



FORMULATION AND INVESTIGATION OF VAGINAL DOUBLE LAYER SUPPOSITORIES CONTAINING LACTOBACILLI AND HERBAL EXTRACTS

Pashayan M.M.

Department of Drug Technology, Yerevan State Medical University, Yerevan, Armenia

Abstract

The objective of this study is to develop vaginal double layer suppositories containing probiotics in core and antibacterial drugs in the outer layer for simultaneous treatment of bacterial vaginosis and vagina recolonization by lactobacilli. The suppositories are specially developed in a way that the herbal extract releases from the outer layer first and exerts action on pathogen microorganisms, then the core slowly releases lyophilized lactobacilli, and while they are revitalized the concentration of an extract decreases below minimal inhibitory concentration being not able to kill lactobacilli.

Four kinds of double layer vaginal suppositories containing lyophilized *L. delbrueckii* MH-10 in core and dried extract of *Achillea millefolium* (Yarrow) in the outer layer were prepared using specially designed metallic molds. Novata ABPH and Suppocire A-25 bases were used for the cores; for the outer layer Witepsol-H15 and Oleum Cacao were applied. The release kinetics of lactobacilli and herbal extract from different bases was determined by rotating basket dissolution method and agar diffusion method, respectively. The highest release of the herbal extract was observed from Witepsol H-15; although Oleum Cacao melts rapidly, drug release is not complete. Novata ABPH base gave the highest release of *L. delbrueckii* MH-10 and was microbiologically stable after storage at 2-8°C over the period of 12 months.

Thus, combination of Novata ABPH in the core and Witepsol H-15 in the outer layer as bases is a more suitable vehicle for preparation of double layer vaginal suppositories with lactobacilli and herbal extract.

Keywords: vaginosis, colonization, probiotic *lactobacilli*, herbal extract, double layer suppository, suppository bases.

INTRODUCTION

Bacterial vaginosis (BV) is one of the most common causes of infection in the female reproductive system. BV is believed to be the result of displaced vaginal *lactobacilli*, which are replaced by a range of unwanted species of pathogen microorganisms. Under normal circumstances, there is a fine balance between the two and the normal flora contributes to the maintenance of vaginal health. BV can be treated with broad-spectrum antibiotics, but the main problem of antibiotics therapy is the rapid emergence of candidosis and vaginal disbacteriosis [Uehara S. *et al.*, 2006]. Current social trends in health care show a definite movement toward the use of bacterial and herbal natural remedies and away from chemothera-

peutic regimens [Reid G., 1999]. Recently there is an enhanced focus on the use of probiotics such as *Lactobacillus spp.* for prophylaxis and treatment of BV [Slaver C., 2008]. *Lactobacilli* are an important part of the normal vaginal flora. Various species of *lactobacilli* play a protective role by producing compounds, such as hydrogen peroxide, lactic acid, and bacteriocin, which inhibit the growth of potential pathogens. They also protect the female urogenital tract from pathogen colonization by competitive exclusion of pathogens from the cell surface, co-aggregation with certain pathogenic bacteria adhering to epithelial cells. Reduction or elimination of *lactobacilli* increases the risk of BV and, in general, urinary tract infection [Wagenlehner F., Naber K., 2006]. In general, the treatment of BV consists of two stages: antibiotic treatment and recolonization of vaginal ecosystem by probiotics. However, this treatment mode takes a long time and is not conve-

Address for Correspondence:

Department of Drug Technology
Yerevan State Medical University after M. Heratsi
2 Koryun Street, 0025, Yerevan, Armenia
Tel.: (374-10) 622 010, (374-91) 214 381
E-mail: mariapashayan@yahoo.com

nient for patients. Therefore, development of vaginal suppository containing both antibiotics for pathogen inhibition and *Lactobacillus* for colonization would be more effective.

The aim of this study is to design vaginal double layer suppositories containing lyophilized probiotics in the core and herbal extract with high antibacterial and antifungal activities in the outer layer, to investigate their physical properties, the release of active substances from the suppository and the viability of microorganisms.

MATERIAL AND METHODS

Hydrogen peroxide producing strains *L. delbrueckii* MH-10 (INMIA 9617) was isolated earlier from healthy human donor, identified by its cultural, morphological, physiological-and-biochemical properties, 16S rRNA and α -subunit structural genes PCR amplification, deposited in RCDM (Republican Center Depository of Microorganisms) [Pashayan M., Hovhannisyan H., 2009]. As test microorganisms, we used *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp. and *Candida albicans* obtained from RCDM.

