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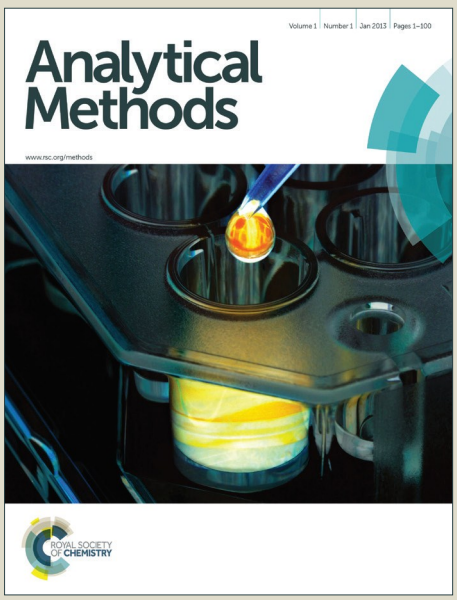

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ARTICLE

Evaluation of Botulinum Toxin Type A by Bioassays and a Validated Reversed-Phase Liquid Chromatography Method

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Biological potency of Botulinum toxin type A (BoNTA) in biotechnology-derived products was assessed by the mouse LD_{50} bioassay (MBA) and the T47D cell culture assay. A stability-indicating reversed-phase liquid chromatography (RP-LC) method was validated to determine the content of BoNTA. The method was carried out on a Zorbax 300SB-C_{18} column (150 mm x 4.6 mm i.d.), maintained at 45 °C. The mobile phase consisted of 0.05 M sodium phosphate buffer solution, pH 2.8, and acetonitrile, run isocratically at a flow rate of 0.3 mL min^{-1}, and using a photodiode array (PDA) detection at 214 nm. Separation was obtained with a retention time of 11.4 min, and was linear over the concentration range of 0.2 - 100 U mL^{-1} (r^2 = 0.9999). Specificity was established in degradation studies, which also showed that there was no interference of the excipients. The accuracy was 100.31% with bias lower than 0.80%. The method was applied to the content assessment, and the results were compared to those of the MBA, giving mean values of 1.16% lower, demonstrating potential to improve the characterization and quality consistency of the products.

1. Introduction

Botulinum neurotoxins (BoNT) are recognized as some of the most potent toxins. Botulinum toxin A (BoNTA) is one of the seven different serotypes produced from broth-culture by Clostridium botulinum, and the first to receive attention clinically as a potent muscular paralytic agent. Exert it paralytic effect at the neuromuscular junction by inhibiting the release of acetylcholine, and has proved to be effective and safe for treatment of disorders associated with inappropriate muscle contractions. Recently, it was expanded to include excessive involuntary movements, muscle spasms, pain, migraine, cerebral palsy, hyperhydrosis and is increasingly used for cosmetic purposes.[1-3]

BoNTA is naturally expressed as inactive single peptide chain with 1296 amino-acids and 150 kDa relative molecular mass neurotoxin, that is post-translationally cleaved by endogenous proteases into a fully active, disulfide-linked (C430-C454) dichain toxin containing a ~54 kDa light chain and a ~97 kDa heavy protein.[4-6]

Biological potency of BoNTA has been assessed by the mouse LD_{50} bioassay (MBA) and the labelled unit of activity is expressed in mouse LD_{50} units.[7,8] Alternative assays based on the local flaccid muscular paralysis with resultant abdominal ptosis or toe-spread reflex have been tested in mice.[9-11] An assay based on the endopeptidase activity towards immobilised specific synthetic substrate SNPA-25 was validated and the results were compared with the in vivo bioassay.[12]An immunobiochemical assay based on dual-coated plate was developed showing to distinguish native and denatured toxin molecule.[13]An alternative cell culture bioassay based on the key steps of action was developed using differentiated human neuroblastoma SiMa cell line (ATCC 24 cell line), evaluating the results by a sandwich ELISA.[14] Another in vitro cell culture bioassay based on the effect on proliferation and apoptosis was studied using the T47D (ATCC HTB-133) breast cancer cell line, assessing the responses by MTT, and showing greater cytotoxicity in comparison with MCF10A cells.[15] A cell-based functional assay using a fluorescence reporter expressed in a cell model was developed for the detection of BoNTA and type E.[16]Excipients and formulation composition of BoNT in clinical use were described with regard to the effects on products delivery, stability and safety.[17]

