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PHYSICOCHEMICAL AND MICROBIOLOGICAL ASSESSMENT OF SOME COMMERCIALLY AVAILABLE STERILE WATER FOR INJECTION BRANDS IN NIGERIA.

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ABSTRACT

Sterile water for injection used in pharmaceutical and clinical practices are expected to comply with standard specifications. The purpose of this study was to conduct physicochemical and microbiological assessment of commercially available sterile water for injection found in Nigeria market. The physicochemical tests were carried out following standard procedures while the determination of microbiological contaminants of each sample was preceded by 48h incubation at 37^{o}C using plate count agar method. The microbial contaminants were expressed as colony forming unit per millilitre (cfu/ml). Limulus Amoebocyte-lysate (LAL) test was used for pyrogen test to determine the presence of bacterial endotoxin. The results showed that almost all the samples analysed were within the acceptable limits of physicochemical parameters. The tests for bacterial endotoxin showed that the concentration of endotoxin in all the samples were within the acceptable concentration of ≤ 0.25 EU/mL. Sample E, however, did not conform to the local regulatory registration requirement as stipulated by NAFDAC. Although it passed the chemical and microbial tests, but, it fell short of the physical assessment test. In conclusion, the different brands of sterile water for injection analysed met the official specifications. These brands could thus be safely interchanged one in place of another in the reconstitution of pharmaceutical injectable products.

KEYWORDS: Sterile water for injection, physicochemical quality, microbial quality.

INTRODUCTION

Drugs are administered parenterally when they cannot be given orally, due to the unconscious or uncooperative state of the patient or due to inactivation or lack of absorption in the intestinal tract.[1] Up to 90-95% of patients in the hospital receive some type of intravenous therapy.[2] These parenteral products have been documented to contribute to the source of life threatening infections and are on record as one of the strongest factors for morbidity and mortality associated with nosocomial infections in hospitals all over the world.[3] Contaminated fluids have been reported in United States to be the largest and most lethal causes of outbreak of hospital acquired infections known.[4]

The use of water in pharmaceutical industry is indispensable, especially in pharmaceutical liquid preparations.[5] It serves many purposes such as an ingredient, solvent, excipients, for reconstitution of product, during synthesis, cleaning agents and other purposes in the production, processing and formulation of pharmaceutical products. Water as a universal solvent is able to dissolve, absorb, adsorb or suspend many different compounds. These include contaminants that may represent hazards in themselves or that may be able to react with intended product substances, resulting in health hazard.[6]

Sterile water for injection (SWFI) should contain no added substances and must pass the test for total organic carbon (TOC) and water conductivity (WC) test. It is also expected to meet the requirement of the test for bacterial endotoxin as well as all the requirements of the test recommended under sterile purified water (SPW).[7] It is generally mandatory that microorganisms or their product must not be present in sterile pharmaceutical products throughout its shelf life.[8][9] It is very important that these parameters are properly assessed because a lot of scientific researchers have reported various health risks associated with them.[10-13]

MATERIALS AND METHODS

Sampling

The brands of sterile water for injection used for this study were procured at random from different drug markets and pharmacies in Nigeria between August and November, 2014. They were stored under appropriate conditions. The sampled brands represent the largest proportion easily accessible in Nigeria.

Table 1: Description of sterile water for injection tested

Sample code	Sample name	Country Manufacturer	NAFDAC* Registration No	Batch No	Manufacture date	Expiry date
A	Sterilized water for injection BP.	India.	A4-3874	2501276	April 2013	March 2018
B	Sterilized water for injection BP.	Nigeria	04-7474	02W09	June 2010	May 2015
C	Aquadee sterilized water for injection BP.	India	A4-5831	3541213	Dec 2013	Nov 2018
D	Geneith sterilized water for injection BP.	India	A4-9893	0442323	Aug, 2008	July 2017
E	Mini-plasco water for injection.	Germany	NA+	11314011	4th Aug, 2014	Jan, 2017
F	Juhel sterilized water for injection BP.	Nigeria	04-6442	55DE09	May, 2014	April 2017
G	Marck sterilised water for injections BP.	India.	A4-4141	2T543135	Aug, 2013	July 2016

