th, 2017; 10,082 entries), with a tolerance of 10 ppm for precursor ions, 0.02 Da for fragment ions, and a maximum of 1 missed cleavage for protein identification. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine was chosen as a variable modification. Both peptide and protein identifications were filtered at a maximum of 1% false discovery rate. Raw data for LNBio and MS Bioworks may be provided on request. Results from Butantan Institute can be found by entering the code 15705121420402650000000001247715 in the following link: http://pje1g.trf3.jus.br:80/pje/Processo/ConsultaDocumento/listView.seam

2.4. N-Terminus Region Analyses

The raw data from MS Bioworks were processed using Mascot Distiller 2.3 and the resulting MGF file was used in Mascot server 2.3 (Matrix Science Ltd) to search for non-specific cleavages using the same parameters described above. The scoring function model was not specific to any particular digestion enzyme, so that all peptides (including but not restricted to those generated by trypsin) were accessible.

2.5. L-Asparaginase Activity

L-Asparaginase (EC 3.5.1.1) activity in Leuginase®, Aginasa®, and murine plasma samples was measured as described previously (Lanvers et al., 2002). Leuginase® and Aginasa® were diluted in Tris buffer, pH 7.3 (0.015 M), supplemented with 0.015% (w/v) bovine serum albumin fraction V (BSA) at concentrations of 5 IU/L, 10 IU/L, 20 IU/L, and 30 IU/L. For the determination of L-asparaginase activity, 20 μL of each enzyme dilution was mixed with 180 μL of 2 mM L-aspartic beta-hydroxamate (AHA) solution dissolved in Tris/BSA buffer (64.5 mM Tris, pH 7.3, 0.15 mg/mL Bovine Serum Albumin fraction V). Assays were performed in triplicates, in 96-well plates. After incubation at 37 °C for 30 min, the reaction was stopped by addition of 60 μL trichloroacetic acid (24.5%, w/v), and the samples were centrifuged for 5 min at 2500 rpm. Fifty microliters of the supernatant were transferred to a new well and 200 μL of freshly made Oxin reagent (1 vol. of 2% 8-hydroxyquinoline in ethanol and 3 vol. of 1 M sodium carbonate solution) was added. After heating at 95 °C for 1 min and cooling down the plate for exactly 10 min, absorbane was measured at 690 nm in a Synergy H1 Hybrid Reader (Biotek).

2.6. Beta-Lactamase Activity

Beta-lactamase (EC 3.5.2.6) activity was assessed indirectly by a modification of the antibiotic sensitivity assay. Briefly, antibiotic discs (10 μg to 30 μg) were impregnated with Aginasa® (50 to 100 IU) or Leuginase® (0.5 to 100 IU) or control and placed on agar plates inoculated with *Escherichia coli* (ATCC 25922). After 18 h incubation at 37 °C inhibition rings were compared.

2.7. Malate Dehydrogenase Activity

Malate dehydrogenase (EC 1.1.1.37) activity was assayed in the forward direction (NADH production) as previously described (Dasika et al., 2015). Mass inference was obtained by comparison to a standard curve made with recombinant *E. coli* malate dehydrogenase (Sigma-Aldrich, cat# SRP6105). Reactions were assembled in 96-well flat-bottom plates kept on ice. Sixteen microliters of different amounts of Leuginase®, Aginasa® and recombinant *E. coli* malate dehydrogenase (Sigma-Aldrich, cat# SRP6105) were added to 100 μL of a reaction mixture containing 1 mM NAD, 1 mM Malic acid, 90 mM KCl, 100 mM Tris, pH 8.9. NADH concentration was immediately measured by fluorescence at 470 nm with excitation at 340 nm, at 37 °C, using a Synergy H1 Hybrid Reader (Biotek). Data were measured over time. Data presented refer to 2:08 min, when a plateau was reached for the highest concentrations.

2.8. L-Asparaginase Bioavailability and Immunogenicity Assays in Mice

The study was registered and approved by CEUA/UNICAMP under #4556-1/2017. Animals were maintained with food and drink *ad libitum* in ventilated racks. Ten Balb/c female mice (6 to 8-week-old) were distributed randomly in two groups of five animals and received injections of 525 IU/kg of Leuginase® or Aginasa®. Blood was collected in EDTA

tubes for the study of asparaginase bioavailability and anti-asparaginase antibody titration as shown in Fig. 5A.

To determine L-asparaginase activity 20 μL of each plasma sample diluted at 1:5, 1:6 and 1:10 were mixed with 180 μL of 2 mM AHA solution and the reaction procedure was performed as described above, in triplicates.

