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# PREPARATION AND EVALUATION OF L-ARGININE CEFATOXIME PESSARY (ALCECO®) (*Part I*)

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## SUMMARY

Aim of our present study was to formulate the L-arginine Cefatoxime pessary (Alceco®) by using direct compression of the formulation technique to improve the physical, chemical and pharmaceutical properties of pessary. We were used the L-arginine Cefatoxime as a core drug preparation, as a vaginal tablet shape suitable in applicators it has a display swelling index slightly decrease as compared with pre storage time for 14 hours. On the other hand pre and post storage period were not displayed differences in solubility, So no changes were seen in cumulative release L-arginine during storage period as compared with pre stability study period and L-arginine viability. Finally stability of their properties programed really sophisticated in as vaginal application.

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**KEYWORDS:** L-arginine, Cefatoxime, Pessary, ALCECO, Swelling index, Vagina, ewe.

## INTRODUCTION

L-arginine is an essential amino acid had numerous pivotal activities in the body system and especially reproductive performance. It assistances the body get rid of ammonia (a waste product); Arginine is synthesized from citrulline by the actions of arginosuccinate synthetase and arginosuccinate lyase[1,2] and catabolized by arginase.[3,4]

Importantly, L-arginine is the only bio-physiological nitrogenous simplistic precursor for NOS-catalyzed reactions; hence availability of this vital substrate could determine amounts of NO generation. Arginine synthesis in addition to its transference into the cells can also influence NO synthesis.[5,6]

Nitric oxide (NO) is a messenger molecule is now known to be produced by various cells in different organs, including smooth muscle cells, NO regulates smooth muscle cell tone, cell growth, apoptosis, neurotransmission as well as infection-induced immune reactions.[7] Because these processes are also associated with the biology, physiology and pathophysiology of multiple reproductive and sexual function with tasks, it is highly likely that NO plays an important role in reproduction. Indeed, in the recent twenty year NO had been recognized as a molecule that importantly regulates the biology and physiology of the reproductive system.[8,9]

Preliminary researches and thesis had found that L-arginine may help with achievement the improvement organ status when blood vessels are vasodilated periodically or long term, such as erectile dysfunction these mediated by Nitric oxide, NO was involved in several tasks as the regulation of penile erection[10], ovulation[12] or transfer of oocytes from the ovaries to the oviducts[13] and estrous cycle synchronization with superovulation. Centrally, NO had an important role in controlling male and female sexual behavior[14], Finally, NO provoked the release of gonadotropin releasing factor (GnRH).[15] The action on NOS is mediated by the aromatization of Testosterone into 17$\beta$-estradiol (E2).[16] Moreover, the NO-c.GMP system is part of an intracellular signaling pathway to regulate the facilitator effect of $\alpha_1$-adrenoreceptors on lordosis and sensual behavior in female rats. To further explain the interrelationships between NO and gonadal hormones, and modified the estrous cycle under provocation of NO and amplify the hypothalamic and limbic system burden of female mice, with up regulation of functional focus on nuclei involved in the control of reproduction.[17]

The main aim of this study was to preparation and develop of L-arginine-Cefatoxime pessary and *in vitro*

challenge test procedure which is close to the physiological conditions of vagina thus to be able to give some predictive working of the *in vivo* drug characteristics.

## MATERIALS AND METHODS

### Manufacturing of L-arginine-Cefatoxime pessary (AlCeco®)

Pessary containing 250 L-arginine and 500 mg Cefatoxime and weighing 500 mg were prepared by direct compression of the formulation in a tablet machine (minipress-1674, Rimek, India) fitted with round; flat-faced 1.7 mm. Table 1 displays the composition of the uterine tablets formulations. A particle size of less than 180 µm for all components was selected to avoid any fractional segregation[18] and several steps undergo to obtain a homogenous blend prior to compression and achieve final formulation of intrauterine pessary, the protocol of preparation steps is listed as follows.