*****NAFDAC: National Agency for food and drug administration and control

+NA: Not available

Reagents and solvents used

The freshly prepared and analytical grade reagents used in carrying out the research include: standard buffer solution of 4.00, 7.00, and 9.10, phenol red solution, 0.01M sodium hydroxide, 0.01M hydrochloric acid, dilute sulphuric acid, 0.02M potassium permanganate, chloride standard solution (5 ppm Cl), distilled water, dilute nitric acid, dilute hydrochloric acid, barium chloride, normal saline (0.9% sodium chloride), plate agar (Lab M limited, UK), *Genscript* ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (32 rxn; Cat. No L00350; Lot C50091310) which contains Limulus amoebocyte lysate (LAL), LAL reagent water, *E. Coli* endotoxin standard, chromogenic substrate, buffer S for colour-stabilizer #1, colour-stabilizer #1, colour-stabilizer #2 and colour-stabilizer #3.

Apparatus and instruments used

The following apparatus and equipment are used in carrying out the research experiment: Test tubes, glass funnel, beakers, test tube rack, pH meter (PHS -25 pH Meter, China), water bath (Techmel, USA), electric oven (New life DG-9023A, England), Analytical weighing balance, crucibles, petri dish, incubator (Memmert 100-800), pipettes, autoclave (LD2X-40), electronic weighing balance (HCK by Dipse), 1liter Erlenmeyer flask, graduated cylinder, stirring rods, vortex mixer (XH-D by Jiangsu Kangjian), laminar flow chamber (ESCO Tech. Inc, USA), and UV/Visible spectrophotometer (JENWAY 6405, UK), AAS, Biotech Engineering, UK Phoneic-986, conductivity meter (winLab 1807, Germany) and lamotte spectrophotometer USA.

Physicochemical analysis

Physical assessment

Under suitable condition of visibility the clarity of the samples were examined. The packaging and labelling were examined very carefully to check for required information such as manufacturer's address, manufacturing dates, batch numbers, expiry date, and the National Agency for Food and Drug Administration and Control (NAFDAC) registration number.

Physicochemical Tests

Physicochemical tests for the determination of pH, acidity/alkalinity, conductivity, oxidizable substance level; concentration of chloride, carbon dioxide, sulphates, ammonia and residue on evaporation were carried out according to the procedures outlined by official compendia.[14, 15]

Nitrate content was determined with the Lamotte smart 3 spectrophotometer using the cadmium reduction method. 10mL of the sample was measured and poured out into the sample cell and the content of one Lamotte Nitra Version 5 (Nitrate reagent powder pillow), which is gentistic acid, was added. A five- minute reaction time was allowed after which the concentration of Nitrogen-Nitrate was read with the spectrophotometer at a wavelength of 400 nm using de-ionized water as reagent blank. Results obtained were in mg/L Nitrate-nitrogen. Nitrate concentration (mg/L) in water sample was obtained by conversion factor of 4.43 as stated below.

Calculation

$$NO_3\ (mg/L) = NO_3\text{–N (mg/L)} \times 4.43$$

The levels of aluminium, calcium, magnesium, lead, chromium, mercury, cadmium, and manganese were determined using atomic absorption spectrophotometer. 25mL of each sample was measured into a glass beaker and 3ml of nitric acid was added. The mixture was placed on a hot plate in a fume hood and heated gently until production of white fumes and the volume reduced to 1/3 of the original volume which indicates that digestion is complete. The digest was allowed to cool to room temperature and made up to mark with deionised water to 25mL mark volumetric flask. The sample was stored in an Erhlemenyer bottle and analysed using atomic absorption spectrophotometer.

The digestate was aspirated by the atomizer of the atomic absorption spectrophotometer into the nebulizer where it went into the flame that ionizes it. The absorbance of the sample is a function of the intensity of the flame produced when the sample was nebulized. The concentration of the analyte was calculated from the linear calibration graph of the atomic absorption spectroscopy.