Physicochemical techniques alone can not predict biological activity yet, but they can produce information about structure and composition, in attempts to improve methods for characterization and identification of biologicals.[18,19]A gradient RP-LC method using C18 columm with fluorescent detection was employed to detect cleavage products generated by formulated BoNT products.[20]An UPLC method was developed using a C_{18} column and applied for quantitative determinations of botulinum neurotoxin proteolytic activity with the sensitivity of 7 pmol of the peptide substrates for BoNT.[21]Capillary electrophoresis method was used to measure catalytic activity of different serotypes of BoNT.[22] Mass spectrometry methods were developed to detect and differentiate

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active BoNTs based on the products derived from the endopeptidase activities with target specific peptides, and applied also for the analysis in complex matrices.[23,24]

The aim of the this article was to develop and validate a specific, sensitive and stability-indicating RP-LC method for the characterization and content assessment of BoNTA; to correlate the results with the MBA and the T47D cell culture bioassays; thus contribute to establishing alternative assay, while ensuring quality and safety of the biological product.

2. Experimental

2.1 Chemicals and reagents

The Reference Botulinum Toxin Type A (R-BoNTA) was obtained from the Medytox Inc. (Seocho-Gu, Seoul City, South Korea) and it was supplied by BSG Pharma Co., Ltd (Dohwa-Dong, Seoul City, South Korea). Batches of Botulift® Bergamo (São Paulo, Brazil), and of Botox® Allergan (São Paulo, Brazil) containing 100 U/vial were acquired from commercial sources within their shelf life period. Hydrogen peroxide (30%) in aqueous solution, human serum albumin, sodium chloride, tetramethylammonium hydroxide, formic acid, acetonitrile, trifluoracetic acid (TFA) and sodium phosphate monobasic were purchased from Merck (Darmstadt, Germany). All the others chemicals used were of HPLC grade or special analytical grade. For all of the analyses, ultrapure water was purified using an Elix 3 coupled to a Milli-Q Gradient A10 system from Millipore (Bedford, MA, USA).

2.2 Apparatus

Liquid Chromatography (LC) method was carried out on a Shimadzu LC system (Kyoto, Japan) equipped with a CBM-20A system controller, a LC-20 AD pump, a DGU-20A_5 degasser, a SIL-20AC_{HT} autosampler, CTO-20A column oven and a SPD-M20A PDA detector. The peak areas were automatically integrated by computer using a LC Solution version 1.22 SP1 software program.

2.3 Samples and Standard Solution

Working standard and sample solutions of BoNTA were prepared daily by diluting the R-BoNTA and the samples of biopharmaceutical formulations in ultra pure water, to a final concentration of 25 U mL^{-1}, for RP-LC; in 0.9% sodium chloride solution to final concentrations between 6.02 and 10.52 U mL^{-1}, for the MBA; and in RPMI-1640 Sigma-Aldrich (St. Louis, MO, USA) medium containing 2% (v/v) fetal bovine serum Sigma-Aldrich to final concentrations between 3 and 81 U mL^{-1}, for T47D cell culture bioassay.

2.4 Procedures

2.4.1 Reversed-Phase Liquid Chromatography Method (RP-LC). The experiments were performed on a reversed-phase Agilent (Santa Clara, CA, USA) Zorbax 300 SB C_{18} column (150 mm x 4.6 mm i.d., with a particle size of 5 µm and pore size of 300 Å) maintained at 45 °C. A security guard holder was used to protect the analytical column. The mobile phase consisted of 0.05 M sodium phosphate buffer, pH 2.8, and acetonitrile (80:20, V/V), run at a flow rate of 0.3 mL min^{-1}. PDA detection at 214 nm was used. The mobile phase was filtered through a 0.22 µm membrane Millipore filter (Bedford, MA, USA). The injection volume was 50 µL for both the reference substance and samples.

