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Preparation and Evaluation of Injectable Dosage Form for Pemetrexed-Monoclonal Antibody Conjugate

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Abstract

Antibody-drug conjugates (ADCs), a powerful type of pharmaceutical medications that combine immunotherapy and chemotherapy. Pemetrexed is a multitarget antifolate agent that inhibits essential enzymes involve in purine and pyrimidine synthesis. Serious toxicities of pemetrexed are due to lack of selectivity despite its broad antitumor activity in a wide variety of solid tumors. The final I.V formulations were characterized by Zeta sizer (particle size analyzer), LAL test, sterility test, toxicity test, and by HPLC-UV for drug content and stability detection. The work revealed that our prepared AtZ-Pem conjugate products (both using PEG and gamma amino butyric acid GABA as linkers) showed different retention time by HPLC-UV, the particle size of the I.V dosage form revealed that AtZ-GABA-Pem had smaller size (465nm) and more uniform size distribution (PDI 0.451), while the particle size and PDI for AtZ-PEG-Pem were (1297nm) and (0.9912) respectively. The two prepared I.V preparations for AtZ-PEG-Pem and AtZ-GABA-Pem showed good stability after six months' storage at 6 °C, unacceptable stability results when both formulas stored at room temperature. Both I.V preparations for the conjugates were not toxic, sterile, and pyrogen free. The prepared injectable preparation could be used as a potential alternative to the available injectable preparations for Pemetrexed to improve its targeting and selectivity, hence reducing its serious side effects.

Keywords: Antibody, I.V, Pemetrexed, Atezolizumab, stability.

Introduction

The oral routes of administration for protein formulations are frequently problematic owing to issues like enzymatic degradation, poor permeation, variable pharmacokinetic and pharmacodynamic profiles, and low bioavailability, therefore parenteral administration, which now account for approximately eighty five percent of all biological delivery, are preferred [(1)]. Certain of these items are intended for subcutaneous self-injection by patients and thereby offer a variety of alternative sites for those patients needing more than dose [(2)], for example, Hizentra® (human immunoglobulin used for primary and secondary immunodeficiency syndromes) available as solution for subcutaneous injection in pre-filled syringe 5mL, 10mL, and 20mL in a concentration of 200 mg/mL [(3)]. The prefilled syringes created as liquid dosage forms for protein products have major limitations, such as their high instability due to physical and chemical deterioration, requirement for cold storage, and restrictive transportation. Based on decreased molecule mobility and disintegration in the dry state, the lyophilized form presents a preferable alternative to enhance stability [(4)].

The conventional methods in the formulation of tiny molecular weight pharmaceuticals are frequently inapplicable to the development of high molecular weight one (protein products), challenges encountered formulations of such products are related to pharmaceutical presentation form, size of dose, route of administration, and stability issue of such products [(5,6)].Antibody-drug conjugates (ADCs), a powerful new type of pharmaceutical medications that combine immunotherapy and chemotherapy. The German physician (Paul Ehrlich) first proposed the concept of ADC, assessed the antibody to a "magic bullet" that can recognize its target. Ehrlich's theory was assumed later when methotrexate was combined with an antibody to target leukemia cells [(7)]. The ADCs constructed by linker, covalently bind with antibody from one side, and from the other side covalently bind with the chemotherapy [(8)]. Most ADCs are formulated as lyophilize injectable I.V dosage form

to be reconstituted just immediately before injection [9, 10]. Pemetrexed (Pem) is partly lipophilic [11], practically insoluble in water with a half-life of 3.5hr. Due to lack of selectivity, it is a DNA damaging affecting normal and cancer cells. It is used to treat different cancers either as monotherapy or in conjunction with other chemotherapeutic drugs [12].

Atezolizumab (AtZ) is a humanized monoclonal antibody, exhibits antibody-mediated cancer cells destruction, recommended dose is 1200 mg (fixed dose) given as intravenous infusion over one hour every 21 day [13]. AtZ solubility is 50mg/mL, isoelectric point 6.6-7.2 [14].

The present study aims to formulate our previously prepared conjugates for AtZ and Pem as I.V injectable dosage forms and evaluate them to be a potential candidate to improve Pem selectivity towards lung cancer cell depending on the targeting of the conjugated monoclonal antibody (AtZ).

Materials and Methods

The two conjugates (AtZ-PEG-Pem, and AtZ-GABA-Pem) were prepared previously in our laboratory and characterized [15]. Acetonitrile, Trifluoroacetic acid, and Methanol, all are purchased from Alpha Chemika Co., India. Phosphate Buffer Saline purchased from Avonchem Co. limited, UK. Gel Clot Endotoxin Test Kit, Bioendo Co., China.

Methods

Preparation of pemetrexed-atezolizumab (Pem-AtZ) as I.V injection

Our previously prepared chemical conjugates of Pemetrexed (Pem) with monoclonal antibody Atezolizumab (AtZ) by using two types of linkers (PEG, and gamma amino butyric acid (GABA); each linker separately) named as AtZ-PEG-Pem and AtZ-GABA-Pem respectively were obtained as dispersion in 4mL PBS pH 7.4 [15], each obtained dispersion was transferred to a previously autoclaved glass vial container. Mannitol and sucrose were added in a ratio of 4:1 % (W/V) [16]. The glass vials were lyophilized using a CHRIST lyophilizer (ALPHA 1-2 LD plus, Germany) at -55°C and 0.07 mbar for approximately 7-8 hr [17].

