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IN-VITRO QUALITY EVALUATION OF TEN DICLOFENAC SODIUM AMPOULE BRANDS IN YEMEN

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ABSTRACT: The aim of this study is to evaluate the quality of ten brands diclofenac sodium ampoule marketed in Yemen. Each brand was tested according the pharmacopeia's requirements of parenteral dosage forms. The tests were performed in this study: the physical tests, pH, sealing, volume, sterility, and assay test. The results revealed that most brands have an acceptable results compared to the reference product. The results of the physicochemical tests were complied with the requirements in most tests including general appearance were acceptable except DIC6. Solution volume results were confirmed the specifications except in DIC6 all ampoules were not filled to the labeled volume to be withdrawn and DIC7 all ampoules were not filled to the labeled volume. The pH values were acceptable except the brand DIC2 (pH = 9.24). Particulate matter test results were acceptable except DIC2, DIC10 and DIC6 have one, one, and two black particulates matters, respectively, in one ampoule of each. Assay test has showen all targeted products were satisfied and within the pharmacopoeias' limits (as general 95-110%). Finally, sterility test including pyrogen test, all products have passed successfully the LAL test, that is mean all products were pyrogen-free as well as free from microbial growth. Also all the results of leaker test were acceptable according to the pharmacopeia determinations.

KEYWORDS: Diclofenac sodium, ampoule, evaluation, brands, Yemeni Markets.

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1.INTRODUCTION

Parenteral dosage forms are preparations intended for injection through the skin or other external boundary tissue, so that the active substances they contain are administered using gravity or force directly into a blood vessel, organ, tissue, or lesion. Parenteral products are prepared scrupulously by methods designed to ensure that they meet pharmacopeias' requirements for sterility, pyrogens, particulate matter, and other contaminants, and, where appropriate, contain inhibitors of growth of microorganisms [1]. Diclofenac, 2(2, 6-dichloroanilino) phenyl acetic acid, has a poor aqueous solubility and is commonly used as sodium, potassium and diethyl amine salts [2]. It is an important member of a class of drugs known as nonsteroidal anti-inflammatory drugs, which is widely used for the treatment of musculoskeletal disorders, arthritis, toothache, dysmenorrhea and symptomatically relief of pain and inflammation [3-6]. It is available in the various formulations such as injections, tablets, gel, suppositories and powdered form [7]. Sodium salts of diclofenac is used to making aqueous solutions. Diclofenac sodium tends to precipitate from aqueous solutions in a crystalline form even when the concentration is below the limit. Thus to improve its solubility, various solubilizers like hydroxypropyl-β-cyclodextrin and polyoxyethylene-35-castor oil, [8] n-octenylsuccinate starch, [9] and α-tocopheryl polyethylene glycolsuccinate [10] have been employed. In aqueous acidic solutions, diclofenac undergoes cyclization to indolinone [11]. In a study conducted earlier, 1-(2,6-dichloro-phenyl)-indoline-2-one, [2-(2,6-dichlorophenyl)- amino-phenyl] methanol and 2-[(2,6-dichlorophenyl)amino]-benzaldehyde were detected as degradation products, in ophthalmic solutions of diclofenac exposed to accelerated testing conditions of 60℃ for 9 weeks [12]. Thermal stability of diclofenac sodium and its inclusion complex with β-cyclodextrin have been characterized in the solid state [13] and in aqueous solutions [14]. It has been observed that formation of inclusion complex of diclofenac with β-cyclodextrin improves the thermal stability of diclofenac sodium in solid state as well as in aqueous solutions. Diclofenac is susceptible to photochemical oxidation [8]. In photolytic degradation studies of diclofenac conducted by exposing aqueous solutions to solar radiation, it has been observed that diclofenac undergoes cyclization to carbazole derivatives [15]. The quality maintenance in a pharmaceutical industry depends on the number of atmospheres including personnel qualifications, active pharmaceutical ingredients quality, validation of the manufacturing process and the area etc. [16, 17]. The purpose of stability testing is to provide evidence of how the quality of a drug substance or formulated product varies with time under the influence of a variety of environmental factors such as temperature, light and humidity to allow the establishment of recommended storage conditions, retest periods and shelf lives [9]. Stability is an essential factor of quality, safety and efficacy of a drug product. A drug product, which is not of sufficient stability, can result in changes in physical as well as chemical characteristics. The chemical stability of drug is of great importance since it becomes less effective as it undergoes