The herb *Achillea millefolium* (Yarrow) was collected from Dilijan region of Armenia, dried, extracted by percolation method with 70% ethanol and lyophilized in our laboratory.

The following suppository bases were used: Witepsol H-15 (Condea Chemie GmbH, Germany); Novata ABPH and Suppocire-25 (Huls AG, Germany); *Oleum Cacao* (Grand Candy Co., Armenia).

The preparation of double layers suppositories, which consist of core and outer layer, was done using two metal molds with different hole size (Figure 1). The core bases have higher temperature of melting than bases of the outer layer. To prepare cores for 10 suppositories, 5 g Novata ABPH or Suppocire suppository base was melted over the water bath. Then, 250 mg $\sim 10^{10}$ colony forming units (CFU) (for 10 suppositories) of lyophilized *L. delbrueckii* was added into the melted base at ~ 40 - 45°C with gentle stirring until a homogeneous mass was obtained. The mixture was poured into the mold with small holes (Figure 1 b) at a temperature just above the congealing point of the suppository base and cooled. For preparation of the outer layer 2.5 g lyophilized extract of the plant was added to 20 g melted base Witepsol H-15 or *Oleum Cacao* and homogenized. Then, the cores were fixed on the nee-

dles in the centre of the large mold (Figure 1 a), outer layer mixture ($\sim 40^\circ\text{C}$) was added and then cooled. All the prepared suppositories were kept in the refrigerator for further studies.

Disintegration Test: The disintegration test of *Lactobacillus* vaginal suppositories was modified from the method described in British Pharmacopoeia [British Pharmacopoeia, 2001] using tablet disintegrator. The suppository was placed in a cylindrical glass container with perforated ends and immersed in 1,000 mL citric acid/phosphate buffer solution (pH 4.4) at $37 \pm 0.5^\circ\text{C}$. The cylindrical glass container was moved up and down in the buffer. The time for disintegration was noted when the suppository has completely melted in the medium. The mean values were calculated from six parallel measurements.

Biopharmaceutical investigations of *Lactobacilli* release from cores was studied by the use of a rotating basket dissolution apparatus at 100 rpm and 37°C . One hundred milliliter of citric acid/phosphate buffer solution at pH 4.4 (modeling the vaginal pH) was used as the medium. Each suppository was placed in the basket and immersed into a flask containing dissolution medium. At appropriate time intervals, 4 mL samples were withdrawn and fresh buffer solution maintained at experimental temperature was used to replace the same volume of withdrawn sample. The count of viable *Lactobacilli* is determined by plating on the MRS agar and incubation at 37°C for 48 h.

The release of herbal extract from suppositories was determined by agar diffusion method [Pankruseva T. et al., 2003]. As inoculums overnight culture suspensions of test microorganisms containing 10^9 CFU/mL were used. The sample of melted suppository (0.1g) was placed in center of the standard size cylinder on the agar surface sowed by 10^6 cell suspension of test-strains *S. aureus*, *E. coli*, *K. pneumoniae* and *C. albicans* incubated at 37°C [Tentsova A., 1993]. Aqueous suspension of lyophilized herbal extract was used for control.

To study viability and stability, the vaginal suppositories containing *L. delbrueckii* MH-10 (formulation No. 1-4) were kept in glass containers at 2 - 8°C for 12 months. At appropriate time intervals of 0; 1 month; 3 months and 6 months, the survival of *Lactobacilli* was determined by plate method using MRS agar medium.



Figure 1. The metallic molds for preparation of suppositories core (above) and outer layer (below).
a) metallic mold for outer layer; **b)** metallic mold for core.

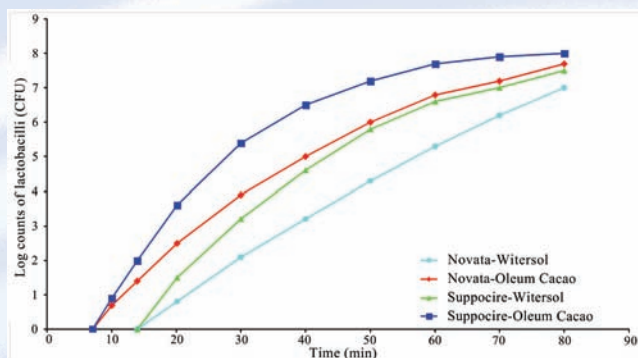


Figure 2. Release profiles of *L. delbrueckii* MH-10 from vaginal suppositories.