The determination of Total Organic Carbon (TOC) was carried out as follows: 1mL of the sample was measured into a beaker and 20mL of potassium chromate was added into it. 10mL of sulphuric acid was added into the mixture.100ml of distilled water was added to the mixture and was allowed to stand for 30mins to cool down to room temperature. The sample was read on a smart 3-spectrophotometer at a wavelength of 610nm. The result obtained from the calibration graph and was expressed in mg/L.

Microbiological Analysis

Sterility Test

Under aseptic condition, serial dilution of 1 in 10 mL was made with the sample in normal saline. A 1 ml portion of each serial dilution was mixed thoroughly with the corresponding freshly prepared and cooled plate count agar and transferred into a labelled petri dish. The agar was allowed to solidify and incubated at 37^{o}C for 48 hours. The number of colonies was counted and expressed as bacterial cells per ml. This was carried out in triplicate for all the samples.

Bacterial Endotoxin Test

Reagents preparation

Lyophilized lysate was constituted by adding 1.7 ml of Limulus Amoebocyte lysate reagent water and gently swirled for 30 seconds to avoid foaming. Chromogenic substrate was

reconstituted by adding 1.7 ml of Limulus amoebocyte lysate reagent water. Colour-stabilizer #1 (stop solution) was reconstituted by adding 10 ml of buffer S. Colour stabilizer #2 and #3 was also reconstituted by adding 10 ml of limulus Amoebocyte lysate reagent water to each. The Lyophilized endotoxin standard (20 EU) was dissolved with 2 ml of limulus Amoebocyte lysate reagent water and thoroughly mixed for 15 minutes using a vortex mixer. 1 EU/ml was prepared from the endotoxin solution to make four standard serial dilutions.

Test procedure

A 0.1 ml portion each of samples, standard and LAL reagent water was carefully dispensed into different endotoxin free vials and labelled properly. A 0.1 ml of reconstituted LAL was added to each vial, capped and mixed properly by swirling gently. The rack with all the vials was incubated at $37^{o}C \pm 1^{o}C$ using water bath for 12 minutes. After proper incubation, 0.1 ml of reconstituted chromogenic substrate solution was added to each vial. This was gently mixed with a vortex mixer. The vials were incubated for 6 minutes at $37^{o}C \pm 1^{o}C$ using water bath. A 0.5 ml of reconstituted stop solution (colour stabilizer #1) was added to each vial and swirled gently to mix well. Then, 0.5 ml of colour stabilizer #2 was added to each mixture and mixed properly. Finally, 0.5 ml of reconstituted colour stabilizer #3 was added to each vial and gently swirled to avoid foaming for 3 seconds. The absorbance of the resulting solution in each vial was read at 545nm. The LAL reagent water was used as the blank.

RESULTS

The results of the physicochemical analyses of the sterile water for injection samples are presented in Tables 2 & 3.

Table 2: Physical assessment of sampled sterile water for injection

S/N	Brand code	NAFDAC Reg No	Mfg. & Exp. Date	Batch No	Colour	Clarity	Remark
1	A	Yes	Yes	Yes	Colourless	Clear	Passed
2	B	Yes	Yes	Yes	Colourless	Clear	Passed
3	C	Yes	Yes	Yes	Colourless	Clear	Passed
4	D	Yes	Yes	Yes	Colourless	Clear	Passed
5	E	No	Yes	Yes	Colourless	Clear	Failed
6	F	Yes	Yes	Yes	Colourless	Clear	Passed
7	G	Yes	Yes	yes	colourless	Clear	passed

