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STABILITY OF CO-AMOXICLAV RECONSTITUTED INJECTABLE SOLUTION

Abubakr O. Nur[1*], Alharith A. A. Hassan[1], Elrasheed A. Gadkariem[2], Zuhair Osman[1], Gamal K. M. Ali[3]

[1]Department of Pharmaceutics, Faculty of Pharmacy, University of Khartoum, Sudan.

[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Khartoum, Sudan.

[3]Central Medical Supplies, Khartoum, Sudan.

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***Correspondence for Author**

Abubakr O. Nur

Department of Pharmaceutics, Faculty of Pharmacy, University of Khartoum, Sudan.

ABSTRACT

Background: The use of co-amoxiclav injectable form among neonates and infants necessitates the usage of a part of the reconstituted drug solution or suspension, based on the relative low weights of this group of patients, with the remaining part of the reconstituted form being discarded and not used for subsequent doses due to unreliability in dosage form stability. This practice increases the cost of the treatment. **Objective:** This study aims at evaluation of the stability of reconstituted co-amoxiclav injectable solutions in order to use it in the most cost-effective manner. Methods: Physical, chemical and microbiological stability of reconstituted co-amoxiclav injectable solutions were evaluated at 4 different storage conditions which assembled by iterating each of temperature and lighting condition at two possible levels. Responses measured were degradation rate, colour and pH changes, shelf life and sterility of reconstituted drug solutions. **Results:** Degradation of both Clavulanic acid and Amoxicillin was found to enhance with increasing of storage temperature whereas only the degradation of Clavulanic acid appeared to be affected by storage light conditions. Time dependent changes in colour and pH were observed in reconstituted solutions under all storage conditions, especially in samples stored at 30˚C in indoor room lighting. Best storage conditions for reconstituted co-amoxiclav injectable solutions was determined as refrigerated at 4-8˚C in dark with associated shelf life of 1 hour. All reconstituted solutions complied with the test for sterility. **Conclusions:** Reconstituted co-amoxiclav injectable solutions should either be used

immediately after reconstitution or within a maximum of 1 hour of reconstitution if stored protected from light at 4-8˚C.

KEYWORDS: Co-amoxiclav, injectable solution, stability, shelf life, sterility.

INTRODUCTION

The stability of a medicinal product relates to its resistance to the various chemical, physical, and microbiological reactions that may change the original properties during transport, storage, and use. The term "stability" is often expressed in quantitative term of shelf-life which is the time during which the medicinal product is predicted to remain fit for its intended use under specified conditions of storage.[1, 2]

Co-amoxiclav, which is known to enhance the activity of Amoxicillin due to the incorporated Clavulanic acid as a beta-lactamase inhibitor,[3, 4] is among the widely used antibiotics in hospitalized neonates and infants suffering from severe infectious diseases based on the relative safety and wide spectrum of activity of the drug combination.[4, 5, 6]

In most neonates and infants, just a part of the reconstituted solution or suspension is used for a single dose due to their relatively low weights with the remaining part of the solution or suspension is usually discarded and not used for subsequent doses because of the unreliability in dosage form stability.[7, 8] This practice increases the cost of the treatment on patient; putting in the consideration this antibiotic is relatively expensive. So there is need for finding out the most cost-effective manner for using this antibiotic.[8]

Many of the cited reports on stability of combined Amoxicillin and Clavulanic acid in reconstituted drug preparations recommended the use of the reconstituted preparations within four hours at ambient temperature or within eight hours if stored at 4°C which is in accord with a published report of Medical and Healthcare products Regulatory Agency (MHRA) dealing with the same matter.[9, 10, 11]

In Sudan, however, the use of this effective antibiotic faced challenges represented by the medication cost in addition to the practice in paediatric hospital that results in increasing further the medication cost as a consequence of using a new vial for each dose.

This work aims to investigate the effects of temperature, indoor light, and medium pH on stability and sterility of reconstituted co-amoxiclave injectable solutions using the shelf-life

criteria in order to determine the best storage conditions and, therefore, the most cost-effective manner of using such drug combination.