For ELISA, plasma samples were diluted 10-fold and 50 μL and assayed in Aginasa®-coated 96-well plates. Briefly, microtiter plates (Nunc, Denmark) were coated with 50 μL per well of 4 IU/mL Aginasa® dissolved in coating buffer (0.2 M sodium carbonate, 0.2 M sodium bicarbonate, pH 9.6). After overnight incubation at 4 °C, wells were washed three times with 150 μL of washing buffer, consisting of: 0.05% Tween-20 in phosphate buffered saline (PBS). Next, wells were blocked with 300 μL of blocking buffer (1% BSA in PBS) for 1 h at room temperature and then washed three times with 150 μL of washing buffer. Assays were performed in duplicates by addition of 50 μL of plasma samples diluted at 1:10 (v/v) in blocking buffer, followed by 1 h incubation at room temperature and washings as above. Two-fold serial dilutions of a commercially available anti-L-asparaginase antibody were included as a positive control. The presence of reactive antibodies was analyzed by adding 50 μL/well of horseradish peroxidase-conjugated secondary antibodies (anti-mouse for the test of plasma samples and anti-rabbit for the positive control curve), followed by 1 h incubation at room temperature, washings (3×) and addition of 100 μL of substrate solution (1 mg/mL of 3,3′,5,5′-tetramethylbenzidine in 0.05 M phosphate-citrate buffer, pH 5.0, 0.03% sodium perborate). After a short incubation of 15 min at room temperature in the dark, 100 μL of 1 N HCl was added to stop the reaction. The enzymatic product was measured at 450 nm using the Synergy H1 Hybrid Reader (BioTek).

2.9. Statistical Methods

Results are presented as means ± SEM. The non-parametric Mann Whitney test was used to compare non-normally distributed datasets. p-Values ≤ 0.05 were considered evidence of significant differences. Statistical analyses were performed using the GraphPad Prism version 5.0 (GraphPad Software).

3. Results

3.1. Leuginase® Contains Several Host Contaminating Proteins

LC-MS/MS analyzes of Leuginase® and Aginasa® were performed at LNBio (Campinas, SP, Brazil) and MS Bioworks (Ann Arbor, MI, USA) laboratories. LNBio analyzed one vial of Aginasa® and two Leuginase® vials, all in triplicate. The MS Bioworks laboratory analyzed one vial of each asparaginase once. In response to a specific demand from the Brazilian Federal Police, both L-asparaginases were analyzed also by the Special Laboratory for Applied Toxinology at Butantan Institute (Results available in case file number 5002151-51.2017.4.03.6105 from Federal Court of the 3rd Region - 1st Degree). LNBio and Butantan laboratories analyzed both L-asparaginases after treatment with trypsin and chymotrypsin, whereas the MS Bioworks used only trypsin digestion. Both Leuginase® and Aginasa® had the expected active principle, *i.e.*, *Escherichia coli* L-asparaginase 2. Between 80% (LNBio and MS Bioworks) to 90% (Butantan) of the amino acid sequence of Leuginase® was covered by these analyses, with no detected mutations (data not shown).

All three LC-MS/MS analyzes revealed that Leuginase® contains host-cell contaminating proteins (HCP), *i.e.* other *E. coli* proteins in the formulation, whereas Aginasa® contains at most one or two spurious proteins in a very low amount, as evidenced by the number of identified peptides.

In Table 1 we show the total of L-asparaginase 2 and HCP identified in both Leuginase® and Aginasa®, as well as the number of corresponding peptides for each protein. Despite the differences in methodology and LC-MS/MS equipments, there was agreement among the three independent analyses, both in protein identity as well as in relative quantity of peptides of each protein. At least 12 spurious proteins were detected in all three analyses. In two analyses 19 common HCP were found in Leuginase® (Table 1). The main discrepancies between the three independent LC-MS/MS analyses reflect differences in bioinformatics methodology. For example, beta-lactamase, code BLAT_ECOLX, was the most abundant contaminating protein in the LNBio analysis but was absent in the tests conducted by the other two laboratories. Questioned about this discrepancy, MS Bioworks laboratory reworked the bioinformatics analysis. Instead of restricting the analysis to the *E. coli* K12 strain database (codes with final _ECOLI), as was done the first time, the analysis was then performed using the *Escherichia coli* Taxi database 562, which includes other strains of E coli. The new analysis revealed unquestionably the presence of beta-lactamase with 62% coverage (Fig. 1A).

Peptides have different ionization efficiencies and therefore are not detected with equal probability by mass spectrometry. However, the relative quantity of peptides gives an approximation of the total protein content. As shown in Table 1, the relative number of peptides of the HCP ranges from 19 to 37% of the total peptides.

3.2. Identification of Product-Related Derivatives in the Leuginase® Formulation

In addition to the identification of HCP, LC-MS/MS analysis allowed us to identify N-terminal differences in L-asparaginase 2 between Leuginase® and Aginasa®. In Aginasa® the signal peptide (1–22 amino acids) is followed by a single leucine (L) N-terminal residue, while in Leuginase® three possible N-terminal residues: leucine, phenylalanine (F) and Serine (S) were verified (Fig. 1B). N-terminus modifications in a biopharmaceutical protein are considered product-related impurities (Eon-Duval et al., 2012).

3.3. Orthogonal Identification of Beta-Lactamase and Malate Dehydrogenase HCPs in the Leuginase® Formulation

Beta-lactamase is a protein with enzymatic activity that degrades beta-lactam antibiotics such as penicillins, cephalosporins, cephamycins, and carbapenems. To confirm the presence of this spurious protein in Leuginase® we performed an antibiotic sensitivity test in which the diffusion disks were impregnated with different amounts of Leuginase® or Aginasa®. The presence of beta-lactamase was inferred by the interference in the beta-lactam antibiotic activity against a susceptible *E. coli* strain (ATCC 25922).

