

## **RESEARCH PUBLICATIONS & PRESENTATIONS OUT OF THE PRESENT STUDY**



### **Paper published:**

- Aliasgar Shahiwala, Ambikanandan Misra. Nasal delivery of Levonorgestrel for contraception: An experimental study in rats. Fertility and Sterility, 81(1), 893-898, 2004.
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### **Paper presented:**

- Presented a poster on "Novel method of optimization of liposomal drug entrapment efficiency", at 5th International Symposium on Innovations in Pharmaceutical Science and Technology at Mumbai, Feb' 2003.
- Presented a paper in State level paper presentation competition on "Pulmonary Absorption of Liposomal Levonorgestrel", at Shree S.K. Patel College of Pharmaceutical Education & Research, Ganpat Vidyanagar, Sponsored by Gujarat Council of Science & Technology, Government of Gujarat, October' 2003.
- Presented a paper in State level paper presentation competition on "Nasal Delivery of Levonorgestrel for Contraception: An Experimental Study in Rats", at Shree S.K. Patel College of Pharmaceutical Education & Research, Ganpat Vidyanagar, Sponsored by Gujarat Council of Science & Technology, Government of Gujarat, October' 2003.
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# Nasal delivery of levonorgestrel for contraception: an experimental study in rats

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**Objective:** To ascertain the nasal bioavailability of Levonorgestrel and change formulation components to provide long-term effective concentrations of the drug in blood. An experimental study was conducted on rats to reduce dose and/or frequency of drug administration to reduce expected side effects reported in humans.

**Design:** In vivo study in rats

**Setting:** Centre of Relevance & Excellence in new drug delivery systems, Pharmacy Dept., G.H. Patel Bldg., Faculty of Technology and Engineering, M.S. University of Baroda, Hatchgunj, Vadodara, Gujarat, India

**Animal(s):** Rats of proven fertility

**Intervention(s):** Formulations containing 10- $\mu$ g of levonorgestrel were administered in rats via the nasal route. Similarly, a 10- $\mu$ g drug suspension was administered orally. The influence of the mucoadhesive agents chitosan and carbopol 934p on nasal absorption of the drug was also evaluated.

**Main Outcome Measure(s):** Plasma drug concentration and pharmacokinetics

**Result(s):** Relative bioavailabilities of 29.93%, 32.14%, and 25.97% were observed after nasal administration of plain drug, physical mixture, and liposomal formulations, respectively. Mucoadhesive agents in nasal formulations were found to produce a threefold increase in drug bioavailability. Bioavailability was improved from 29.93% to 101.70% and 99.42% respectively, for chitosan (0.5%) and carbopol 934p (0.5%) formulations, with a significantly improved plasma half-life from 7.0 hours to 55.7 hours and 52.9 hours respectively. Pharmacodynamic studies indicate that the dosing interval can be changed from daily oral administration to nasal administration once every 2 days without changing the dose.

**Conclusion(s):** Levonorgestrel with mucoadhesive agents administered nasally in rats is superior for maintaining effective drug concentrations over an extended period of time when compared with the presently available orally administered form (Fertil Steril® 2004;81(Suppl 1):893-8. ©2004 by American Society for Reproductive Medicine).

**Key Words:** Levonorgestrel, nasal, liposomes, mucoadhesive, contraception

New materials and new technologies have stimulated pharmaceutical researchers to identify and use alternatives to the classic oral and injection routes. One of the alternatives currently being studied is drug administration via the nasal route. In selected drugs the pharmacokinetics relating to drug absorption and metabolism via the nasal route are more favorable (1-3). It has recently been shown that the bioavailability of the steroidal drugs  $P_4$ ,  $17\beta$ -E<sub>2</sub>, and  $17\alpha$ -ethynylestradiol when given via nasal route in rats is greatly superior to that via the oral route (4, 5). A study involving nasal administration of norethisterone also showed the superiority of this route (6). The bioavailability of T was found to be similar to that of the intravenous route when given nasally (7).

Liposomes are phospholipid vesicles composed of lipid bilayers enclosing one or more aqueous compartments. Liposomes provide an efficient delivery system because they are biocompatible, biodegradable, and relatively non-toxic (8). As a drug-delivery system, liposomes can significantly alter the pharmacokinetics and pharmacodynamics of entrapped drugs, for example, by enhancing drug uptake, delaying rapid drug clearance, and reducing drug toxicity (9-11). Liposomes are attracting considerable interest for drug delivery to the nasal mucosa. In fact, they are known to sustain the release of the entrapped drugs owing to their surface viscosity. Their action on nasal mucosa is related to the incorporation of phospholipids in the membrane, opening "new pores" in the paracellular tight junction (12).

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To facilitate absorption, suitable penetration enhancers have to be developed that only allow for paracellular absorption and that are nontoxic. Weakly cross-linked polyacrylates (e.g., polycarbophil and carbomers, which are approved by the U.S. Food and Drug Administration) have been found to fulfill these requirements. By  $\text{Ca}^{2+}$  complexation they are able to trigger the reversible opening of the tight junction between the cells and to allow the paracellular transport of peptides (13, 14). Chitosan and such chitosan derivatives as trimethyl chitosan have been shown to have similar properties to reversibly open the tight junctions. This mechanism is thought to occur by ionic charge transfer between the positive charge of the chitosan molecule and the negative charges (sulfate and sialine groups) of the glycocalyx (15). Carbomer and chitosan, being mucoadhesive agents, prolong the drug residence time with the nasal mucosa.