**Table 1: list of active ingredients assemble to L-arginine-Cefatoxime (AlCeco®) pessary as well as the quantity used of each amount.**

| Composition | Amount mg/pessary | percentage |
|---|---|---|
| L-arginine hydrochloride | 300 | 30 |
| Cefatoxime | 250 | 25 |
| Povidone | 30 | 3 |
| Crystalline cellulose | 125 | 12.5 |
| Colloidal Silicon Dioxide | 5 | 0.5 |
| Methylcellulose | 280 | 28 |
| Magnesium stearate | 10 | 1 |

The protocol of processing L-arginine-Cefatoxime pessary was starting as follows.

**Step 1_** all ingredients were screened in a #20 mesh screen and magnesium stearate was screened in #30 mesh screen, and avoids granulation.

**Step 2_** first half amount of povidone was dry mixed with L-arginine and Cefatoxime for 3 minute and the second half povidone was dissolved in purified distilled water then add by spring manually during granulation for 7 minute.

**Step 3_** the wet granules were milling for 2 minute and screen size (180), dried, milling.

**Step 4_** the dried granule blended with crystalline cellulose and colloidal silicon dioxide for 30 minute 50 rpm.

**Step 5_** magnesium stearate blended was add to the blender contains mixture and blend for 20 minute 25 rpm.

**Step 6_** finally, the blend was compressed into Pessaries with a target weight of 300 mg of L-arginine and Cefatoxime 500 mg disc shape, compress a pessary to a hardness about 7 kp.

### Ovine mucus collection

Mucus specimen were collected from the posterior region of vagina of each ewe adult cycling using sterile ewes vaginal speculum and intubation “12" for 8-12” cm for 15 minute, the mucus pass on sterile polyethylene tube by shallow intermittent vacuum machine possessed for different time. Samples were collected immediately prior to the introduction of the device, at the time of device withdrawal, The pH of the vaginal mucus was recorded using pH meter (Merck, Germany), Collected mucus pooled and put in tight, sealed, opaque container and stored in 4 C◦ until used, the mucus was thawed before used for 30 minute.[19,20]

### Evaluation of pessary

### Swelling index studies

L-arginine-Cefatoxime formulation AlCeco® pessary were weighed as an initial mass and immersed in beaker containing the set of dissolution ovine mucus, the dissolution mucus media incubated in water bath at 37.8 C°. The protocol of evaluation of swelling test of AlCeco® were done at end point time 1, 2, 4, 8, 12 and 24 hours, each time were replicated for five time to achieve reproducibility.[21,22] The pessary was pickup via small basket and swollen weight of each pessary was determined. The swelling index was calculated using following formula.

**S.I = Wt –Wo/Wo x 100**

S.I = Swelling index

Wt = Weight of tablet at time “t”

Wo = Weight of tablet before placing in beaker.

### Solubility studies

Solubility studies of AlCeco® L-arginine-Cefatoxime pessary were carried out by shaking an excess amount of drug with 5 ml of the following dissolution medium simulated vaginal: fluid consequently, the specific "recipe" for 1 liter of this simulant given as mixture compound and amount (g) was as follows: NaCl, 3.51; KOH, 1.40; Ca (OH)2, 0.222; bovine serum albumin, 0.018; lactic acid, 2.00; acetic acid, 1.00; glycerol, 0.16; urea, 0.4; glucose, 5.0. The simulant was planned to have the alike “physical and chemical properties as those known to influence intra-vaginal fluid efficacy”. The samples were incubated in a 37°C water bath (Korea) at 60 rpm for 24 hours. After 24 hours, the samples were centrifuged in Eppendorf centrifuge (Model 5810 R, Germany) at 10,000 rpm for 10 minutes.[23] The supernatants were collected and filtered through 0.45 µm filters and estimated L-arginine concentration by spectrophotometer.[24]

