

Original Research Article

Bioassay-guided optimization of lipid-based erythromycin microparticles

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Abstract

Purpose: To optimize erythromycin microparticles by *in vitro* bioassay methods based on its antibacterial activity.

Methods: The microparticles were produced by high shear homogenization. The effects of different lipid-to-surfactant ratios were studied. The hydrodynamic size of the different batches was evaluated using dynamic light scattering while bioactive drug load per batch was assessed in agar using bioassay methods. The antimicrobial activities of selected batches were tested *ex vivo* by determination of reduction in bacteraemia following administration of the microparticles to infected animals.

Results: All batches had particles with hydrodynamic sizes < 8.5 microns. Batch 7 with a 2: 5: 2.5 (drug: surfactant: stearic acid) ratio, represents the optimized batch with a hydrodynamic size of 2281 nm, a bioactive drug loading capacity (BLC) of 4.67 ± 0.70 % and bioactive drug entrapment efficiency (BEE) of 10.51 %. The "microparticle MIC" against *Staphylococcus aureus* was 1.74×10^{-3} µg/ml. Despite containing lower amounts of erythromycin than the pure sample, the microparticles achieved comparable reduction in bacteraemia, with the optimized batch exhibiting lower variation in bacteraemia than the pure drug.

Conclusion: Erythromycin microparticles have been successfully optimized with the aid of bioassay methods which has the advantage that only the bioactive drug concentration is factored in. This method eliminates problems posed by inadequate or non-discriminating chemical assay methods.

Keywords: Microparticles, Erythromycin, Gastrointestinal, Bioavailability Antimicrobial, Bioactivity, Encapsulation

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INTRODUCTION

Erythromycin is an insoluble drug used in many infectious disease conditions including atypical pneumonia [1-3]. In addition to poor solubility, erythromycin is also unstable, thus requiring

storage at low temperatures. Being basic, it is easily protonated at low pH, which causes loss of activity. This instability has been associated with gastrointestinal side effects and hepatotoxicity [4-6]. In addition, it has an unpleasant taste, and a rather low half-life of 1.5 h [3]. As such, it is

normally prepared as film or enteric-coated tablets to protect it from gastric fluid. However, such film coating often leads to incomplete absorption. Furthermore, enteric coating of erythromycin has been associated with increase in the incidence of side effects [7], hence the conversion to other analogues like azithromycin which is aimed in part to overcome this challenge [6]. Solid lipid microparticles (SLMs) are readily prepared using simple homogenizing equipment and have the capacity for achieving high drug loadings [8-9] given their fairly bigger particle sizes in comparison with nanoparticles. They are therefore better suited for sustained release effect where desired because drug release can occur over longer periods. As has been demonstrated with liposomes, large particles may be used to selectively target cells of the mononuclear phagocyte system [10]. The use of suitably-sized SLM may confer other advantages such as limiting the distribution of the drug following administration by a particular route. There have been attempts before now to optimize erythromycin colloidal formulations using such methods as surface contour and factorial design methods [3,11]. However, the optimizations tended to lay too much emphasis on particle size, encapsulation efficiency or drug loading obtained by instrumental methods, whereas *in vitro* physicochemical data may fail to correlate with *in vitro* or *in vivo* antibacterial performance. Determination of drug loading by instrumental methods can often overestimate the total biologically effective and diffusible drug concentration (referred to here as bioactive drug load), since with fairly large size systems such as microparticles, total erosion of the matrix may not occur even in the GIT if insoluble matrices such as lipids are used. Moreover, due to severity of conditions encountered during preparation of such particles, such as high-pressure homogenization or high homogenization temperatures, a fraction of the drug load may convert or degrade to inactive forms and polymorphs. For drugs such as erythromycin, microbiological assay remains a method of choice for assay [12]. The aim of the current study is to use *in vitro* bioactivity for selecting an optimum drug:surfactant:lipid ratio for delivery of erythromycin as solid lipid microparticles.

EXPERIMENTAL

Materials

Erythromycin stearate was received as a gift from Juhel Nigeria Limited, Enugu, Nigeria. Both *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* were obtained from stock cultures maintained in the Department of

Pharmaceutical Microbiology of Nnamdi Azikiwe University, Awka. Wistar albino rats of both sexes weighing between 80 - 120 g were obtained from the animal house of the Department of Pharmacology & Toxicology of the same Institution.

