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This paper is published in Journal of pharmaceutical and biomedical analysis, 2022, vol 210, p 114543

https://doi.org/10.1016/j.jpba.2021.114543

Abstract

Idarubicin (IDA) is an antineoplasic drug commonly used to treat hematologic diseases. Because of its relative lipophilic properties, this anthracyclin is also used in transarterial chemoembolization (TACE) as part of hepatocellular carcinoma (HCC) treatment. But TACE requires injected volume to be reduced to restrict systemic diffusion and side effects. The aim of this study was to determine the stability of a highly concentrated aqueous solution of IDA, stored in polypropylene syringes between 2°C to 8°C. Analyses were performed with an HPLC system combined with UV detector and mass spectrometer. Forced degradation was used to investigate potential degradation products. This study demonstrated 7 days of stability after storage in previously mentioned conditions. This conservation is long enough to anticipate drug preparation and facilitates pharmacy organization.

Keywords: Idarubicin, HPLC, stability study, mass spectrometry, antineoplasic

1. Introduction

Idarubicin (IDA) is an antineoplasic drug mainly used to treat myelogenous leukemia and chronic lymphocytic leukemia [1]. As other anthracycline drugs, IDA prevents DNA synthesis by interfering with the topoisomerase II enzyme. Structurally close to daunorubicin, the absence of 4-methoxy on the first aromatic cycle gives it more lipophilic properties (Log P IDA 1.9 *vs*. Log P Daunorubicine 1.73) (Figure 1) [2,3]. In fact, IDA demonstrates its superior cytotoxicity on hepatocellular carcinoma (HCC) cell lines over other chemotherapeutic drugs such as doxorubicin [4]. That is why it is currently used in transarterial chemoembolization (TACE) of HCC. TACE remains the first-line treatment in the intermediate-stage of unresectable HCC [5]. It consists of a chemoembolic injection directly in the artery feeding the tumor site. The injected substance is a water-in-oil emulsion of an antineoplasic drug mixed with appropriated oily phase (frequently lipiodol *i.e* poppy-seed oil) [6]. There are several advantages of TACE as compared to regular drug injection: the temporary embolic effect on the tumor-feeding artery and the longer retention of the drug in the vincinity of the tumor [7]. Moreover, radio-opacity of the emulsion can be used as an imaging biomarker.

Nevertheless, TACE requires some specific features. The injected volume has to be equivalent to the tumor volume. Indeed, final volume must remain around 10mL of injection according to clinical studies [8]. Beyond 20mL, some side effects have been reported, because of systemic diffusion of the cytotoxic drug [7]. Proportion of emulsion components has also to be respected. Boulin *et al.* [9] showed that the most stable emulsion was constituted with idarubicin and lipiodol with a 1:2 v/v ratio. This information implies that volume of the aqueous phase has to be reduced to ensure stability of emulsion along with a lower final volume. However, concentration of commercialized IDA solution is fixed at 1mg/mL meaning that the volume of drug solution to obtain a sufficient dose is unfortunately not compatible with the low requested volume. IDA

powder vials (Pfizer, NYC, USA) can be used to prepare a solution of higher concentration. However, manufacturers have not reported the stability of IDA at concentration higher than 1mg/mL. In this conditions drug stability is 48 hours after storage between 2°C to 8°C; or 24 hours at temperature up to 25°C as stated by the manufacturer.

The aim of this study was to determine stability of a high-concentrated aqueous solution of IDA, in specific storage conditions. On oncologist's request, we decided to especially study a 4 mg/mL concentration. Alike storage data from the manufacturer, storage was fixed between 2°C to 8°C in handlier packaging, in a polypropylene syringe to ease the future administration.

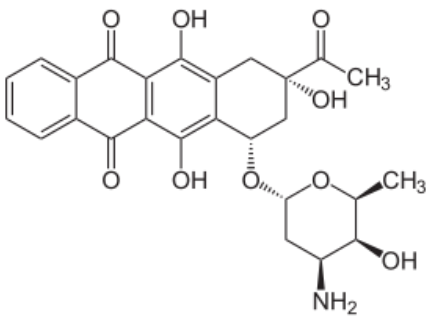

Figure 1: Chemical structure of Idarubicin.

2. Materials and method

2.1 Material and Reagents

IDA samples were all prepared from Zavedos® 10mg vials (Pfizer, NYC, USA). Vials were reconstituted with water for parenteral injection (Aguettant, Lyon, France).

Solvents used for chromatography were of HPLC analytical grade, including acetonitrile (VWR, Fontenay sous Bois, France), formic acid, and ammonium formate (Thermo Fischer Scientist, Waltham, USA). Water was obtained from a Prima reverse osmosis system (Elga Labwater, Antony, France). Chemicals used to prepare solutions for forced degradation studies were hydrochloric acid solution 37% (VWR, Fontenay sous Bois, France), sodium hydroxide (Merck, Darmstadt, Germany), and hydrogen peroxide (Thermo Fischer Scientist, Waltham, USA). For concentration measurements with the HPLC method, a Waters Alliance® e2695 HPLC system was used (Mildford, USA). It was composed of an autosampler, a gradient pump, a Waters photodiode array detector (PDA 2998) operating between 200nm to 400nm, and a mass spectrometer detector (QDA) operating for m/z between 50 to 1250 Daltons. Acquisition and operation data were acquired using Waters® Empower® 2 software (v9.3). Development of stability indicating method was performed with several types of equipment to obtain forced degradation of IDA. Indeed, light degradation was accomplished with a UV-A exposition of 366nm under a 300µW/cm² intensity (Chromato-Vue system model CC-20, Ultra Violet Product, Upland, California). Heat degradation was performed with a lab oven at 80°C (Memmert, Schwabach, Germany).

The samples were stored in a refrigerator (Liebherr, Kirchdorf an der Iller, Germany) at a temperature between 2°C to 8°C equipped with a temperature monitoring system (Sirius software, MMS, Bezons, France) fully qualified and validated according to the International Council for Harmonization (ICH) (*ICH guidelines*).