Aginasa®-impregnated antibiotic disks resulted in the formation of inhibition rings compatible with those observed in the control disc, even when Aginasa® was used at the dose of 100 IU per disc. In the case of Leuginase® there was a clear absence of ring formation around Cefalotin, Ampicillin and Amoxilin beta-lactam antibiotics, at concentrations of 10 IU per disk (Fig. 2A). Ampicillin activity was affected by Leuginase® even at concentrations of 0.5 IU (Fig. 2B) and 2 IU for ampicillin/sulbactam (data not shown), which suggests that the amount of contaminating beta-lactamase in the Leuginase® formulation is not low. Interference with the activity of other antibiotics, some of them commonly used during ALL treatment, were also detected (Fig. 2C and Table 2).

Malate dehydrogenase (MDH) converts malate into oxaloacetate using NAD+ (forward reaction) and *vice versa* using NADH (reverse reaction). As indicated by the mass spectrometry data, Leuginase® contains malate dehydrogenase. To confirm the presence of this spurious protein in Leuginase® an enzymatic test for MDH was performed. The comparison to a standard curve generated with known amounts of recombinant MDH allowed us to determine the concentration of the spurious MDH to be approximately 250 ng/μL of reconstituted Leuginase® (5 IU/μL; Fig. 3), *i.e.* a total of 500 μg of MDH in the vial. Considering that the Leuginase® vial contained approximately 21 mg of protein,

Table 1
Protein profiling of Leuginase® and Aginasa® samples using solution digestion with trypsin, followed by LC-MS/MS analyses. Data from three independent laboratories (LNBio, MS Bioworks, Butantan). Full list of proteins identified (including known contaminants) and their molecular weight and spectral counts are presented.

Accession	Identified proteins	Spectral peptide counts			KDa	Subcellular location
		LNBio[a]	MS Bioworks	Butantan[b]		
Leuginase®						
P00805	L-Asparaginase 2	657.7	214	674[c]	36.8	Peri
P61889	Malate dehydrogenase	44.5	44	26	32.3	Cyto, extr memb
P23843	Periplasmic oligopeptide-binding protein	39.8	13	32	60.9	Peri
P33363	Periplasmic beta-glucosidase	37	7	9	83.4	Peri
P0A867	Transaldolase A	35.7	12	12	35.6	Cyto
P45523	FKBP-type peptidyl-prolyl cis-trans isomerase FkpA	21.8	13	9	28.9	Peri
P0AF93	2-iminobutanoate/2-iminopropanoate deaminase	14.3	9	6	13.6	Cyto
P0AFH8	Osmotically-inducible protein Y	13.5	6	5	21.1	Peri
P0ACI6	Regulatory protein AsnC	13.2	9	7	16.9	Cyto
P0A955	KHG/KDPG aldolase	10.8	4	5	22.3	Cyto
P40120	Glucans biosynthesis protein D	8.3	4	4	62.7	Peri
P0ABZ6	Chaperone SurA	3.8	2	4	47.3	Peri
P0AEG7	Thiol:disulfide interchange protein DsbC	4.3	2	1	25.6	Peri
P23847	Periplasmic dipeptide transport protein	29.3		14	60.3	Peri
P33790	Protein TraG	11.5		3	102.4	Inner memb
P0A9B2	Glyceraldehyde-3-phosphate dehydrogenase A	9.7		5	35.5	Cyto
P05458	Protease 3	9.3		3	107.6	Peri
P0AE22	Class B acid phosphatase	4.3		3	26.1	Peri
Q46845	Disulfide-bond oxidoreductase YghU	4		3	32.4	Unknown
A8A391	Autonomous glycyl radical cofactor	6.3		3	14.3	Cyto
P62593	Beta-lactamase TEM	50.8			31.5	Peri
P00448	Superoxide dismutase [Mn]	5.2			23.1	Cyto
P0AGD3	Superoxide dismutase [Fe]	3			21.3	Cyto, memb
P19926	Glucose-1-phosphatase	3			45.7	Peri
B7L799	Adenylate kinase	2.8			23.6	Cyto
P0ADU2	Probable quinol monooxygenase YgiN		2		11.5	Cyto
P0A870	Transaldolase B			4	35.2	Cyto
P0AFM2	Glycine betaine/proline betaine-binding periplasmic protein			2	36.0	Peri
Q47537	Taurine-binding periplasmic protein			2	34.3	Peri
	Total peptide counts	1043.9	341	836		
	Total HCP peptide counts	386.2 (37%)	127 (37%)	162 (19%)		
Aginasa®						
P00805	L-Asparaginase 2	743	861	509[c]		
P0ABZ6	Chaperone SurA		3		47.3	Peri
P05793	Ketol-acid reductoisomerase		3		54.1	Cyto
P0A9G6	Isocitrate lyase			2	47.5	Cyto
	Total peptide counts	743	867	601		
	Total HCP peptide counts	0	6 (0.7%)	2 (0.3%)		

Subcellular locations: Peri, periplasm; Cyto, cytoplasm; Memb, membrane, Inner memb, inner membrane; Extr memb, extracellular to membranes.

[a] Number represents the mean of two biological replicates of trypsin digestion run in triplicates in LC-MS/MS.