Note: without herbal extract in the outer layer.

RESULTS

The drug release from studied suppositories can be divided in five stages: melting of the suppository; spreading of the melted mass; sedimentation of the drug particles; passage of the solid particles through the oil/water interface; dissolution of drug particles in the vaginal fluid.

Four kinds of double layers suppositories were prepared by means of special molds (Figure 1) with different bases, and their physical properties were studied (Table 1). The cores of suppositories had diameter of 6 mm, length 20 mm and mass 0.5±0.01 g. The obtained double layer vaginal suppositories had diameter of 11 mm, length 26 mm and mass 2.5±0.2 g. The physical characteristics of suppositories were studied. The color of suppositories with Witepsol H-15 was white, while that with *Oleum Cacao* in the outer layer was dark yellow.

The disintegration test determines, whether suppositories become soften or disintegrate within a prescribed time, when placed in an immersion fluid. According to the British Pharmacopoeia (BP) requirement, disintegration occurs in no more than 60 minutes. From this study, the disintegration time for the suppository formulations No. 1, 2, 3 and 4 was 24±1.3, 17±1.1, 26±1.1, and 19±1.0 min, respec-

tively. Therefore, all suppositories were found to satisfy the BP requirement for disintegration.

For release determination we prepared two types of double layers suppositories with 4 subtypes each. The first type contained *lactobacilli* in core without herbal extract in the outer layer, and the second contained herbal extract in the outer layer without *lactobacilli* in core. The release of *L. delbrueckii* MH-10 from the suppositories is shown in Figure 2.

Maximum ~ 10⁸ CFU release of *lactobacilli* from all formulations was approximately the same, but the release from core with Novata ABPH (formulations No. 1, 2) was slower than from Suppocire bases (formulations No. 3, 4). The release of *lactobacilli* from formulations 1 and 3 started at 13.6 min and from formulations 2 and 4 – at 7.0 min. The observed delay of *lactobacilli* release was caused by difference of melting time of outer layer bases: Witepsol H-15 or *Oleum Cacao*.

Herbal extract release from outer layers was studied by agar diffusion method (Table 2). As a criterion of release, the zone of inhibitions on microbial lawns was used. The analysis of inhibitory zone signifies to the rate of herbal extracts release from the outer layer bases. The maximal inhibition zones

Table 1.

Compositions of vaginal suppositories containing *Lactobacilli* and herbal extract

Formulation No.	Core		Outer layer	
	Suppository Base	Active substance	Suppository Base	Active substance
1	Novata	<i>L. delbrueckii</i> MH-10	Witepsol H-15	Herbal extract
2	Novata	-/-	<i>Oleum Cacao</i>	Herbal extract
3	Suppocire	-/-	Witepsol H-15	Herbal extract
4	Suppocire	-/-	<i>Oleum Cacao</i>	Herbal extract

Table 2.

The release of herbal extract from double layer suppositories

Formulation No.	Inhibition zone (mm)			
	<i>E. coli</i>	<i>S. aureus</i>	<i>K. pneumonia</i>	<i>C. albicans</i>
1*	34.83 ± 0.84	28.4 ± 0.56	32.50 ± 0.96	18.5 ± 0.47
2*	30.17 ± 1.09	25.24 ± 0.86	28.67 ± 0.75	100 ± 0.59
3*	33.25 ± 1.09	26.8 ± 0.86	32.2 ± 0.84	15.8 ± 0.51
4*	30.00 ± 0.84	24.8 ± 0.86	27.02 ± 0.73	10.00 ± 0.73
Control**	37.32 ± 0.96	30.35 ± 0.94	35.1 ± 0.93	20.05 ± 0.63

Note: *- core without lactobacilli;

** - aqueous suspension of lyophilized herbal extract used for control. n=6; p ≤ 0.05

were observed for suppository formulations No. 1 and 3 with Witepsol H-15 in the outer layer. The smaller inhibition zone around suppository formulations No. 2 and 4 was due to incomplete release of herbal extracts from *Oleum Cacao* base.

The viability of *lactobacilli* and stability of vaginal suppositories were determined during 12 months storage at 4°C (Table 3).

As obvious from results presented in Table 3, all vaginal suppositories containing *L. delbrueckii* MH-10 have a 1 log reduction in counts after preparation (day 0). This may be attributed to the heating process during preparation. Type 1 and 2 suppositories, on Novata base, were showing decrease in the viable counts of about 1 log over the 6 months period of storage, but suppositories type 3 and 4, with Suppocire base, were showing decrease of about 2 log.

