2.

MATERIALS, EXPERIMENTAL, RESULTS & DISCUSSION

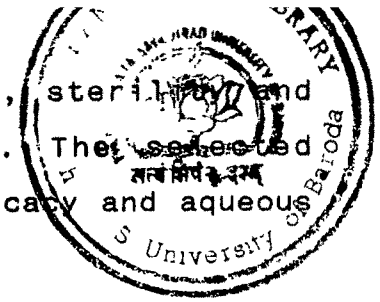
2.1 EXPERIMENTAL DESIGN

Ketorolac tromethamine (KT) is a new, potent nonsteroidal anti-inflammatory agent. It has been approved by US-FDA, for the treatment of itching due to seasonal allergic conjunctivitis, and also for prophylaxis and reduction of inflammation and associated symptoms following ocular surgery. Eventhough, ophthalmic drops of KT are marketed, the technology is not disclosed. These drops need to be administered 4-6 times a day. Hence the study was designed into approaches dealing with the development of conventional as well as long acting formulations of KT for ophthalmic use.

A methodical approach was designed in consonance with the characteristics of the drug. By keeping the objectives of this investigation in mind the experiments were designed in the following manner.

Preformulation studies were carried out systematically which included: i) Standardization of the drug, characterization of additives and packaging components. ii) Analytical method development for the drug and preservative. iii) Compatibility studies of drug with additives and packaging materials. iv) Selection of suitable sterilization procedure for the drug and its formulations.

By utilizing the collected and generated preformulation data various prototype formulations were developed. These prototype formulations were subjected to accelerated stability studies. The stable formulations were scaled upto 1 litre or 1 kg batch,



which were further evaluated for stability, sterility and antimicrobial preservative effectiveness. The selected formulations were evaluated for safety, efficacy and aqueous humor drug concentration in rabbits.

A well designed preformulation studies were required, since there was no much literature available on i) analysis of ketorolac in presence of its degradation products and additives, ii) compatibility of the drug with commonly used ophthalmic additives, packaging components and iii) suitable sterilization technique for the drug.

Preformulation studies were started with standardization of samples of KT. A stability-indicating HPLC and HPTLC methods were developed for analysis of the drug from the prepared formulations. A HPLC method was also developed for analysis of the preservative used in the formulations. To analyze drug from *in-vitro* release medium, a UV-spectrophotometric method was developed.

Since KT is not stable in aqueous solution, a study was designed to evaluate the effect of various stabilizers possessing different mechanism of protecting a drug. The stabilizers evaluated included antioxidants, non-ionic surfactants to improve clarity, chelating agent and a complex forming agent.

Compatibility of aqueous solutions of KT with commonly used additives, preservatives, polymers, closures and containers were studied at various temperatures viz. 60°C, 45°C, ambient temperature and 5°C for a period of 3 months.

Freedom from microbial contaminants (sterility) is an important

criterion for all ophthalmic dosage forms. Based on the objectives of our study, different methods of sterilization such as dry-heat and ethylene oxide for the drug in solid form and autoclaving & membrane filtration for the drug in aqueous solution were evaluated.

To develop long acting ophthalmic formulation of the drug, the polymers selection were based on their biocompatibility, regulatory status and compatibility with the drug. For the preparation of viscous solution of the drug, hydroxypropyl methylcellulose E4M (HPMC E4M) was selected, because of its safety and wide use in such products. The concentration of HPMC required in the formulation was selected based on viscosity and stability of the formulation.

The long acting gels of KT was prepared by incorporating either Carbopol 940 or Poloxamer-407. Carbopol 940 is approved by US-FDA for ophthalmic use, and is being used in ophthalmic gel and tear substitute formulations. Poloxamer-407 has a unique property of thermoreversibility, it exists as liquid below 15°C and as semisolid above 20°C. The concentration of Carbopol 940 or Poloxamer-407, to be incorporated in the formulation was selected based upon stability, viscosity and *in-vitro* release of the drug from the gels.

Ophthalmic ointment of KT is not available in India or abroad, therefore a technology was developed to dispense KT in ointment form, using a very commonly used ointment base.

The prepared formulations in their final pack were subjected to a six month accelerated stability studies at 5°C, 25°C, 37°C and 45°C, in addition ointment was subjected to accelerated humid

condition at 37°C/75% RH also. A room light stability studies, at ambient temperature, were also carried out simultaneously for a period of 6 months. The various parameters evaluated before and during the stability studies were drug content, preservative content, pH, clarity, sterility and antimicrobial effectiveness test for all the formulations. Additional studies such as viscosity measurements and *in-vitro* release were carried out for gels and ointment. It is a regulatory requirement that the sterile ophthalmic multiple dose formulation should meet specification for sterility and preservative effectiveness tests and hence these studies were carried out.