MATERIALS AND METHODS

Materials

The following materials were utilized in the present study

Phosphoric acid(Bernd Kraft GmbH- Germany), hydrochloric acid(VMR International Ltd. England), sodium dihydrogen orthophosphate anhydrous (Surechem Products Ltd. England) and sodium hydroxide(Fisher Scientific Ltd. U.K.) were analytical grade and were used as purchased. Tablets of phosphate buffer pH 7 and phthalate buffer pH 4 were products of Fisher Scientific Ltd. UK and were donated by CMS. Sudan. HPLC-grade methanol was a product of ScharlauChemie S.A. Spain. Other materials are different grades obtained from different commercial sources.

Amoxicillin trihydrate and Clavulanate Potassium were working standard of Shin Poong Pharmaceutical Co., Ltd. (Korea) with potency of 86% and 605Mg/ mg, respectively, and were donated by GMC. Ltd. Sudan.Co-amoxiclav vials for injection of the brand name Julmentine® were kindly provided by Gulf Pharmaceutical Industries, UAE. Labeled content claim per vial is sterile 1g of Amoxicillin as Amoxicillin sodium and 200mg of Clavulanic acid as potassium Clavulanate.

For sterility test materials, Thioglycollate Medium USP (culture medium Lot No. 967542), Tryptone Soya Broth USP (Soybean-Casein Digest Medium, Lot No. 983099) and USP Buffered 0.1% w/v solution of sodium chloride-peptone pH 7 were products of Oxoid Ltd., Hampshire, England. The first two medium were used as culture medium and prepared according to manufacturer instructions while the later was utilized as a diluent.

Methods

Experimental design

Based on the study objectives, 2^2 full factorial design was selected where two factors, namely, light condition and temperature were investigated at two possible levels each for their influence on stability of reconstituted Co-amoxiclav injections. Dark and indoor room light were set as levels for light factor whilst 30°C and 4-8°C were selected as levels for temperature factor. The design composed of four possible storage conditions (refrigerated at 4°C-8°C in indoor light, refrigerated at 4°C-8°C in dark, at 30°C in indoor light and at 30°C

in dark) and within each experimental run, four vials were examined. Selection of vials and the order of runs were done randomly.[12]

Samples preparation and processing

For each storage condition, vials were reconstituted with 20 ml of sterile water for injection (according to the manufacturer's instructions). Directly after reconstitution, 200 µl from each of the four vials of the nominal concentration 50 mg/ ml of Amoxicillin and 10 mg/ ml of Clavulanic acid were withdrawn using micropipette, transferred into 50 ml volumetric flasks and completed to volume using distilled water. 20 µl of the last formed solution were used in duplicates for the HPLC based drug assay. Vials were then introduced into a cooled incubator (ES110 nÜve, Turkey) adjusted to the required conditions of temperature and light.

The source of light consists of five cool white fluorescent lamps of one foot length (Tazen, China) that fixed inside two cooled incubator at a distance of about 17 cm from vials to give the highest possible illumination in order to simulate lighting of pharmacies, especially hospital pharmacies. Both light brightness (measured in Lux unit), wavelength and energy (measured in watt.hrs/ m^2) were determined at the beginning and at the end of the storage periods (4 hrs) for samples stored in light at either 4-8˚C or 30˚C by using photometer (Model 450-1 with multiprobe 550-2, USA) and spectrometer (Ocean Optics Inc. USB 2000 VIS/IR, with Ocean Optics software, USA) apparatus.

The pH of the Co-amoxiclav solutions in the vials was measured (Inolab®,level1,TÜV, Germany) immediately after the reconstitution and at the end of study. The vials in the other groups were manipulated by the same way with difference in the storage conditions in the cooled incubator and the process of samples collection for analysis was repeated at every predetermined time interval following the same manner.