2.4.2 Mouse LD_{50} bioassay. Female 7 to 8 weeks-old Balb/c mice weighing between 18 and 23 g were housed in air-conditioned, controlled conditions (room temperature 22 ± 2 °C, relative humidity of 50 – 60%, and artificial illumination, 12 h per day). They were given free access to food and water. The bioassay was performed as described previously,[7] and modified accordingly. Briefly, the animals were allocated to sample, reference, and control groups in a fully randomized order with 8 mice per treatment group, respectively. Reference and samples were diluted to concentrations between 6.02 - 10.52 U mL^{-1} with 0.9% sodium chloride solution, and a single dose of 0.1 mL injected intraperitoneally. The mortality observed per treatment group during 72 hours was used as a probit transformed response, and analyzed by the parallel line assay method. All of the assays were conducted in accordance with the National Protection Laws on Animal Welfare and the UFSM ethical committee (protocol 23081.018819).

2.4.3 T47D cell culture bioassay. The bioassay was performed as described elsewhere [15] and adjusted. The T47D cells (breast cancer cells, ATCC number HTB-133) were maintained in culture medium RPMI-1640 Sigma-Aldrich (St. Louis, MO, USA) containing 10% (v/v) fetal bovine serum Sigma-Aldrich, seeding at approximately 2.0-4.0 x 10^6 cells mL^{-1}. The assay was performed in triplicate, the cells were seeded in 96-well cell culture plates BD Biosciences (San Jose, CA, USA) at a density 2.0 x 10^5 cells mL^{-1} (1.0 x 10^4cells/well) and dosed on seeding by using four concentrations with two fold dilution series of BoNTA between 3 - 81 U mL^{-1}. The R-BoNTA was used as standard and the negative control was RPMI-1640 culture medium. Briefly, the plates were incubated at 37 °C, 5% CO_2 for 24 h. Then 50 µL per well of MTT solution 5 mg mL^{-1}, was added per well, and the plates were incubated for a further 4 h. Following the addition of 100 µL per well of dimethyl sulfoxide (DMSO) for a further 3 h, the absorbance was assessed at 570 nm, using a microplate reader Thermo Scientific Multiskan FC (Vantaa, Finland).

2.4.4 *In vitro* Cytotoxicity test. The *in vitro* cytotoxicity method was performed as described elsewhere,[25] based on a neutral red uptake (NRU) assay, with the exposure of NCTC Clone 929 cell line (mammalian fibroblasts, ATCC number CCL-1) to degraded samples, the pH of the samples was adjusted to 7.0, and positive and diluents control were included in the assay together with R-BoNTA solution. The NRU assay was performed on 96-well microplates, maintained at 37 °C in a CO_2 incubator for 24 h, with a cell suspension density of approximately 2 x 10^5 cells mL^{-1}. Neutral red release was evaluated by the addition of an extracting solution, and the absorbance was measured at 540 nm, using a microplate reader.

2.5 Validation of RP-LC method

The method was validated using samples of biopharmaceutical formulations of BoNTA with a label claim of 100 U/vial by determination of the following parameters: specificity, linearity, range, precision, accuracy, detection limit (DL), quantitation limit (QL), robustness, stability, and system suitability test, following the guidelines.[26, 27]

2.5.1 Specificity. The specificity of the method was assessed by subjecting a R-BoNTA solution and a sample of BoNTA biopharmaceutical formulations (25 U mL^{-1}), to oxidative conditions induced by adding 50 µL of 3% hydrogen peroxide for 25 min. Photodegradation was induced by exposing the samples in a photostability chamber to 200 W h m^{-2} of near ultraviolet-light for 4 hours and 30 min. Sample was also subjected to neutral hydrolysis at 80 °C, for 2 h and shaken for 10 min. In addition, interference from excipients of the biopharmaceutical formulations was determined by injection of a sample containing only placebo (in-house mixture of formulation excipients). Then, the specificity of the method was established by determining the peak purity using PDA detector. Additionally, the BoNTA and the degraded samples were subjected to the *in vitro* cytotoxicity test.

2.5.2 Linearity. The linearity was determined by constructing three independent analytical curves, each one with eight concentrations of the R-BoNTA, over the 0.2 - 100 U mL^{-1} range. Three replicate 50 µL injections of the reference solutions were prepared to verify the repeatability of the detector response. The peak areas of the chromatograms were plotted against the respective concentrations of R-BoNTA to obtain the analytical curve. The results were subjected to regression analysis by the least squares method to calculate calibration equation and the determination coefficient.