Other materials were used as procured from their suppliers without further processing. Animal experiments were done according to the Principles of Laboratory Animal Care and in accordance with other guidelines developed by the local ethics committee.

Preparation of erythromycin-loaded SLM

Eight (8) batches of the formulations were prepared using varying ratios of stearic acid, surfactant and drug according to the formula in Table 1. The preparations followed the method of Cavalli *et al* [13], with modifications. Briefly, the needed quantity of lipid for a particular batch was weighed into a beaker and heated to $70 \pm 1^\circ\text{C}$. The drug was gently dispersed in the lipid melt. In another beaker, the required amounts of surfactant and water were mixed together and heated.

Table 1: Composition of microdispersion

Batch	Stearic acid (g)	Tween 80 (mL)	Erythro-mycin (g)	Water to, (g)
1	1	1	2	100
2	1	2.5	2	100
3	1	5	2	100
4	1	7.5	2	100
5	2.5	1	2	100
6	2.5	2.5	2	100
7	2.5	5	2	100
8	2.5	7.5	2	100

A pre-emulsion was obtained by mixing the lipid and the aqueous phases and homogenizing (Stuart, UK) at 1000 rpm for 15 min. This was followed by shock cooling, wherein the pre-emulsion was introduced drop-wise into 500 mL ice cold water, with stirring effected by a magnetic stirrer assembly. The suspensions were then filtered using Whatman No. 1 filter paper and the solid residues collected on a filter paper and dried in a desiccator fused with calcium chloride.

This represents the formula of the microdispersion before dilution with cold water, filtration and drying.

Determination of hydrodynamic size

The hydrodynamic sizes of reconstituted and suitably diluted (with distilled water) samples

were measured by photon correlation spectroscopy at 25 °C in a particle sizer (Zeta Nano ZS, Malvern Instruments, UK), as previously reported [14].

pH stability study

The pH values of the microparticles dispersions were determined over a three-week period with the aid of a pH meter (Jenway, UK).

Evaluation of thermal stability

This was conducted by weighing 1 mg of the microparticles and then sealed in an aluminium pan with a similar empty pan serving as control. The machine was calibrated with indium and purged with nitrogen gas. Heating of the sample was carried out at the rate of 10 °C/min from 30 °C – 400 °C under nitrogen flow rate of 20ml/min followed by cooling back to 30 °C at the same rate. This procedure was done using the Calorimeter-Netzsch DSC 204 FI (Phoenix, Germany) and was carried out at the Nigerian Institute of Pharmaceutical Research and Development (NIPRD) Abuja.

Bioassay-based determination of drug loading

Fifty (50) mg quantities of microparticles were introduced into 9 bijou sample bottles. In a conical flask, 10 mL of Tween 80 was added to 90 mL of distilled water with stirring to ensure homogenous mixing. The aqueous surfactant solution was added to the samples held in the bijou bottles to obtain 10 mg/mL concentrations of microparticles. These dispersions were further diluted 2-fold to obtain other concentrations: 5, 2.5, 1.25, and 0.625 mg/mL of microparticles. Agar plates (24) were arranged in rows of three per row. To each dish, 20 mL of Müller Hinton Agar was added. The molten agar was allowed to solidify and a standard culture of *Staphylococcus aureus* was streaked on the petri dishes using a swab stick. Five holes were bored per dish using a sterile cork borer. The separate dilutions of pure drug or each batch (20 µL of each) were introduced into the holes (one concentration per hole). The petri dishes were then inverted and incubated overnight and the inhibition zone diameters were measured and converted to concentration units using the calibration curve obtained for the pure drug. The bioactive drug loads and encapsulation efficiencies were calculated from these concentrations using Equations 1 and 2 respectively. Standard dilutions of pure erythromycins were similarly prepared.

$$\text{BLC (\%)} = \frac{C_{\text{bioactive drug}}}{\text{amount of microparticle}} \times 100 \dots\dots\dots (1)$$

where the BLC is the bioactive drug loading capacity, the $C_{\text{bioactive drug}}$ is the concentration of bioactive drug (determined by bioassay).

Similarly, the bioactive drug entrapment efficiency (BEE) was computed as in Eq 2.

$$\text{BEE (\%)} = \frac{\text{BLC} \times 100}{\text{theoretical loading capacity}} \dots\dots\dots (2)$$

where BLC is the bioactive drug loading capacity determined in Eq 1.