[b] Data obtained from http://pje1g.trf3.jus.br:80/pje/Processo/ConsultaDocumento/listView.seam. Only proteins identified by ≥2 unique peptides were considered. Identification based on a single peptide have a high probability of being false positive and, usually, is not accepted by the scientific community (Carr et al., 2004). Only the Thiol:disulfide interchange protein (DsbC) was retained because it was found in the other two analysis.

[c] Sum of L-asparaginase-derived peptides from 2 trypsin plus 2 chymotrypsin readings.

MDH corresponds to approximately 2.4% of the total mass. This value is quite similar to the estimated amount of MDH-derived peptides in the LC-MS/MS analysis (4%, Table 1). This suggests that the relative quantitation made by mass spectrometry was correct. Therefore, total HCP contamination in Leuginase® is likely indeed to be in the range of 19 to 37% (Table 1). These levels certainly exceed the purity threshold of all pharmacopoeias, including the Chinese one.

3.4. Leuginase® Showed a 3 Times Lower Plasma Bioavailability as Compared to Aginasa®

Leuginase® and Aginasa® had similar *in vitro* enzymatic activities (Fig. 4). However, when injected intraperitoneally (i.p.) or intramuscularly (i.m.) in Balb/C mice, Leuginase® resulted in at least 3 times lower L-asparaginase plasma activity compared to Aginasa®, regardless of the administration route and time point (Fig. 5A). Mice injected i.p. with Leuginase® and Aginasa® showed at 24 h an average of 41 ± 10 IU/L and 157 ± 36 IU/L ($p = 0.0079$) and at 42 h 13 ± 5 IU/L and 40 ± 4 IU/L, respectively ($p = 0.0119$). By i.m. administration, the average plasma L-asparaginase activity for Leuginase® and Aginasa® was 46 ± 17 IU/L and 137 ± 22 IU/L ($p = 0.0079$) at 12 h and 20 ± 4 IU/L and 60 ± 13 IU/L ($p = 0.0079$) at 24 h, respectively. Although the experiment that had just been described was not designed to measure the half-life of the drugs, what can be inferred from the only two time-points suggest them to be quite similar.

3.5. Leuginase® is More Immunogenic Than Aginasa®

It is conceivable that the HCP in Leuginase® could act as an adjuvant to elicit a higher antibody response against L-asparaginase. Indeed, Balb/C mice injected i.m. with Leuginase® developed higher anti-asparaginase antibody titres than those injected with Aginasa® (Fig. 5B). Antibodies against Leuginase® were detected as early as the third injection, with a plateau achieved after the 5th injection. Only background levels were detected in mice injected with Aginasa® throughout the experiment. It is worth noting that the two animals that developed the highest anti-asparaginase titre (animals #1 and #5 in the Leuginase® group) had no asparaginase activity detectable 12 h after the 5th injection of the drug (on day 50), suggesting the occurrence of antibody-driven L-asparaginase inactivation/clearance (Fig. 5B and C).