Oral delivery of contraceptives includes combined estrogen/progestogen pills and progesterone-only pills. Progesterone-only pills are free from estrogenic adverse effects, including myocardial infarction, stroke, venous thromboembolism, and breast cancer. However, progesterones are associated with various progestogenic adverse effects, including irregular bleeding, mastalgia, headaches, amenorrhea, breast cancer, cervical neoplasia, and rarely hepatocellular carcinoma. We attempted to develop formulations of levonorgestrel (LN) for nasal delivery and evaluate them for their contraceptive efficacy. Our objectives were to enhance and sustain drug bioavailability with liposomes and to reduce reported adverse effects. The mucoadhesive agents carbopol and chitosan were also incorporated to retain the formulation at the administered site and to enhance the drug bioavailability via the nasal route.

## MATERIALS AND METHODS

Levonorgestrel was provided as a gift (German Remedies Pvt. Ltd., Mumbai, India), as was phosphatidylcholine (type-E 80) (Lipoid GmbH, Ludwigshafen, Germany). Cholesterol was purchased (S. D. Fine Chemicals, Mumbai, India). Carbopol and chitosan were purchased (B.F. Goodrich Company, Cleveland, and Sigma Chemical, St. Louis, MO). All other solvents and chemicals used were of analytic grade unless otherwise specified.

### Preparation of LN Formulations

#### *LN Suspension (LN1)*

Levonorgestrel (10 mg) was weighed accurately and transferred to a 10-mL volumetric flask. Water was added, and volume was made up to the mark. The resulting suspension was sonicated so as to obtain particle sizes in the range of 10  $\mu\text{m}$  to 15  $\mu\text{m}$ .

#### *LN Liposomes (LN2)*

Liposomes of LN were prepared by the reverse-phase evaporation method (16) in a drug: phosphatidyl choline

cholesterol (PC:CHOL) molar ratio of 1:4:1 and dissolved in diethyl ether (organic phase) in a glass tube (Quick fit neck B-24). Distilled water was injected rapidly into the lipid solution through a 23-gauge hypodermic needle from a 5-mL syringe. The tube was closed with a glass stopper and vortexed for 5 minutes. It was then attached directly to a rotary evaporator to dry the contents at 40°C under vacuum (500 mm Hg) until a gel was formed. The vacuum was released, and the tube was removed from the evaporator and subjected to vigorous mechanical agitation on vortex mixture for 5 minutes. When the gel collapsed to fluid, it was again fitted to a rotary flash evaporator for the removal of organic solvent. A cycle of 10 minutes drying and 5 minutes vortexing was again repeated twice. The final liposomal suspension was subjected to complete removal of the last traces of organic solvent in a rotary flash evaporator under vacuum (500 mm Hg) for 15 minutes. The resulting suspension was frozen at -40°C for 2 hours and then thawed at room temperature for 15 minutes to obtain optimum percent drug entrapment.

Measurement of percent drug entrapment of liposomes was carried out by separation of untrapped drug from liposomes by centrifugation at 2000 rpm for 15 minutes and analysis according to the method described in USP'26 NF (17). The percent drug entrapment of the prepared liposomes was  $98.3 \pm 0.211$  (mean  $\pm$  SEM). Size analysis of the liposomes was performed with a microscope (Olympus BX 40F4, Olympus, Tokyo, Japan) under 1000 $\times$  magnification. Particle size distribution of prepared liposomes was of 10  $\mu\text{m}$  to 15  $\mu\text{m}$ .

#### *LN Physical Mixture (LN3)*

Levonorgestrel (10 mg) along with liposomal constituents, namely egg phosphatidyl choline (PC) and cholesterol, were weighed accurately and transferred to a 10-mL volumetric flask. Water was added, and the volume was made up to the mark. The resulting suspension was sonicated so as to obtain particle sizes in the range of 10  $\mu\text{m}$  to 15  $\mu\text{m}$ .

#### *LN Suspension with Chitosan (LN4)*

Levonorgestrel (10 mg) was weighed accurately and transferred to a 10-mL volumetric flask. Water (5 mL) was added, and the volume was made up to the mark with 1% chitosan solution (0.01% acetic acid). The resulting suspension was mixed well and stored in a refrigerator until required. The pH of the suspension was 3.8.

#### *LN Suspension with Carbopol 934 Hydrogel (LN5)*

Levonorgestrel (10 mg) was weighed accurately and transferred to a 10-mL volumetric flask. Water (5 mL) was added, and the volume was made up to the mark with 1% carbopol 934 dispersed in double-distilled water. The resulting suspension was mixed well and stored in a refrigerator until required. The pH of the suspension was 3.9.