Drug release studies

An *in vitro* release was conducted for 2 hours separately using buffers of pH 4.0 and 7.0. For the release study, a 10 % shellac coating solution was prepared. An empty gelatin capsule was filled with 100mg of the microformulation and immersed in the coating solution using a forcep and then withdrawn to dry. When dried, the capsule was dropped into the dissolution medium prepared with 1 % tween 80 in the appropriate buffer

After 30 min, 4 ml of the solution was withdrawn in triplicates and their absorbance read at the wavelength of the drug, 320nm. This process was repeated at 30 min interval for 2 hours. Conversions were done using a calibration curve obtained in the respective media.

In vivo activity studies

Animal experiments were done according to the Principles of Laboratory Animal Care and in accordance with other guidelines developed by the local ethics committee. Wistar albino rats (80 - 120 g) were randomly divided into four groups of five rats each. A suspension of *E. coli* (0.1 mL of 0.5 McFarland standard concentration) was inoculated into groups 1 to of the animals intra-peritoneally (IP) to establish infection. Blood (1 mL) was collected from each animal for total viable count (TVC) using pour plate method before the inoculation to establish base-line systemic colony forming unit. This was repeated after inoculation to confirm bacteremia. All groups received equal amounts (25 mg/kg) of either pure erythromycin or the microparticles (Batch 6, 7, or 8). The treatment was maintained for three days while determining the TVC based on healthy animals only to ascertain the effect of the pure drug sample and also that of the formulations. A fifth group of animals were similarly infected but not treated.

Statistical analysis

Statistical analysis was carried out with Microsoft Excel and GraphPad 6 software. The latter was used for two-way RM ANOVA with Bonferroni's multiple comparisons test. Statistical significance was tested in groups of animals ($n = 5$), and P-values of less than 0.05 were considered significant.

RESULTS

Physicochemical properties

Table 2 shows the hydrodynamic sizes and polydispersity indices of the samples. The hydrodynamic diameters of the particles varied between 2.28 μm and 8.15 μm . The batches were all slightly alkaline.

Thermal properties

The thermogram of erythromycin (Fig.1) showed a strong endothermic peak at 86.2°C with a second broad transition at 297°C which may imply the presence of an amorphous fraction. Stearic acid showed an endothermic peak at 61.8 °C (Fig. 2) which suggested it would produce relatively firm microparticles. Three endothermic peaks (Fig. 3) were observed in the thermogram of the erythromycin-loaded microparticles. These peaks represent the components of the microparticles including erythromycin and stearic acid. The appearance of the peak of erythromycin in the formulation thermogram shows that the erythromycin is dispersed in the stearic acid matrix and the microparticles exists more as solid suspensions since the peak of erythromycin was still very much pronounced in the microparticles thermogram.