Calibration curve for Pem by HPLC-UV

The HPLC experiments were performed by SYKAM (German) chromatographic system encompassed with a quaternary pump, thermal adjustment column compartment and a variable wavelength UV–VIS detector. Separation was performed on Octadecyl-silica (ODS) C18 (150 x 4.6 mm) column which is manufactured by Chemical Evaluation and Research Institute (CERI, Tokyo, Japan). Pemetrexed(Pem) stock solution was prepared in acetonitrile at a concentration of 2 mg/mL. Different working standard solutions in acetonitrile (2.5, 5, 7.5, and 10 ppm) were prepared. 100µL of each Pem standard solution was injected into the column. The calibration curve was constructed by plotting the obtained peak area versus each standard concentration, a value of correlation coefficient (R^2) near to 1 was evidence of an acceptable fit for the data to the regression line [18, 19].

Characterization of the prepared antibody-drug I.V. injection

Content assay determination by HPLC-UV detector

The HPLC analysis was carried out for pure Pem, PEG linker, GABA linker, pure AtZ, physical mixture (Pem with AtZ) to detect retention time for each one and compare it with retention time of both prepared AtZ-PEG-Pem and AtZ-GABA-Pem conjugates. Samples (100µl of each in acetonitrile) were injected into column of HPLC-UV detector. The flow rate 1mL/minute, temperature of the column was maintained at 37ºC, and the mobile phase was as follow:

For pemetrexed (Pem) and PEG linker (each one separately), isocratic mobile phase 0.03% Trifluoroacetic acid (TFA): Acetonitrile (ACN) (50:50) was used and detected by UV at 228 and 210 nm respectively [20,21]. For GABA linker, analysis: isocratic mobile phase Methanol: disodium hydrogen phosphate (40:60) was used and detected by UV at 280 nm [22].

For pure AtZ, analysis as follows: isocratic mobile phase 0.03%TFA: ACN (90:10) was used and detected by UV at 290nm [23].For physical mixture (Pem and AtZ) as well as for both (AtZ-PEG-Pem and AtZ-GABA-Pem) conjugates: gradient mobile phase, 0-3minute 50% aqueous TFA:50% ACN, 3-10 min 90% aqueous TFA:10%ACN. Detection by UV at 228 and 290 nm.

The concentration of Pem in each prepared conjugate sample was detected by dividing the obtained area under Pem peak (in each conjugate) to that of standard Pem peak and multiplied with concentration of standard Pem according to Equation 1 below:

$$\textit{Conc. of Pem in sample} = \frac{\textit{Pem area in sample}}{\textit{Pem area in stand.}} \times \textit{Conc. of stand. Pem} \quad \text{(Equation 1)} \ [24]$$

Particle size, and polydispersity index analysis

The average particle size (average particle diameter) and polydispersity index (size range of particle) were measured for the AtZ-PEG-Pem, and AtZ-GABA-Pem conjugates in comparison with pure Pem, and pure AtZ, after reconstitution the vial in normal saline, placed in 1cm diameter disposable cuvette, and measured immediately at room temperature and 90° fixed angle to give a suitable scattering intensity [25]. The work was done using Zetasizer-ZS, Malvern instrument (Malvern, UK).

Sterility test

It is done for the two prepared AtZ-Pem conjugates injectable preparation vials to ensure that these products are free from all living microorganisms. Direct inoculation method was used where each preparation (after reconstitution with 5mL normal saline) drawn aseptically, transferred into a vessel of culture medium, mixed with the medium (Casein Soybean Digest CASO for detecting aerobic bacteria and fluid Thioglycolate Medium for detecting anaerobic/aerobic bacteria, each one separately), medium provides nutritious material, kept at a favorable temperature and incubated for not less than 14 days. The growth of microorganisms in the medium (if present) will be observed visually and can be indicated by a turbidity in the medium [26].

Pyrogen test by Limulus Amebocyte Lysate test (gel clot)

The Limulus Amebocyte Lysate test (LAL) is a blood cells (amoebocytes) aqueous extract, obtained from the horseshoe crab (*Limulus polyphemus*). LAL test is commended in pharmacopeial monographs as the authorized, not time consuming, easy to perform, and accuracy test for detection bacterial endotoxins (Gram-negative and Gram-positive). Materials are Gel Clot Lysate, Gel Clot Standard, and LAL reagent water.

The test was as follows: tested preparations (AtZ-PEG-Pem, AtZ-GABA-Pem; each one separately) mixed with Gel Clot Lysate, another test tube contains only positive control (Gel Clot Standard), and third test tube contains only reagent water as a negative control. The three test tubes were incubated at 37°C for one hour. After incubation, check for the gel by turning over the test tube. If the material remains firm in the bottom of the test tube, it means gel clot has formed (positive, containing pyrogen) and if the material stays as clear solution, it means gel has not formed (negative, pyrogen free) [27].

pH measurement

It is done for the two prepared formulations to follow pH after reconstitution of the lyophilize products.

Stability study

A-Stability of the prepared conjugates in plasma by gel electrophoresis

The 300µL of AtZ-Pem conjugates (AtZ-PEG-Pem, and AtZ-GABA-Pem, each one separately) were mixed with 700 µL human plasma, then 100 µL from each mixture was added to 100 µL water and incubated at standard incubation condition (5 % CO_2, 95 % humidified air at 37 ° C) for continuous 7 days. After each day incubation time, the sample was withdrawn, quickly frozen in a deep freezer and prior to analysis the samples were thawed at 37 ºC [28]. The samples were examined using our SDS- PAGE gel electrophoresis method previously published [15].