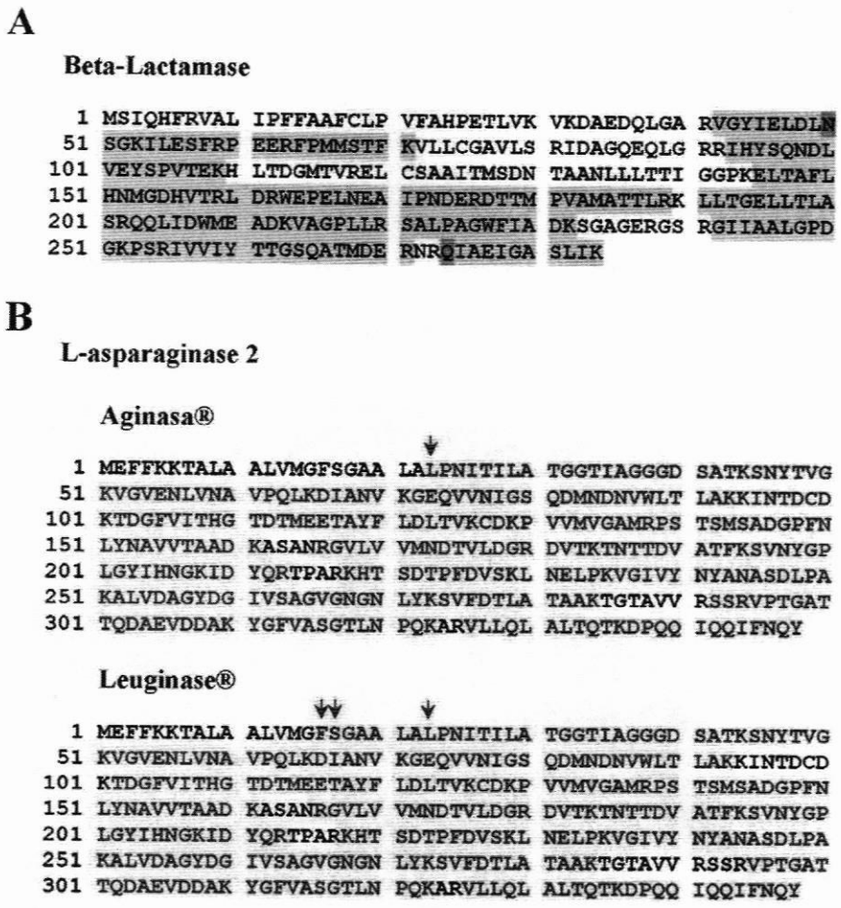

Fig. 1. Beta-lactamase and N-terminus isoforms of L-asparaginase. (A) Amino acid sequence of the beta-lactamase protein (A0A0U2Q1H4_ECOLX) identified in Leuginase® by LC-MS/MS. Analysis performed by MS Bioworks laboratory using the *Escherichia coli* Taxi database 562. Amino acid sequence "covered" by mass spectrometry analysis is represented in yellow and corresponds to 14 exclusive unique peptides, 17 exclusive unique spectra, 30 total spectra, and 175/284 amino acids (62% coverage). (B) N-terminus region isoforms of the L-asparaginase 2 (ASPG2_ECOLI) protein found in Aginasa® and Leuginase®. Analysis performed on the MS Bioworks raw data. Amino acid sequence "covered" by mass spectrometry analysis is represented in red letters. Arrows represent the N-terminus residues found in Aginasa® and Leuginase®.

4. Discussion

The purification process is one of the most critical and costly steps for biopharmaceutical production (Eon-Duval et al., 2012; Wolter and Richter, 2005). The downstream process must remove all contaminants, including host cell material such as DNA and protein. Even after several purification steps these contaminants can be copurified along with the drug substance, with potential detrimental clinical consequences. L-asparaginase manufacture must be flawless considering its importance as a clinically life-rescuing medicine for children with ALL.

In the present study, Leuginase® was found to have unacceptable levels of host contaminant proteins (HCP). Mass spectrometry analyses performed in three different laboratories revealed unambiguously >12 different *E. coli* proteins. Although the amount of peptides identified by mass spectrometry cannot be extrapolated exactly to relative amounts due to different ionization probabilities, an estimation of about 19 to 37% contamination can be made. This estimation was supported by the direct quantitation of one specific contaminant (malate dehydrogenase) through an enzymatic method. It is noteworthy that 4 out of the 7 most abundant HCP in Leuginase® have a molecular mass similar to that of L-asparaginase 2 (Table 1), which would make it difficult to discriminate as well as to purify them by HPLC/size exclusion chromatography. Based on these results, the authors suggest the inclusion of mass spectrometry for purity analysis in replacement of the traditional HPLC methods, adopted currently in the pharmacopoeias. Of note, the Chinese pharmacopoeia preconizes not <95% purity for injectable asparaginase, as measured by HPLC (science and press, 2015).

The most abundant contaminating protein, beta-lactamase, was shown to retain its antibiotic degrading activity and thus is likely to have a deleterious effect in patients who need antibiotic therapy, which is the case of children with chemotherapy-induced immunosuppression. The high level of beta-lactamase suggests strongly that L-asparaginase is of recombinant origin in Leuginase®. Regarding the remaining contaminants, there is no information about their potential medium- and long-term risk posed to patients. This could only be addressed in clinical trials.

It is well known that *E. coli* L-asparaginase triggers hypersensitivity reactions in about 30 to 70% of patients, due to anti-asparaginase antibody production. Besides discontinuation caused by overt allergic reactions, these antibodies may also cause inactivation of L-asparaginase in a 'silent' fashion, also resulting in suboptimal asparagine depletion and poorer clinical outcome (van der Sluis et al., 2016). The presence of multiple HCP in Leuginase® is likely to enhance the intrinsic immunogenicity of L-asparaginase. Earlier use of insulin and hGH, for instance, when the preparations were relatively impure, were associated with more adverse immunologic reactions than nowadays (Lundin et al., 1991; Schernthaner, 1993). HCP can act not only as antigens but also as