degradation. In addition, drug decomposition may yield toxic products that are harmful to the patient. Microbiological instability of a sterile drug product could also be hazardous [18]. Pre-requirement of drug products that should be chemically and pharmaceutically equivalent must be identical in strength, quality, purity, active ingredient release profile and in the same dosage form, for the same route of administration [19]. Because of the widespread use of this drug, quality control testing should be done for diclofenac marketed products to ensure safety; efficacy; accepted quality; rationality of use to protect public health [20]. In-vitro testing or quality control of drugs is a set of studies or experiments undertaken during production in process and occasionally ought to be undertaken post production by regulatory agencies and researchers. Routine laboratory testing of drugs in the market is a crucial to protect public health especially in developing countries where counter-fit and substandard drugs have become a major challenge to health care services [21]. Generic products need to be therapeutically equivalent to the brand innovator products. This can be achieved only when bioequivalent study is conducted to show whether a generic product is interchangeable with brand product or another generic product. The aim of this study is to assess the quality of ten brands of diclofenac sodium injections available in Yemeni markets.

2. MATERIALS AND METHODS

1.1.Materials

All substances that used in this search including: Methanol 96%; Hydrochloric acid, Acros-Germany; Diclofenac sodium standard, Biopharm company in Yemen; limulus amebocyte lysate water and powder,Cape Cod-United States; Fluid Soyabean-Casein Digest; Fluid Thioglycollate Medium, Himedia. Microorganisms that used in the sterility test of diclofenac sodium ampoules including *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Clostridium sporogenes, Candida albicans,* and *Aspergillus brasiliensis* from Cape Cod Company in the United States.

1.2.Methods

1.2.1.Sample collection

Ten different ampoules of 75mg/3ml diclofenac sodium products randomly collected from retail pharmacies of targeted area in Yemen (Dhamar city), which characterized by low humidity and low temperature areas, About 20 ampoules 75 mg/3ml have the same batch number of each brand were collected for the analysis. All the analytical methods were done in the quality control laboratories of Yemen Biopharm Company.

Table 1: Diclofenac sodium brands used in this study

Product code	Batch No.	Manufacturing date	Expiry date	Company	Country
DIC1	S2135	06/2012	05/2014	Novartis	Switzerland
DIC2	0711213	07/2011	07/2014	Sedico	Egypt
DIC3	282A	07/2012	07/2015	Biopharm	Yemen

DIC4	S2105	05/2012	04/2014	Novartis	Turkish
DIC5	110911	04/2011	09/2014	Shanghai	China
DIC6	A0032VP	12/2010	11/2013	Lupin	India
DIC7	23013D	01/2012	01/2015	Alpha	Syria
DIC8	17087	11/2011	10/2014	Denk	Germany
DIC9	1201988	03/2012	03/2015	E.I.P.I.CO.	Egypt
DIC10	XD1031	11/2011	10/2014	Cipla	India

1.2.2. Calibration Method

Preparation of standard calibration curve

The standard calibration curve was prepared according to method of analysis, which depend on the final concentration of the samples preparation for analysis (i.e. 15 µg/ml), by preparing series of standard solutions with different concentration of reference diclofenac sodium e.g. 1.87, 3.75, 7.5, 15, and 30 µg/ml as following: The stock solution of STD diclofenac sodium was prepared by weighing accurately a quantity of diclofenac sodium reference equivalent to about 75 mg diclofenac sodium using analytical balance. Then, it was transferred to 250 ml volumetric flask, and dissolve in 10 ml methanol 96% then complete the volume by distilled water. After that, 20, 10, 5, 2.5, and 1.25 ml of stock solutions were taken in a series of separate 200 ml volumetric flask by pipettes and volume was adjusted to 200 ml by distilled water and mixed well. Absorbance was taken by UV-Visible spectrophotometer at 276 nm against distilled water as blank solution for each sample. Finally, the previous steps were repeated for 3 days and the absorbance was recorded. The average of measured absorbance was taken and plotted against the respective concentration of reference.