TABLE 1

Pharmacokinetics of different formulations after oral, and nasal administration of levonorgestrel in rats

Formulation	Route	Area under curve (ng-h mL)	Bioavailability	$t_{\max}$ (hours)	$C_{\max}$ (ng mL)	$t_{1/2}$ (hours)
Plain drug (10- $\mu$ g suspension)	Oral	261 408		2.1	14.4	16.9
Plain drug (LN1)	Nasal	78 245	29.9321	4.2	7.13	7.0
Physical mixture (LN2)	Nasal	84 007	32.1364	4.6	6.1	11.9
Liposomes (LN3)	Nasal	67 901	25.9751	4.6	5.24	9.40
0.5% chitosan (LN4)	Nasal	265 862	101.7038	4.4	4.73	55.7
0.5% carbopol 934 p (LN5)	Nasal	259 888	99.4185	5.0	4.70	52.9

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For nasal and oral administration of the formulations, doses of 10  $\mu$ g per rat were used.

### Animal Studies

Six female albino rats ( $250 \pm 20$  g) for each group were used unless otherwise specified. Three male albino rats ( $250 \pm 20$  g) for each group were used for mating studies. Only animals with proven fertility were selected for the studies. All animals were housed in polypropylene cages with free access to palletized chow and tap water with a 12-hour light/dark cycle. Animal experiments were approved by the Ministry of Social Justice and Empowerment, Government of India, New Delhi, India.

### Methodology

#### Nasal Administration

At the time of administration, animals were partially anesthetized with anesthetic ether, and the formulations were administered to the nasal cavity by means of micropipette.

#### Oral Administration

For oral administration, a 10- $\mu$ g drug suspension was given by mouth with a 28-gauge long blunt needle.

#### Blood Sampling

For all animals, 100- $\mu$ L blood samples were drawn from the tail vein at 2, 4, 6, 12, 24, 48, and 72 hours and subjected to drug analysis.

#### Analytic Method

To determine intact LN in blood, a specific spectrofluorometric method was used, as described earlier (18). Plasma samples of 20  $\mu$ L were taken and extracted with 2 mL and 1 mL methylene chloride two times. Methylene chloride was again extracted with 80% sulfuric acid in ethanol, and samples were analyzed at an excitation wavelength of 460 nm and an emission wavelength of 520 nm. Blood samples were collected, and the calibration curve of LN in plasma was prepared by adding the known quantity of drug

in blood, and the procedure was carried out as described above.

### Pharmacokinetics

The drug plasma concentrations at each sampling time point were plotted against time in hours. Maximum plasma concentration ( $C_{\max}$ ), time in hours to achieve  $C_{\max}$  ( $t_{\max}$ ), and drug plasma half-life ( $t_{1/2}$ ) were determined from drug plasma concentration-time curve from best fit curve using major and minor gridlines with  $\pm 0.2$  unit accuracy. The area under the plasma level curve was calculated by the trapezoidal rule. Data were compared with analysis of variance; differences at  $P < 0.05$  were considered significant.

### Pharmacodynamic Studies

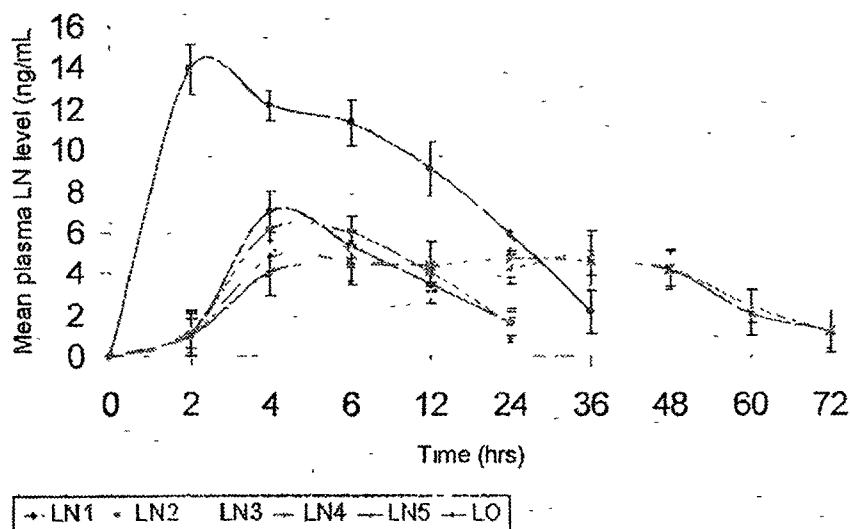
Various LN preparations were administered for a maximal period of four consecutive estrous cycles. Each female was inspected every morning for evidence of mating (the presence of vaginal plugs or sperm). The orally administered 10- $\mu$ g drug suspension and formulations LN1, LN2, and LN3 were administered daily, whereas LN4 and LN5 were administered once every 2 days. During the LN formulations administration period, if any animal had mated, it was removed from the mating cage immediately after observation of one or both coital signs (day 1) and killed on the postcoital day 9, and the number of implantations was counted. The remaining animals, in which coital signs were not observed, were also killed 9 days after the last night's cohabitation and inspected for any sign of implantation.