The eye is a delicate organ and the formulation intended to be used in the eye should cause no irritation and it is a regulatory requirement. Therefore, the stable formulations were evaluated for eye-irritation studies as per Draize test guidelines, by comparing with normal saline for a period of one week in New Zealand rabbits.

The stable and non-irritating ophthalmic formulations of KT were evaluated for anti-inflammatory activity in New Zealand rabbits, by comparing with the innovators product (Acular^{R}). A sodium hydroxide induced ulcer model was used to induce inflammation. The alkali burn model, provides various parameters to evaluate a anti-inflammatory agent, hence it was selected.

The efficacy studies were time consuming and required large number of animals. Therefore, once the efficacy of the prepared conventional ophthalmic solution was confirmed, further comparison was made by measuring the aqueous humor drug concentration at various time points. In case of viscous solution, ointment and gels, only aqueous humor drug

concentration-time profile was compared with the conventional formulation, after administration of a single dose. Since the objective of preparing gels were to reduce the frequency of administration of available KT drops, a multiple dose of conventional formulation was compared with a single dose of both Carbopol 940 and Poloxamer-407 based gel. The *in-vivo* studies were carried out in New Zealand rabbits.

2.2 MATERIALS:

2.2.1 DRUGS AND ADDITIVES:

Benzalkonium chloride 50% w/solution: Spectrochem, India

Brij 35: Loba Chemie, India

Carbopol 940 NF and 971P: B.F. Goodrich Co, USA

Cholesterol GR: Loba Chemie, India

Cremophor EL NF: BASF, Germany

Decarboxy anologue of ketorolac: Courtesy, Syntex Research, USA.

Dextrose AR: Qualigens, India

Disodium EDTA AR: Qualigens, India

Glycerol IP: United Chemicals, India

Hydroxy analogue of ketorolac: Courtesy, Syntex Research Corporation, USA

HPMC E4M USP: Colorcon, UK

Hydroxypropyl-β-cyclodextrin: Wacker Chemie, Germany

Ketamine hydrochloride: Ketmin^{R} injection, 50 mg/mL, Themis Pharmaceuticals Ltd., India

Ketorolac tromethamine USP: Courtesy, Cadila Laboratories and Ranbaxy Laboratories, India.

Keto analogue of ketorolac : Courtesy, Syntex Research Corporation, USA

Mannitol, puriss: Spectrochem, India

Poloxamer-407 NF: BASF, Germany

Propylene glycol AR: S.D. Fine Chemicals Ltd. India,

Sorbitol GR: Fluka, Germany

Sodium chloride AR: Qualigens, India

Sodium metabisulfite LR: S.D. Fine Chemicals Ltd., India

Thiomersal IP: Courtesy, Cadila Pharma, India

α-tocopherol: BASF, Germany

Tween 80: Prolabo, France

White soft paraffin: Omega Labs., India

Water: Freshly double distilled from an all-glass distillation still

Xylazine hydrochloride: Courtesy, Cadila Pharma, India

Xylocaine: Lidocaine^{R} topical, 4% w/v solution, Astra-IDL, India

2.2.2 SOLVENTS AND CHEMICALS USED FOR ANALYSIS:

Absolute alcohol (Aldehyde free alcohol, AFA): Alembic Chemical Works Co. Ltd., India