HPLC method for drug assay

Chromatographic analyses of drug components in the combination was performed following USP official HPLC method[13] with some modifications in the flow rate. The HPLC system (Shimadzu, Kyoto, Japan) was equipped with Inertsil® ODS-SP column (4.6 mm x 15 cm, 5µm, GL Sciences Inc. Tokyo, Japan), a pump (LC-20AB, Prominence), CTO-20A, Prominence Column Oven, SIL- 20A, Prominence Auto sampler and a UV-VIS detector operating at 220 nm (SPD-20A, Prominence).

Mobile phase used consists of mixture of Methanol and pH 4.4 Sodium dihydrogen Orthophosphate buffer (5:95). This mixture was passed through a filter having 0.5 µm porosity and then degassed using the Ultrasonic device (Retsch, UR-275 D, GS, Type: T570, Germany). The buffer was prepared following USP method.(1) For equilibration of mobile phase with stationary phase, the flow rate was increased gradually from 0 to 1 ml/min in 0.1 ml increments. The equilibration needs approximately 10 column volumes (around 20 minutes).

Eluted peaks and corresponding specifications of Calvulanic acid and Amoxicillin were traced on a PC using LC-Solution software and terminal HP LaserJet printer.

Validation of the HPLC assay method

Since the official HPLC method for drug components analysis was performed with some modifications, the method was validated for linearity, selectivity, accuracy, reproducibility and suitability as stability indicating.

For linearity, 20 µl samples of standard solutions ranging in concentration of Amox:Clav from 20:2 to 500:100 µg/ml were injected directly into the chromatographic system using the autoinjector (SIL- 20A). Peak responses were integrated and calibration curves relating peak areas to the corresponding component concentrations were generated and inspected for linearity.

Concerning method selectivity, different solutions of standard Amoxicillin, Clavulanic acid and a mixture of both were prepared and subjected to acid hydrolysis with 1M HCl, thermal degradation on a water bath and photodegradation using a UV lamp at short wave length (254 nm). Samples of degraded solutions were chromatographed and the resulting chromatograms were inspected for appearance of additional peaks and changes in concentration, retention time and peak shape of the drug components peaks in relation to a fresh, non degraded samples.

The accuracy of the method was tested using standard addition method and the average % recovery was calculated. In addition, the reproducibility of the standard curves were tested over 5 days and system suitability criteria (theoretical plates, tailing, and resolution) were developed and evaluated to ensure consistent chromatographic performance, according to accepted analytical guidelines.[13, 14]

Sterility studies

Immediately after the end of stability study the remaining solutions of each group of vials were tested for their microbiological stability. Sterility test was performed using Membrane Filtration as a technique suitable for the product.[13]

Thioglycollate USP culture medium USP was used primarily for the culture of anaerobic bacteria, but also detects aerobic bacteria and was prepared by suspending 15 g in 1L of distilled water and boiling on water bath to dissolve completely. Five screw capped borosilicate glass bottles were filled each with about 80 ml of the medium and tightly closed. 15 g of USP Tryptone Soya Broth was dissolved in 500 ml of distilled water, mixed well, and distributed into 5 screw capped borosilicate glass bottles as final containers. The medium was used for the culture of aerobic bacteria and fungi.

14.63 g of Peptone diluent was dissolved in one liter of purified water, mixed well to form 0.1% w/v solution and distributed into 6 screw capped borosilicate glass bottles as final containers. The diluent was used for washing the membrane to remove any residue of the antibiotic.

Prepared culture media, Sartorius filtration apparatus (Sartorius AG 37070 Goettingen, Germany) and the membranes were autoclaved (LS-B50L, China) either at 121°C for 30 minutes (for the culture medium) or at 121 °C for 15 minutes (for filtration apparatus and filter).

Solutions of the co-amoxiclav vials were filtered aseptically under a Class A laminar-air-flow (BIOAIR Instruments, AURA VF 48, Siziano (PV), Italy) located within a Class B clean-room. The membranes were washed with the diluent (0.1 % w/v Peptone).The membranes were removed and cut aseptically into two equal parts. A one half was transferred to Thioglycollate Medium and the other to Tryptone Soya Broth. Then both media were incubated for 14 days at 33°C± 2 for Thioglycollate Medium and at 22.5°C ± 2.5 for Tryptone Soya Broth.