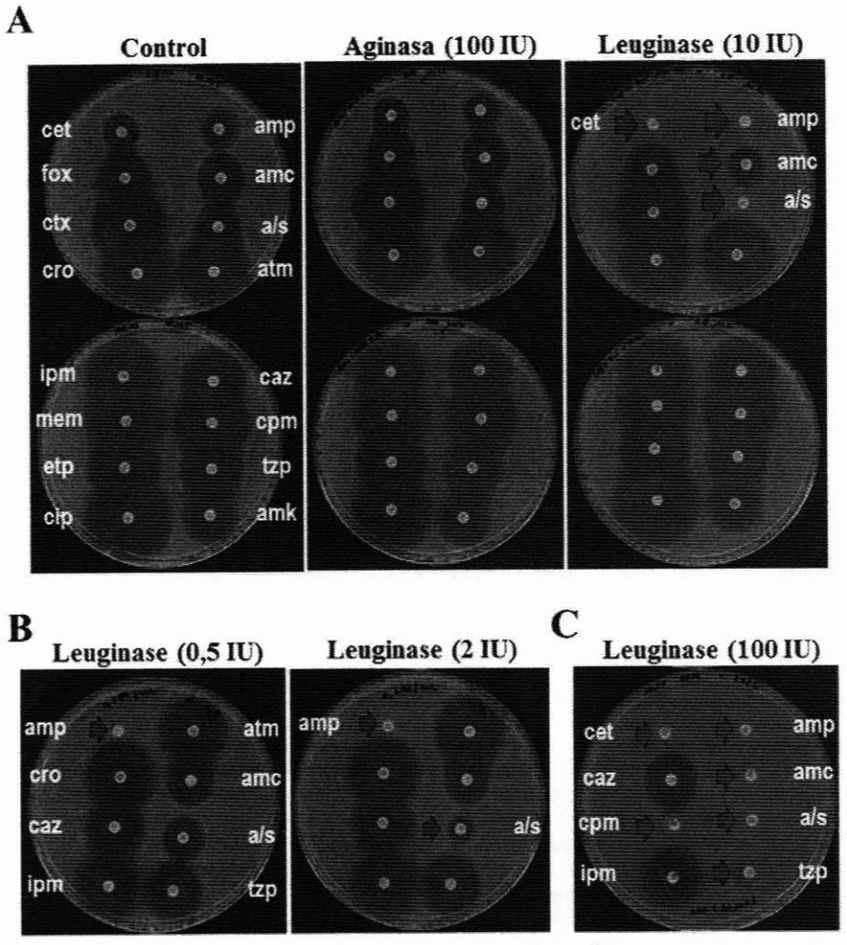

Fig. 2. Verification of beta-lactamase activity in the Leuginase® preparation by means of a modified antibiotic sensitivity test. (A) Petri dishes inoculated with *E. coli* in the presence of antibiotic disks spotted with Leuginase® or Aginasa®. In Control, the discs were used without the addition of L-asparaginase. The upper left antibiotic legend indicates the panel adopted for the three upper dishes. (B) Assay with lower concentrations of Leuginase®. The lower left antibiotic legends indicate the panel adopted for both Leuginase® 0.5 IU and Leuginase® 10 IU. (C) Assay with higher concentration of Leuginase® (100 IU). Plates were incubated at 37 °C for 18 h. Arrows indicate antibiotics affected by Leuginase®. Antibiotic disks were purchased from CECON (Sao Paulo, Brazil). Cet, cefalotin (30 μg); fox, cefoxitin (30 μg); ctx, cefotaxime (30 μg); cro, cefatriaxone (30 μg); amp, ampicillin (10 μg); amc, amoxicillin/clavulanate (30 μg); a/s, ampicilin/Sulbactam (20 μg); atm, aztreonam (30 μg); ipm, imipenem (10 μg); mem, meropenem (10 μg); etp, ertapenem (10 μg); cip, ciprofloxacin (5 μg); caz, ceftazidime (30 μg); com, cefepime (30 μg); tzp, piperacillin/Tazobactam (110 μg); amk, amikacin (30 μg).

adjuvants to elicit an immune response to the drug product itself (Sharma, 2007). Accordingly, Leuginase®-injected mice developed higher titres of antibodies against L-asparaginase. Although murine models are not always reflective of human immune reactions, they still hold value when addressing the relative immunogenicity between products (Brinks et al., 2011).

Lastly, Leuginase® was shown to have 3 times lower plasma bioavailability in mice compared with Aginasa®. In clinical practice the lower the blood asparaginase activity the lower the depletion of asparagine amino acid and, consequently, the lower the anti-leukemic efficacy of the drug. Although older studies found some variation in the bioavailability of different brands of L-asparaginase (Schwartz et al., 1970; Asselin et al., 1993; Boos et al., 1996; Ahlke et al., 1997), no explanation was given other than putative biological differences due to mutations. In fact, differences in even a single amino acid may have a drastic effect in L-asparaginase bioavailability. For example, modifications of the N24 residue confers resistance to asparaginyl endopeptidase degradation (Patel et al., 2009). This explanation does not apply in the present case, because most of the amino acid sequence of both L-asparaginases were covered by LC-MS/MS with no mutation detected, including at N24. However, there are N-terminal L-asparaginase isoforms in Leuginase® that may have shorter half-lives *in vivo*.

On the other hand, an issue that has seldom been taken into consideration, namely preparation purity, may impact L-asparaginase bioavailability. A recent study on the distribution of radiolabeled L-asparaginase in mice identified a central role of phagocytic cells in L-asparaginase depletion. It reaches plasma peak levels within 2 h after infusion, with accumulation in liver, spleen and bone marrow. Over time, L-asparaginase levels decrease in the circulation, remain constant in bone marrow and increase in spleen and liver – the residence of most phagocytic cells of the reticuloendothelial system (van der Meer et al., 2017). L-Asparaginase degradation occurs possibly by means of plasmatic and intracellular proteases, such as lysosomal cysteine proteases B cathepsin and asparaginyl endopeptidase, which are expressed in macrophages (Patel et al., 2009; van der Meer et al., 2017). Macrophage depletion using clodronate prolongs the L-asparaginase half-life significantly, revealing the central role of these cells in the pharmacokinetics of this drug (van der Meer et al., 2017). The adjuvant effect of impurities that is likely to underlie the higher anti-asparaginase antibody titres observed in animals injected with Leuginase® may also be operative in

Table 2
Inhibition of different antibiotic activities by Leuginase®. A modified antibiotic sensitivity test was used to address the inhibition caused on different antibiotics by different doses of Leuginase®. Shown is the amount of Leuginase® that when spotted in the antibiotic disc caused a reproducible decrease in the diameter of the zone of inhibition of the bacteria growth.