2.2 Sample preparation

Considering the potential exposure to cytotoxics, vials containing drug powder were all reconstituted with water for parenteral injection (Aguettant, Lyon, France) in a class III isolator system. Then, all dilutions and sample preparations were carried out in a class II chemical, fume hood, with single-use materials. Contaminated wastes were thrown out in adapted chemical trash.

For validation of the analytical method, forced degradation and stability studies, it has been decided to work in the same conditions. Thus, Zavedos® 10mg vials were reconstituted with 2.5mL water for parenteral injection to obtain a stock solution used to prepare all samples.

For stability study this solution was transferred into a 10mL polypropylene syringe (Becton Dickinson, Franklin Lakes, USA) closed with a plug.

2.3 Study design and sample storage

At predetermined time points (0, 2, 4, 20, 24, 48, 72, 168 hours) one sample per time condition was tested in triplicate analysis. Thus, eight syringes were prepared at the same time and then stored following ICH Q1A guidelines conditions (*ICH guidelines*), thus at 5°C ± 3°C in a refrigerator, and protected from light for seven days.

2.4 Chromatographic assay

2.4.1 Chromatographic conditions

IDA was analyzed by a reversed-phase high-performance liquid chromatography method adapted from Kaushik and *al*. [11]. IDA and the degradation products were resolved on a C18 BDS Hypersil Phenyl column (100mm x 4.6mm; particle size 3µm), which was purchased from Thermo Fisher Scientific (Waltham, USA). The mobile phase was composed of 20mM ammonium formate pH3 buffer (A) and acetonitrile (B), degassed in an ultrasonic bath. Gradient elution was set up as follows: 80% (A) and 20% (B) from the start to 5 minutes, 70% (A) and 30% (B) from 5 to 10 minutes, 60% (A) and 40% (B) from 10 to 25 minutes, then returning to 80% (A) and 20% (B) from 25 to 30 minutes. The applied flow rate was 0.7mL/min, and the injection volume of all samples was 20µL. The temperature of the injection chamber was set at 15°C, and the column temperature was maintained to 30°C.

2.4.2 Detection conditions (UV and Mass spectrometers)

For UV detection and quantification, wavelength was set at 254nm and 280nm. Then, QDA mass spectrometer with an electrospray ionization interface operating in the positive polarity (+ESI) was used to detect IDA, with a recording by mass scan in the range of 50 to 600m/z. Nitrogen (50psi) was used as both the curtain and nebulization gas.

2.4.3 Forced degradation experiments

Degradation products of IDA were studied after various stress conditions namely hydrolysis, oxidation, heat and photolysis. IDA (4 mg/mL) stock solution was used to prepare all samples. Areas of the obtained peaks were compared with 100µg/mL control solution injected the same day.

Degradation assays were performed as follows:

Alkaline hydrolytic degradation: 1mL of a 400µg/mL IDA solution was diluted with 1mL NaOH 0.01M, stored at 80°C for 30min, then neutralized with 1mL HCl 0.01M.

Acidic hydrolytic degradation: 1mL of a 400 µg/mL IDA solution was diluted with 1mL HCl 2M, stored at 80°C for 90min, then neutralized with 1mL NaOH 2M.

Oxidative degradation: 1mL of a 200 µg/mL IDA solution was diluted with 1mL H_2O_2 30%, stored at 25°C for 20 hours.

Temperature degradation: a solution of 200 µg/mL was stored in a lab oven regulated at 40°C for 25 days.

Photolytic degradation: a solution of 200µg/mL was exposed under a light source UV-A exposition of 366nm under a 300 W/cm² intensity during 25 days at 25°C.

2.5 Other analyses performed

2.5.1 Organoleptic inspection

The appearance and color of the preparation were assessed. Visual examination was performed to detect a potential precipitation or color change.

2.5.2 pH determination

The pH measurements were performed using a HI-1330 pH-meter (Hanna Instruments, Lingolsheim, France). Considering the potential toxicity of IDA, only two measures were carried out on the first day of the study, and seven days after.

3. Calculation, data analysis and acceptability criteria

3.1 Method validation for high liquid chromatographic study

The analytical validation method was performed following ICH Q2A guidelines (*ICH guidelines*). Quantitative assays were performed from 1mg/mL IDA solution, successive dilutions were carried out to obtain five concentrations of working solutions: 50, 75, 100, 125, 150µg/mL. The calibration curves were performed on three different days to demonstrate linearity of response. The method was considered linear if the correlation coefficient was over 0.99 for a mean standard curve.

The accuracy was performed using 9 determinations of three different concentrations levels (75, 100 and 125 µg/mL), measured three times a day, for three days. The accuracy was measured as the difference between the mean and the accepted true value. Then the percentage of accuracy was calculated from the ratio of the difference between true and observed value over the true value. Accuracy, for each concentration, had to be less than 5% to be accepted. The repeatability was assessed by measuring a 100µg/mL solution six times. The intermediate precision was evaluated by measuring six times a day for three days this concentration. Repeatability and intermediate precision were determined using the standard deviation of the repeated assays, the threshold value of acceptability was 5%.

3.2 Stability study criteria

IDA samples are considered stable if physical and chemical characteristics have not significantly changed. The pH was considered stable if its measure was found between plus or minus 0.2 units of initial pH measurement (pH meter precision: +/- 0.1 pH unit).

After sample dilution to reach 100µg/mL (theoretical concentration), concentrations of IDA solutions obtained during stability study were compared with initial concentration. The solutions were considered stable if IDA concentration did not fall under 90% of initial concentration, without any specific degradation products found according to forced degradation

4. Results

4.1 Chromatographic assays

4.1.1 HPLC method parameters

LC-UV chromatogram obtained following injection of IDA solution display a sharp and symmetric peak; the retention time was around 11min (Figure 2).