## RESULTS

Drug particle size in all the formulations was kept between 10  $\mu$ m and 15  $\mu$ m, because particles of 10  $\mu$ m to 20  $\mu$ m are all deposited in the nasal cavity, whereas particles smaller than 1  $\mu$ m pass with inspired air into the lungs (19). Encapsulated liposomes, physical mixture, and a suspension of 10- $\mu$ g LN were administered nasally in rats. Similarly, a 10- $\mu$ g drug suspension was administered orally. Blood sam-

**FIGURE 1**

Plot of mean plasma levels of levonorgestrel versus time after oral and nasal administration. Black line, LN1; red line, LN2; yellow line, LN3; blue line, LN4; purple line, LN5; brown line, 10- $\mu$ g suspension (oral)



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ples were collected at specific time points, and plasma LN concentrations were estimated. The data for drug plasma concentrations are shown in Figure 1, from these data various pharmacokinetic parameters were calculated, and these are shown in Table 1. Plasma levels of LN after a single oral dose indicate that a considerably higher level of LN ( $C_{max}$  14.4 ng/mL) occurs with  $T_{max}$  of 2.1 hours as compared with plain drug (LN1), physical mixture (LN2), and liposomal (LN3) formulations given intranasally ( $C_{max}$  7.13 ng/mL, 6.1 ng/mL, and 5.24 ng/mL, respectively) at  $T_{max}$  of 4.2 hours, 4.6 hours, and 4.6 hours, respectively. Levels of LN fall precipitously to less than 1 ng/mL in all the cases.

When the areas under the curve for nasally administered LN1, LN2, and LN3 were compared with that of oral plain drug administration, significant differences were observed. Bioavailability of these formulations was found to be significantly less (29.93%, 32.14%, and 25.97% for LN1, LN2, and LN3, respectively). When the drug was formulated with the mucoadhesive agents chitosan (LN4) and carbopol 934p (LN5), significant improvements in the bioavailability of the drug were observed (101.70% and 99.42% respectively). Plasma half lives ( $t_{1/2}$ ) were also significantly increased, from 7.0 hours to 55.7 hours and 52.9 hours, respectively. However, the  $T_{max}$  values were 4.4 hours and 5.0 hours with  $C_{max}$  of 4.73 ng/mL and 4.70 ng/mL, respectively, for the LN4 and LN5 formulations.

Pharmacokinetic studies were followed by pharmacodynamic studies, in which the animals were administered with different formulations intranasally for 4 weeks and allowed

to mate during the treatment period. Numbers of implantations in mated female rats were as shown in Table 2. Treatment with LN1, LN2, and LN3 formulations failed to show contraceptive efficacy, perhaps because of the short plasma half lives of the drug. However, in cases of LN4 and LN5 100% contraception was observed, even when formulations were administered on alternate days.

## DISCUSSION

The large number of fenestrated capillaries just below the surface epithelium might well contribute to absorption (20). However, the mucociliary clearance under normal conditions rapidly clears the applied material because there is little time of contact between the drug and the mucosa. This is what we observed in the cases of the LN1, LN2, and LN3 formulations delivered nasally. Prolonging the contact time of the drug with the absorptive surfaces by means of appropriate mucoadhesive agents contributed to increases in the bioavailability of the intranasally administered drug. The clearance of the administered drug was delayed by the use of the mucoadhesive polymers chitosan and carbopol. Chitosan acts by opening the tight junction between epithelial cells (21). It might also enhance the absorption of drugs by being a useful bioadhesive and slowing mucociliary transport (22). Carbopol hydrogel is a thin liquid at acidic pH, but it gels at physiologic pH and thus has great potential for nasal delivery of drugs (23).

When the  $t_{1/2}$  value of orally administered formulations was compared with that of nasally administered mucoadhe-

TABLE 2

Contraceptive effects with different formulations of levonorgestrel

Formulation	Route	No of rats mated	No of rats pregnant	No of implantations
Control		4/4	4/4	1-10, 2-9, 3-9, 4-8
Plain drug (10- $\mu$ g suspension)	Oral	4/4	0/4	
Plain drug (LN1)	Nasal	4/4	1/4	1/4
Physical mixture (LN2)	Nasal	4/4	1/4	1-3
Liposomes (LN3)	Nasal	4/4	2/4	1-1, 2-3
0.5% chitosan (LN4)	Nasal	4/4	0/4	
0.5% carbopol 934p (LN5)	Nasal	4/4	0/4	

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sive formulations, significant increases in  $t_{1/2}$  were observed (from 16.9 hours to 52.9–55.7 hours). The results clearly indicate that the dosing interval can be changed from daily oral administration to once every 2 days nasally without changing the dose. The reduction in the drug dose and the maintenance of a therapeutic concentration of the drug in blood plasma for at least 48 hours is expected to reduce the reported adverse effects in humans and probably the cost of the therapy because of the lower dose. These results depend on the deposition of the instilled dose within the nose. The deposition and absorption of sprays in the nose are different from those of drops. The nasal spray deposits anteriorly in the nasal atrium, whereas drops are dispersed throughout the length of the nasal cavity. Nasal sprays deposit more anteriorly, resulting in slower clearance of sprays than of drops (24). However, due to the impracticality of giving nasal sprays in rats, experiments were only performed with the drops. There is a chance that studies in larger animals with the spray form of these formulations might result in different deposition and absorption patterns; this will ultimately help in the decision of which type of dosage form should be used in humans.