Antibiotic (amount in the disc)	Leuginase® inhibitory dose (quantity spotted in the disc)
Ampicillin (10 μg)	<0.5 IU
Ampicillin/Sulbactam (20 μg)	2 IU
Cefalotin (30 μg)	<10 IU
Amoxicillin/Clavulanate (30 μg)	10 IU
Cefepime (30 μg)	>40 IU
Piperacillin/Tazobactam (110 μg)	>80 IU
Imipenem (10 μg)	No effect (max. tested 100 IU)
Ceftazidime (30 μg)	No effect (max. tested 100 IU)
Meropenem (10 μg)	No effect (max. tested 20 IU)
Ertapenem (10 μg)	No effect (max. tested 20 IU)
Ciprofloxacin (5 μg)	No effect (max. tested 20 IU)
Amikacin (30 μg)	No effect (max. tested 20 IU)
Cefoxitin (30 μg)	No effect (max. tested 10 IU)
Cefotaxime (30 μg)	No effect (max. tested 20 IU)
Cefatriaxone (30 μg)	No effect (max. tested 20 IU)
Aztreonam (30 μg)	No effect (max. tested 20 IU)

the activation of the innate immune cells of the reticuloendothelial system, therefore contributing to faster L-asparaginase elimination.

5. Conclusion

The cost of oncologic drugs has been a matter of serious debate as pointed out recently in the American Society of Clinical Oncology Position Statement on Addressing the Affordability of Cancer Drugs (American Society of Clinical Oncology, 2017). We hope this manuscript may help in the design of new policies to better address the challenge of offering oncologic drugs at fair price with high clinical efficacy and safety.

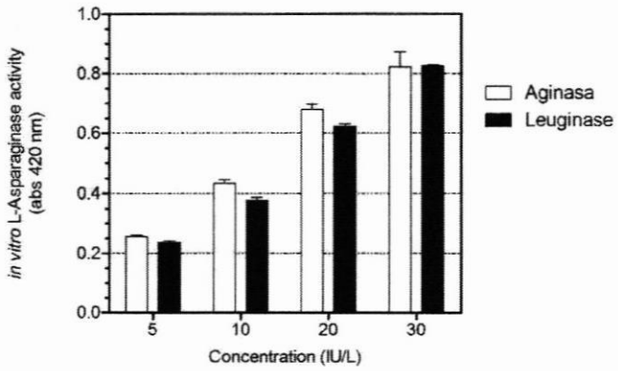

Fig. 4. *In vitro* enzymatic activity of Leuginase® and Aginasa®. Different volumes of Leuginase® and Aginasa® vials reconstituted at 5 IU/μL were assayed for L-asparaginase activity *in vitro*. A standard curve was prepared with Aginasa®.

Authors' Contributions

PPZ and NAM performed the biochemical and *in vivo* studies and contributed equally to this work. RAMS did the antibiotic sensitivity assays. FCG analyzed mass spectrometry data. NMC, PPZ, NAM, POdCL, and JAY analyzed data. POdCL, FCG, JAY, and SRB wrote the manuscript. JAY and SRB jointly coordinated the work.

Declaration of Interests

The authors declare no conflicts of interest.

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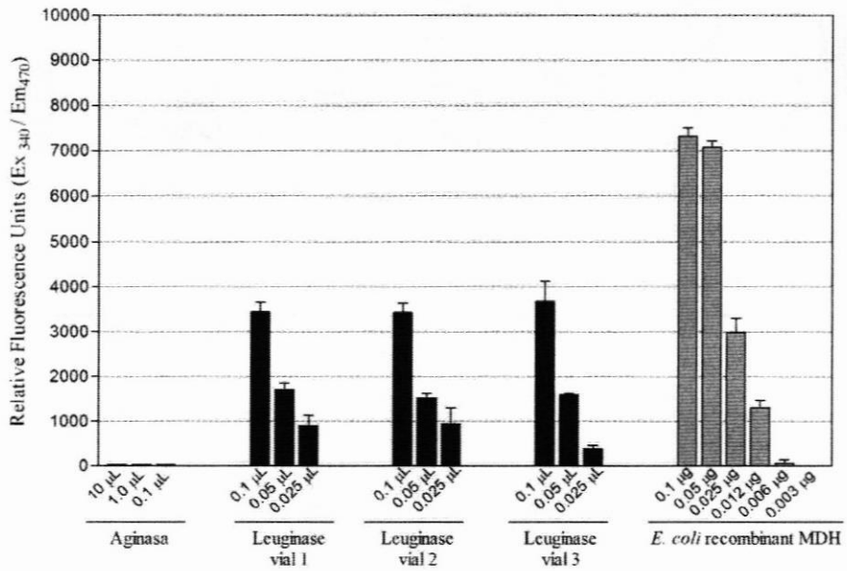

Fig. 3. Detection and quantitation of malate dehydrogenase activity in the Leuginase® preparation. Graph shows the malate dehydrogenase (MDH) activity measured in terms of NADH production after 2:10 min at 37 °C, for different amounts of Leuginase® (5 IU/μL) and Aginasa® (5 IU/μL), and known quantities of recombinant *E. coli* malate dehydrogenase obtained from Sigma-Aldrich. Bars represent mean and SD of technical triplicates. Three different vials of Leuginase® were tested. The malate dehydrogenase activity in 0.1 μL of Leuginase® equals that of 0.025 μg of the recombinant MDH.

