Original Article

Microbiological Assay of Two Selected Products of Ceftriaxone Powder for Injection from Pharmaceuticals' Market in Sudan

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ABSTRACT

Background: Different techniques have classically been used to evaluate and assure the quality of medicines circulated on the market; one of the commonly uses is chemical analysis. However, some evidence has shown that there are other important indicators (e.g. bioequivalence, relative potency, etc.) that should also be considered when evaluating the quality of pharmaceutical products.

Materials and Methods: A microbiological assay was conducted to compare the relative potency of two Ceftriaxone products (with a third one used as standard product) from the market using 3 reference bacteria including *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. Serial dilutions were made with the corresponding 1, 4, 8, 16 and 32-fold Minimum Inhibitory Concentration (MIC) of Ceftriaxone against the bacteria under investigation.

Results: The relative potency of one product compared to the standard product was estimated to be within the acceptable range of bioequivalence (89.6%), while the other product showed unacceptable relative potency (72.3%).

Conclusions: The microbiological assay is an effective and simple method for comparing the equivalency of injectable products. A complaint reporting system about quality and effectiveness problems needs to be considered as a priority source of such information to inform decision-makers.

Keywords: Microbiological assay, Ceftriaxone, quality assurance, relative potency and generic medicines.

Different techniques have classically been used to evaluate and assure the quality of medicines circulated on the market. One of the most important techniques is chemical analysis, in which different methods of analysis can be used to assure quality. However, measurements of chemical content alone do not provide sufficient indication about the quality of drugs. Evidence has shown that there are other important indicators (e.g. bioequivalence, relative potency, etc.) that should also be considered when evaluating the quality of pharmaceutical products[1].

In 2009, a comprehensive study was conducted to evaluate the quality of medicines distributed on the private market in Khartoum, Sudan. The main method used for this assessment was chemical analysis. Approximately 10% of the samples tested in this study failed to comply with reference standards specifications in terms of chemical analysis of Active Pharmaceutical Ingredients content. The results obtained in this unpublished quality survey from chemical tests were not sufficient, alone, to answer the complaints continuously reported from health professionals (doctors and pharmacists) regarding the quality issues related to these products. One of the products within this category which was often reported was Ceftriaxone powder for injection. Ceftriaxone is one of the third generation cephalosporins. It is administered intravenously or intramuscularly and has a broad spectrum of activity against Gram-positive and Gram-negative aerobic, and some anaerobic,

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bacteria. From the results, we noted that Ceftriaxone was the most frequently reported generic that doctors took decisions about related to alterations in their patients' treatment. These decisions were varied; there was either a shift to similar products (from different sources) or a shift to different generics. We also noted that among the circulated Ceftriaxone (powder for injection) products on the market, there was one specific product reported in the majority of complaints received during the survey. The majority of the cases were referring to products imported from low income countries.

Based on one study conducted previously in this area by Zuluaga and colleagues, it was recommended to benefit from the potential use and application of microbiological assays to determine the pharmaceutical equivalence of generic intravenous antibiotics[2]. The approach was found effective by other similar series of studies conducted by a team of researchers from the Wayne State University of Michigan - USA, to assess the usefulness of relative potency of pharmaceutical products[3]. This approach was adopted to investigate these complaints about the quality and effectiveness of Ceftriaxone further. Based on that, the main aim of this study was to verify the hypothesis that the difference in potency (equivalence) of Ceftriaxone products is a contributing factor to this problem in Sudan. To the best of our knowledge this was the first time to adopt this approach in Sudan, in large scale, for the purpose of monitoring the quality of pharmaceutical products.

MATERIALS AND METHODS:

Preparation of culture media:

The media used for antibacterial screening tests were nutrient agar and nutrient broth. Twenty six grams of nutrient agar (from Scharlau Chemie, Spain) was suspended in one litre of distilled water and heated in boiling water bath to dissolve the media completely; this solution was then divided into 20 ml portions in small vials. Thirteen grams of nutrient broth (from Fine-Chem. LTD. India) was dissolved in one litre of distilled water, heated in a water bath to dissolve the media and divided into 10 ml portions in small vials. The prepared nutrient agar and nutrient broth media were sterilised by autoclaving at 121 degrees (at atmospheric of 15 pounds) for 15 minutes.