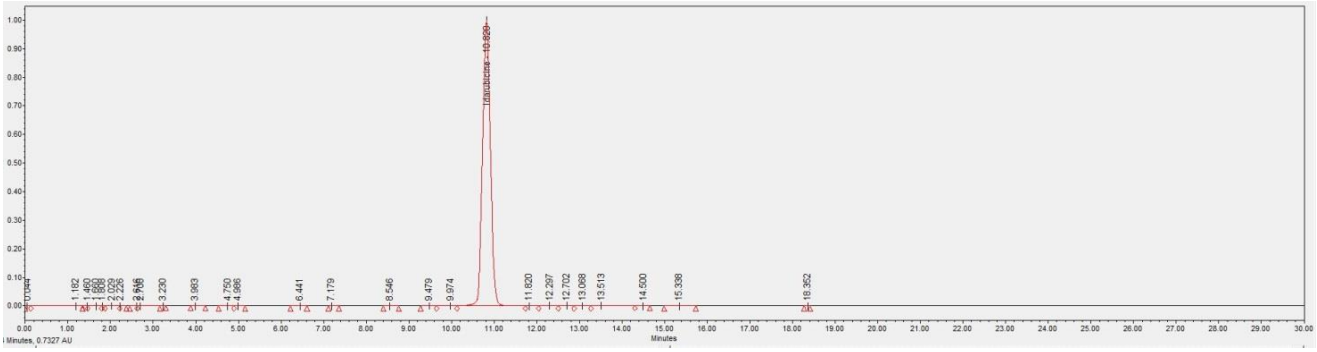

Figure 2: Chromatogram of IDA solution (100 µg/mL), UV detection at 254nm, from 0 to 30 minutes.

For the calibration curve the correlation coefficient (r^2) was found greater than 0.9999, so the method was considered as linear between 50 to 150µg/mL. The repeatability was approved with 0.19% of average variability. Variation ratings obtained were 1.15%, 1.05% and 0.41% for 75, 100 and 125µg/mL samples respectively. HPLC parameters and method were considered accurate since repeatability and intermediate precision were found lower than 5%. Limit of quantification (LOQ) determination is not necessary in the case of a stability study whose objective is to quantify near 100 % of IDA concentration, but for information estimated LOQ was estimated at 0.4µg/mL using the signal to noise ratio method.

4.1.2 Forced degradation

Forced degradation study is essential to approve a stability-indicating analytical method, indeed it is necessary to ensure that IDA is adequately distinguished from its degradation products. Figure 3 represents chromatograms obtained after various degradation conditions. As described in previous studies [11], IDA seems to be very unstable in alkaline medium and unstable in oxidative and strong acidic conditions. Dry heat and photolytic conditions do not engender chemical degradations but could induce physical modifications.