The results of the pharmacodynamic studies were found in agreement with pharmacokinetics, which further confirms the contraceptive efficacy of proposed formulations for prolonged periods of time.

Levonorgestrel, an orally active progestogenic derivative, is associated with various adverse effects, perhaps because of the initial very high  $C_{max}$  achieved, which is significantly greater than the therapeutic window of the drug (active therapeutic window, 4–6 ng/mL). Nasal delivery with mucoadhesive agents, however, enables extended release of the drug over a long period without resulting in initial higher plasma concentrations. This might further reduce the frequency of dose administration. This study demonstrates prolonged LN absorption closely following zero-order kinetics in rats after nasal administration. Maintenance of an effective drug concentration in the blood for prolonged periods is expected to reduce the dose and/or frequency of drug ad-

ministration and probably the adverse effects, provided similar findings are demonstrated in humans. However, the role of the formulations developed in this investigation can only be determined after detailed pharmacokinetic and pharmacodynamic studies in at least two more animal models in a larger number of animals, followed by experimental trials in humans and then clinical evaluation.

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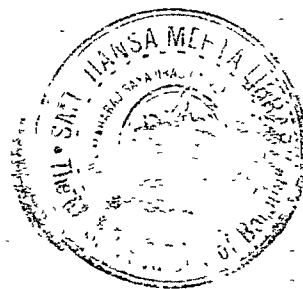
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## Pulmonary Absorption of Liposomal Levonorgestrel

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### ABSTRACT

The purpose of these studies was to achieve desired bioavailability after pulmonary administration of Levonorgestrel (LN) and to provide prolonged effective concentration of the drug in plasma and to reduce reported side effects of orally administered drug. The plain drug suspension, physical mixture (plain drug with liposomal constituents), and drug-encapsulated liposomes containing 10 µg of drug were instilled intratracheally in rats. Similarly, 10-µg drug suspension (LO) was administered orally. The blood samples were withdrawn at specific time intervals and were subjected to LN analysis by spectrofluorimetric technique. The plasma drug concentration data of both the treatments were plotted, and pharmacokinetics data were calculated and compared with that of oral administration. Percentage relative bioavailability ( $F^*$ ) of 97.6%, 98.6%, and 109.9% were observed after pulmonary administration of plain drug formulation (LP1), physical mixture (plain drug along with constituents of liposomes [LP2]), and liposomal (LP3) formulations of the drug, respectively. Following oral administration,  $C_{max}$  of  $14.4 \pm 0.6$  ng/mL was observed at  $2.1 \pm 0.2$  hours followed by subtherapeutic concentration beyond  $30 \pm 0.2$  hours, while after pulmonary administration of LP1, LP2, and LP3 formulations,  $C_{max}$  of  $4.4 \pm 0.4$  ng/mL,  $4.2 \pm 0.5$  ng/mL, and  $4.4 \pm 0.6$  ng/mL were observed at  $6.0 \pm 0.2$  hours,  $7.0 \pm 0.2$  hours, and  $6.8 \pm 0.2$  hours, respectively, followed by maintenance of effective plasma drug concentration up to  $60 \pm 2$  hours. These studies demonstrate superiority of pulmonary drug delivery with regards to maintenance of effective therapeutic concentration of the LN in the plasma over a period of 6 to 60 hours. Hence, the pulmonary delivery is expected to reduce frequency of dosing and systemic side effects associated with oral administration of LN.

**KEYWORDS:** pulmonary, liposomes, Levonorgestrel pharmacokinetics

### INTRODUCTION

It is well known that contraceptive needs change during a couple's reproductive life because of changing cultural, religious, and reproductive needs. In addition, many couples do not use modern methods for the fear of side effects. To meet the unmet needs of couples, a wider range of fertility regulation methods should be made available. Research is going on worldwide for the development of improved versions of existing technologies as well as the development of new methods.

An important feature of all oral contraceptive drugs is the interference with production and action of endogenously synthesized steroid hormones.<sup>1</sup> Indeed, changes in oestradiol metabolism after administration of exogenous hormones have been reported.<sup>2</sup> As the majority of contraceptives are administered by mouth, the hepatic first-pass effect may result in induction of hepatic enzymes, including liver microsomal cytochromes P450.<sup>3</sup> Therefore, an important side effect of oral contraceptive drugs is the interference with potency and duration of other medications such as anticoagulants,<sup>4</sup> antibiotics, or anticonvulsant drugs. In addition, orally administered steroids interfere to different degrees with hepatic protein synthesis of procoagulatory and fibrinolytic proteins.<sup>5</sup> Interference with liver function also explains why some oral contraceptive users develop a fatty liver as a consequence of long-term treatment. It is also likely that factors originating from or due to hepatic metabolism of exogenous steroids play a role in hypertension and dyslipidaemia, side effects frequently observed with oral contraceptive treatment.