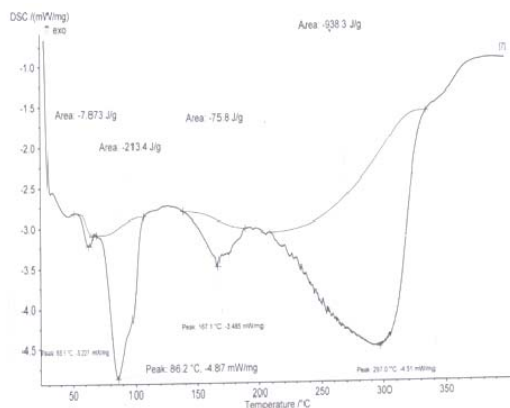


Figure 1: DSC thermogram of erythromycin

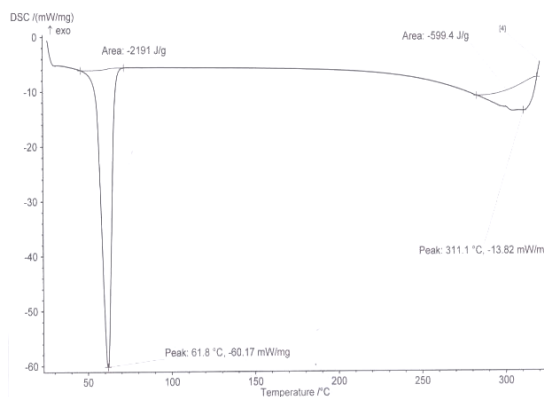


Figure 2: DSC thermogram of stearic acid

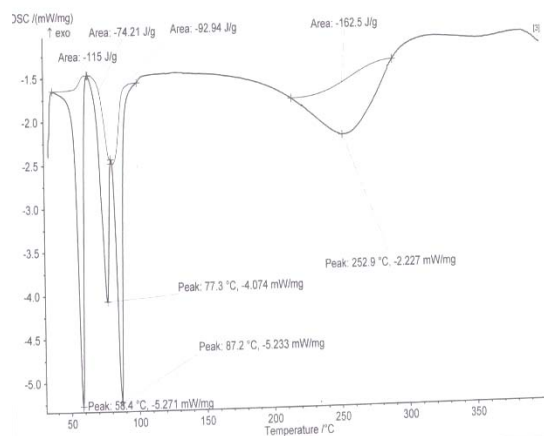


Figure 3: DSC thermogram of erythromycin SLM (Batch 8)

“Bioactive” drug loading capacity

The inhibition zone diameters and concentration of erythromycin have been fitted into a simple logarithmic equation in Figure 4, with correlation coefficients (R^2) presented below. The conversions to concentration and hence BLC were done using a linear calibration curve of IZD against $\text{Log}[\text{erythromycin}]$, with ($y = 5.8846x - 1.0072$, $R^2 = 0.9924$). The squared inhibition zone diameters (IZD^2) were further plotted against the $\text{Log}[\text{microparticles}]$ to derive the corresponding MIC values for the batch of microparticles. The MIC of the pure erythromycin was calculated as 4.00 $\mu\text{g/ml}$. MIC values derived for the microparticles were much lower than for erythromycin powder, with batch 4 giving an MIC of 6.25×10^{-6} $\mu\text{g/ml}$, while batch 7 gave an MIC of 1.74×10^{-3} $\mu\text{g/ml}$. On the other hand, Batch 2 exhibited a high MIC of 4.01 $\mu\text{g/ml}$ which was comparable to that of pure erythromycin (4.00 $\mu\text{g/ml}$).

The calculated bioactive drug load data are presented in Table 3.

Drug release

The enteric-coated capsules exhibited negligible release of drug in low pH medium (pH 4.0) over the two-hour test period, with cumulative release being just above 2 %. More drug was released in to the medium at higher pH (7.0), achieving 48 % release in 2 hours. The release profiles in the two media are presented in Figure 5.

In vivo activity

Following a three--day administration of the microparticles at a dose of 25 mg/kg, there were differences in i reduction in bacteraemia. Colonies were counted at the start of the experiments (Day 0, Day 3 and Day 7).

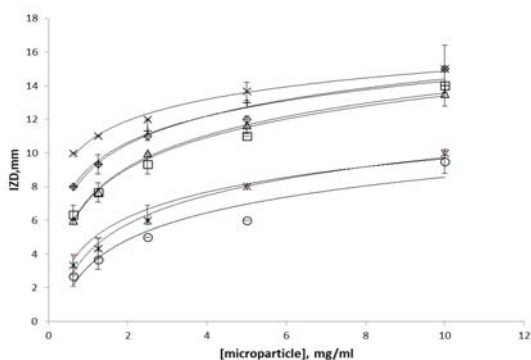


Figure 4: Fitting of IZDs produced by batches of microparticles in a logarithmic equation.

Key: \diamond = batch 1 ($y = 2.405 \ln(x) + 8.8623$, $R^2 = 0.9660$); \square = batch 2 ($y = 2.6935 \ln(x) + 7.198$, $R^2 = 0.9740$); \triangle = batch 3 ($y = 2.7411 \ln(x) + 7.2563$, $R^2 = 0.9970$); \blacktriangle = batch 4 ($y = 1.8279 \ln(x) + 10.659$, $R^2 = 0.9890$); \blacklozenge = batch 5 ($y = 2.454 \ln(x) + 4.0834$, $R^2 = 0.9853$); \circ = batch 6 ($y = 2.3069 \ln(x) + 3.2542$, $R^2 = 0.9207$); $+$ = batch 7 ($y = 2.2607 \ln(x) + 9.0605$, $R^2 = 0.9897$); \blacksquare = batch 8 ($y = 2.164 \ln(x) + 4.6831$, $R^2 = 0.9829$). IZD denotes inhibition zone diameter