At intervals during the incubation period and at its conclusion, the media were examined for macroscopic evidence of microbial growth (turbidity).

Statistical data analysis

Mean, standard deviation and relative standard deviation (coefficient of variation) were employed to describe the HPLC assay method validation data. The calibration plots and the assay data were subjected to regression statistics for fitting and predicting product-moment correlation coefficient (r) and coefficient of determination (r^2) for model fitting and predicting regression coefficients in determination of order of degradation kinetics.

Inferential statistics based on two-way analysis of variance (ANOVA) was performed to analyze data derived from the 2^2 factorial design in order to estimate the effects of temperature and lighting condition on degradation of drug components. Computations were aided by software computer package STATISTICA 8 (Statsoft Inc., USA) and in all analysis, a probability p=0.05 was considered as a cutoff point for significant measures.

RESULTS AND DISCUSSION

Validation of HPLC stability- indicating assay method

HPLC chromatogram for simultaneous determination of Clavulanic acid (10 µg/ml) and Amoxicillin (50 µg/ml) shows corresponding sharp and well separated peaks for the two drugs at R_t of 3.98 and 6.88 min, respectively (Figure 1).

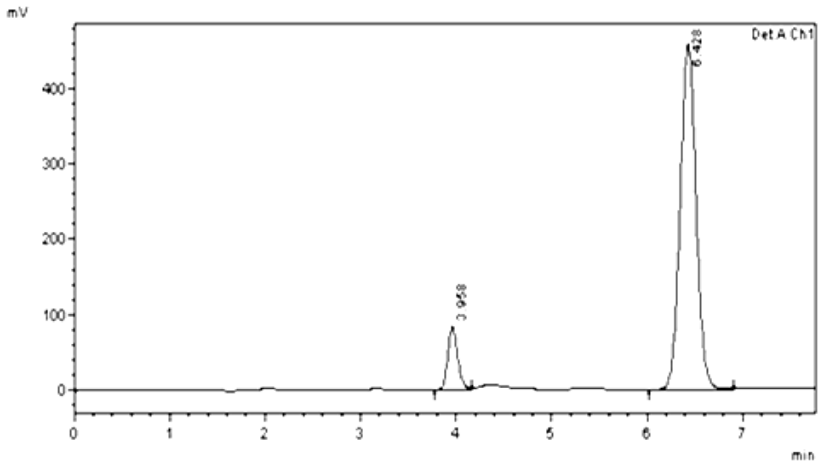
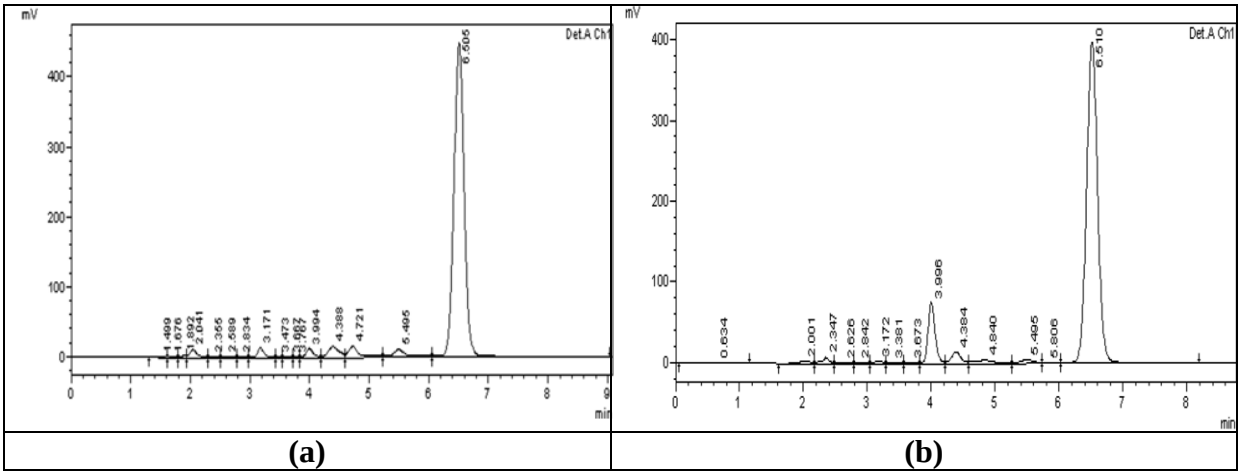