1.2.3 Quality control tests for diclofenac sodium ampoules

a) Physical tests

i.General appearance of ampoule

Testing of general appearance involves measurement of attributes such as production date and expiry date; packaging material; the shape and dimensions of ampoule; solution volume; the color; quality of sealing; odor; pH values were determined by pH meter; and particular matter, all product containing clear solution should be inspected against a black or white background using a special light source.

ii.**Leaker test (integrity test)**

This test was performed by producing a negative pressure within all ampoules by using autoclave; while the ampoules was submerged entirely in a deeply colored dye solution, approximately 1% methylene blue solution is employed. After carefully rinsing the dye solution from the outside, color from the dye will be visible within a leaker. Leakers, of course, were discarded. Opened containers of solutes, capable of supporting the growth of microorganisms invite such contamination [22].

b) Chemical test (official)

1. Identification test

Using UV spectrophotometer, we identify all samples against reference standard of diclofenac sodium. The standard was prepared by weighing 75mg of diclofenac sodium powder into 250ml volumetric flask, then 10ml methanol 96% and the volume was completed by distilled water, then 10ml was transferred into 200ml volumetric flask and completed to volume by distilled water. The final concentration of standard was 0.015mg/ml. The sample solutions were prepared by use five diclofenac sodium ampoules from each brand product were emptied into small beaker and equivalent to 3 ml solution was transferred to 250ml volumetric flask. Next, 10ml methanol 96% and the volume was completed by distilled water, then 10ml was diluted to 200ml by distilled water then applied to UV system for the analysis. After adjustment of the specific absorbance of spectrophotometer at 276 nm, the absorbance was taken for the previously prepared standard solution then the absorbance values of each prepared sample of diclofenac sodium ampoules was taken. Finally, the results were compared between reference standard and each sample.

2. Assay test

Using the previous prepared solutions of diclofenac sodium reference and samples, and measured by calibrated UV-spectrophotometer at 276 nm. The assay percent of each sample recorded as shown in table (2).

c) Biological tests

1. Sterility test (Microbial growth testing)

A. Preparation of the media

a) Preparation of thioglycollate medium

29.25g of dehydrated medium was suspended in 1000ml purified water and heated to boiling until completely dissolve. The prepared medium was sterilized by autoclave at 15 ibs pressure and 121℃ for15 minutes; the solution would have pH of 9 to 7.3. Fluid thioglycollate medium is to be incubated at 30–35°C. The preparation carried out under aseptic condition by using laminar sterile airflow cabinet.

b) Preparation of soybean casein digest medium:

30g of soybean casein medium was suspended in 1000ml purified water and heat to boiling until dissolve. After that, it was sterilized by autoclave at 15 ibs pressure and 121℃ for15 minutes after sterilization the solution would have pH of 7.1 to 7.5. Soya-bean casein digest medium is to be incubated at 20–25°C.

B. Procedure of microbial growth testing

All glassware and other heat-stable materials were sterilized using a hot-air oven at 250ºC for 120 minutes. The test was gained carefully in a perfect aseptic manner and suitable conditions.

At intervals during the incubation period, the media for macroscopic evidence of microbial growth was examined. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination. 14 days after the beginning of incubation transfer portions (each not less than 1 ml) of the medium to fresh vessels of the same medium and then the original and transfer vessels for not less than 4 days were incubated.

C. **Growth promotion test of aerobes, anaerobes and fungi**

Each batch of medium prepared from dehydrated medium was tested. Next, suitable strains of microorganisms were obtained. Then portions of fluid thioglycollate medium was inoculated with a small number (not more than 100 CFU) of the following microorganisms, using a separate portion of medium for each of the following species of microorganisms: *Clostridium sporogenes, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* Moreover, portions of soya-bean casein digest medium was inoculated with a small number (not more than 100 CFU) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *Aspergillus brasiliensis, Bacillus subtilis, and Candida albicans.* Meanwhile, incubated period for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi. Finally, the media are suitable if a clearly visible growth of the microorganisms occurs.

D. **Negative controls**

The purpose of negative control samples is to verify the sterility of the medium before, during, and after the incubation period of the sterility test. If microbial growth is detected with a negative control, the medium was not sterilized properly. Negative controls consist of containers of culture media as the following: three portions of each fluid thioglycollate medium and soya-bean casein digest medium were inoculated without addition of product sample or microbial challenge.