Levonorgestrel (LN) has been used for many years both alone (in low doses) in the progestogen only pill (POP) and in combination with estrogen in combined oral contraception (COC) preparations. There have been many fewer studies on the safety of long-term use of the POP than of the COC, but the existing data are largely reassuring.<sup>6</sup>

New materials and new technologies have stimulated pharmaceutical researchers to identify and use alternatives to the classical oral and injectable routes. The pulmonary route is

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being used for the effective delivery of drugs into the systemic circulation. For a long time, the lung has been used for the administration of drugs for the treatment of local conditions. However, more recently, spurred on by the advent of novel delivery devices, there is a growing interest in the use of the lung for the systemic delivery of challenging molecules, such as peptides and proteins, as well as analgesic agents and even vaccines.<sup>7</sup> The larger surface area of the lung is well known, although, interestingly, the permeability of the lung tissue in itself is not that different from other mucosal surfaces, it is the large area that provides for the rapid absorption. Liposomes are phospholipid vesicles composed of lipid bilayers enclosing one or more aqueous compartments. Liposomes provide an efficient delivery system because they are biocompatible, biodegradable, and relatively nontoxic.<sup>8</sup> As a drug delivery system, liposomes can significantly alter the pharmacokinetics and pharmacodynamics of entrapped drugs, for example, by enhancing drug uptake, delaying rapid drug clearance, and reducing drug toxicity.<sup>9-11</sup>

In the present study an attempt was made to develop formulations for pulmonary administration of LN and to establish pharmacokinetic parameters and comparable relative pulmonary bioavailability to oral route. It was an objective to enhance and maintain effective therapeutic concentrations of the drug for a prolonged period of time in the development of a pharmaceutically rational pulmonary drug-delivery system for maximizing the therapeutic index, reducing the dose/frequency of dosing and systemic side-effects, and thereby reducing the cost of therapy.

## MATERIALS AND METHODS

LN was obtained from German Remedies Pvt Limited, Mumbai, India. Phosphatidylcholine (PC) (type-E 80) was a gift sample from Lipoid GmbH, Ludwigshafen, Germany. Cholesterol (CHOL) was purchased from S. D. Fine Chemicals, Mumbai, India. All other reagents and chemicals used were of analytical grade unless otherwise specified.

### Preparation of Levonorgestrel Formulations

#### Levonorgestrel Suspension (LP1)

One hundred milligrams of drug was weighed accurately and transferred to a 10-mL volumetric flask. Water was added and volume was made up to the mark using distilled water, and the resulting suspension was sonicated.

#### Levonorgestrel Physical Mixture (LP2)

One hundred milligrams of drug along with liposomal constituents, namely, egg PC and CHOL, were weighed accu-

rately and transferred to a 10-mL volumetric flask. Water was added and volume was made up to the mark with distilled water, and the resulting suspension was sonicated.

#### Levonorgestrel Liposomes (LP3)

Liposomes of LN were prepared by reverse phase evaporation method (REV method)<sup>12</sup> with Drug:PC:CHOL molar ratio of 1:4:1 and dissolved in diethyl ether (organic phase) in a glass tube (Quick fit neck B-24, Durga Scientific Pvt Ltd, Vadodara, India). Distilled water containing 100mmol/L sucrose was injected rapidly into lipid solution through a 23-gauge hypodermic needle from a 5-mL syringe. The tube was closed with a glass stopper and vortexed for 5 minutes. The tube was then attached directly to a rotary evaporator to dry the contents at 40°C under vacuum (20" of Hg) until a gel was formed. Vacuum was released and the tube was removed from the evaporator and subjected to vigorous mechanical agitation on vortex mixture for 5 minutes. When the gel collapsed to fluid, it was again fitted to rotary flash evaporator for the removal of organic solvent. A cycle of 10 minutes drying and 5 minutes vortexing was again repeated twice. Final liposomal suspension was subjected to complete removal of last traces of organic solvent in a rotary flash evaporator under vacuum (20" of Hg) for 15 minutes. The liposomal dispersion so formed was subjected to ultrasonic downsizing in an ice bath for 30 minutes. Resulting suspension was frozen at -40°C for 2 hours and then thawed at room temperature for 15 minutes to get optimum percent drug entrapment (PDE) (i.e., 98.3%). The dispersion was analyzed for PDE in liposomes and in the preparation of liposomal dry powder inhaler (LDPI); the liposomal dispersion was diluted with sufficient hydrating medium to obtain a lipid:sugar ratio of 1:1. An equivalent proportion of sorbolac, calculated to have a final strength of 10 µg entrapped drug (in purified liposomal dispersion) per 20 mg formulation, was dispersed into the liposomal dispersion. The paste so formed was frozen at -40°C overnight and dried under negative displacement pressure (model DW1 0-60E, Heto Drywinner, Bukerod, Denmark) for 24 hours. The porous cake thus formed was sized successively through no. 120 and no. 240 sieves.<sup>13</sup> Capsules (size "2") were filled with individually weighed powder (20 mg) containing 10 µg LN and packed under nitrogen atmosphere in high-density polyethylene (HDPE) bottles containing silica bags as desiccant. The bottle with desiccant was sealed with polyvinyl chloride-coated aluminum foils and stored in a refrigerator until further use. A fraction of the powder was rehydrated with triple-distilled water with gentle, occasional agitation. The rehydrated liposomal dispersion was separated from the leaked drug by centrifugation and analyzed for PDE and percentage free drug in the LDPI formulation.