2.5.3 Precision and accuracy. Assay precision was determined by means of repeatability (intra-day) and intermediate precision (inter-days). Repeatability was examined by eight evaluations of sample of BoNTA, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by analysis of two samples of the pharmaceutical formulations on three different days (inter-days) and also by submitting the samples to analysis by other analysts in the same laboratory (between-analysts). The accuracy was assessed applying the proposed method to the analysis of the in-house mixture of the excipients with known amounts of the biomolecule, to obtain solutions at concentrations of 20, 25 and 30 UmL^{-1}, equivalent to 80, 100 and 120% of the nominal analytical concentration, respectively. Accuracy was calculated as the percentage of the drug recovered from the formulation; it was expressed as the percentage relative error (bias%) between the measured mean concentrations and the added concentrations.

2.5.4 Limits of detection and quantitation. The detection limit (DL) and the quantitation limit (QL) were calculated, as defined by ICH,[26] by using the mean values of three independent analytical curves determined by a linear-regression model, where the factors 3.3 and 10 for the detection and quantitation limits, respectively, were multiplied by the ratio of the standard deviation of the intercept and the slope. The QL was also evaluated in an experimental assay.

2.5.5 Robustness. The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters; it provides an indication of its reliability for the routine analysis. The robustness was determined by analyzing the same samples (25 U mL^{-1}) under a variety of conditions of the method parameters, such as: sodium phosphate concentration, percent of acetonitrile, buffer pH in mobile phase, flow rate, temperature and wavelength. To assess the stability of the BoNTA samples, analysis were performed after their storage at 2 – 8 °C for 24 h, and also placed into the auto sampler, at room temperature, for 24 h. To study the stability of these solutions the experiment was run and any changes in the chromatographic pattern as compared with the fleshly prepared solutions were noted.

2.5.6 System suitability test. The system suitability test was also carried out to evaluate the resolution and repeatability of the RP-LC system. Five replicate injections of a R-BoNTA 25 U ml^{-1} were used. The peak area, retention time, theoretical plates, and tailing factor (peak symmetry) were measured using the LC Solution Version 1.22 SP1 software.

3. Results and discussion

3.1 Optimization of chromatographic conditions

Tests on different amounts of 0.05 M sodium phosphate buffer solution, acetonitrile, tetramethylammonium hydroxide buffer, formic acid and TFA-acetonitrile solution mixed with water were carried out to achieve which mobile phase would lead to satisfactory selectivity and sensitivity within a short separation time. Next, evaluation of C_{18} columns with lengths of 250 and 150 mm and different pore sizes showed that the Zorbax column measuring 250 mm length provide longer retention times. For the selection of the best wavelength detection a PDA detector was used. The optimized conditions of the LC method were validated for the analysis of BoNTA in biopharmaceutical formulations.

Typical chromatograms obtained by the proposed method, demonstrating the resolution of the symmetrical peak, corresponding to BoNTA, with retention time of 11.4 min, are shown in Fig 1(a,b).

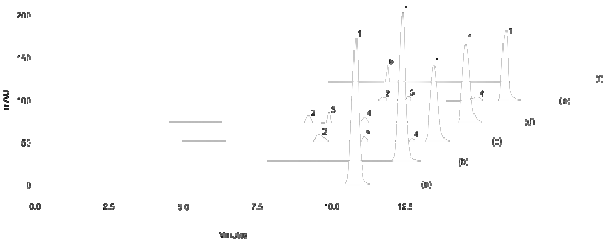

Fig. 1 Representative chromatograms showing peak 1=Botulinum toxin type A; peak 2=deamidated; peaks 3-4=sulphoxides; peak 5=human serum albumin. (a) Reference of BoNTA; (b) Sample of biopharmaceutical formulation, and (c) after degradation by hydrogen peroxide; (d) after photodegradation; (e) after neutral hydrolysis; (f) placebo.