Figure 1. HPLC chromatogram for simultaneous analysis of Clavulanic acid (10µg/ml) and Amoxicillin (50µg/ml)

Calibration curves relating the different concentrations of standard Amox: Clav solutions to their respective peak areas were found linear with significant high correlation coefficients for both drugs at different concentration levels investigated (r ranged 0.9985-0.9995, $F_{cal}>>F_{crit}$).

HPLC chromatograms of different solutions of standard Amoxicillin and Clavulanic acid that subjected to acid hydrolysis and thermal degradation show sharp peaks of drugs separated well from their degradation products (Fig. 2a and b) and the same was observed with samples that subjected to photo-degradation. Such findings support the ability of the applied HPLC method to separate the degradation products (if any) from the parent drugs without overlapping or broadening of the peaks (specificity).



(a) **(b)**

Figure 2. Chromatograms for Co-amoxiclav solutions that subjected to (a) HCl hydrolysis for 2 hrs and (b) thermal degradation for 1 hr on a water bath.

On another hand, the demonstrated high values of % recovery of spiked drug components and the low values of relative standard deviation (RSD) associated with determination of that % recovery has guaranteed the accuracy of the method (99.2- 100.2% with RSD of 0.33% and 97.9-100.3% with RSD of 0.96% for Clavulanic acid and Amoxicillin, respectively).

Moreover, values of RSD associated with five determinations of both drugs within and between days were found to be < 2% and <5% respectively, authenticating precision, repeatability and reproducibility of the applied method of analysis.[12, 14]

Furthermore, values for resolution between drugs' peaks (7.34), the average theoretical plates of the two drugs (6262 and 6773 for Clavulanic acid and Amoxicillin, respectively) and the tailing factors determined from 7 runs for both drugs (<1.5) appear within the required limit of the official USP method for system suitability.[13]

Content of drug components in reconstituted Co-amoxiclav injectable solutions

Degradation profiles of drug components in reconstituted Co-amoxiclav injectable solutions that stored under different storage conditions are depicted in Figures 3 and 4.

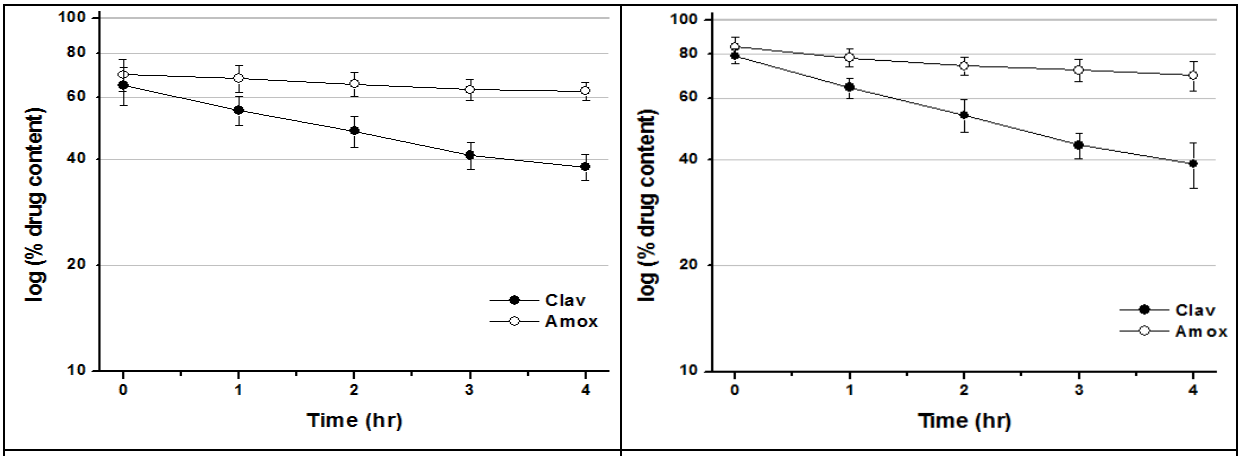


Figure 3. Degradation profiles of Clavulanic acid and Amoxicillin from reconstituted co-amoxiclav injection that stored at 4-8˚C in (a) dark and (b) indoor room light for 4 hours