Table 3: Chemical characteristics of the samples of sterile water for injection

PARAMETERS	SAMPLES							
	A	B	C	D	E	F	G	Standard
pH (Mean ± SEM)	5.23 ± 0.003	6.14 ± 0.005	5.43 ± 0.003	5.16 ± 0.003	5.03± 0.006	5.60± 0.003	5.04± 0.005	5.0 – 7.0
Conductivity (µS/cm) (Mean ± SEM)	0.7 ± 0.05	0.9 ± 0.03	0.9 ± 0.05	0.8 ± 0.03	0.9 ± 0.03	1.0 ± 0.05	0.7 ± 0.03	Max 25
TOC (mg/L)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	Max 0.5
Acidity	N	N	N	N	N	N	N	N
Alkalinity	N	N	N	N	N	N	N	N
Dissolved CO_2	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Nitrates (mg/L)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	Max 0.2
Ammonia (mg/L)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	Max 0.2
Sulphates	-	-	-	-	-	-	-	-
Chloride	-	-	-	-	-	-	-	-
Oxidizable Substance	Faintly pink	Faintly pink	Faintly pink	Faintly pink	Faintly pink	Faintly pink	Faintly pink	Faintly pink
Residue on Evaporation (%w/v)	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Max 0.004%

KEY: N = Neutral; - = No change in appearance

The results of heavy metals levels obtained using atomic absorption spectroscopy are presented in Table 4. The heavy metals analysed include; Aluminium, calcium, magnesium, lead, cadmium, chromium, mercury, and manganese.

Table 4: Levels of metals (mg/L) in the samples of sterile water for injection

Metals			Samples				
	A	B	C	D	E	F	G
Calcium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Aluminium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Magnesium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Chromium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cadmium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lead	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Mercury	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Manganese	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002

MICROBIOLOGICAL ANALYSIS

Microbial analyses were carried out on the samples to determine the presence of microorganism and endotoxins. The result of the sterility test is presented in table 5.

Table 5: Total Bacterial viable count of the samples in colony forming unit per mL (cfu/mL)

SAMPLES	Cfu/ml x 10^1	Remark
SWFI- A	0	No Microbial growth
SWFI-B	0	No Microbial growth
SWFI-C	0	No Microbial growth
SWFI-D	0	No Microbial growth
SWFI-E	0	No Microbial growth
SWFI-F	0	No Microbial growth
SWFI-G	0	No Microbial growth

Table 6: Absorbance values of known concentration of standard Endotoxin solution

S/N	Concentration	Absorbance at 545nm	ΔAbsorbance
1	LAL reagent water (blank)	0.722	-
2	1.00EU/mL standard	1.577	0.855
3	0.50EU/mL standard	1.147	0.430
4	0.25EU/mL standard	1.007	0.287
5	0.125EU/mL standard	0.924	0.199

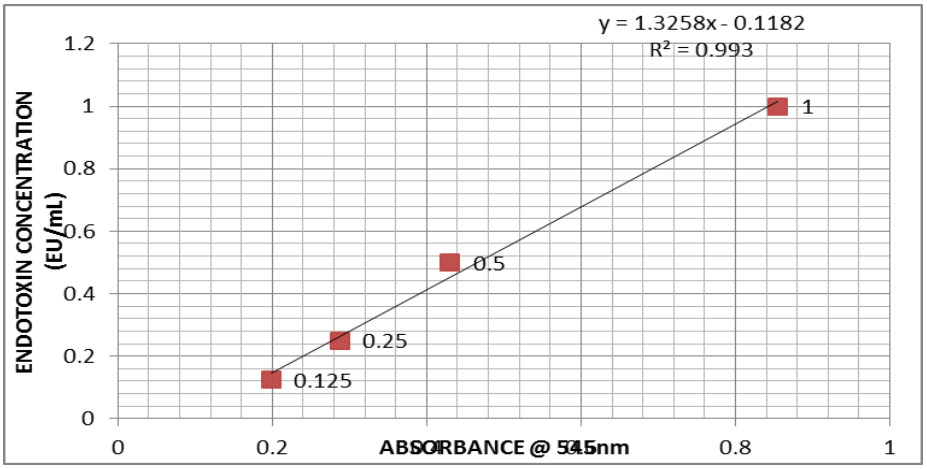

Fig 1: Standard Curve for the Quantification of Endotoxin in the samples of sterile water for injection

Table 7: Level of Bacterial Endotoxin in the different brands of sterile water for injection sampled.