3.2RP-LC Method validation

Specificity of the method for the biological product was evaluated under oxidative conditions showing reduction of 13% of the main peak, generating additional peaks of related proteins with retention times at 7.27, 8.45 and 10.19 min as shown in Fig 1 (c). Photodegradation resulted in reduction of 17% of the main peak, and three additional peaks with retention times at 5.66, 6.35 and 7.46 min, respectively, as shown in Fig. 1 (d), as also cited.[17] Neutral hydrolysis showed reduction of approximately, 19% of the main peak area, and three additional peaks were detected with retention times at 6.85, 7.63 and 10.12 min, (Fig 1, e). Moreover, the injection of a sample containing only in-house mixture of the formulation excipients, showed only one peak of human serum albumin with retention time at 6.23 Fig 1 (f). Then the specificity was established by determining the peak purity in the R-BoNTA and the samples using a PDA detector.

The analytical curves constructed for R-BoNTA were found to be linear over the range of 0.2 - 100 U mL^{-1}. The value of the determination coefficient calculated (r^2 = 0.9999), y = (100937 ± 128.99)x + (17295 ± 1641.66), where, x is concentration and y is the peak absolute area, indicated linearity of the analytical curve for the method.

The precision was studied by calculating the relative standard deviation (RSD%) for eight analyses at a concentration of 25 U mL^{-1}, performed on the same day and under the same experimental conditions. The obtained RSD value was 0.09%. The intermediate precision was assessed by analyzing two samples of the biopharmaceutical formulation on three different days (inter-days), giving for the content RSD values of 0.20% and 0.27%, respectively. Between-analysts precision was determined by calculating the mean values and the RSD after analysis of two samples of the same pharmaceutical formulations by two analysts; the mean values were found to be 99.40 and 99.93 and the RSD values found to be 0.25% and 0.54%, respectively, as given in Table 1.

Table 1. Inter-days and between-analysts precision data of RP-LC for botulinum toxin type A in biopharmaceutical formulations

	Inter-days			Between-analysts		
Sample	Day	Recovery[a], %	RSD[b], %	Analysts	Recovery[a], %	RSD[b], %
1	1	99.56	0.20	A	99.23	0.25
	2	99.27		B	99.58	
	3	99.66				
2	1	100.27	0.27	A	99.55	0.54
	2	99.85		B	100.32	
	3	99.64				

[a] Mean of three replicates. [b] RSD, relative standard deviation.

The accuracy was assessed from three replicate determinations of three solutions containing 20, 25 and 30 U mL^{-1}, respectively. The absolute means obtained with a mean value of 100.31% and a bias lower than 0.80% (Table 2), show that the method is accurate within the desired range.

Table 2. Accuracy of RP-LC for botulinum toxin type A in the formulations

Nominal concentration/ U mL^{-1}	Mean concentration measured[a]/ U mL^{-1}	RSD[b], %	Accuracy, %	Bias[c], %
20	20.16	0.23	100.80	0.80
25	25.02	0.23	100.09	0.08
30	30.01	0.05	100.03	0.03

[a] Mean of three replicates. [b] RSD, relative standard deviation. [c] Bias, [(Measured concentration - Nominal concentration)/Nominal concentration] x 100.

The DL and QL were calculated from the slope and the standard deviation of the intercept determined by a linear-regression line, by using the mean values of the three independent calibration curves. The obtained values were 0.04 and 0.16 U mL^{-1}, respectively.

The robustness of the analytical procedure was determined by analyzing samples of the R-BoNTA solution containing 25 U mL^{-1} in triplicate by the one-variable-at-a-time (OVAT) approach. The results and the experimental range of the selected variables evaluated are given in Table 3, together with the optimized values. Additionally, the robustness was also evaluated and compared by the multi-variable-at-a-time (MVAT) approach[28] at three levels (1 unit per parameter up or down around optimized values). This procedure gives results for minimum changing of the maximum number of parameters at a time, and is a very useful, rapid and efficient approach for a robustness determination. The results for the OVAT and MVAT procedures were within the acceptable deviation (RSD < 2 %), and an analysis of the variance showed non-significant differences (p > 0.05). The analysis performed with a wider level of variations of the solution pH, acetonitrile percentage and flow rate, showed changes of the retention time related to the optimized conditions. Moreover, the peak symmetry values were also evaluated, showing non-significant differences (p > 0.05). The stability of reference and sample solutions was assessed showing non-significant changes (< 2%) of the main peak relative to freshly prepared samples.