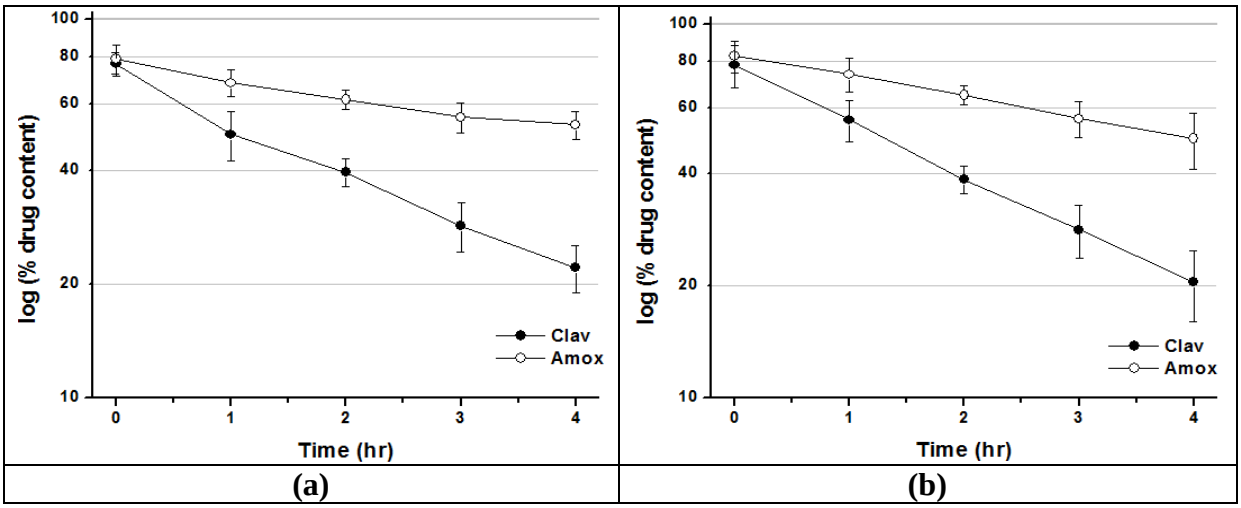


Figure 4. Degradation profiles of Clavulanic acid and Amoxicillin from reconstituted co-amoxiclav injection that stored at 30˚C in (a) dark and (b) indoor room light for 4 hours

From the two figures, it might be apparent that none of the drug components remained within the official limits (loss ≤ 10%) after one hour in all studied conditions. Moreover, it appears that the loss in Clavulanic acid is higher than that of Amoxicillin.

In fact, both components share the β-lactam ring which is believed to degrade by cleavage as a consequence of hydrolysis.[15] However, in contrast to Clavulanic acid, presence of large group side chain [2-amino-2-(p-hydroxyphenyl) acetamido] attached to a fused ring system β-lactam thiazolidine of Amoxicillin at the 6-position renders it more resistant to be attacked

by either water or hydroxide ion by virtue of steric hindrance. This observation agreed with previous works which consider Clavulanic acid as the determinant component for stability of this combination.[9, 10, 16, 17]

It is worth mentioning that Amoxicillin in injectable Co-amoxiclav is found in form of sodium salt which is very soluble in water while in oral form is found as trihydrate which is slightly soluble in water.[4] This makes Amoxicillin more available and liable to hydrolysis in injectable form and explains the marked difference in its stability between injectable solution and oral suspension form.[16, 17] In addition to that, pH of Amoxicillin sodium salt solution is basic, in contrast to that of trihydrate which is acidic, causing the hydrolysis to be more aggressive.