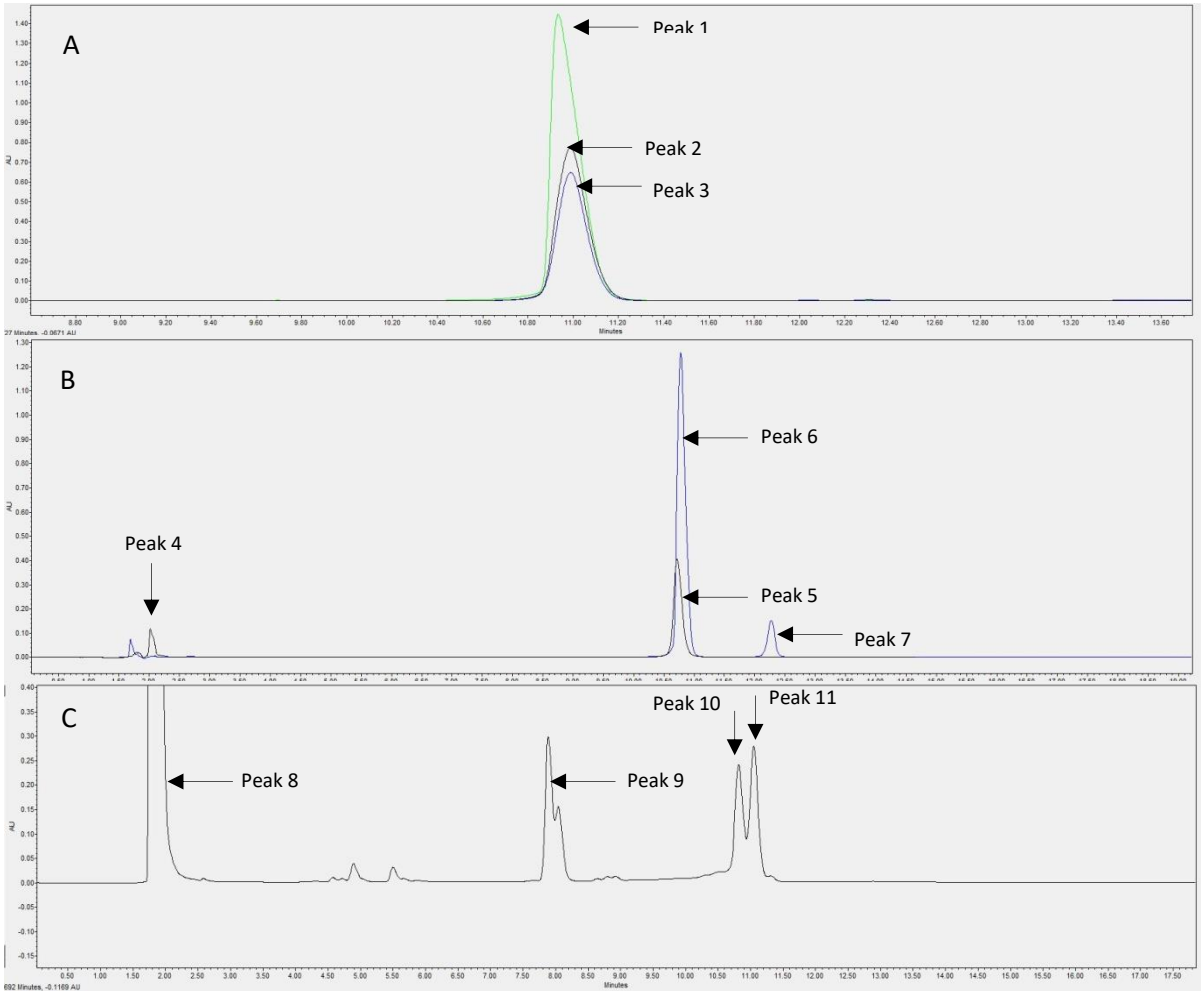

Figure 3: Chromatograms of IDA at 254nm after various degradation conditions. Panel 3A: stacks of control sample (Peak 1) and chromatograms obtained after photolytic or thermal degradation (Peak 2 and 3). Panel 3B: stacks of chromatograms obtained after alkaline or acidic exposition (degradation products Peaks 4 and 7 respectively) and remaining IDA (Peaks 5 and 6). Panel 3C: Chromatogram obtained after after oxidative conditions, IDA (Peak 11), H_2O_2 (Peak 8), degradation products (Peak 9 and 10).

Mass spectrometry was used to determine degradation products and pathways after forced degradation. The standard spectrum of mass analysis is presented in Figure 4.

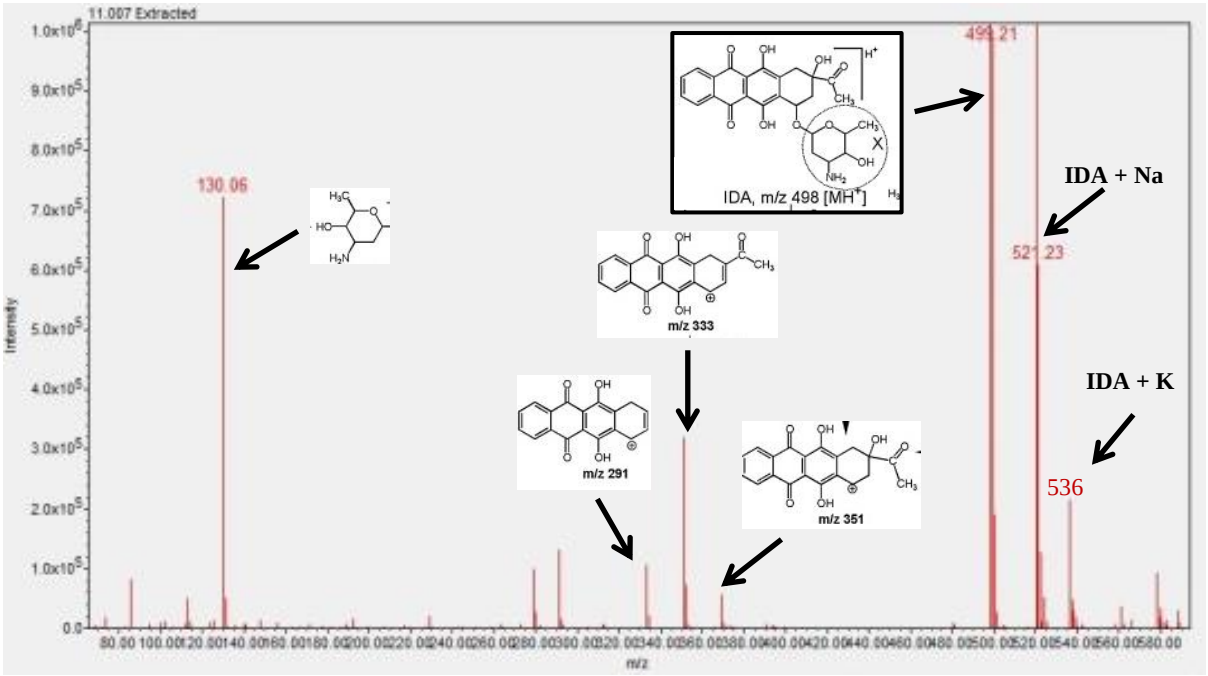

Figure 4: Mass spectra of IDA after 15V ionization energy.

In alkaline conditions, a product corresponding to *Peak 4* on Figure 3B was determined on spectra mass analysis (Figure 5) showing that the ether links with aminosugar was removed.