B-Effect of storage temperature on the stability of the prepared conjugates injectable preparations

The vials containing 250mg of each lyophilized conjugates (AtZ-PEG-Pem, and AtZ-GABA-Pem) conjugates injectable preparations of AtZ-Pem were stored at 6°C, and at room temp 25 °C, for a period of 6 months [29]. Stability was determined through assessment of the peak of analyte, monitor any degradation products using previously mentioned HPLC-UV method.

In-vivo preliminary toxicity test

Calculation of animal dose

The dose was calculated according to Pemetrexed therapeutic dose which is 500mg/m^2 [30] (Pem human therapeutic dose regarding the human average weight equal 60kg and height 1.62m) [31], animal dose was calculated by using animal (mouse) and human correction factor which is equal to 3 and 37 respectively. The calculated animal dose was found to be 166mg/kg as explained in Equation 2 below [31].

$$\boldsymbol{HED}\left(\frac{\boldsymbol{mg}}{\boldsymbol{kg}}\right) = \boldsymbol{Animal\ dose}\left(\frac{\boldsymbol{mg}}{\boldsymbol{kg}}\right)\boldsymbol{X}\frac{\boldsymbol{Animal\ Km}}{\boldsymbol{Human\ Km}} \quad \text{(Equation 2)}$$

Where HED: Human Equivalent Dose, Km: Correction factor specific for each species

Work design

The work was done according to the ethical committee in Iraqi Center for Cancer Research, Mustansiriyah University to check mortality if it is caused by giving preparations or not., using 3 months age mice. The test was done for the AtZ-PEG-Pem and AtZ-GABA-Pem conjugates to follow any sign of toxicity for 96hrs [32].Fifteen mice weighing ≈ 25g were divided into three groups (each group with five mice). Group1, and group 2 received injectable dose equivalent to 166mg/kg (after reconstitution with normal saline) of AtZ-PEG-Pem and AtZ-GABA-Pem respectively. Group 3 was a negative control (without treatment). All mice were kept in the animal house under environmental temperature of 25 ºC, mice had free access to water and food and terminated at the end of experiment.

Results and Discussion

Calibration curve for Pem by HPLC-UV detector

The results showed a linear correlation between Pem peak area and concentration with correlation coefficient (R^2) 0.999 as illustrated in Figure. 1

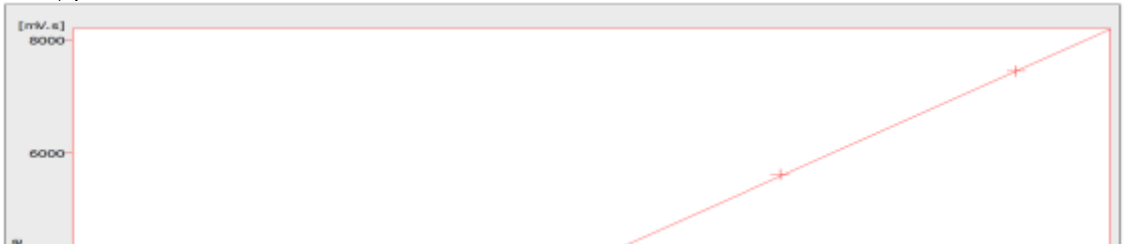


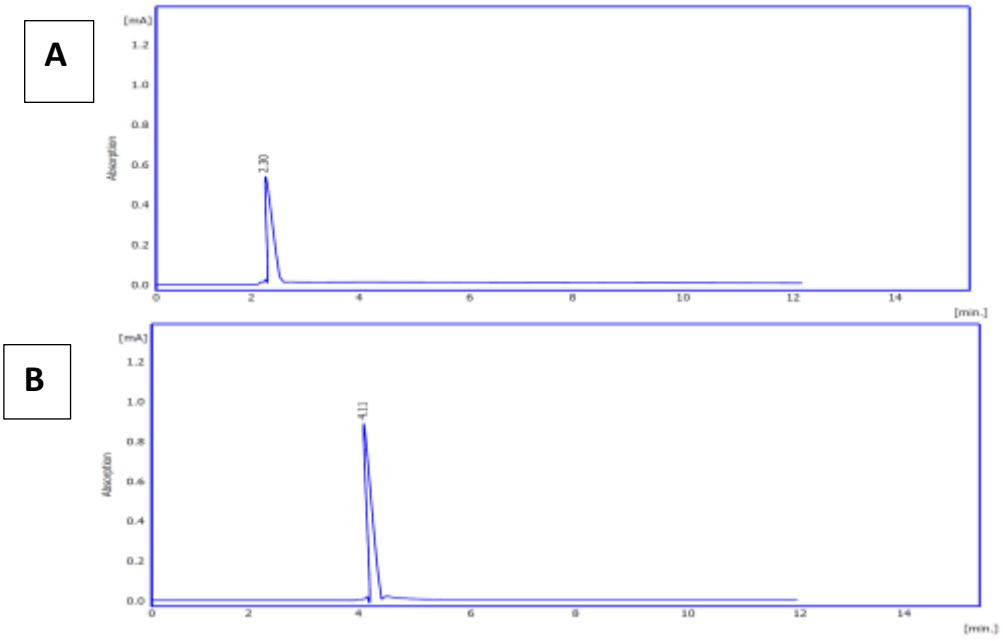
Figure 1. Calibration curve of pemetrexed by HPLC-UV

The concentration of Pem in the prepared conjugates (AtZ-PEG-Pem and AtZ-GABA-Pem) was calculated by injecting 100µL of a 20 ppm of each sample into the column, the obtained peak area was (965.88 mAU.s) for AtZ-PEG-Pem conjugate and (650.19 mAU.s) for AtZ-GABA-Pem conjugate.