Preparation of standard bacterial suspensions:

Each 10 ml portion of sterilised nutrient broth was inoculated with a loopful of each bacterial slant agar culture and incubated for 18-24 hours at room temperature. A 10% dilution of each liquid culture was prepared in sterilised normal saline and kept in a refrigerator.

Preparation of serial dilutions of Ceftriaxone:

Serial dilutions with corresponding 1, 4, 8, 16 and 32-fold Minimum Inhibitory Concentration (MIC) of Ceftriaxone against the bacteria under testing were prepared. In table 1 the MIC90 for different bacteria is shown which was used to prepare the serial dilutions[3].

Table (1): Minimum Inhibitory Concentration of targeted microorganisms.

Organism	MIC90 mg/ml
Streptococcus pneumoniae	0.060
Klebsiella pneumonia	0.125
Staphylococcus aureus	4.000

The equivalent amount of powder from different products was dissolved in distilled water to obtain the required dilutions.

Antibacterial assay:

The cup-plate agar diffusion method adopted in this study was that of Murray and others with some minor modifications, which was used to assess the antibacterial activity of the products[4]. From each of the standard stock suspensions, 0.1 ml was thoroughly mixed with 20 ml of sterile testing media in Petri dishes and left to solidify on a plain surface. Then, four cup-shape wells (10 mm diameter/each) were made in each plate using a sterile cork-borer (no.7). The agar discs were removed and

alternate cups were filled with 0.08 ml sample of each concentration from the solution of one product at a time. The fourth alternate cup was filled with the solvent used (water) for control purposes using a sterile adjusted pipette. The plates were then incubated in the upright position for 18-24 hours at room temperature. Three replicates were used for each solution against each tested organism. After the incubation periods indicated, the inhibition zone diameters were measured and the mean value was tabulated.

The products:

Three products of Ceftriaxone sodium (1 gm powder for injection) were included in this study. One of these was the originator's product, "*Rocephin G03C10T02*", which is manufactured by Roche – Switzerland; for the purposes of this study, it was selected as the reference product. The other two products were chosen based on random selection process. The details of the products are shown in the table (2).

Table (2): Details of Products under testing

Trade	Company	Country of origin	Origin classification
G03C10T01	C20	Switzerland	High income level
G03C10T02	C10	India	Low income level
G03C14T03	C14	Jordan	Lower-middle-income level

Statistical analysis:

The range of concentrations of each product was statistically satisfactory to produce a wide range of points sufficient for producing clear statistical data. The concentrations prepared and used in this assay include 0.125, 0.5, 1.0, 4.0, 16.0 and 32.0 mg/ml (according to the MIC of the targeted bacteria). The assay was repeated using three concentrations of each antibiotic in triplicate for each microorganism.

For the purpose of this analysis, the linearity and precision of the method used were all determined. This was achieved by plotting the log-transformed concentrations of each product against the inhibition zone in mm. The data followed a linear statistical model which was confirmed by the values of the intercept and slope of the best straight line when applied in the equation ($y = b + mx$, where b is the y-intercept and m is the slope). The x-intercept (log10 mg/L) and slope of the regression line with 95% confidence intervals (95% CI) were both calculated and used for regression analysis to determine the statistical significance of these variables[2].

The equivalencies of products from this experiment were concluded by comparing the slope and intersect of each product using a symmetrical parallel-line assay. In this analysis, and in accordance with the purpose of the study, potency was defined as the slope of the linear regression and concentration. In this case, and if we assume that the test products and the innovator are equivalent, then the products must show a trend of a parallel and overlaid curve with the curve of the innovator. At the same time, when there are parallel curves with different intercepts, this may indicate the existence of the same active ingredient but at different concentrations above or below that of the innovator product. The relative potency for each product to the potency of the innovator was calculated using the distance between the innovator line and that of the test product. The response values (diameter of inhibition zones) were calculated using the standard deviation and slope method and these were stated in term of means +/- the standard deviations and with the calculated coefficients of variation. To test the accuracy of the test, i.e. its ability to detect significant differences in the concentration, a standard curve was obtained using different concentrations of Ceftriaxone working standard solution with a potency of 98.6%/L. This curve was compared with the curve of all products being investigated.

RESULTS:

Table (3) shows the potency estimates and other parameters derived from linear regression with their statistical comparison of the test product versus innovator using Curve Fitting Analysis.