**Density measurement of tablet**

Density of tablets was measured from their volumes and masses in triplicate set. The volume V of the cylindrical tablets were calculated from their dimensions height h and radius r by a venire tool "caliper" using the precise of a cylinder equation (V=л×r2×h). The pessary with ~1g/cm$^3$ density or less was chosen for further studies.[25,26]

**Stability studies**

The time challenge for stability of the verified formulated vaginal tablets was assessed by packing them in aluminum foil sheath, sealed tightly and stored for 1 year at 30 C◦ ± 2 at 5% RH. The tablets were analyzed for drug content and drug release on pre and post 1 year storage time.[27]

**Determination of L-arginine concentration**

In order to determine the lowest possible determine able concentration of arginine, the conditions needed to be optimized. Therefore, the dependence of the ninhydrin reactions on the concentration percent of each reactants was performed as follows.[24]

**Extraction and sampling of different biological fluids**

The extraction and sampling of pessary was done as follows: - The reaction was carried out in the following way, in since the samples are complex compound, it was necessary to combine several methods for extraction and estimation of arginine. 1$^{st}$, it was separated using 80% ethylalcohol and the proteins were deposited by chloroform; 2$^{nd}$, arginine was separated from and 0.5 g of the sample was placed into an Erlenmeyer flask and 4 ml of deionized water was added both with16 ml of absolute alcohol. The yield solution were shaken for half an hour and transferred into a 100 ml separation funnel where 60 ml of chloroform was added and all was vortexed the upper water.[4] A warm concentrated solution of $Ag_2SO_4$ in a small surplus was added to the water solution Arginine were deposited out of the solution by adding Ba $(OH)_2$. The impurity or bind material were then separated on the basis of the different properties of their respective silver salts at pH= 7, whereas arginine remains in the solution. Silver ions were removed by adding (0.1) mol L-1 $Na_2S_2O_3$ solution which binds silver ions into a stable complex. Employing multiple chloroform extractions, complex of Ag+ with $S_2O3$-$_2$ was extracted from the water solution.

**Procedure**

Several steps were done to determine concentration of L-arginine according to[5] with modification steps.[19]

**Step 1: Preparation of phosphate buffer solution**

The phosphate buffer solution at PH 9.0 was prepared by mixing appropriate volume of 0.2M disodium hydrogen phosphate (50) ml and 0.2 M sodium dihydrogen phosphate (0.35) ml.

**Step 2: Preparation of ninhydrin solution**

Ninhydrin reagent (2%) was prepared by dissolving 2.0g of ninhydrin in phosphate buffer, PH 9.0 (100) ml.

**Step 3: Preparation of L-arginine standard solution**

The L-arginine stock solution 3mg/ml was prepared by dissolving 30 mg of L-arginine in 10.0 of phosphate buffer, pH 9.0. Then, 1.0 ml of solution was transferred into 25 ml volumetric flask and diluted to the mark set point with the same buffer (0.12) mg/ml.

**Step 4: Preparation of calibration solution of L-arginine**

Serial dilutions of L-arginine were prepared by transferring (0.3, 0.35, 0.4, 0.45, 0.50, 0.55, 0.60) mg/ml, of standard solution of L-arginine (0.12) mg/ml separately in to series of the test tubes, then the test tubes were diluted to (2) ml with phosphate buffer PH 9.0 and plus 1.0 ml aliquot of ninhydrin reagent was added and finally the end concentration were became (0.012, 0.014, 0.016, 0.018, 0.020, 0.022 and 0.024). The resulting mixtures were heated in water bath at 80C$^\circ$ for 15min. After cooling, they were subjected to spectrophotometric analysis at λ=404nm against the mixture of 2 ml of phosphate buffer, PH 9.0 and 1ml of ninhydrin blank set and the optical density values of these mixtures were recorded. A calibration graph was obtained by regression of the standard L-arginine concentration against absorbance of certain concentration. This calibration graph was used to calculate the concentration of L-arginine in unknown samples.