Acetonitrile AR: S.D. Fine Chemicals, India

Ammonia solution sp. gr. 0.91, AR: Qualigens, India

Chloroform AR: Qualigens, India

Dichloromethane AR: S.D.Fine chemicals, India

Hydrochloric acid AR: Qualigens, India

Karl Fischer reagent: Qualigens, India

Litmus indicator powder: Qualigens, India

Monobasic ammonium phosphate AR: Qualigens, India

Methanol AR: Qualigens, India

Nitric acid AR: Qualigens, India

Ninhydrin AR: Spectrochem, India

Nitrogen, zero air: Poonam Oxygen Ltd., Ahmedabad, India

Orthophosphoric acid AR: S.D. Fine Chemicals, India

Potassium dihydrogen orthophosphate AR: Qualigens, India

Potassium iodide AR: S.D. Fine Chemicals Ltd., India

Potassium iodate AR: S.D. Fine Chemicals Ltd., India

Potassium nitrate AR: Qualigens, India

Potassium permanganate LR: S.D. Fine Chemicals, India

n-propyl alcohol AR: S.D. Fine chemicals Ltd., India

Silica gel GF254: Merck, India

Sodium dihydrogen orthophosphate AR: Qualigens, India

Sodium hydroxide AR: Qualigens, India

Toluene AR: Qualigens, India

Triethyl amine AR: S.D fine chemicals, India

Tetrahydrofuran, HPLC grade, stabilized: Spectrochem, India

Water: Freshly double distilled from an all-glass distillation still

2.2.3 MATERIALS USED IN MICROBIOLOGICAL STUDIES:

Bacteriological agar: Qualigens, India

Beef extract: Qualigens, India

Fluid thioglycollate medium: Himedia, India

Membrane filters (0.22µm, GV): Millipore,

Microbial cultures: Courtesy Cadila Pharma, India

Peptone, bacteriological: S.D. Fine Chemicals Ltd., India

Polysorbate 80, for bacteriology: Himedia, India

Soyabean casein digest medium: Himedia, India

2.2.4 APPARATUS, EQUIPMENT etc.

Aluminium crimps: M/s Jayantilal Mehta & Sons., India

G-4 filter: Borosil, India

Grey butyl rubber stoppers 20mm: Shree products, India

Grey bromobutyl rubber stoppers 20 mm : Shree products, India

Lacquered aluminium tubes: Chotalal Ind., India

Membrane filters 0.22 µm (GV): Millipore

Presterilized LDPE stoppers: Vijay Bakelite, India

Type-I Vials: Gujarat glass, India

Analytical balance: Mettler AE 240, Switzerland

Gas Chromatograph: Chemito, model 8570, India
- Column: Porapak Q, 1.5m X 4mm i.d.
- Data processor: Chemito, model 5000, India
- Detector: Flame ionization type

HPLC:

- Column: Finepak^R, C_8, 10µm, 25cm X 4.6mm i.d.
- Guard column: Waters, C_{18}, 35-45µm Corasil^R, 3cmX4 mm i.d.
- Injector: Rheodyne 7125, with 20µL loop
- Integrator: Chromatocorder 21, System Instruments Co. Ltd. Japan
- Solvent delivery pump: Jasco, model 880-PU, Japan
- UV-Vis detector: Jasco, model 875-UV, Japan

HPTLC

- Applicator: Linomat IV
- Scanner: CAMAG TLC scanner 3 with software CATS-4.01
- Plates: Kieselgel 60F254 HPTLC (20cm X 20cm) plates, Merck, Germany
- UV light cabinet: Camag, UV cabinet II, Switzerland

Infrared spectrophotometer: Buck Scientific, model 500, USA

Light microscope: Leitz, Laborlux 12, Germany

pH meter: Elico, model LI-120, India

pH meter electrode: Elico, model CL-51, India

UV-Visible spectrophotometer (double beam): Jasco, model 7850, Japan

Viscometer: Brookefield, type LVDV-II+, Brookefield Instruments Inc, USA

2.3 PREFORMULATION STUDIES

Preformulation is a systematic investigation of the physico-chemical properties of drug alone and when combined with other additives and packaging components. Preformulation studies, if properly designed, have a significant part to play in anticipating formulation problems and identifying logical paths in formulation development. The successful formulation of a stable and effective dosage form depends on the careful selection of the additives used to improve stability, preserve and promote bioavailability of the drug.

Extensive literature survey provided important physico-chemical properties of the drug. However, to generate useful information for developing a stable, acceptable, safe and efficacious KT ophthalmic dosage forms, the following preformulation studies were carried out:
(i) Standardization of the drug and additives (ii) Development of analytical methods (iii) Compatibility studies of aqueous solution of KT with additives, closures & containers and (iv) Selection of suitable sterilization techniques for KT and its formulations.

2.3.1. STANDARDIZATION OF DRUG AND ADDITIVES:

The quality of the product depends upon the quality of raw materials used in the formulation. All the raw materials, used in manufacturing of ophthalmic products, must be of the highest quality available. Complete raw material specifications for each component must be established and verified. It is a regulatory

requirement that, the drug used in the formulation should meet the official specification. Therefore, to develop zero defect product, the drug and additives used in the formulations were standardized. The methods used for standardization of each of the ingredient is given below.