Table 3: Summary of relative potencies and other parameters of Ceftriaxone products

Product	r2	Intercept ± SD	P-value	Slope ± SD	P-value	Potency estimate (%)
Organism: *Staphylococcus aureus*						
G03C20T01	0.97	2.401 ± 0.205	0.000	0.027	0.000	100.0%
G03C10T02	0.91	2.223 ± 0.187	0.000	0.026	0.000	84.3%
G03C14T03	0.78	2.172 ± 0.164	0.000	0.027	0.000	70.%
Organism: *Klebsiella pneumoniae*						
G03C20T01	0.97	2.198 ± 0.172	0.000	0.023	0.000	100.0%
G03C10T02	0.91	2.023 ± 0.124	0.000	0.025	0.000	89.4%
G03C14T03	0.78	2.089 ± 0.166	0.000	0.023	0.000	74.1%
Organism: *Streptococcus pneumonia*						
G03C20T01	0.41	2.351 ± 0.311	0.000	0.022	0.000	100.0%
G03C10T02	0.52	2.325 ± 0.324	0.000	0.024	0.000	51.4%
G03C14T03	0.48	2.305 ± 0.452	0.000	0.024	0.000	47.1%

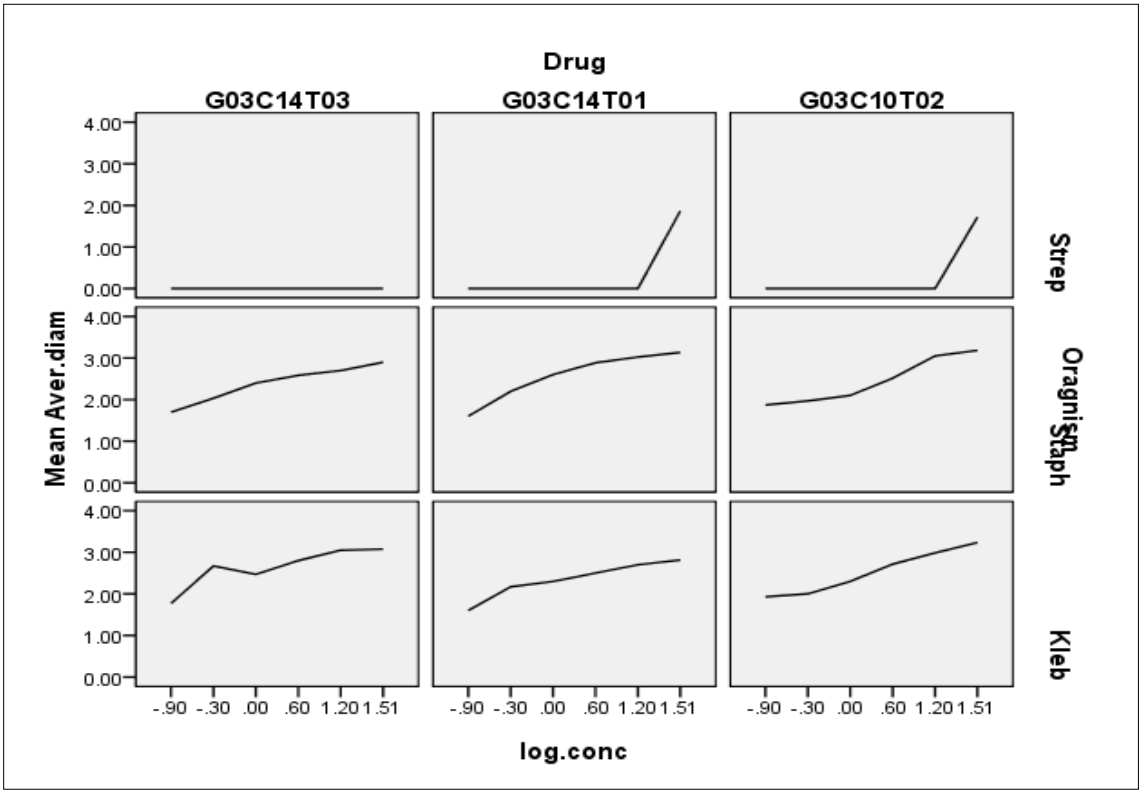

Figure (1): Single dose-response curves for Ceftriaxone products against targeted organisms**.**