The calculated Pem concentration was 1.29ppm and 0.871ppm for AtZ-PEG-Pem and AtZ-GABA-Pem conjugates respectively, which mean 155mg AtZ-PEG-Pem conjugate contained approximately 10mg of Pem, while 229mg AtZ-GABA-Pem conjugate contained approximately 10mg of Pem.

The HPLC chromatograms (each sample at their λ max) for AtZ, Pem, GABA linker, PEG linker, physical mix of Pem and AtZ, AtZ-PEG-Pem conjugate, and AtZ-GABA-Pem conjugate, are demonstrated in Figure. 2 Physical mixture (Pem and AtZ) displayed two peaks, first peak at 2.8min (at 228nm λ max), and the second one at 4.31min (at 290nm λmax), these two peaks are related to pure Pem and pure AtZ respectively indicating no interaction between them in the physical mixture.

The conjugate product AtZ-PEG-Pem displayed a peak in retention time 2.08 min at 228nm, and the second peak in retention time 6.10min at 290nm, same result was obtained for AtZ-GABA-Pem conjugate at 2.15min and 6.2min respectively, it was apparent that the newly conjugates products (AtZ-PEG-Pem and AtZ-GABA-Pem) displayed different retention time than their pure molecules; indicating that HPLC method used is efficient for analysis of our prepared conjugates. This result is in accordance with another study in which Trastuzumab-Emtansine conjugate product showed two peaks at 252nm (Emtansine) and 280nm (Trastuzumab) [33].



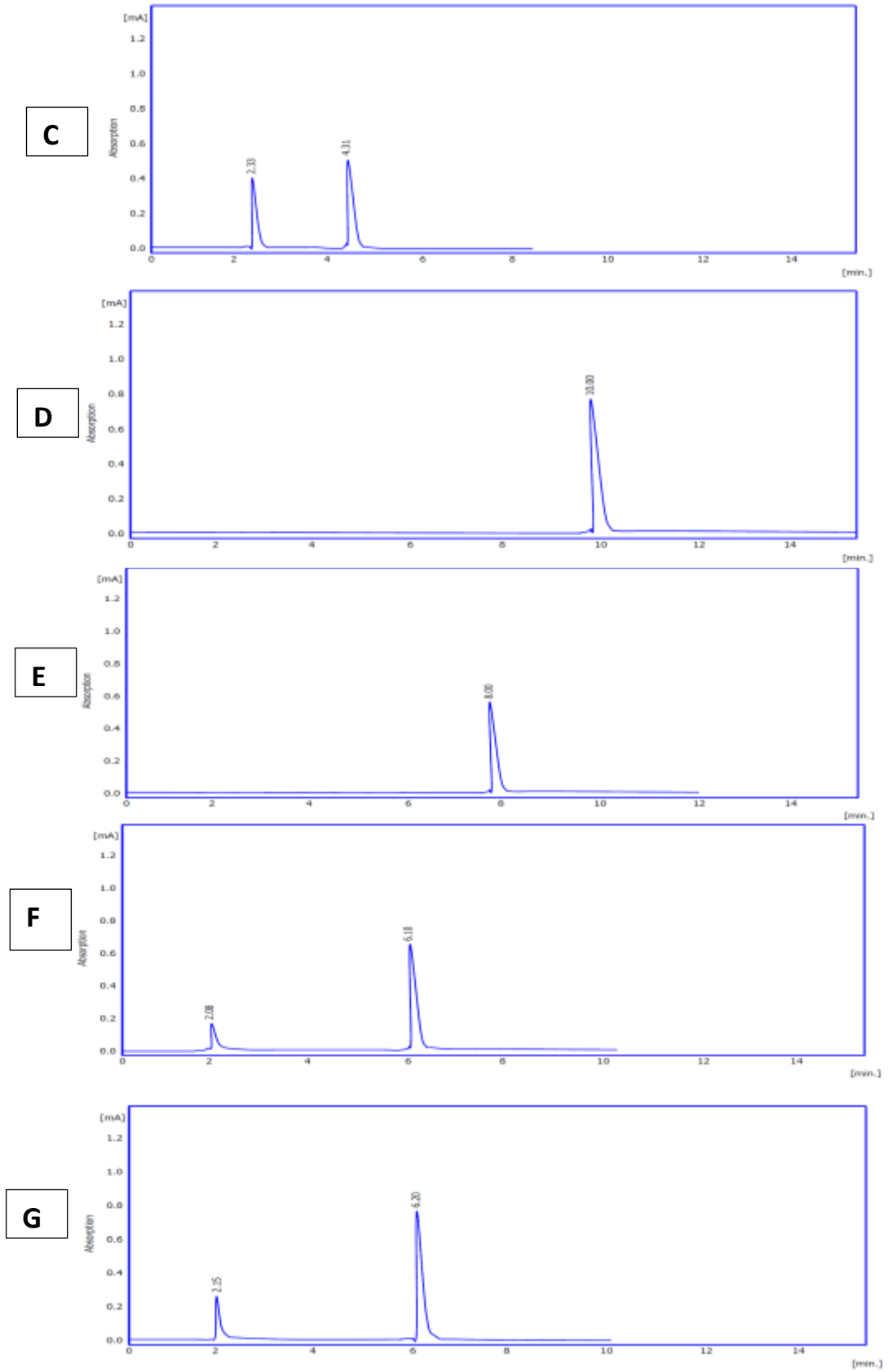


Figure 2. Representative HPLC chromatograms for (A) pure pemetrexed, (B) pure antibody, (C) physical mixture of pemetrexed ana antibody, (D) pure PEG, (E) pure GABA, (F) AtZ-PEG-Pem conjugate, and (G) AtZ-GABA-Pem conjugate.