Evidenced by Figs. 3 and 4 that Clavulanic acid is more affected by changing in storage temperature rather than light condition where upon storage at 30˚C for 4 hours, Clavulanic acid measured a content of 20-22% (Fig. 4a and b) which is almost half that displayed with storage at 4-8˚C (refrigerated) (38-39%) for the same time period regardless the light conditions (Fig. 3a and b). This, in turn, is confirmed by statistical analysis of effect estimate for factors in the 2^2 factorial design which support that storage temperature has a more profound influence (p= 0.0232) on % content of Clavulanic acid than that of light condition (p= 0.0452), though both factors are influential whereas the effect of the pooled linear influence of both factors appears of no consequence (p= 0.6336).

Similarly, effect estimate for the two factors encourages the assumption that storage temperature has a considerable influence on % content of Amoxicillin (p= 0.0116) followed by the linear interactive influence of both temperature and light (p= 0.0341) with light condition, as an individual factor, receiving the least influential effect (p= 0.0484). Therefore, refrigerated Amoxicillin solution stored in light exhibited 70% content (Fig. 3b) which account for 1.4 times that measured by drug solution stored in light at 30˚C (Fig. 4b) and 1.1 times that showed by drug solution stored refrigerated (4-8˚C) but in dark (Fig. 3a).

Degradation kinetics of Clavulanic acid and Amoxicillin in reconstituted injectable solutions

Data of % content-time profile for both drugs were fitted to zero (linear) and first order (exponential) decay models in a search for degradation kinetics characterizing the two drugs (Table 1). Based on the associated high values of correlation coefficient (*r* ranged 0.984-

0.999), it might be assumed that both drug components follow the first order decay model (Table 1). For both drug components, refrigerated samples (4-8˚C) that stored in dark show the lowest degradation rate constant (k= 0.136 and 0.029/hr for Clavulanic acid and Amoxicillin, respectively) as compared to those stored at 30˚C in light which measure highest degradation rates (k= 0.336 and 0.129/hr for Clavulanic acid and Amoxicillin, correspondingly).

Table 1: Estimated parameters of the degradation kinetics of drug components in injectable solutions stored under different storage conditions.

Drug component	Storage Conditions	Zero order model		First order model		Estimated $T_{90\%}$
		K (%/hr)	R	K (hr^{-1})	R	
Clavulanic acid	In dark at 30˚C	13.01	0.962	0.304	0.995	12 min
	In light at 30˚C	14.24	0.978	0.336	0.999	10 min
	In dark at 4-8˚C	6.76	0.985	0.136	0.993	52 min
	In light at 4-8˚C	10.02	0.985	0.179	0.997	29 min
Amoxicillin	In dark at 30˚C	6.46	0.988	0.101	0.990	49 min
	In light at 30˚C	8.36	0.998	0.129	0.999	47 min
	In dark at 4-8˚C	1.90	0.986	0.029	0.988	217 min
	In light at 4-8˚C	3.47	0.975	0.046	0.984	116 min

K and r stands for order rate constant and correlation coefficient of fitting to the decay model, respectively; $T_{90\%}$ stands for time to reach 90% of the initial drug content

In contrast to many reported works held previously to assess stability of components in Co-amoxiclav drug combination, the investigated source of light in this study provides light that is similar in brightness and energy to the ambient indoor room lighting. This might be important because any substance that is susceptible to photolysis is affected by certain wavelengths that confer specific activation energy required for that photolysis reaction.[18, 19] Within each storage condition, degradation rate constant demonstrated by Clavulanic acid is greater than that of Amoxicillin at least by 2-folds, supporting the assumption made before that Clavulanic acid is the least stable component of this drug combination.