2.Pyrogen test (Endotoxin test)

i. Manual LAL (limulus amebocyte lysate) test procedure

Using sterilized glassware under the previously mentioned conditions, two ampoules were emptied into sterilized tube; the pH of the reaction mixture should be between 6.0 and 8. Therefore, the pH was adjusted by addition of sterile, endotoxin-free, 0.1N hydrochloric acid. Then 100µL of samples was taken into another test tubes and mixed with100 µL of LAL water then reconstituted LAL powder (0.125EU/ml) was mixed thoroughly and is placed immediately in a dry-block incubator at 37˚C± 1˚C for 60 ± 2 minutes. The tube was removed from the incubator and inverted. If a gel has formed and remained intact in the bottom of the reaction tube after an inversion of 180 degrees, the test is positive. Any other state of the reaction mixture constitutes a negative test.

ii.Control standard endotoxin (CSE)

5ml of LAL was added to the vial, seal the vial with parafilm, the concentration obtained 100ng/ml then shaken vigorously for one minute, at 5-10 minute intervals over a 30-60 minute period at room

temperature. After that, 100ml of CSE was reconstituted with LAL powder. Reaction solution was mixed thoroughly and placed immediately in a dry-block incubator at 37˚C± 1˚C for 60 ± 2 minutes.

3. RESULTS AND DISCUSSION

1.3. Results of standard calibration curve

Table 2: Results of standard calibration curve

Soln.NO	Conc.µgml^{-1}	A1	A2	A3	$\bar{X}$	$\bar{X}$±SD	%RSD	r²
Soln.1	30	0.985	0.984	0.989	0.986	0.986±0.0026	0.26	
Soln.2	15	0.503	0.501	0.501	0.502	0.502±0.0012	0.24	
Soln.3	7.5	0.144	0.134	0.139	0.139	0.139±0.005	3.6	
Soln.4	3.75	0.257	0.262	0.263	0.260	0.260±0.0032	1.23	
Soln.5	1.87	0.078	0.071	0.078	0.076	0.076±0.0040	5.3	0.9998

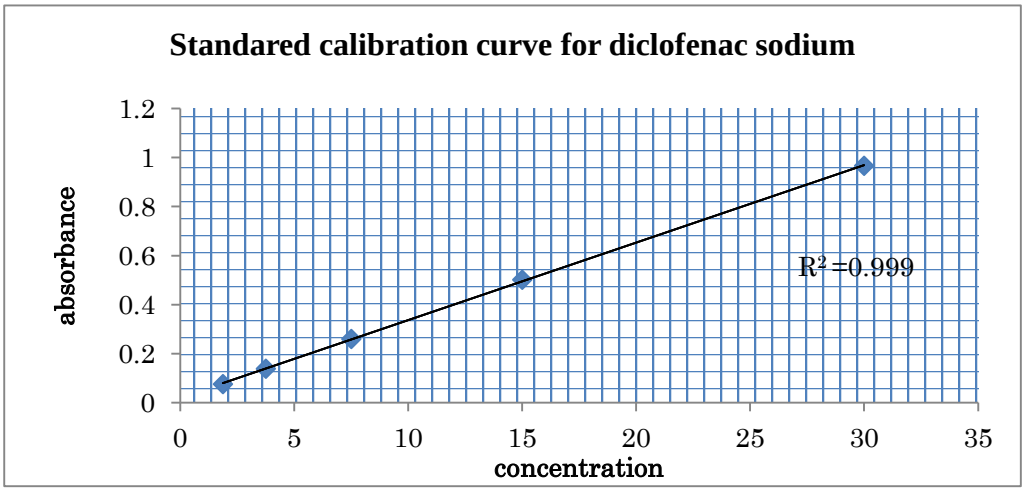

Figure 1: Calibration curve for working standard of diclofenac sodium

From the table above the average of absorbance were calculated and plotted against the concentration and calculated the correlation coefficient and the results of standard calibration curve were proving the main three points:

A. Linearity.

B. Correlation coefficient (r^2) is equal 0.9998, the limit (0.95–1).

The relative standard deviation percent (RSD %) is 5.3% (the lower limit of concentration RSD% ≤ 10%).

C. The line ran about through the origin.

These results indicated the selectivity, linearity, accuracy, and precision of the analysis method, and the method was validated.

1.4.Results of physicochemical tests

Table (3) gives information about the results of physical quality control tests of ten brands of diclofenac sodium ampoules, which included in this study.