**Step 5: Determination of L-arginine in Pharmaceutical Preparations**

Phosphate buffer solution (2) ml of PH 9.0 and 1ml of ninhydrin solution were transferred separately into sequence of the test tube. 0.1-0.3 ml of the different biological extracts from plants and animals fluids was added to the contents of each test tube. The resulting mixtures were heated in water bath at 80C$^\circ$ for 15 min. After cooling they were subjected to spectrophotometric analysis at λ=404 nm against the mixture of 2 ml of phosphate buffer, PH 9.0 and (1) ml of ninhydrin reagent as the blank and the absorbance of these mixtures were recorded.

**RESULTS**

**AlCeco® pessary pharmaceutical form**

The dosage form of L-arginine-Cefatoxime (AlCeco® as trade name) where chosen the disc figure 1 (diameter 1.6 cm and Thickness 0.7 cm) after pilot study for perfect dosage form, creamy color, suitable for many animal species. It is fitting for vaginal applicator and easy intrauterine introducing.

**Figure 1: L-arginine-Cefatoxime pessary (AlCeco®) intrauterine dosage form.**

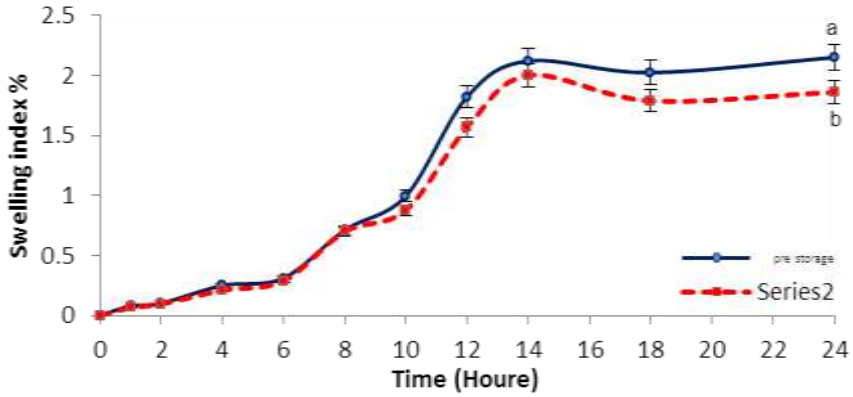


**Figure (2): Swelling index of L-arginine-Cefatoxime [1]AlCeco® pessary comprising of pre and post one year storage period.**

**Swelling index**

Swelling index was calculated with respect to 24 hours. Maximum swelling achieved after 14 hours in both pre and post one year storage time whereas, the post storage time was display swelling index slightly significant P<0.05 decrease as compared with pre storage time (figure 2).

[1] (AlCeco®) L-arginine 200 mg Cefatoxime 500 mg per pessary.
Values presented as mean±SE; different letters: (P < 0.05) vs. differences between stored time.
n = 10 Pessaries

**Solubility**

The solubility of L-arginine-Cefatoxime AlCeco® was in simulated vaginal fluid showed significant P<0.05 superior solubility results were observed of the drugs. Hence, the solubility of the drugs in pre and post storage period were not displayed significant P>0.05 differences in solubility value at simulated vaginal fluid was evaluated (Table 2).

**Table (2): Solubility of L-arginine-Cefatoxime [1]AlCeco® Data in Simulated Vaginal Fluid.**

| Media of solubility | Concentration mg/5 ml at pessary preparation | Concentration mg/5 ml after 1 year of pessary preparation |
|---|---|---|
| Simulated Vaginal Fluid | 243.54±0.230 a | 239.95±0.157 a |

[1] (AlCeco®) L-arginine 200 mg Cefatoxime 500 mg per pessary
Values presented as mean±SE; Letters: (P < 0.05) vs. differences between stored time.
n = 10 Pessaries

**Pessary physical properties**

Pessaries of L-arginine-Cefatoxime AlCeco® were prepared successfully and complied with BP in respect to drug content (97% - 101%) and reliable appearance with diameter fitness to multiple utero-vaginal capacities for different animals and having the physical characteristics as described in Table (3). L-arginine-Cefatoxime AlCeco® pessary, being acceptable physical behaviors, so the pessaries did not significant P>0.05 complain alteration in physical properties test after one year storage period as compared with pre-storage physical parameter.