Characterization of the prepared antibody-drug conjugates as injectable preparations

The two types of the prepared AtZ-Pem conjugates injectable preparations (using PEG or GABA linker) were characterized as follow:

Particle size, and polydispersity index analysis

Table 1 shows that the average particle size of the prepared AtZ-GABA-Pem were notably smaller than the average particle size of AtZ-PEG-Pem, this result could be due to the difference in molecular organization resulted from linker effect, the increase in molecular weight is associated with increase in particle size [34]. In addition, PEG conjugation to antibody masks the surface of the protein and is associated with increases in the molecular size [35]. Both preparations were within the acceptable approximate particle size for drug via injectable preparation [36,37].

The Polydispersity index (PDI) value of (0.05-0.7) is desirable for uniform distribution of particles [38], while PDI value more than 0.7 to less than 1 is deemed to have a broad distribution of particle size [39]. The prepared AtZ-GABA-Pem conjugates showed a uniform distribution for particle size.

Table 1. Particle size, and PDI

Sample	Average diameter (nm) ±SD	PDI ± SD
Pemetrexed	2111±36.08	6.494±0.11
Lyophilize Atezolizumab	750.5±14.71	0.7452±0.0145
AtZ-PEG-Pem	1297±19.86	0.9912±0.0152
AtZ-GABA-Pem	465.5±4.26	0.451±0.004

Sterility test

Both CASO (Casein Soybean Digest) medium and Fluid Thioglycolate medium was clear without turbidity observed, indicating that the prepared injectable preparations of AtZ-PEG-Pem conjugate and AtZ-GABA-Pem conjugate were free from living microorganism (anaerobic/aerobic bacteria) [40].

Pyrogen test

The incubated test tube of each conjugation formula showed no gel formation and remained as a flow down liquid, indicating that the prepared two injectable preparations passed the test successfully.

pH measurement

The pH of the two prepared injectable preparations of both conjugates (AtZ-PEG-Pem, and AtZ-GABA-Pem; each one separately) was measured after reconstitution with water and was found to be (7.4±0.044) and this is compatible with pH of body fluid.

Stability study

A-Stability of the prepared injectable preparations for both antibody-drug conjugates in human plasma by SDS-PAGE electrophoresis

The results in Figure. 3 show that AtZ-PEG-Pem conjugate remained stable for up to 7 days (bands of conjugate available at protein ladder 180KDa) while AtZ-GABA-Pem conjugate remained stable for 3 days indicating the contribution of PEG in stabilizing the conjugates was higher than GABA. GABA is a small molecule that is susceptible to proteolysis, whereas PEG is a polymer known for its stability and resistance to enzymatic degradation [41]. Therefore, GABA linkers are more prone to cleavage in the presence of proteases, resulting in fragmentation of the ADC on SDS-PAGE, while PEG linkers remain intact.

B-Effect of storage temperatures on the stability of injectable preparations of both conjugates

The pure AtZ chromatogram (as received) showed one peak at 4.2min which is the same peak after 6 months storage at 6 °C and this indicating good stability, while pure AtZ chromatogram at room temperature revealed a degradation product after 4 weeks which are illustrated by black arrows in (Figure. 4) and this agreed with previous reported data [42]. Pure Pemetrexed showed to be stable at room temperature and refrigerator temperature with no degradation products as in Figure. 5 similarly to that reported data [43].

The stability chromatogram of AtZ-PEG-Pem and AtZ-GABA-Pem conjugates (Figure. 6) showed no degradation products or appearance of a new peak after 6 months storage at 6 °C indicating good stability of both prepared injectable conjugates at refrigerator temperature. While degradation was observed (black arrow Figure. 6) when AtZ-PEG-Pem and AtZ-GABA-Pem injectable conjugates preparations stored at room temp, these results indicating that the antibody drug conjugates degradation could be generated within short term storage at room temperature because ADC in general bearing instability issues which may be related to linker degradation when stored out of refrigerator [44]. Accordingly, both injectable preparations for both conjugates should be stored at refrigerator.

Toxicity test

After 4 days, there was no mortality among the tested mice after receiving 64.325 mg AtZ-PEG-Pem and 95.035mg of AtZ-GABA-Pem (equivalent to 4.15 mg pem dose) indicating safety of the injectable preparations for both prepared conjugates.