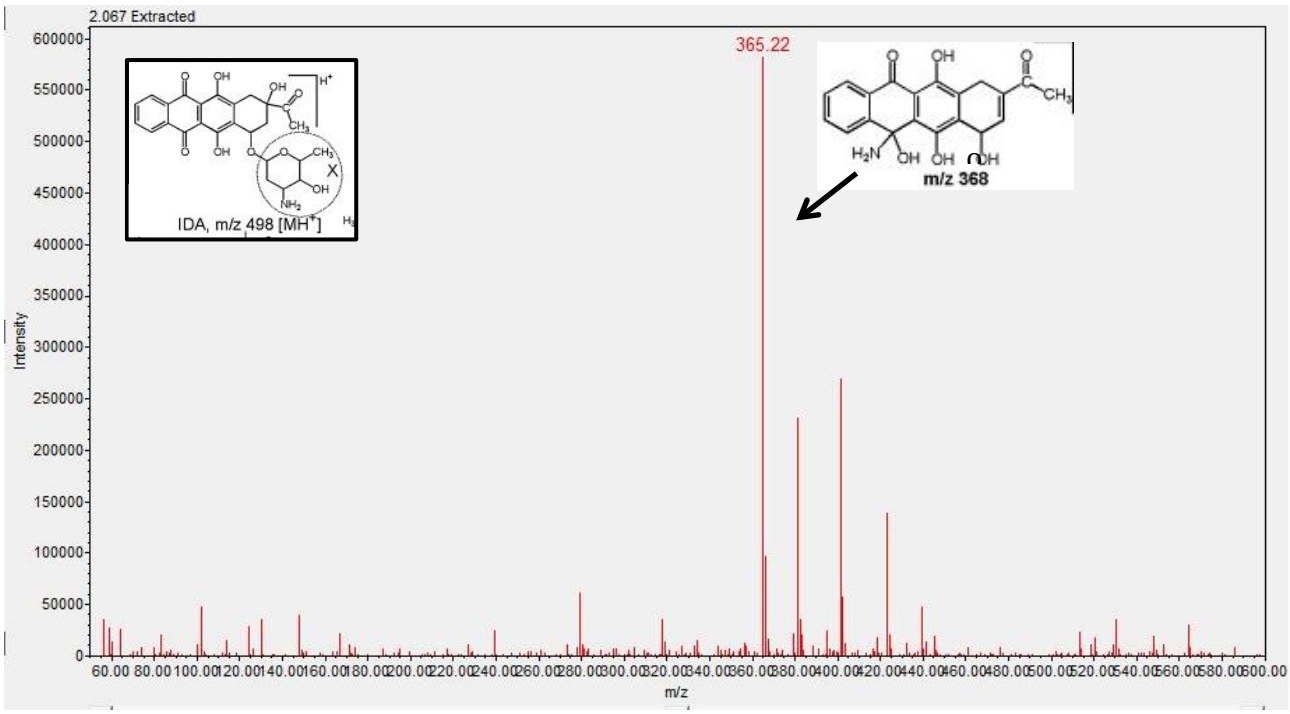

Figure 5: Mass spectra of IDA after alkaline conditions of degradation at 15V ionization energy (2.067 min).

In acidic condition a product corresponding *to* peak 7 was determined on spectra mass analysis (Figure 6).

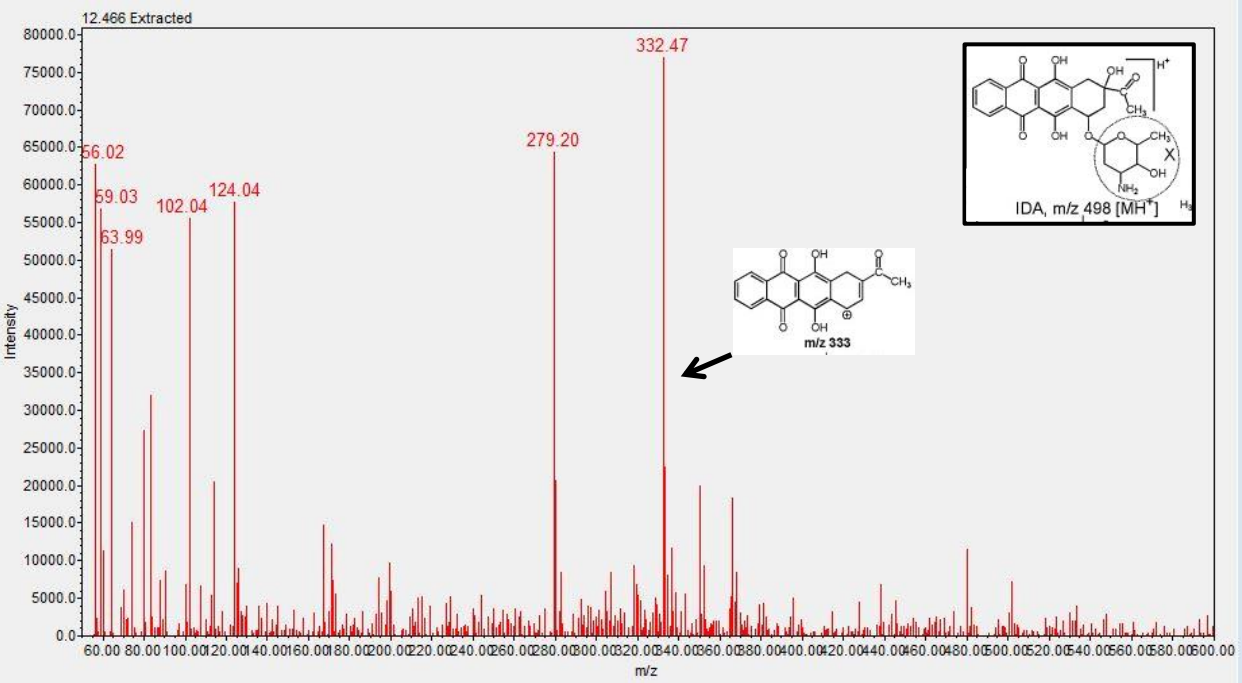

Figure 6: Mass spectra of IDA after acidic conditions of degradation at 15V ionization energy (12.466 min).

In oxidative degradation conditions, two peaks appear on Figure 3C (*Peaks 9* (RT=8 min) and *10* (RT=10.5 min)), their spectra analysis gives the following spectra on Figure 7 and Figure 8.