Table 3: Results of physical tests of all targeted brands of the study

Test	Product samples									
	DIC1	**DIC2**	**DIC3**	**DIC4**	**DIC5**	**DIC6**	**DIC7**	**DIC8**	**DIC9**	**DIC10**
Production and expiry date	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.
Packaging	Elegant	IOL	Elegant	Elegant	Elegant	IOL	Elegant	Elegant	Elegant	IOL
Shape	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.
Color	Conf.	Conf.	Conf.	Conf.	Not	Conf.	Conf.	Conf.	Conf.	Conf.
Odor	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.
Sealing	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Non	Conf.	Conf.	Conf.
Easy to	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.
Solution	3.25	3.15	3.25	3.25	3.15	3	2.7	3.25	3.25	3.25
pH	8.14	9.24	8	8.01	7.80	8.31	8.05	8.41	8.05	8.4
Particulate	NP	1	NP	1	NP	2	NP	NP	NP	1

*IOL.: Impurities on label, Conf.: confirmed, (NP): no particulate matters, DIC: sample code.

The identification test for all products were determined by UV spectrophotometer and the observed results, indicated all products have absorbed at the same specific absorptivity of reference standard of diclofenac sodium. As shown in table 3, all the results of packaging material accepted according to USP, however, the brand DIC2 was not clean on label this is may be due to improper storage. The results of general appearance was accepted except the brands DIC6 and DIC10 were not clean on label. Turning to sealing quality was good except in DIC7 was difficult to broken at used. The results of solution volume were found in the ampoules was measured in (ml) for all brands were good enough according to USP except DIC6 brand; all ampoules were not filled to the volume must be in slight excess of the labeled volume to be withdrawn and DIC7 all ampoules were not filled to a labeled volume. This overfill permits the ease of withdrawal and administration of the labeled volumes [1]. The results of pH for all products were acceptable except the brand DIC2 that was out of the limit (pH = 9.24). Parenteral products should be formulated with a pH close to physiological pH; the ideal pH of parenteral products is pH 7.4, unless stability or solubility considerations preclude this. Often the pH selected for the product is a compromise between the pH of maximum stability, solubility and physiological acceptability. Many products are formulated at a slightly acidic pH because of solubility or stability considerations, and the vast majority of licensed products have a pH between 3 and 9. A pH outside this range should be avoided if possible, since a pH of greater than 9 can cause tissue necrosis, whereas a pH of less than 3 may cause pain and phlebitis [23]. The results of the particulate matter for all products approved according to USP except DIC2 and DIC10

have a black particulate matter in one ampoule for each, the product (DIC6) has two black particulates matters in one ampoule, particulate contamination may originate from the production process and its variables (e.g., environment, equipment, personnel) [1]. This particulates matter in injections can be harmful when introduced into the bloodstream. The contamination of parenteral fluids and drugs by particulate matter has been recognized as a potential health hazard and adverse reactions may include vein irritation and phlebitis [24]. Assay test used for determination of the percentage of the active ingredient (i.e. diclofenac sodium) in ampoule dosage form according to USP 30 specification within shelf life as shown in table (4).

Table 4: Results of assay test of all targeted brands of the study

Assay Test	Product samples									
	DIC1	**DIC2**	**DIC3**	**DIC4**	**DIC5**	**DIC6**	**DIC7**	**DIC8**	**DIC9**	**DIC10**
Absorbance	0.485	0.532	0.505	0.522	0.491	0.522	0.521	0.509	0.519	0.508
Mean assay%	107.23	102.92	104.34	100	104.34	102.19	100.84	103.08	102.30	100.07
SD	2.44	1.66	2.80	1.04	2.80	1.92	0.98	2.56	1.16	1.71
RSD%	2.28	1.61	2.68	1.04	2.68	1.88	0.97	2.48	1.13	1.71
Label amount	75 mg/3ml									
API amount	80.42	77.19	78.26	75	78.26	76.64	75.63	77.31	76.73	75.05