Table 3. Chromatographic conditions and range investigated during robustness testing with the one-variable-at-a-time (OVAT) procedure.

Variable	Range investigated	BoNTA[a], (%)	RSD[b], (%)	Optimized value
Sodium phosphate concentration (M)	0.02	97.57	0.05	0.05
	0.05	98.47	0.04	
	0.08	97.35	0.10	
Acetonitrile (%)	10	98.92	0.24	20
	20	99.33	0.04	
	30	98.62	0.10	
Buffer pH in mobile phase	2.6	99.05	0.07	2.8
	2.8	99.07	0.00	
	3.0	98.38	0.73	
Flow rate (mL min^{-1})	0.1	99.90	0.04	0.3
	0.3	100.11	0.02	
	0.5	99.73	0.07	
Temperature (°C)	40	98.52	0.10	45
	45	99.11	0.02	
	50	98.71	0.70	
Solution stability	Autosampler 24h	97.20	0.63	-
	2 – 8 °C, 24 h	98.16	0.03	-
Wavelength (nm)	210 - 320	-	-	214

[a] Mean of three replicates. [b] RSD, relative standard deviation.

The system suitability test was performed and RSD values calculated for the retention time, peak symmetry and peak area were 0.89, 0.87 and 0.63%, respectively. The number of theoretical plates was about 10,125, with RSD of 0.67%. The experimental results showed that the parameters tested were within the acceptable range (RSD < 2.0%), indicating that the system is suitable for the intended analysis.

3.3Mouse LD_{50} bioassay

Biological potency of BoNTA was assessed by the MBA relative to the reference, and the statistical analysis of the data was performed using the CombiStats® software- EDQM (Strasbourg, France), giving the values shown in Table 4. There is no International Standard, and a suitable reference preparation that does not have to be of identical formulation or composition as clinical lots, provided it has active relevant component of defined activity and stability, gives substantially greater consistency of results.[8]The units of toxin per vial are product specific, but they can be compared as demonstrated for the samples tested in Table 4.

3.4T47D cell culture bioassay

The potency of BoNTA was assessed by the T47D cell bioassay based on the dose-dependent cytotoxicity curve of the cells, evaluating the response with MTT. The statistical analysis of the assay was performed by a parallel line method, using CombiStats®. Biological activity of biopharmaceutical products was assessed by the T47D cell bioassay, which showed mean values 0.22% higher, compared to the MBA, as shown in Table 4, with non-significant difference, as calculated by the Student's t-test ($p > 0.05$). But, the excipient composition as well as the potential presence of complex (or other) proteins may affect the sensitivity of the assay. However the assay represents a contribution that can be explored and standardized as described.[29]

3.5Method application

The validated RP-LC method was applied for the determination of BoNTA in biopharmaceutical formulations, giving contents within 97.64 and 104.45% of the stated potency as shown in Table 4, with mean values 1.16% lower compared to the MBA. Samples diluted and/or spiked to obtain concentrations of 50% and 150%, were also analyzed (Table 4), showing the stability-indicating capability of the RP-LC. In addition,samples subjected to photodegradation and neutral hydrolysis conditions, were evaluated by the MBA and the cell culture assay which showed non-significant ($p > 0.05$) effects on the potencies (Table 4), but with changes in the chromatographic profiles that demonstrated the potential and importance of the RP-LC method. Moreover, peak 1 separated by the chromatographic method (Fig. 1, a, b), was collected and subjected to the MBA, showing typical effects of BoNTA. Thus the agreement between the results of the bioassay and the RP-LC method may represent an improvement, in attempts to develop alternative assays, to evaluate the profiles and the consistency of batches in clinical use.

Table 4. Comparative content/potencies evaluation of botulinum toxin type A in biopharmaceutical formulations, by the mouse LD_{50} bioassay, RP-LC method and T47D cell bioassay.