**Table 1.** Analytical Profile of Levonorgestrel Formulations\*

	LN Suspension (LP1)	LN Physical Mixture (LP2)	LN Liposomes Before Dehydration	LN Liposomes (LP3) After Rehydration
Drug Entrapped in Liposomes (%)	—	—	98.30 ± 0.21	98.17 ± 0.18
Size (μm) D [4,3]	2.7 ± 0.01	4.8 ± 0.02	2.5 ± 0.01	2.5 ± 0.01
Span (polydispersibility)	3.26 ± 0.01	16.20 ± 0.01	1.55 ± 0.01	1.97 ± 0.01

\* D [4,3] indicates volume mean diameter, and LN, Levonorgestrel. Data are the mean ± SEM (n = 3).

### Characterization

Measurement of PDE of liposomes was carried out by separating untrapped drug from liposomes by centrifugation at 2000 rpm for 15 minutes and analyzed according to the method described in *United States Pharmacopeia* 26-NF. Results obtained are recorded in Table 1.

The mean vesicle size of rehydrated liposomes was determined by a laser light scattering technique using Mastersizer (Malvern Instruments, London, UK). The particle size of the formulations was described by the volume mean diameter ( $D[4,3]$ ). The polydispersity of the powder was expressed by the span:  $\text{Span} = [D(v,90) - D(v,10)]/D(v,50)$ , where  $D(v,90)$ ,  $D(v,10)$ , and  $D(v,50)$  are the equivalent volume diameters at 90%, 10%, and 50% cumulative volume, respectively. The results are given in Table 1.

### Animal Studies

Six female Albino rats (250 ± 20 g) for each group were used in this study. All animals were housed in polypropylene cages with free access to palletized chow and tap water. The animals were exposed to alternate cycles of 12 hours light and darkness. Animal experiments were approved by Social Justice and Empowerment Committee, Ministry of Government of India, New Delhi, India.

### Methodology

#### Pulmonary Administration

The method of Enna and Schanker<sup>14</sup> for measurement of absorption rates of instilled compounds from the lungs of anesthetized rats was modified to allow measurements in conscious animals for periods of up to 72 hours after instillation. Animals were anesthetized using urethane intraperitoneally. Anesthetized animals were placed in supine position on a 45° slanted support, and a small middle incision was made over the trachea. The trachea was exposed by blunt dissection of the sternohyoideus muscle. A small hole was made in the trachea between the fifth and the sixth tracheal rings using a 20-gauge needle. A short (10- to 15-cm) length

of PE50 tubing was inserted into the hole and advanced to the bifurcation of the trachea. Formulations of LN (0.1 mL) were slowly instilled over a 1-minute period using a 1-mL syringe attached to the PE50 tubing. Following instillation, the tubing was withdrawn and a small drop of cyanoacrylate adhesive was placed over the hole to seal the opening. The skin was clothed with 3-0 Dexon sutures. The animal was removed from anesthesia and allowed to recover under a heating lamp. After recovery, animals were housed in individual plastic cages with access to food and water for the remainder of the study.

#### Oral Administration

For oral administration, the drug was instilled through the mouth using a 28-gauge, long, blunt needle.

Blood was sampled from tail vein at 2, 4, 6, 12, 24, and 36 hours after oral administration and further at 48, 60, and 72 hours after pulmonary administration.

### Analytical Methods

To determine intact LN in blood, a specific spectrofluorimetric method was used as described earlier.<sup>15</sup> Ten microliters of plasma samples were taken and extracted with 2 mL and 1 mL methylene chloride, twice. Methylene chloride was again extracted with 80% sulphuric acid in ethanol, and samples were analyzed at excitation wavelength of 460 nm and emission wavelength of 520 nm. Blood samples were collected and calibration curve of LN in blood was prepared by adding known quantity of drug (after dissolving in ethanol, volume not exceeding 1% of the blood volume) in blood, and the procedure was performed as described above.

### Pharmacokinetics

The AUC (area under the blood LN concentration time curve) of both orally administered and intratracheally instilled LN formulations was calculated by the trapezoidal rule.<sup>16</sup>  $C_{\text{max}}$ ,  $t_{\text{max}}$ , and Half-lives of plasma drug disappearance  $t_{1/2}$ , were determined from plasma LN level-time curves. Data were compared using analysis of variance.