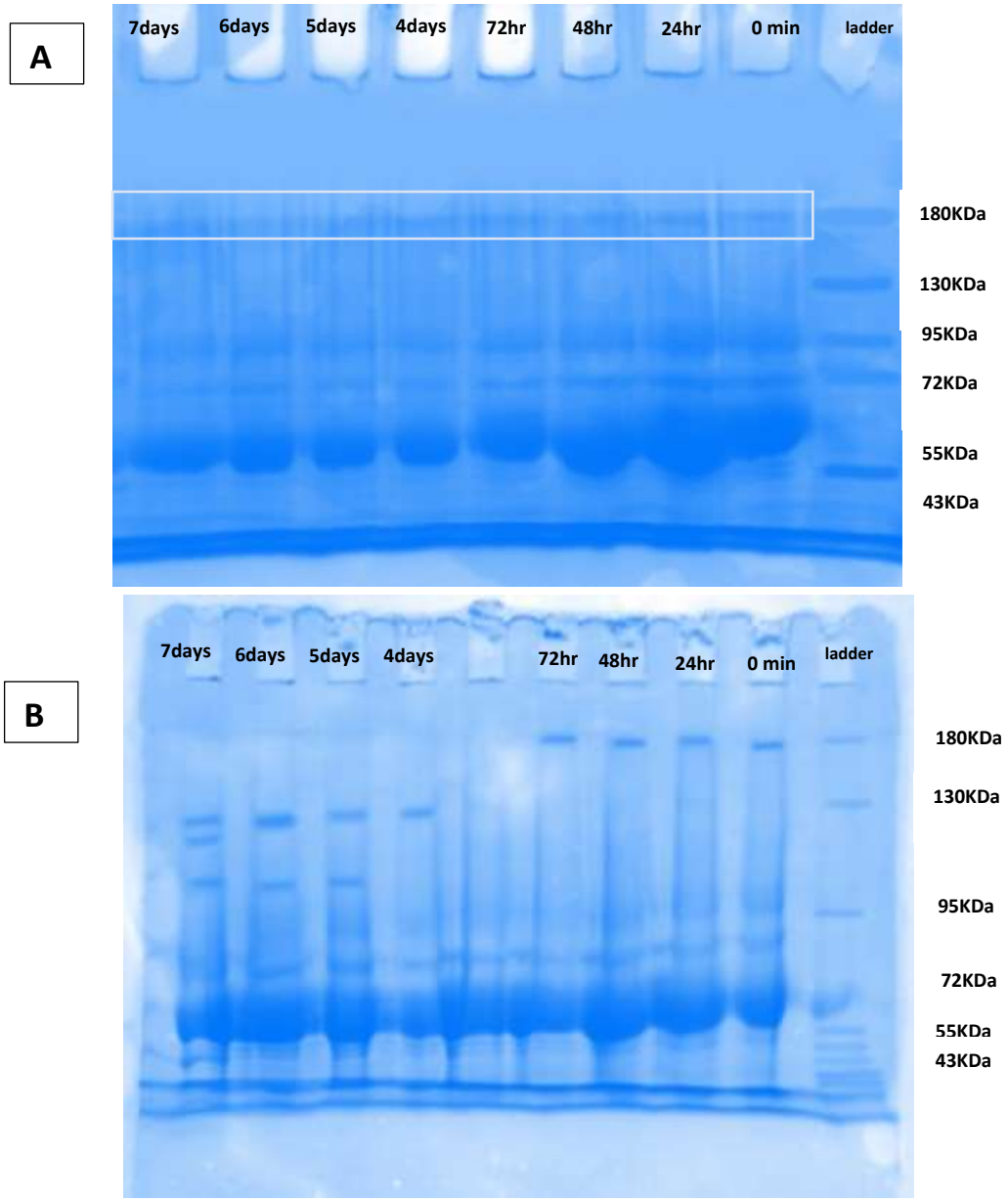
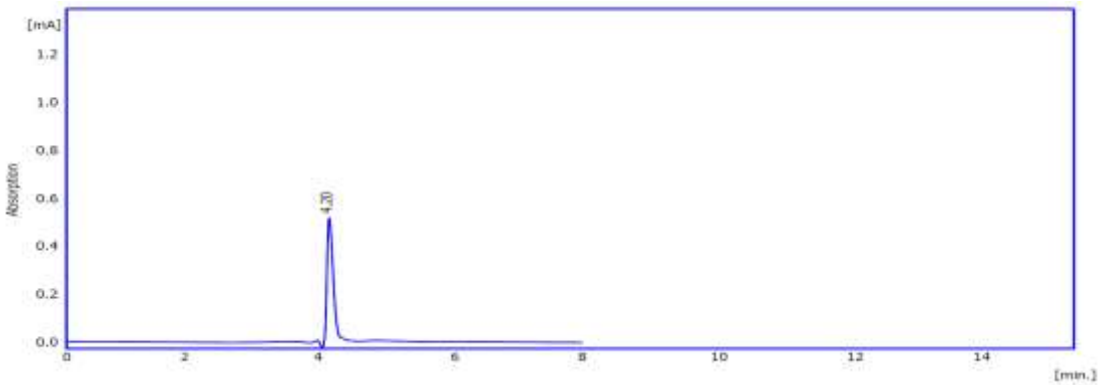


Figure 3. Stability of Pem-AtZ conjugate in human plasma by SDS- PAGE electrophoresis: (A) AtZ-PEG-Pem conjugate and (B) AtZ-GABA-Pem conjugate in which, Lane1(Ladder) Lane2(the conjugate at 0 min before storage) Lane3(conjugate at 24h) Lane4(conjugate at 48h) Lane5(conjugate at 72h) Lane6(conjugate at 4 days) Lane7(conjugate at 5 days) Lane8(conjugate at 6 days) Lane 9 (conjugate at 7 days).



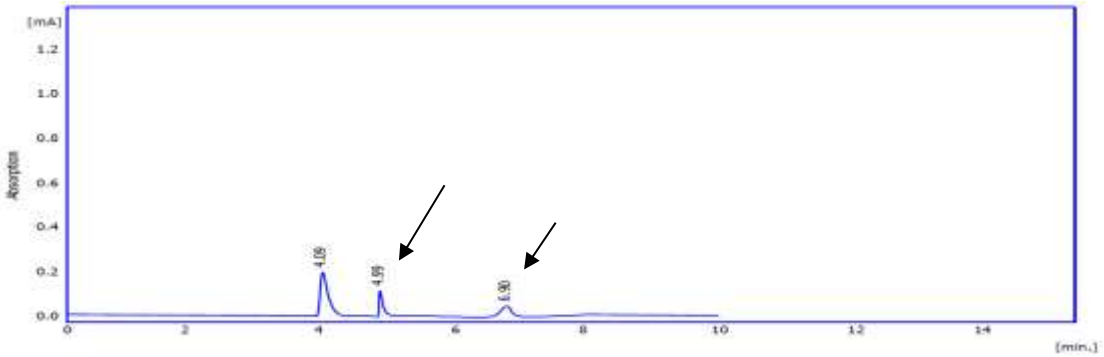


Figure 4. The effect of storage temperature on the stability of pure AtZ, arrows represent appearance of new peaks (degradation of original product)