Sample		LD_{50}bioassay		RP-LC[a]		T47D bioassay[a]	
	Theoretical amount (U)	Potency%	Confidence intervals (P=0.95)	Main peak, %	Deamidated/ sulphoxides, %	Potency%	Confidence intervals (P=0.95)
1	100	100.04	88.10 – 112.10	99.19	0.76	99.24	91.40 – 109.20
2	100	103.89	89.20 – 114.70	101.43	0.66	102.37	93.70 – 110.50
3	100	98.14	86.30 – 109.30	98.18	0.48	101.06	89.90 – 109.60
4	100	99.23	85.70 – 110.20	97.64	1.16	101.18	93.50 – 109.70
5	100	103.30	91.50 – 115.90	101.72	0.83	104.13	94.20 – 112.40
6	100	101.45	92.10 – 111.90	100.36	0.99	99.17	92.40 – 107.30
7	100	98.26	87.10 – 113.10	99.95	0.79	100.16	93.20 – 108.50
8	100	107.93	96.00 – 122.80	104.45	1.34	106.70	97.60 – 114.40
Mean	-	101.53	-	100.37	0.88	101.75	-
SD[b]	-	3.37	-	2.18	0.28	2.59	-
9[c*]	-	53.17	41.10 – 64.40	52.36	26.41	53.23	44.70 – 61.80
9[d*]	-	148.16	133.7 – 166.30	151.87	0.12	151.44	147.70 – 172.30
9[e*]	-	104.22	91.60 – 119.60	82.26	12.16	101.72	93.60 – 110.60
9[f*]	-	99.34	87.30 – 112.80	66.74	18.37	101.57	95.30 – 108.20

[a] Mean of three replicates. [b] SD, standard deviation. Content of altered samples*: [c] 50%, [d]150%, [e] UV degraded, [f] Neutral hydrolysis.

3.6 Cytotoxicity evaluation

The cytotoxicity test was performed on degraded forms versus the intact molecule, in order to detect possible effects resulting from the instability of the samples during storage, showing responses for the NCTC Clone 929 cell line with mean values of IC_{50}=1.37 ± 0.02 U mL^{-1} and IC_{50}=0.87 ± 0.03 U mL^{-1} for the sample of BoNTA and for the photodegraded molecule, respectively, with significant differences (p < 0.05). Such evaluations are now necessary, mainly due to the recent concerns related to possible human undesirable effects of the degraded forms.[30]

4. Conclusions

The results of the validation studies show that the RP-LC method is specific, accurate, and sensitive with a QL of 0.16 U mL^{-1}. Separation of the BoNTA was achieved with a retention time of 11.4 min, and the method was successfully used for the analysis of products, showing comparable profiles and mean values of the estimated content 1.16% lower, compared to the MBA. The proposed method has the potential to reduce and refine animal assays, can be applied in high-throughput screenings to process control and to monitor degraded products, contributing to improve the characterization and quality consistency of the biological products.

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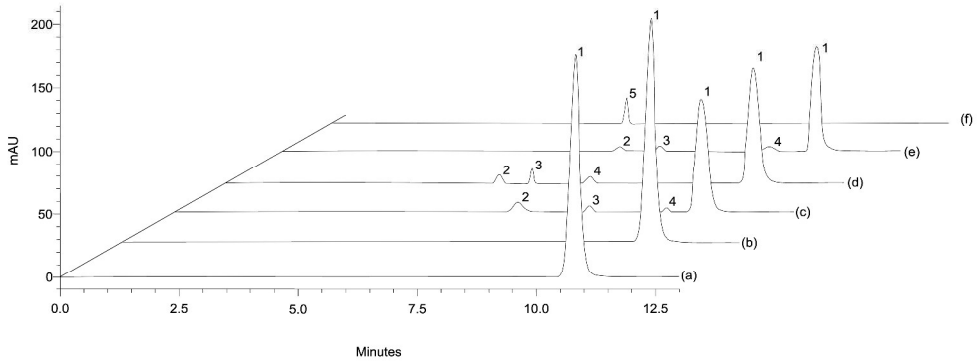

Representative chromatograms showing peak 1=Botulinum toxin type A; peak 2=deamidated; peaks 3-4=sulphoxides; peak 5=human serum albumin. (a) Reference of BoNTA; (b) Sample of biopharmaceutical formulation, and (c) after degradation by hydrogen peroxide; (d) after photodegradation; (e) after neutral hydrolysis; (f) placebo.
804x338mm (300 x 300 DPI)