**Table 2.** Pharmacokinetics of Different Formulations Following Oral and Pulmonary Administration of Levonorgestrel in Rats\*

Formulation	Route	AUC (ng-h/mL)	P*	T <sub>max</sub> (hours)	C <sub>max</sub> (ng/mL)	T <sub>1/2</sub> (hours)
Plain Drug (LO)	Oral	261.41 ± 12.36	—	2.1 ± 0.2	14.4 ± 0.6	16.9 ± 0.2
Plain Drug (LP1)	Pulmonary	255.16 ± 9.87	97.6 ± 1.2	6.0 ± 0.2	4.4 ± 0.4	61.2 ± 0.2
Physical Mixture (LP2)	Pulmonary	257.63 ± 10.15	98.6 ± 1.4	7.0 ± 0.2	4.2 ± 0.5	61.4 ± 0.2
Liposomes (LP3)	Pulmonary	287.24 ± 11.29	109.9 ± 1.4	6.8 ± 0.2	4.4 ± 0.6	64.4 ± 0.2

\*AUC indicates the area under the blood LN concentration time curve

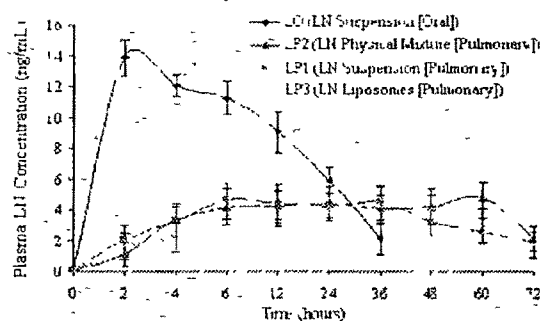
(ANOVA), and the difference at  $P > 0.05$  was considered significant.

## RESULTS AND DISCUSSION

The data of drug plasma concentration are shown in Figure 1, and from the figure various pharmacokinetic parameters were calculated and recorded in Table 2. The AUC following oral and pulmonary administrations of formulations was found to be significantly different. However, no significant difference was observed in AUC after pulmonary administration of these formulations. The  $F^*$  values after pulmonary administration were 97.6%, 109.88%, and 98.55% for LP1, LP2, and LP3 formulations, respectively. The  $T_{max}$  for these formulations were found to be 6.0, 6.8, and 7.0 hours for pulmonary administration with a  $C_{max}$  of 4.40, 4.42, and 4.20 ng/mL, respectively, followed by a plateau up to 48 hours, while for oral administration of LN suspension,  $T_{max}$  and  $C_{max}$  were 2.1 hours and 14.4 ng/mL, respectively. Following oral drug delivery,  $C_{max}$  of 14.4 ng/mL was followed by decline in plasma concentration with  $t_{1/2}$  of 16.9 hours. In contrast, pulmonary delivery gave effective plasma drug concentration for the period of 56 to 60 hours with the zero-order release kinetics following  $C_{max}$  of 4.40, 4.42, and 4.20 ng/mL for LP1, LP2, and LP3 formulations, respectively.

The rate and extent of lung uptake depend on drug physicochemical properties such as degree of ionization and lipophilicity.<sup>17-21</sup> Pulmonary delivery of all 3 formulations resulted in similar pharmacokinetic behavior because of the similarity in lipophilicity and size of the drug and liposomes.

Solubilization and diffusion of the drug and drug from liposomes into alveolar fluid before absorption into systemic circulation through transcellular uptake may be responsible for prolonged and zero-order absorption of LN (up to 60 ± 2 hours). It has also been reported that liposomally encapsulated drug remains in the lung for a prolonged period of time.<sup>22</sup> Slow and prolonged absorption of the drug after pulmonary delivery significantly reduces  $C_{max}$  and is also expected to reduce dose-dependent progestronic side effects associated with orally administered LN.

**Figure 1.** Plasma LN concentration (ng/mL) after oral and intratracheal instillation up to 72 hours

Different portions of the bronchopulmonary tree possess different characteristics, it is possible that drug release from liposomes is affected by the distribution of formulation achieved during administration and later altered by mucociliary transport and other mechanisms. Animal studies to date have utilized instillation of liquid formulations in order to obtain accurate dosimetry.<sup>23-24</sup> Such results are dependent upon the spreading of the instilled dose within the lung and their interpretation may be complicated by the presence of components capable of affecting the spreading process. The distribution and absorption of inhaled aerosols in the lungs and airways are different from those of instilled liquids,<sup>25-27</sup> and it is possible that release kinetics of drug from instilled formulations in animals and from inhaled aerosols may be significantly different. In addition, the size and aerodynamic behavior of the powder through human airways may result in a significantly different distribution and rehydration of aerosolized liposomes compared with rodent test systems, which may affect observed release kinetics, duration, onset, and intensity of effect.

LN, an orally active progestronic derivative, is associated with various side effects possibly due to the initial very high plasma concentration ( $C_{max}$ ) achieved, which is significantly higher than the therapeutic window of the drug (active therapeutic window, 4-6 ng/mL). Pulmonary dosage forms, however, give an extended release of the drug over a long period of time without resulting in initial higher plasma con-

centrations. This may further reduce the frequency of dose administration. While more work is needed to extrapolate these findings to better contraceptive efficacy by pulmonary route, the present study clearly indicates the important role of this route as an alternative to oral administration with regards to sustainability, and slow zero-order release kinetics may help in reduction of various side effects of oral contraceptives. The role of the formulations developed in this investigation can only be settled after pharmacodynamic studies and clinical investigations.

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