In this study the assay test for diclofenac sodium ampoule 75mg/ml is achieved by calibrated UV-spectrophotometer, ($r^2 = 0.9998$ and the RSD % was 2.54), and the obtained results are shown in the table (2) and in the figure (1). USP specifications is diclofenac sodium ampoule is not found but as general range the ampoule must contain the equivalent of not less than 95.0 percent and not more than 110.0 percent of the labeled amount of diclofenac sodium [1]. The results pointed to that some of the targeted products of diclofenac sodium ampoule 75mg/3ml under various brand names were complied with pharmacopeias' specifications, and some of the targeted brands of diclofenac sodium ampoule 75mg/3ml were not comply with pharmacopeias' specifications. The study indicated all the products were within the limit (i.e. not more than 110% and not less than 95%). Furthermore, other studies were determined the amount of diclofenac sodium in different formulations of diclofenac sodium (ampoule, tablets, eye drop, suppositories, and gel) by HPLC described in USP 30 monograph for diclofenac sodium and the results were complied with specification [25]. More studies determined the amount of diclofenac sodium and benzyl alcohol of diclofenac sodium ampoule by the UV spectrophotometer described in USP 30. The value of standard deviation showed that the batches value are very close to the mean value of assay.

Table 5: Results of assay test of all targeted brands of the study

Test	Product samples									
	DIC1	**DIC2**	**DIC3**	**DIC4**	**DIC5**	**DIC6**	**DIC7**	**DIC8**	**DIC9**	**DIC10**
LAL test	PF	PF	PF	PF	PF	PF	PF	PF	PF	PF
Leaker test	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.	Conf.
Sterility test	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG

*PF: pyrogen free, NG: no microbial growth

Regarding to pyrogen testing, all the results above approved according to USP that indicates all products passed successfully LAL test, that is mean all products were pyrogen-free. The state of the reaction mixture constitutes a negative test, which indicates an endotoxin concentration less than the pyrotell sensitivity [1]. All results of leaker test were reliable according to USP as showed in table (5). Moreover, results of sterility test, all targeted products pass successfully the sterility test and acceptable according to USP, there is no evidence of microbial growth was found, the product to be examined complies with the test for sterility that is mean all products microorganism-free [1].

2.Results of growth promotion test of aerobes, anaerobes and fungi

Table 6: the final review of growth promotion test of aerobes, anaerobes and fungi results

Name of microbial challenge	Type of media	Results
Clostridium sporogenes,	Thioglycollatemedium	Positive
Pseudomonas aeruginosa	Thioglycollatemedium	Positive
Staphylococcus aureus	Thioglycollatemedium	Positive
Aspergillus brasiliensis	Soya-bean casein digest	Positive
Bacillus subtilis	Soya-bean casein digest	Positive
Candida albicans	Soya-bean casein digest	Positive

All the results above adhered to USP requirements which indicate that all microbial challenge was grown in the two medias (thioglycollate medium and soya-bean casein digest medium) that is mean the media are suitable for growth of the microorganisms so that suitable for sterility test for diclofenac sodium sample [1].

2.1. Results of negative controls

Table 7: Results of negative control of sterility test

Number of sample	Type of media	Results
Sample1	Thioglycollatemedium	Negative
Sample2	Thioglycollatemedium	Negative
Sample3	Thioglycollatemedium	Negative
Sample4	Soya-bean casein digest medium	Negative
Sample5	Soya-bean casein digest medium	Negative
Sample6	Soya-bean casein digest medium	Negative

The results above are satisfied according to USP, which indicate the medium was sterilized properly; the purpose of negative control samples is to verify the sterility of the medium before, during, and after the incubation period of the sterility test. If microbial growth is detected with a negative control, the medium was not sterilized properly, contamination was introduced accidentally during the test procedure, or there exists an inefficiency in the container or packaging system [1].

4. CONCLUSION

In this study, ten targeted brands of diclofenac sodium ampoules collected from Dhamar city, Yemen, by following pharmacopeia's validated analytical methods. All targeted products of diclofenac sodium ampoules 75mg/3ml in the study are complied with quality pharmacopeias' specifications. DIC2 brands which be out the limit in pH test which given 9.4 and DIC7 brands in total volume is not the same to the labeled amount, but all the other targeted products were comply with quality (physical test) pharmacopeia requirements. Sterility testing requires high levels of control with regards to colony forming units quality systems requirements, Good Laboratory Practices, environment (aseptic clean room ISO Class 5 or better), and employee practices. Regarding to sterility test, all the targeted brands were microorganisms-free and pyrogen-free. Finally, the local Yemeni manufacturing companies are able to produce medicinal products with high quality, about as compared with original brand Voltaren®.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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