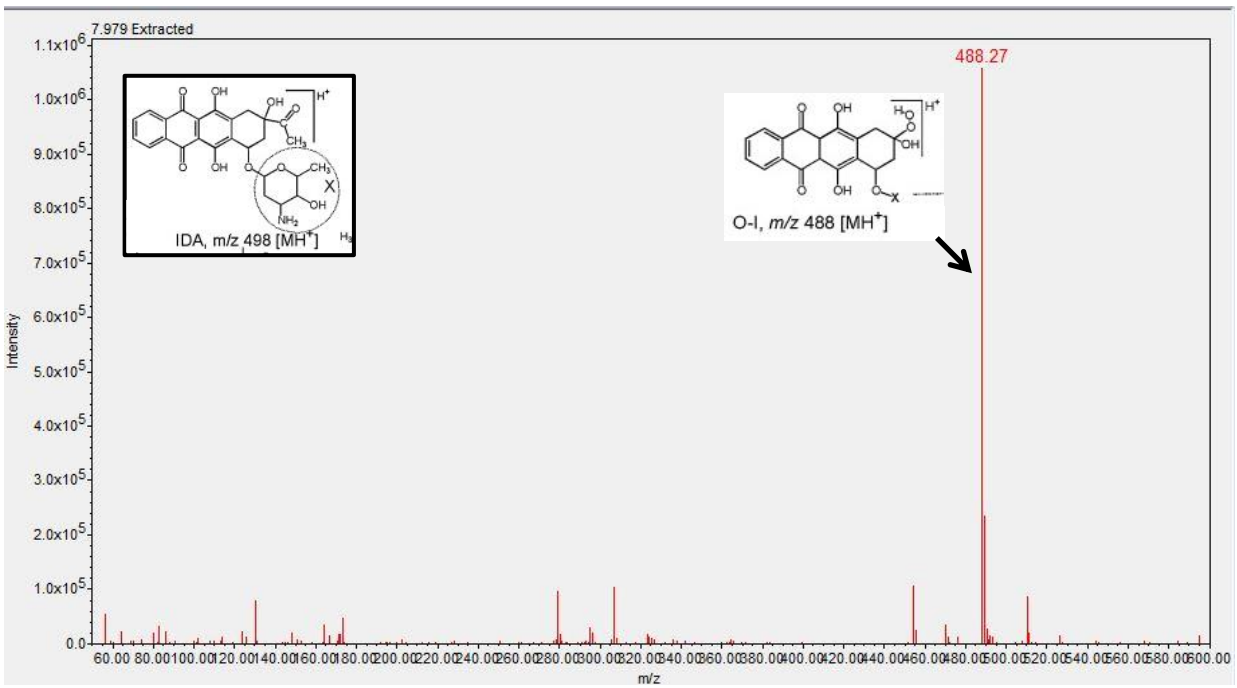

Figure 7: Mass spectra of IDA after oxidative conditions of degradation at 15V ionization energy (7.979 min).

The main degradation product obtained after oxidative conditions (Figure 7) is IDA without aminosugar and methyl function with rearrangement of ketone and hydroxyl function. In Figure 8, other minors degradations products were presented because of a very low-intensity signal.

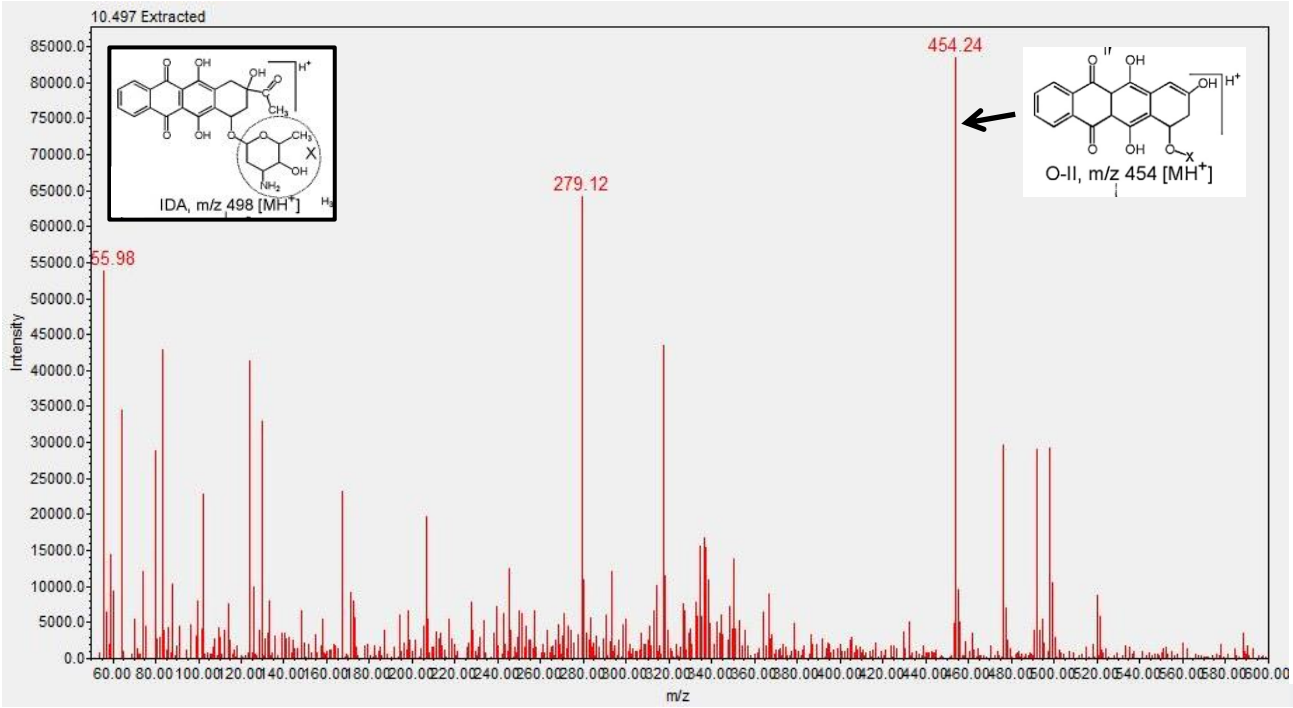

Figure 8: Mass spectra of IDA after oxidative conditions of degradation at 15V ionization energy (10.497 min).

In figure 3C, in the oxidative condition of degradation one supplementary peak appear, *Peak 11* (RT=11.2min) its spectra analysis give the following spectra in Figure 9.