In another occasion, time for decay to reach 90% of initial drug content ($T_{90\%}$) for both drug components under different storage conditions was estimated from k values associated with best fitted first-order equations (Table 1). As degradation rate of Clavulanic acid appears more higher than that of Amoxicillin, stability of the combination is, therefore, expected to be determined mainly by the degradation profile of the former. Calculated $T_{90\%}$ (Shelf-life) of Clavulanic acid, and consequently of Co-amoxiclav injectable solution is about one hour in

the most stable storage condition (4-8˚C in dark) and just 10 minutes in the least stable one (30˚C in indoor room light).

Whilst values of k and $T_{90\%}$ shown in Table 1 clearly ensure the role of temperature changes on the degradation rates of both drugs, the effect of light condition become more profound with the refrigerated samples.

Colour and pH changes in reconstituted Co-amoxiclav injectable solutions

With time, all reconstituted Co-amoxiclav solutions developed physical instability which is manifested in form of colour change in the sequence pale yellow, dark yellow, orange and dark brown without precipitation being observed in any of the stored samples (Table 2).

The results demonstrated that colour change was aggressive and faster in reconstituted drug samples that stored at 30˚C in either dark or light condition. It should be noted, however, that drug samples showed enhanced and profound colour changes are those which characterized by enhanced chemical degradation rates (samples stored at 30˚C in either dark or light condition, Table 2). Accordingly, the observed physical instability (colour change) in these samples could possibly be related to the chemical instability.

Table 2: Colour and pH changes of the reconstituted co-amoxiclav injectable solutions under different storage conditions.

Storage Conditions	Time (hrs)	Colour	pH
In dark at 4-8˚C	0	Pale Yellow	8.60 ± 0.02
	2	Pale Yellow	
	4	Dark Yellow	8.54±0.01
In light at 30˚C	0	Pale Yellow	8.56±0.02
	2	Pale Yellow	
	4	Dark Brown	8.33±0.05
In light at 4-8˚C	0	Pale Yellow	8.51±0.03
	2	Pale Yellow	
	4	Dark Yellow	8.34±0.03
In dark at 30˚C	0	Pale Yellow	8.60±0.01
	2	Dark Yellow	
	4	Orange	8.24±0.01

The average starting pH values for all reconstituted solutions were almost similar and ranged 8.5-8.6 (Table 2). Moreover, under all storage conditions the pH decreases with time but remaining within the acceptable range for intravenous injectable solutions (pH 3-9). These results contradict findings of relevant works on stability of oral Co-amoxiclav suspensions in which an increase in pH with time was reported.[17, 20] In general, the pH of the drug

combination in aqueous solution is influenced by generation of pH-influencing degradants of Amoxicillin and Clavulanic acid in addition to contained degradants of both drug components when initially prepared as a result of the fermentation process by which they are prepared.[20] In the light of such scenario, the dissimilarity in pH changing profile between the present study and other reported ones could possibly be attributed to the unlike chemical form and source of the contained active ingredients of the investigated dosage forms.

It is observed that the least change in pH is associated with samples which stored refrigerated in dark condition (Table 2) and, therefore, change in pH could also be related to chemical and physical instability.

Sterility test

Sterility of parenteral products is important issue of their quality and it might not be surprising that a product could possibly fails the requirement of sterility though there is no evidence of chemical or physical instability.

In this study, no turbidity referring to microbiological instability has been observed in all the bottles of liquid culture media for both fungi and bacteria (Thioglycollate Medium, Tryptone Soya Broth). Therefore, all tested samples under different storage conditions have complied with the official test for sterility.

CONCLUSION

Storage of co-amoxiclav injectable solutions after reconstitution may be hazardous. It undergoes first order rapid hydrolysis especially Clavulanic acid, which is the least stable component, and might lose its stability in no more than one hour even if kept protected from light under refrigeration. The influence of storage temperature on degradation of the two drug components is evidenced whereas that of lighting condition appear to be associated mainly with degradation of Clavulanic acid. Both colour and pH of reconstituted solutions change with time under all storage conditions, especially in solutions stored at 30˚C in indoor room lighting. All reconstituted solutions complied with the test for sterility and best storage conditions for reconstituted co-amoxiclav injectable solutions was determined as refrigerated at 4-8˚C in dark with associated shelf life of 1 hour.

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