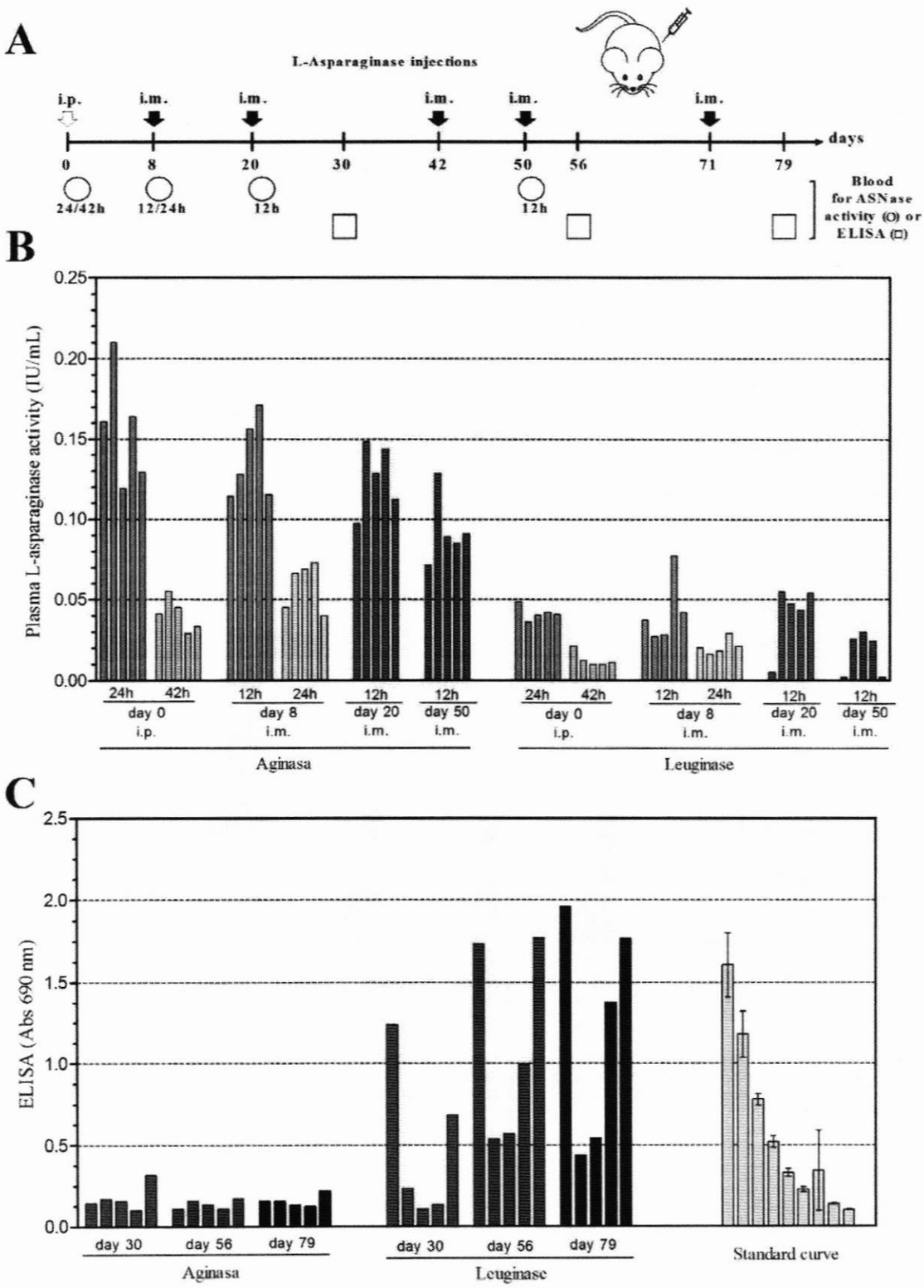

Fig. 5. Plasma bioavailability and immunogenicity of Leuginase® and Aginasa® in Balb/C mice. (A) Timeline of L-asparaginase injections and blood collection. Arrows represents intraperitoneal (i.p.) or intramuscular (i.m.) injection of L-asparaginase at the dose of 525 IU/kg. Circles, blood collected for enzymatic activity analyses; Squares, blood for anti-asparaginase antibody titration by ELISA. (B) Activity of L-asparaginase measured in murine plasma after i.p. or i.m. injections of Leuginase® or Aginasa®. A standard curve with Aginasa® was used to calculate the activity ($r^2 > 0.99$; data not shown) (C) ELISA titration of antibodies against L-asparaginase in plasma samples collected on at least 6 days after Leuginase® or Aginasa® administration. Plates were coated with Aginasa®. The standard curve obtained by using two-fold serial dilutions (from 100 to 0,39 ng/μL) of a commercially available antibody against L-asparaginase is shown. Each bar represents an animal in panels B and C, and the same order was kept.

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