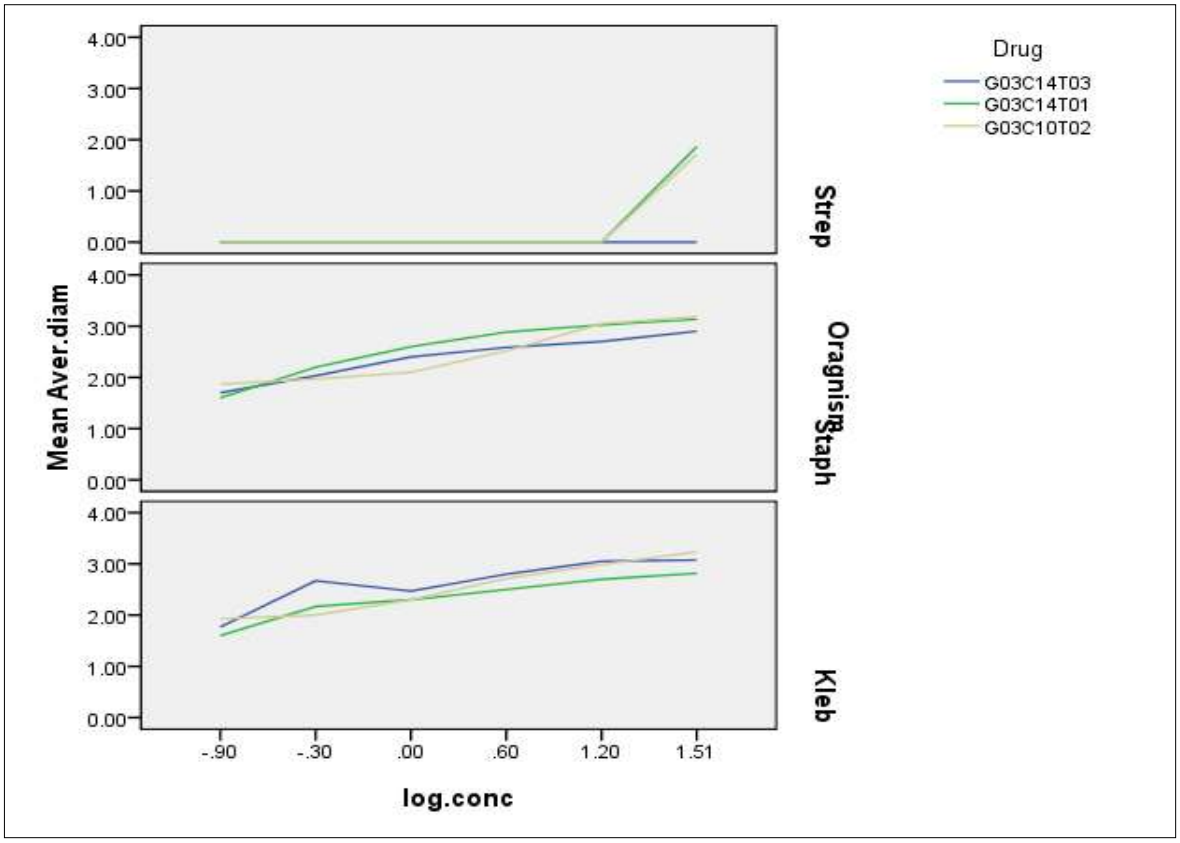

Figure (2): Combined dose-response curves for Ceftriaxone products against targeted organisms

Figures (1) and (2) shows the log concentration-response relationship and the best straight line obtained from data from the microbiological assay of the innovator. In the case of *Klebsiella pneumoniae* and *Staphylococcus aureus,* the figure showed a linear relationship between the logarithm of the concentration (log10 mg/L) and the diameter (mm) of inhibition zones with relatively high coefficients of determination (r2 ranging from 0.97 to 0.78) and a statistically significant intercept and slope ($P < 0.001$ by ANOVA). On the other hand, the results for *Streptococcus pneumoniae* showed different trends. The main observation from the raw data showed that the response of the organism (growth inhibition) only appeared at higher concentrations. This result appeared at a concentration 32 times higher than the MIC90 for this organism, i.e. 2mg/ml, and this was also observed for all products under investigation. This finding was significant on its own due to the fact that growth inhibition was expected to be at relatively lower concentrations. These outputs were not reported in any available studies about the microbiological response of Ceftriaxone against *Streptococcus pneumoniae*.