DISCUSSION

Vaginal suppositories have many advantages: dose uniformity can be maintained, absence of irritation into the vagina without irritation is possible, and large volume of dissolution fluid is not required for the release of the active substance [Kale V. et al., 2005].

Different type conventional and specially designed suppositories for rectal and vaginal use are described in literature. For example, a hollow-type suppository was developed by Y. Watanabe in order to evaluate the effectiveness of the drug when administered rectally [Watanabe Y. et al., 1986]. The mold of this suppository is equipped with a cylindrical tube in the center that forms hollow cavity, into which drugs in the form of powder, liquid, or solid could be placed. Sanae and Nattha Kaewnopparat

used it for design of vaginal suppositories. Instead of a drug, they filled the cavity with powder of different species of *lactobacilli*, and the opening at the hind part of the suppository was sealed with the melted base [Kaewnopparat S., Kaewnopparat N., 2009], but the viability of *lactobacilli* in this types of suppositories is lower than in our suppositories.

There is still another type of double layer rectal suppositories: total drug dose is present in the outer layer and the core acts as a mere vehicle, support [Soliman S. et al., 2000]. It consists of polyethylene glycol (PEG) blend in the outer layer and fatty base Suppocire A 25 in the core for the enhanced release properties. Unlike our double layer suppositories, the core of this suppository does not contain any active substance.

Double-phased rectal suppositories are also known to utilize anesthetic lidocain with Witepsol® H-15 in a front layer and a terminal layer containing Carbopol with white beeswax [Reiko Y. et al., 1999]. *In vitro* release profiles of lidocain from double-phased suppositories were similar to conventional single-phased suppositories containing Carbopol alone. All mentioned suppositories are mono-functional: either core or outer layers play only the role of a container or supporter for the active substance.

In the core of our suppositories, we used *L. delbrueckii* MH-10 H₂O₂ produced active species isolated from healthy women in our laboratory. Hydrogen peroxide (H₂O₂)-producing *Lactobacilli* isolated from vagina are more preferable probiotics for the treatment of bacterial vaginosis and other sexually transmitted diseases, including HIV [Klebanoff S., Coombs R., 1991; Hawes S. et al., 1996].

Yarrow extract included in the outer layer is anti-

Table 3.

Viability of *L. delbrueckii* MH-10 from suppositories stored at 2-8°C

Suppository Type	CFU				
	Storage time (months)				
	0	1	3	6	12
1	5.4±0.20 x10 ⁸	4.8 ± 0.21 x10 ⁸	3.4 ±0.09 x 10 ⁸	9.1±0.15 x 10 ⁷	7.2 ±0.22 x10 ⁷
2	5.2±0.15 x10 ⁸	4.6 ± 0.13 x10 ⁸	3.0 ±0.20 x10 ⁸	9.0±0.09 x10 ⁷	7.0 ±0.20 x10 ⁷
3	4.8±0.17 x 10 ⁸	4.0 ± 0.32 x10 ⁸	1.8 ± 0.13 x10 ⁸	6.8±0.32 x 10 ⁷	3.0 ±0.08 x10 ⁷
4	4.3±0.22 x 10 ⁸	3.5 ±0.20 x 10 ⁸	1.1 ± 0.15 x10 ⁸	6.0±0.23x 10 ⁷	2.8 ±0.12x 10 ⁷

Note: n=6; $p \leq 0.05$

septic (inhibits bacterial growth), styptic (stops bleeding), astringent (makes tissue contract), vulnerary (helps tissue heal), anti-inflammatory, and possibly anesthetic. According to our and literature data, extracts of *Achillea millefolium* possess a broad spectrum of antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella enteritidis* and two fungi *Aspergillus niger* and *Candida albicans*, but express low activity against *Lactobacilli*. The composition of *Achillea millefolium* extract, which showed the strongest activity, was investigated and the structures of isolated compounds were elucidated by spectral means (1D and 2D NMR, UV, IR and MS). The extract yielded alkanes, fatty acids, monoterpenes, guaiane sesquiterpenes (rupicolin A and B, 1-deoxy-1 α -peroxy-rupicolin A and B), and flavonoids (apigenin and centaureidin) [Pashayan.M., Hovhannisyan H., 2009].