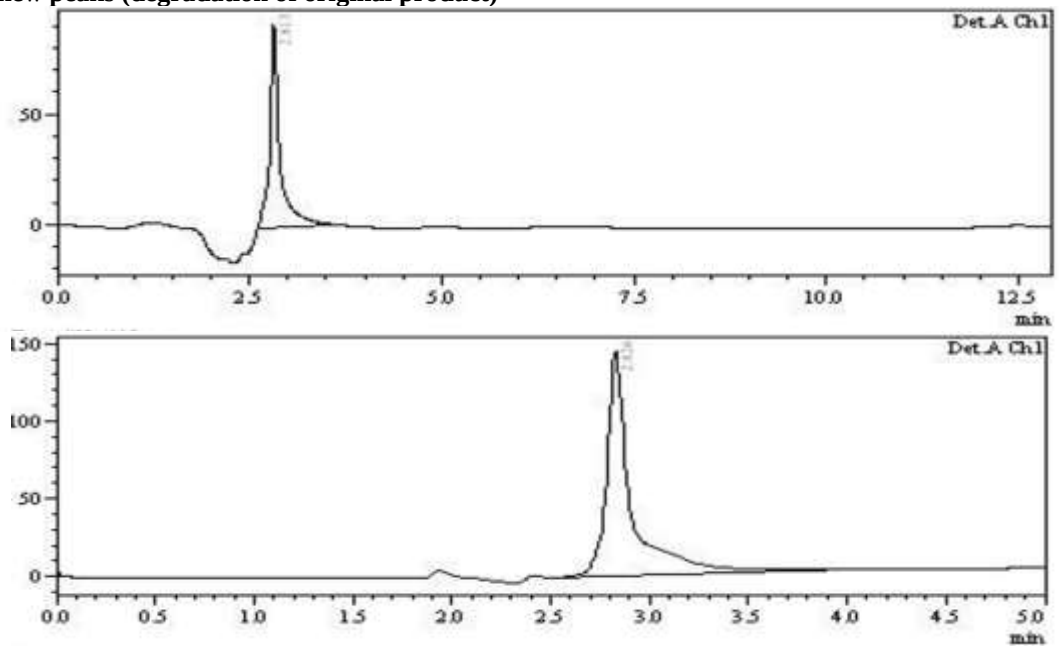
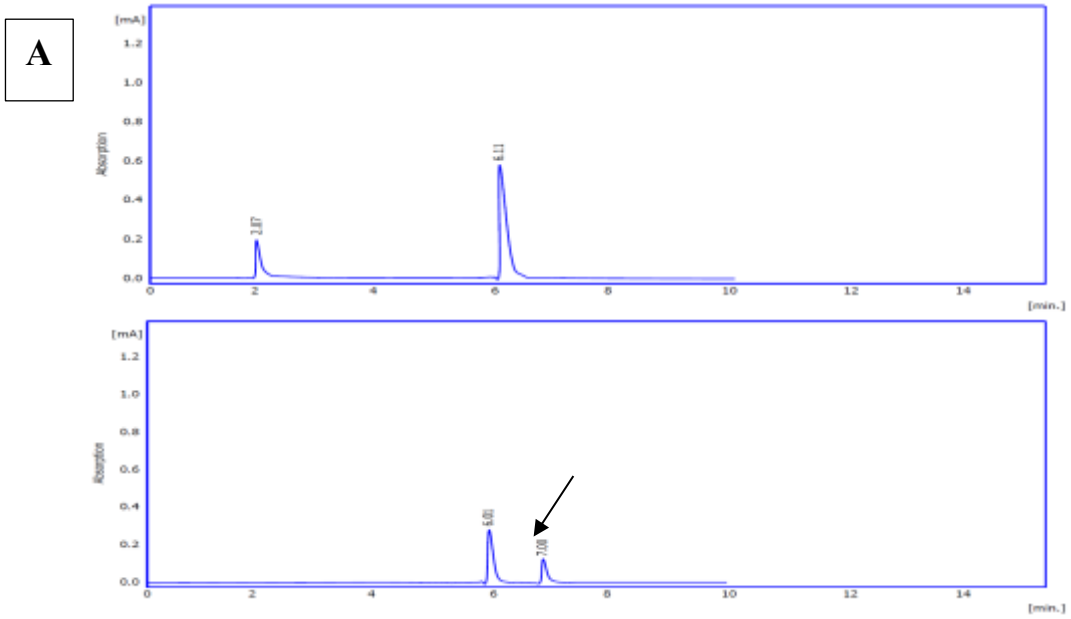