Excluding the data of *Streptococcus pneumoniae* and considering the data from other organisms, all of the products exhibited a different intercept ($P < 0.005$); the log concentration-response relationship for one of the generics (G03C20T02) was parallel and overlaid to the innovator linear curve without any significant difference ($P < 0.005$), while the curve of the other generic (G03C20T03) was parallel but not overlaid with the innovator linear curve. These findings indicate that generic G03C20T02 and generic G03C20T03 had the same biological activity (potency). On the other hand, generic G03C20T02 and generic G03C20T03 showed relative estimated potencies of 86.9% and 72.3% of the originator product, respectively. All of these data indicate that generic G03C20T02 is pharmaceutically equivalent to the originator product while generic G03C20T03 did not show the same association.

DISCUSSION:

The results obtained from the chemical analysis tests used in the quality monitoring survey mentioned previously were not sufficient to answer the complaints from health professionals regarding the quality issues around Ceftriaxone powder for injection (especially for certain products). However, these products complied with reference standard specifications in terms of chemical content limits. This raised many questions regarding the reasons leading to these complaints about that specific product. No single reason prior to this study could be considered evidence for the primary cause of this observation.

The equivalency of pharmaceutical products is an essential and basic criterion for proving the quality of any product[1]. In general, there are different methods that can be used by the regulatory authorities to evaluate these characteristic; including the in vitro /in vivo bioavailability studies and other methods like bioassays. The use of the bioassay method is not new in different pharmaceutical sciences; however, it has been used limitedly, and mainly, for pharmacokinetic studies[5]. Late in the 1980s, the bioassay method drew attention as a possible tool for establishing scientific judgment about the pharmaceutical equivalence of certain categories of products like anti-infectives and vitamins[6]. This method could help to obtain more conclusions about the similarity and the differences between products that are supposed to contain similar and equivalent active ingredients. This is especially true when we consider the fact that any pharmaceutical formulation usually contains the active ingredient in addition to other substances (e.g. preservatives, impurities, etc.). In most of the cases, these materials were not considered during assessment of the quality or equivalency of generic products[7]. The statistical method adopted here was used for a similar study performed using other anti-infective intravenous preparations[2]. It was previously believed that the agar-diffusion assay is less reliable than other chemical methods (e.g. HPLC) because it suffers from a wide variety of biological errors. Still, this method is statistically suitable for use in such kinds of studies (equivalence and potency evaluation studies). The logic behind the statistics used in this study (and similar studies in general) was based on the assumption that: if two Ceftriaxone products were equivalent to each other, by obtaining two symmetrical and straight parallel lines (plotting of the mean zone size against the logarithmic concentration), then the relative potency of the test product to the reference product could be derived by calculating the distance between the two lines[2]. At the same time, these curves derived from the bioassay should not differ significantly from each other.

The results obtained in this study support the reservations about Ceftriaxone products reported in quality monitoring surveys. The product G03C20T03 was not found to be equivalent to the reference product under testing. Accordingly, it is not anticipated to show a similar therapeutic profile and effectiveness. Considering the critical importance of anti-infectives (especially Ceftriaxone) to public health in Sudan and other similar countries, it has now become essential to investigate the assumption about the differences in therapeutic equivalency between the products available on the market. The equivalence and potency of intravenous/parenteral generic products of Ceftriaxone, away from clinical studies, cannot be evaluated using conventional physical and chemical methods alone. After carrying out this study, it was proven that using a well-designed microbiological bioassay should help medicines regularity authorities to perform such re-evaluations to check the quality of different products. In general, this study also highlighted the importance of expanding Post-Marketing Surveillance systems to check the quality and efficacy beyond the physiochemical analysis to include more tests including microbiological assays, bioassays, bioequivalence studies, minilabs, dissolution test, etc. as routine tests.

CONCLUSION:

In conclusion, this simple method of analysis is very important for any regulatory authority to be considered a routine process. The method provides a good tool for the accurate determination of the pharmaceutical equivalence of drugs in injectable dosages. This in fact is one of the considerable innovations for any quality monitoring system in the country related to injectable products. In addition, a complaint reporting system about quality and effectiveness problems needs to be considered as a priority source of such information to inform the decision makers.

Conflict of Interest:

The authors declare no conflict of interest in this work.

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