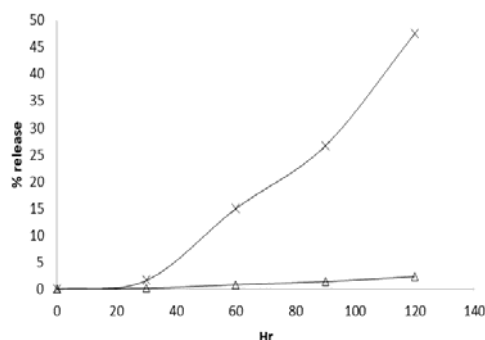


Figure 5: Release of erythromycin microparticles from gelatin capsules enteric-coated with shellac. **Key:** -x- buffer pH 7, -Δ- buffer pH 4

DISCUSSION

The advantage of the method of hot pre-emulsion followed by dilution in ice-cold water is that it can be used in preparing both nano and microparticles by varying the conditions and relative amounts of ingredients without a further need for high pressure homogenization. However, the disadvantage is that it can produce particles having fairly high polydispersity, particularly if the drug being loaded is insoluble in the matrix in that there would be a mixture of microparticles and insoluble drug particles in the system.

The 1:2 lipid-to-surfactant ratio resulted in the optimum particle size of 2281 nm. This corresponds to the optimum ratio we obtained previously using a different drug [14]. Size is important in the stability and pharmacokinetics of nano and microparticle systems. Big particles are constrained in moving through the different anatomical barriers as well as in particle uptake. Low size would be an advantage for improved microparticle kinetics given the number of anatomical barriers to drug uptake. In fact, it has been claimed that the size exclusion limit for uptake of particles in rat was 3 μm [15]. Other reports suggest that though particles bigger than 5 μm can be taken up by Peyer's patches, they

Table 2: Hydrodynamic diameters of different batches of erythromycin-loaded microparticles

Batch	(Drug: lipid): surfactant (g:g)	Theoretical loading (%)	3-week average pH	Z-average (nm)	PDI
1	2: 1: 1	66.67	8.66±0.04	2288	1.000
2	2: 1: 2.5	66.67	8.19±0.06	8145	0.475
3	2: 1: 5	66.67	8.29±0.07	6115	1.000
4	2: 1: 7.5	66.67	8.51±0.02	6573	0.382
5	2: 2.5: 1	44.44	8.41±0.06	6366	1.000
6	2: 2.5: 2.5	44.44	8.00±0.06	6017	0.768
7	2: 2.5: 5	44.44	8.13±0.03	2281	1.000
8	2: 2.5: 7.5	44.44	8.39±0.03	2943	0.970

^aThe density of tween 80 is approximately 1 g/ml

Table 3: Calculated “bioactive” drug loading capacity (BDL) of erythromycin-loaded microparticles

Batch	BDL (%) at each dilution					Av. BDL (%)	BEE (%)	MIC ($\mu\text{g/ml}$)
	10 mg/mL	5 mg/mL	2.5 mg/mL	1.25 mg/mL	0.63 mg/mL			
1	5.24	3.24	4.39	4.57	5.42	4.57±0.86	6.85	8.39 x10 ⁻²⁴
2	3.55	2.19	2.45	2.39	2.85	2.64±0.57	3.96	4.01
3	2.92	2.85	2.97	2.39	2.48	2.72±0.27	4.08	0.87
4	5.25	6.24	6.49	8.77	11.89	7.73±2.66	11.59	6.25 x 10 ⁻⁶
5	0.74	0.68	0.62	0.65	0.87	0.71±0.10	1.60	103.47
6	0.61	0.31	0.42	0.50	0.67	0.50±0.15	1.13	763.13
7	3.55	4.80	5.00	4.57	5.43	4.67±0.70	10.51	1.74 x 10 ⁻³
8	0.74	0.68	0.71	0.84	1.14	0.82±0.19	1.85	20.32

MIC of pure erythromycin against the organisms is calculated as 4.00 $\mu\text{g/ml}$

Table 4: Percentage reduction in bacteremia (*E. coli*) with administration of erythromycin-loaded microparticles

Batch	Initial count (ml)		Reduction in bacteraemia (%)	
	Day 0	Day 3	Day 3	Day 7
Erythromycin powder	132.40±58.76	72.36 ± 16.41	94.66 ± 5.90	
Batch 6	121.6±35.34	51.66 ± 0.06 ^a	89.43 ± 3.97	
Batch 7	284.40±41.15	75.66 ± 5.55	95.61 ± 3.09	
Batch 8	159.00±46.90	58.05± 10.86 ^a	95.65 ± 3.73	

^aSignificantly different from pure erythromycin ($p < 0.05$). 25 mg/kg) of each was used in the test

remain there and do not get into the lymphatic circulation in appreciable amounts [16]. In view of this, having particles less than 3 microns would be an advantage, implying that judging strictly by the hydrodynamic diameters, only Batches 1 and 7 are capable of uptake into Peyer's patches. Disease state can further enhance the uptake of particles in this size range by several fold [17,18].