Figure 5. The effect of storage temperature on the stability of pure pemetrexed



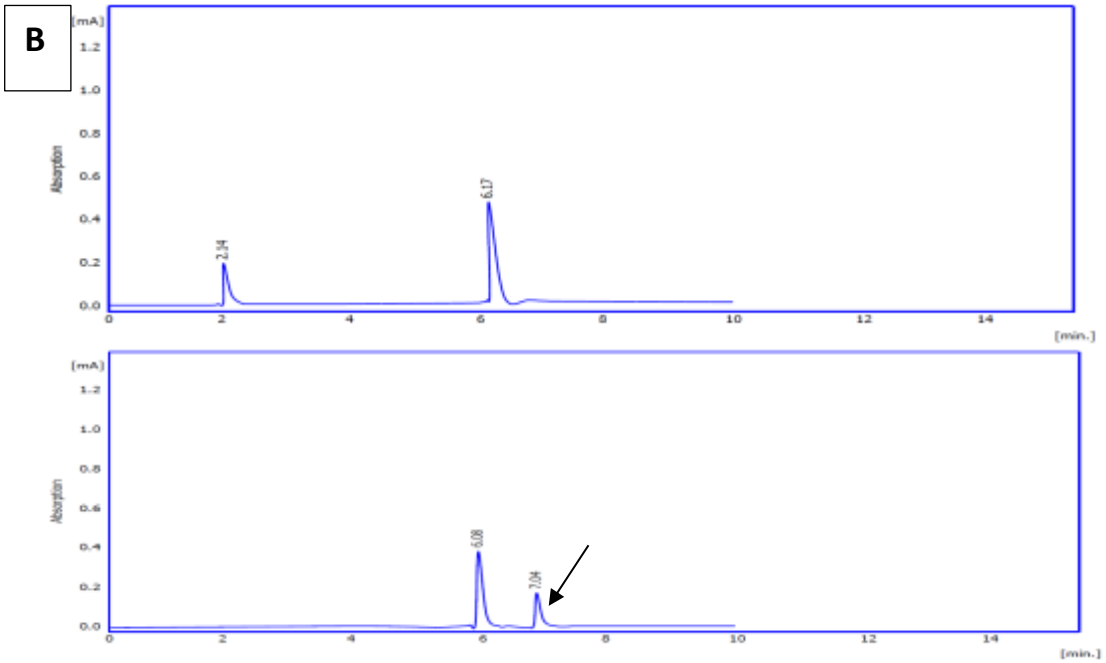


Figure 6. Effect of storage temperature on the stability of (A): AtZ-PEG-Pem conjugate and (B): AtZ-GABA-Pem conjugate, arrows represent appearance of new peaks (degradation of original product)

Conclusion

This work succeeded in the preparation of AtZ-Pem conjugates as injectable dosage forms with good stability, no toxicity, pyrogen free, and sterile. Such preparations may have a good selectivity towards a cancer cell leading to lower side effect for pemetrexed as well as the adjuvant effect of both compounds may improve the effectiveness and reduce the dose and cost of monoclonal antibody.

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Conflicts of Interest

No competing interests were disclosed.

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Ethics Statements

The author(s) declared that there is no need for an ethical approval from an ethics committee.

Author Contribution

The authors confirm contribution to the research paper as follows: study conception and design: **Faten Q. Ibraheem, Nidhal K. Maraie, and Basma Talib Al-Sudani**; data collection: **Faten Q. Ibraheem.**; analysis and interpretation of results: **Faten Q. Ibraheem and Nidhal K. Maraie**; draft manuscript preparation: **Faten Q. Ibraheem and Nidhal K. Maraie[2]**. All authors reviewed the results and approved the final version of the manuscript.

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تحضير و تقييم الاجسام المضادة المرتبطة بعلاج البيمتركسيد بشكل ابر إعطاء وريدي

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الخلاصه

الأدوية المرتبطة كيميائيا بالاجسام المضادة (ADCs)، نوع قوي من الأدوية الصيدلانية التي تجمع بين العلاج المناعي والعلاج الكيميائي. دواء البيمتركسيد المثبط للانزيمات التي تدخل في تركيب القواعد النيتروجينية التي تدخل في تكوين المادة الوراثية للخلايا هو نوع فعال من الادوية الكيميائية لكن بالرغم من فعاليته الا انه يفتقد الانتقائية العلاجية للتاثير على الخلايا السرطانية دون غيرها من الخلايا الطبيعية. كان الهدف من الدراسة هو إعداد Atezolizumab (الاجسام المضادة العلاجية) المرتبطة كيميائيا بدواء pemetrexed (Pem) المحضر سابقا في المختبر كنموذج جرعة قابلة للحقن الوريدي عن طريق استخدام التجفيف بالتبريد. تم تشخيص التركيبات المحضرة بواسطة (HPLC-UV),(particle size) و قد تم دراسة استقرارية التركيبات المحضرة و دراسة كمية المادة التي تحتويها من علاج pemetrexed بالإضافة لدراسة التركيبات المحضرة للتاكد من تعقيمها و خلوها من البكتريا المسببة للامراض. وجدت الدراسة ان التركيبات المجففة بالتبريد تمتلك استقراريةعالية لمركباتها عند خزنها لفتره ٦ شهور في درجة تبريد 6° C بالمقارنه مع نفس التركيبات التي أظهرت تكسر لمركباتها عند خزنها بدرجات حرارة 25 ° C. كما أظهرت الدراسة ان نموذج الحقن الوريدي المعد باستخدام التحضير AtZ-GABA-Pem يمتلك قياس ذرات اصغر بالمقارنه مع التحضير AtZ-PEG-Pem. خلاصة الدراسة هو ان النموذج المحضر بشكل الحقن الوريدي لدواء ال pemetrexed المرتبط بالاجسام المضادة Atezolizumab ممكن ان يكون بديل لدواء البيمتركسيد الوريدي المتوفر مما قد يؤدي الى تحسين انتقاءيته العلاجية للتاثير على الخلايا السرطانية حصرا وذلك يؤدي الي تقليل الاثار الجانبية العديدة لدواء البيمتركسيد.

الكلمات المفتاحية : إعطاء وريدي، اجسام مضادة،بيمتركسيد،استقرارية،الحرارة.