Samples Code	ΔAbsorbance (mean) @545nm	Endotoxin concentration (EU/mL)
A	0.201	0.235
B	0.226	0.260
C	0.181	0.226
D	0.222	0.257
E	0.202	0.242
F	0.193	0.235
G	0.168	0.216

DISCUSSION

Physical assessment result showed that samples A, B, C, D, F and G, were well packaged and properly labelled, with labels containing NAFDAC registration numbers, manufacturing date, expiry date and batch number. Sample E, although well packaged, had no NAFDAC registration number. NAFDAC registration number is one of the regulatory parameters which must be endorsed on the packaging of sterile water for injection because it shows that such drugs have been legally approved for marketing in Nigeria. Under suitable condition of

visibility, none of the samples showed the presence of particles. The result obtained from the residue on evaporation showed that the samples were free from any form of solid concentrate as the percentage of residue on evaporation for each of the samples was 0%. The acceptable limit of residue on evaporation is 0.004%w/v.[10, 14]

The pH value of all the samples complied with the requirement for pH limit of between 5.0 and 7.0.[7, 10] The pH values of all the samples are within the range of 5.03 and 6.14. This is neither too acidic nor alkaline; therefore the tendency of the water to corrode plumbing system or cause leaching is minimal.

The assay of the samples for acidity, alkalinity, conductivity, total organic carbon, ammonia, chloride, sulphates, nitrates, oxidizable substances produced results which were within the acceptable criteria stipulated by the official compendia. Conductivity in water is affected by the presence of inorganic dissolved solids as chloride, nitrates, sulphates, and phosphate anions or sodium, magnesium, calcium, iron, and aluminium cations. The conductivity value of the samples were far below the maximum stipulated limits. This explains the very low levels of inorganic substances found in the samples. In 1996, the oxidizable substance test was replaced by the Total Organic Carbon test for bulk purified water and Water for injection; but the test was retained for testing sterile waters, even as the other chemical tests are been replaced in 2008. The retention of oxidizable substance test was due, in part, to the difficulty in determining an appropriate and safe instrument test method and limit for organic impurities that would arise from packaging and packaging process.

The Atomic absorption spectroscopy determination of metals showed that the samples were free from contaminating metals. The values obtained from analysis showed that the level of calcium, aluminium, magnesium, Lead, cadmium, chromium and mercury was less than 0.001 ppm while the acceptable limit of heavy metals as stated in the USP and BP is 0.1 ppm.[7, 10] The concentration of metals in the sterile water for injection samples were undetectably below the instrument detection limit of measurement.

Microbiological Quality

The bacteriological test result indicated that all the samples were free from microbiological contamination as no microbial growth was observed after 48hours of incubation. This result is of great significance as it is generally mandatory that microorganisms or their product must not be present in sterile pharmaceutical products throughout its shelf life.[10] The results of the

test of bacterial endotoxin in the sterile water for injection samples showed that all the samples passed the bacterial endotoxin test. The endotoxin concentrations of the samples were calculated using the equation of the straight line y = 1.3258x – 0.1182. This equation was obtained from the graph of standard curve for the quantification of Endotoxin in chromogenic assay where the absorbance values of standard endotoxin solutions of known endotoxin concentration were used to plot the standard calibration curve. This produced a standard calibration curve with good linearity; the coefficient of correlation (r) of the graph was 0.993 (this was within the acceptable range of ≥ 0.980 as indicated in the Toxin Sensor ™ Endotoxin Detection system User Manual), slope of 1.3258 and intercept of 0.1182. The endotoxin concentration of different brands was extrapolated from the standard curve. The results obtained showed the all the samples have endotoxin concentration which is within the acceptable limit (< 0.25 EU/mL) as stated in the British and United States Pharmacopoeia.

CONCLUSION

From the analysis carried out on the different brands of sterile water for injection marketed in Nigeria and the result obtained, it can be concluded that the samples were within the specified limits as stated in the United States and British pharmacopoeia in terms of physicochemical and microbiological parameters. Therefore patients might not be exposed to major health risk in using any of the brands. It is recommended that the various brands of sterile water for injection in the market should continue to be monitored on regular basis by the National Agency for Food and Drug Administration and Control (NAFDAC) for proper quality assurance.

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