Erythromycin stearate is a basic drug which is very unstable in acidic media. pH measurements indicated that all the microparticle batches were of alkaline pH, and all exhibited low standard deviation over the 3-week test period. This pH range can be expected to contribute to drug stability. The batches containing higher amounts of the lipid showed a lower pH value than the batches which had higher amounts of surfactants, as expected.

The microbiological assay technique is very suitable for assay of erythromycin and had been used in many preparations of the drug [19]. As shown in Figure 2, there is a logarithmic relationship between concentration and IZD for the microparticles. The bioactive drug loading capacity (BLC) and bioactive drug entrapment efficiency (BEE) were highest for Batch 4, which also recorded the lowest “microparticle MIC” against *Staphylococcus aureus*. Batches 4 and 7 have comparable BEE, but even with the higher BLC of batch 4, batch 7 was chosen as the optimized batch due to a combination of high bioactive drug loading capacity, low

“microparticle MIC” and the low standard deviation of the “bioactive drug load (0.70 vs 2.66 for Batch 4). It also has a low particle size. From the MIC values in relation to pure erythromycin, it can be inferred that delivery as microparticles effectively reduced the MIC of the drug. However, the lowering of MIC may also be solubility related due to the presence of Tween.

Erythromycin is associated with gastro-intestinal side-effects such as irritation of the gastric mucosa, and is also unstable in low pH environment such as gastric fluid, being why some commercial formulations are enteric-coated. Instability at low pH means that all solutions should be analyzed in a very short time to minimize the percentage of degradation. But as noted in preceding sections, enteric coating is associated with its some problems such as incomplete absorption. Low solubility also means that even with ongoing matrix erosion, the recovery of the released drug by the assay method may be incomplete.

E. coli was used in the *in vivo* studies because of its limited sensitivity to erythromycin [20,21]. It was hoped that this would help in discriminating between the antibacterial activities of the microparticles and the pure drug. The percentage reductions achieved by the pure drug and Batch 7 microparticles by Day 3 were 72.36 ± 16.41 and 75.66 ± 5.55 %, respectively. By Day 7, the values were 95.61 ± 3.09 (Batch 7)

and $94.66 \pm 5.90\%$ (pure drug). Despite the relatively low bioactive drug loads as shown in Table 3, the reduction in bacteremia seen in the optimized batch (7) was equal to ($P > 0.05$), and more uniform (lower SD) than for pure erythromycin). The higher variations (SD) seen with the pure drug indicate fluctuations in plasma concentration, which may result in a higher incidence of side effects. Erythromycin has several side effects including gastro-intestinal irritation and liver toxicity, and both the uncoated drug and enteric-coated forms have been associated with these effects [6,7]. In this case, delivery in an inert stearic acid matrix can be expected to minimize this possibility.

This study highlights that, at lower doses of erythromycin delivered as a microparticle, *in vivo* antimicrobial activities are equal to that achieved with higher amounts of the pure drug. This reduction in bacteremia is also more uniform (smaller standard deviation) when compared to the group dosed with pure erythromycin.

The optimization of micro and nanoparticle preparations of antibacterial agents can utilize microbiological assay techniques, particularly if the procedure involves heating or other steps that may cause loss of biological activity which chemical assay procedures may not detect. This is even more compelling for acid unstable and water insoluble drugs like erythromycin which either degrade or are insoluble in several media used in chemical assay.

CONCLUSION

Bioassay-based techniques have been applied in preference to physicochemical techniques in the optimization of erythromycin microparticles, with optimum reduction in bacteraemia in the batch containing drug: surfactant: lipid in the ratio of 2: 5: 2.5 (Batch 7). This method is recommended for antimicrobial agents which either are insoluble in common media used in chemical analysis, or would degrade during analysis. It also factors in the degradation that may be caused by processing conditions such as high temperature, sonication or homogenization.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. ITN conceived the work, supervised it, analyzed data, drafted it and approved the final draft. ACO captured data, analyzed them and prepared the first report of the work, IEO reviewed design of work, and methods, KEE: edited and created further drafts of manuscript while COA reviewed the work, interpreted data, helped in drafting and edition of manuscript. All authors read and approved the manuscript for publication.

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