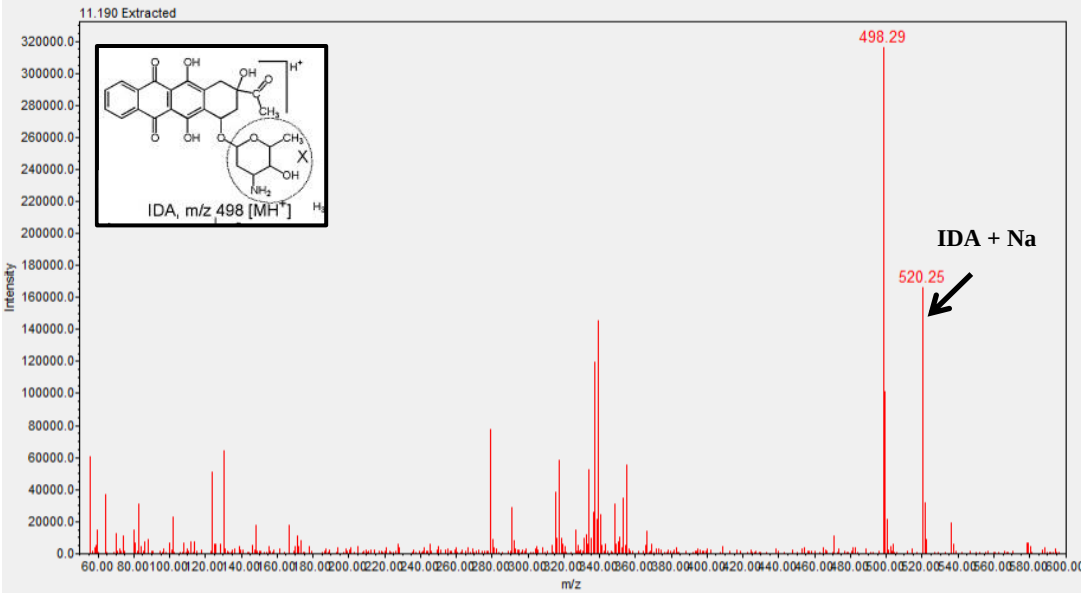

Figure 9: Mass spectra of IDA after oxidative conditions of degradation at 15V ionization energy (11.19 min)

4.2 Relative concentrations and qualitative observations

Relative IDA concentrations were measured and are presented in Table 1. After seven days, there was no statistical difference between concentrations as compared to the first injection (*p= 0.098*). To demonstrate the purity of the peak and the absence of degradation products hidden below the peak, UV spectra comparison and mass spectrometric analysis were executed. UV spectra from 210 to 400 nm extracted at the apex of IDA chromatogram peak were overlaid to the initial solution (Figure 10). No other peak was present in a significant quantity from 0 to 30 minutes (Figure 11).

Table 1: IDA relative concentrations along time after storage between 2°C to 8°C in polypropylene syringe.

Triplicates	Expected Concentration (µg/mL)	Actual concentration (µg/mL)*	% initial concentration along time (in hours)						
			2	4	20	24	48	72	168
Sample 1	100.00	105.75	97.61	102.52	102.14	101.60	100.64	102.49	101.40
Sample 2	100.00	105.65	99.62	102.10	101.13	101.15	101.41	101.67	98.15
Sample 3	100.00	103.35	98.65	101.70	100.85	102.03	99.43	101.17	100.74
Average relative concentrations		104.91 ± 1.36	98.63 ± 1.02	102.25 ± 0.46	101.38 ± 0.70	101.59 ± 0.44	100.50 ± 0.99	101.80 ± 0.65	100.24 ± 1.89

*Calculated from average equation of the standard curves

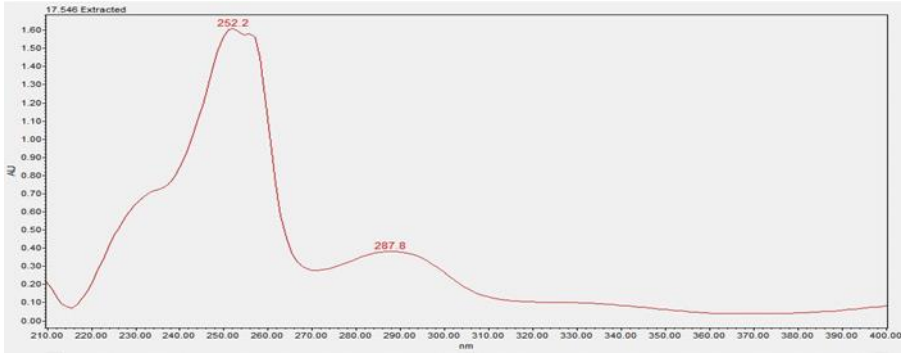

Figure 10: UV spectra of IDA from 210nm to 400nm after 7 days stored in a polypropylene syringe between 2°C to 8°C.

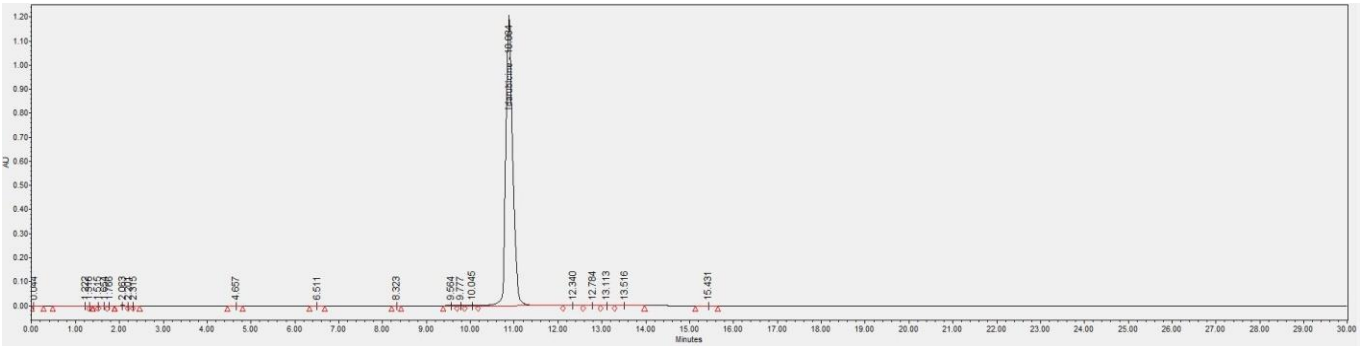

Figure 11: Chromatogram of IDA at 254nm after 7 days stored in a polypropylene syringe between 2°C to 8°C.

After 7 day of storage mass spectra were obtained from the IDA peak and showed few ions products (Figure 12). Products ions identified are m/z 498 [M + H^+] ion corresponding of 497Da (molecular mass of IDA), m/z 520 and m/z 536 are respectively identified as IDA in addition to Na^+ [M + Na^+] and K+ [M + K^+]. Some minor ions are also perceptible, such as m/z 130 probably

corresponding to an amino sugar, m/z 351-333-291 were also described in Kaushik and *al.* [11] from IDA control mass analysis.