Double layer vaginal suppositories containing lyophilized *L. delbrueckii* MH-10 in core and dried extract of *Achillea millefolium* (Yarrow) in the outer layer were obtained.

Investigation on the release profiles of drug and

lactobacilli show that herbal extract is firstly released from the outer layer exerting antibacterial action on pathogen microorganisms followed by the further slow release of *lactobacilli* in freeze-dried condition not susceptible to the extract. The revitalization of freeze-dried *lactobacilli* in MRS broth or milk takes a few hours. Because of the small amount of moisture available in vagina, the revitalization of *lactobacilli* proceeds very long; during this period of time the concentration of drug declined below the minimal inhibitory concentration (MIC), being unable to kill *lactobacilli*. Among all tested bases Novata ABPH is chosen, because the release of *lactobacilli* is more complete, the viability of included *lactobacilli* is highest, and it is stable at ambient temperature (30±5°C) and at 2-8°C during 12 months.

For the outer layer Witepsol is more preferable, because the *Oleum Cacao* is not microbiological stable and the release of drug from this base is very slow and not complete. Thus, the first formulation of suppository with combination of Novata ABPH and Witepsol H-15 as bases is more suitable as a vehicle for preparation of double layer vaginal suppositories with *lactobacilli* and the herbal extract.

REFERENCES

1. *British Pharmacopoeia*. London. The Stationary Office. 2001. P. 2015.
2. Hallen A., Jarstran C., Pahlson C. Treatment of bacterial vaginosis with lactobacilli. Sex. Transm. Dis. 1992; 19: 146-148.
3. Hawes S.E., Hillier S.L., Benedetti J. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. J. Infect. Dis. 1996; 174: 1058-1063.
4. Kaewnopparat S., Kaewnopparat N. Formulation and evaluation of vaginal suppositories containing Lactobacillus. J. World Academy of Science, Engineering and Technology. 2009; 55: 25-28.
5. Kale V.V., Trivedi R.V., Sanjay P. Development and evaluation of a suppository formulation containing lactobacillus and its application in vaginal diseases. Ann. N.Y. Acad. Sci. 2005; 1056: 359-365.

6. *Klebanoff S.J., Coombs R.W.* Viricidal effect of *Lactobacillus acidophilus* on human immunodeficiency virus type 1: possible role in heterosexual transmission. *J. Exp. Med.* 1991; 174: 289-292.
7. *Pankrusheva T.A., Rudko E.A., Nesterova A.V.* [Formulation of the composition, technology and quality assessment of the suppositories with ofloxacin] [published in Russian]. *Vestnik VSU.* 2003; 2: 220-224.
8. *Pashayan M.M., Hovhannisyan H.H.* Isolation of new vaginal lactobacilli cultures against sexually transmitted infections. *Medicine, science and education.* 2009; 1: 123-126.
9. *Reid G.* Potential preventive strategies and therapies in urinary tract infection. *World J. Urol.* 1999; 17: 359-363.
10. *Reiko Y., Hiraku O., Yoshiharu M.* Preparation and evaluation of double-phased mucoadhesive suppositories of lidocaine utilizing Carbopol and white beeswax. *Journal of Controlled Release.* 1999; 61(issues 1-2): 1-8.
11. *Slaver C.M.* *Lactobacillus*: a Review. *Clin. Microbiol. Newsletter.* 2008; 30: 23-27.
12. *Soliman S., Gadalla M.F., Naggat V.F.* Formulation, in vitro and in vivo evaluation of fast release double layer theophylline suppositories. *J. Acta pharmaceutica.* 2000; 50(4): 315-328.
13. *Tentsova A.I.* [Trends and prospects of biopharmaceutical research] [published in Russian]. *Pharmacia.* 1993; 6: 43
14. *Uehara S., Monden K., Nomoto K., Seno Y.* A pilot study evaluating the safety and effectiveness of *Lactobacillus* vaginal suppositories in patients with recurrent urinary tract infection. *Int. J. Microbiol. Agent.* 2006; 285: 530-534.
15. *Wagenlehner F.M.E., Naber K.G.* Treatment of bacterial urinary tract infections presence and future. *Eur. Urol.* 2006; 49: 235-244.
16. *Watanabe Y., Matsumoto Y., Baba K.* Pharmaceutical evaluation of hollow type suppositories. IV. Improvement of bioavailability of propranolol in rabbits after rectal administration, *J. Pharmacobiodyn.* 1986; 9: 526-531