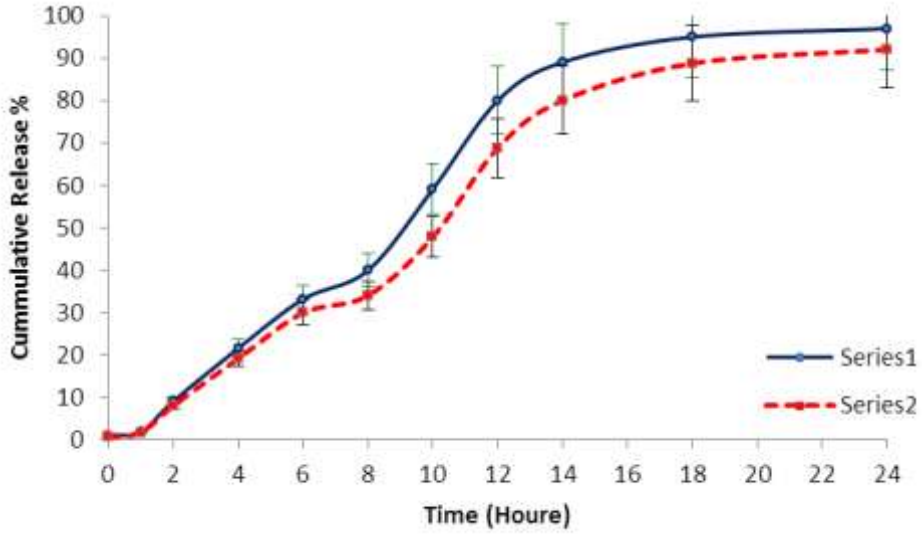


**Figure (3): Cumulative release of L-arginine-Cefatoxime [1]AlCeco® pessary comprising of pre and post one year storage period.**

[1] (AlCeco®) L-arginine 200 mg Cefatoxime 500 mg per pessary
Values presented as mean±SE; different letters: (P < 0.05) vs. differences between stored time.
n = 10 Pessaries

**Table (3) Physical characteristics of L-arginine-Cefatoxime [1]AlCeco® pessary.**

| Physical parameters | Pre-storage | Post 1 year storage |
|---|---|---|
| Average weight (g) | 1.009 ± 0.12 a | 1.008 ± 0.10 a |
| Hardness (horizontal) (Kp) | 7.013 ± 0.14 a | 6.994 ± 0.25 a |
| Thickness (mm) | 7.15 ± 0.01 a | 7.14 ± 0.01 a |
| Moisture content (% w/w) | 1.65 ± 0.07 a | 1.77 ± 0.13 a |
| Density g/ml | 0.699 ± 0.02 a | 0.698 ± 0.04 a |

[1] (AlCeco®) L-arginine 200 mg Cefatoxime 500 mg per pessary
Values presented as mean±SE; Letters: (P < 0.05) vs. differences between stored time.
n = 10 Pessaries

**Cumulative release**

Figures (3) show comparative release profiles of L-arginine-Cefatoxime AlCeco® in pre and post storage period respectively. L-arginine release profile from medium exhibited a light sharp release in 6 hours, forming a plateau after 2 hours then sharp release profile from 10 to 14 hours and coming soon plateau release. However, profile in aqueous organic medium established a steady and semi linear rise in the drug release during the 24 hours study. So and no significant p>0.05 changes were seen in cumulative release L-arginine during storage period as compared with pre stability study period.