No modification of the visual aspect of the test solutions was observed during the stability study. The pH measurements carried out on the first day and the seventh day were similar, with an average value of 4.8 ± 0.1 in ambient temperature.

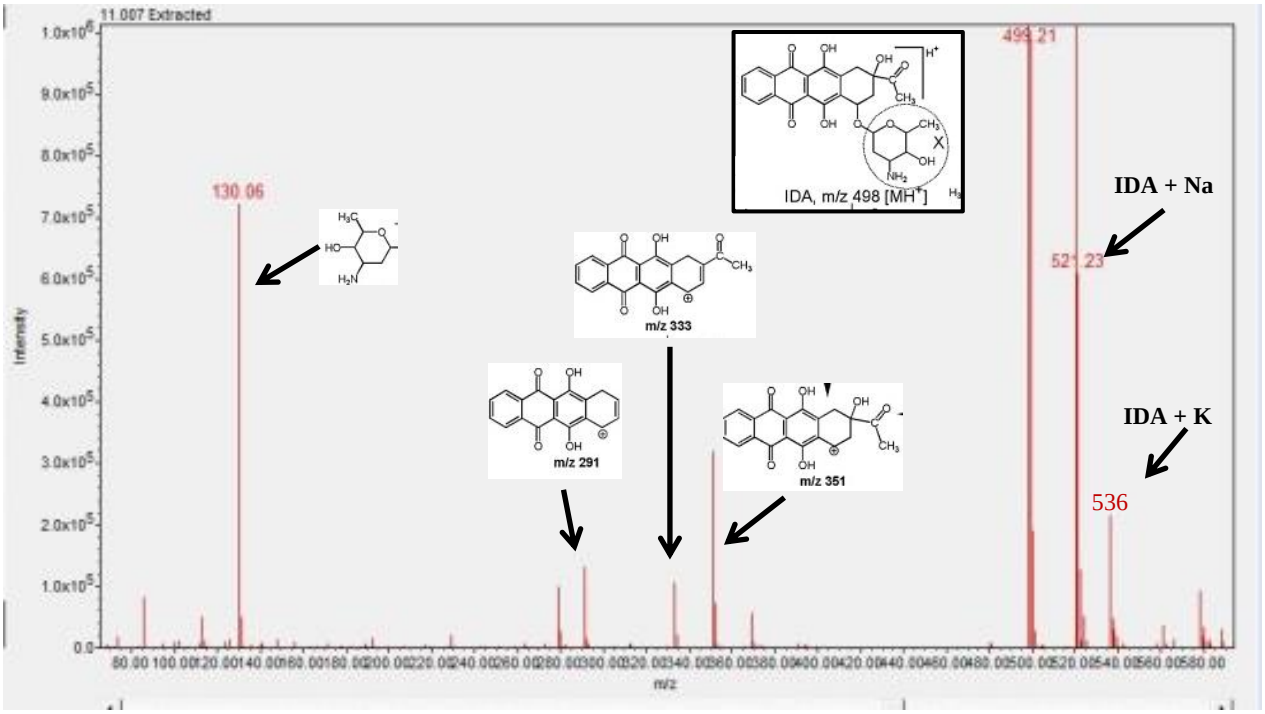

Figure 12: Mass spectra of IDA after 15V ionization energy after 7 days stored in a polypropylene syringe between 2°C to 8°C.

5. Discussion

This study was set up to know if high-concentrated aqueous solution of IDA is stable in polypropylene syringes, in order to facilate drug preparation and pharmacy organization.

All chromatographic parameters meet the acceptance criteria for the IDA assay.

Concerning the degradation products, Kaushik *et al.* performed a specific study on IDA degradation products in ICH conditions [11]. In this study the regular 1mg/mL concentration have been used. The exposure conditions (acidic, alkaline and oxidative and photodegradation) were comparable to ours. In our study, very similar degradation products were obtained (slight differences between molecular weights come from the techniques used to obtain ionized products) . Other studies with several anthracyclin compounds [12,13] were in accordance with our findings. Indeed, it was previously demonstrated that IDA and other anthracyclins follow the same degradation pathways: they are unstable in alkaline medium and susceptible to oxidative and acidic hydrolysis conditions. For example, the glusocamine seems to be always the first subtracted chemical group to the anthracycline main structure independently of the stress origin. Moreover, anthracyclines belong to an homogenous chemical group and only differ by their anthraquinone segment or carbohydrate moiety to modulate antitumor activity and limit cardiac toxicity [14].

Although, for photolysis and thermal degradation conditions, we observed a slight decrease in IDA concentration while this was not described in other studies [11] this may be due to the high concentrations used in our work.

This study constitutes the first step of the development of IDA high concentrated solution for TACE utilization. It could be improved by a microbiological stability study, as recommended by the GERPAC guidelines [15]. However, we could raise two main arguments to avoid a microbiological stability study (i) this preparation will be always made in aseptic conditions in

controlled areas of the pharmacy (ii) the delay of administration of idarubicine high concentrated solution will remain short after preparation (maximum 24 hours). If this delay is longer a microbiological study should be envisioned.

6. Conclusion

The HPLC stability-indicating method with forced degradation has demonstrated its suitability for testing the stability of IDA high-concentrated solutions stored in syringes. This study demonstrates that a 4mg/mL IDA solution remains chemically stable when stored in polypropylene syringes between 2°C to 8°C for at least 7 days. Degradation products following heat stress were also observed. No visual physical modification of the solution was seen during the study. This conservation time is long enough to anticipate drug preparation and facilitates pharmacy organization, including transport service to the wards.

Acknowledgment: The authors would like to thanks to Audrey Bourges, for her technical support.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

Conflict of interest statement: The authors state no conflict of interest. The authors have read the journal's publication ethics and publication malpractice statement available at the journal's website and hereby confirm that they comply with all its parts applicable to the present scientific work.

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