## DISCUSSION

It has been discovered that efficient of L-arginine granules improves the release characteristics benefit pharmaceutical formulation of the compositions of the present study. Further, the present method is advantageous over methods that include safe pharmaceutical preparation as these methods are not need time consuming and inexpensive as well as avoid utilized hormonal program and their risk side effects in animals control fertility programs.

The key to effective and efficient coverage is execution the "granulating, milling and blending steps" of the present study. In a preferred embodiment, pessary are manufactured according a method that includes the steps of granulating, milling, blending of the L-arginine with the remainder of the ingredients and compressing the ingredients to form a pessary. Preferably, the method also includes either or each of the steps of screening the ingredients and/or drying the L-arginine during the milling.

### Swelling index

L-arginine-Cefatoxime AlCeco® were checked as *in vitro* swelling index of intrauterine pessary for this study because of display good swelling characteristics and presumably have been reported to form a layers around drug dosage form core and this interaction of pessary containing compound vehicles or adjuvants results in the formation of a non-destructive layer on the surface of the pessary which aids in minimizing the burst effect of pessary-based systems.[14,15] This interaction enables rapid creation of a firmness layer upon hydration and this has been observed as a crucial first stride in achieving orderly drug release from matrix pessary.[16] As shown in figure 2, at the primary stage, there was a preliminary rapid rise in swelling index due to the entry of water via metastable pores in the vehicles of the pessary. This mechanism, known as swelling hysteresis, was followed by swelling caused by diffusion processes.[17]

The swelling plateau reached at ~14 hour thereafter may be due to the solvent front on each face of the matrix meeting in the central of the pessary, leaving no additional non-hydrated part to hydrate and enlarge and swell.[18] It has also been reported that hydrogen bond interaction between compounds hinders the swelling of pessary.[19] Swelling reached a peak in the AlCeco® pessary after the 24$^{th}$ hour then erosion followed.

### Solubility

Solubility of AlCeco® was determined in simulated vaginal fluid prepared according to Owen and Katz[20] with modified pH virtual reality of utero-vaginal fluid of ewe. However, the degree of solubility results was observed due to partial hydrophilic nature of the drugs, table 2. The disintegrating pessary of AlCeco® dispersed in the small amount of fluid in the vagina simulated vaginal fluid giving a solubility profile of the drug rather than predicting its release characteristics.[21] Enhancer adjuvant has the advantage of containing the pessary in a pocket wherein it is allowed to disperse in small volume of media. Comparative release studies of AlCeco® from pre and post storage period at 50 rpm clearly show that the shape of the two profiles is same. The only slightly difference is the rate of release of the drug which corresponds to the difference in the hydration or complains conventional changes during storage period.[22]

### Pessary physical properties

The formulated Pessaries were subjected for various evaluated physical parameters like average weight (g), hardness (horizontal) (Kp), thickness (mm), moisture content (w/w %) and density g/ml, revealed that the formulated Pessaries was of good quality with regard to result in table 3.[23] No statistically changes in drug of the formulations occurred over the period of the stability of time challenge study. Thus, the additives used did not adversely affect the physical stability of the pessary formulations in ageing. This was able to release the L-arginine more than 92.03% in an optimum time period of storage, that given an impression no physical degradation on pessary properties. Uniformity and weight variability were couple key aspects that had to be taken into through dealing with pessary production. The blends were composed of starkly different yet visibly similar powders.

### Cumulative release

The Cumulative release were coincided with swelling index curve showed the releasing amount is flow up swell of pessary bulk which trapped the drugs between 6-10 hour and reach to maximum at same time of study associated with increase swelling capacity of the pessary that predicted mechanism of diffusional release.[17,23] it can be also explained by the swelling behavior of matrix system and degree of hydrophilic which may be responsible to a high rate of drug diffusion. The greater role of the vehicles content in the pessary of the prepared formulations was determined the drug releasing rates. It has been reported that a matrix tablet as the results in a vary of drug release due the build–up of an excessively viscous material which is very resistant to water penetration and erosion.[24]

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