2.3.1.1 STANDARDIZATION OF KETOROLAC TROMETHAMINE:

Since KT is official only in U.S.P, the drug samples were standardized by carrying out the following tests as per the pharmacopoeial monograph.

EXPERIMENTAL:

a) **Infrared absorption:** The IR spectrum of the drug was recorded on an infrared spectrophotometer by making a KBr pellet of the drug and compared with the USP reference standard of KT.

b) **Ultraviolet absorption of the drug:** A 10 µg/ml solution of the drug in methanol was scanned on a UV/visible spectrophotometer in the range of 220nm to 400nm.

c) **Test for tromethamine:** The test was carried out by applying, 40µl of a 5 mg/ml solution of the U.S.P reference standard of KT, in a mixture consisting of dichloromethane and methanol (2:1) on a 0.25 cm thick layer of chromatographic silica gel. Similarly the drug sample of KT was prepared and applied on the same plate. The plate was developed in a mobile phase consisting of dichloromethane, acetone and acetic acid in the ratio 95:5:2 v/v. After drying at 100°C for 30 minutes, the plates were sprayed with alcoholic ninhydrin solution (30 mg/ml) and heated at about 150°C for 5 minutes.

d) **pH of a 1 % w/v ketorolac tromethamine solution:** 0.25 gm of drug was weighed and dissolved in enough water to make 25 ml. The pH was measured using a calibrated pH meter.

e) **Loss on drying:** About 1 gm of KT was weighed in a tared, dried weighing bottle, and dried it in vacuum oven at 60°C for 3 hours. Percent loss on drying was calculated by the formula:

$$\frac{(W1-W2) \times 100}{W1}$$

W1 = weight of sample before drying
W2 = Weight of sample after drying

f) Chromatographic purity and assay of ketorolac tromethamine: Chromatographic purity of the drug samples was carried out using a liquid chromatograph equipped with a 313 nm detector and a Finepak^R 4.6 mm X 25 cm column that contains 10 µm packing C_8. The mobile phase consisted of monobasic ammonium phosphate buffer adjusted to pH 3.0 and tetrahydrofuran in the ratio 70:30 v/v, respectively. The flow rate was kept at 2.0 ml/minute. The resolution solution was prepared by taking 30 mg of KT in a mixture containing 100 ml each of distilled water and dichloromethane and 1 ml of 1 N hydrochloric acid. Ketorolac was extracted into dichloromethane and exposed to direct sunlight for 10-15 minutes. A one ml aliquot of the dichloromethane layer was transferred to a test tube, evaporated to dryness in a stream of nitrogen. The residue was reconstituted in 1.0 ml of solvent mixture consisted of distilled water and tetrahydrofuran (70:30 v/v), and 10 µl of the solution was injected onto the column to identify the impurity and related substances peaks.

The assay of KT was also carried out by using the above mentioned chromatographic conditions. The solution of KT was prepared in tetrahydrofuran and water (70:30 v/v) to get 400 µg/ml. Similarly the working U.S.P reference standard of the drug was prepared, and 10 µl of each of the solution was injected onto the column separately.

The quantity in, mg, of $C_{15}H_{13}NO_3.C_4H_{11}NO_3$ was calculated using the formula: $100C(r_u/r_s)$, in which C is the concentration in mg/mL of USP ketorolac tromethamine RS in the standard preparation, and r_u & r_s are ketorolac peak response obtained from the assay preparation and standard preparation, respectively.

RESULTS & DISCUSSION:

The results of standardization of KT samples are shown in Table-1. The IR spectrum of the drug is given in Fig. 3. The bulk drug was found to conform to the official specifications.

TABLE-1. RESULTS OF THE STANDARDIZATION OF KETOROLAC TROMETHAMINE

TEST	SPECIFICATION	RESULT
1. Identification		
(a) IR Spectrum	To match with standard	Complies
(b) TLC	To match with standard	Complies
(c) Test for tromethamine	To comply	Complies
2. pH of 1 in 100 solution	5.7 - 6.7	6.2
3. Loss on drying	NMT 0.5 %	0.12% w/w
4. Residue on ignition	NMT 0.1%	0.04%
5. Heavy metals	NMT 0.002 %	Complies
6. Assay	98.5-101.5% (anhydrous basis)	99.16 w/w
7. Chromatographic purity	Sum of all impurity peaks NMT 1.0 %	0.22 %
1-keto analog	NMT 0.1 %	0.07 %
1-hydroxy analog	NMT 0.1 %	0.05 %
Other impurities	NMT 0.5 %	0.20 %

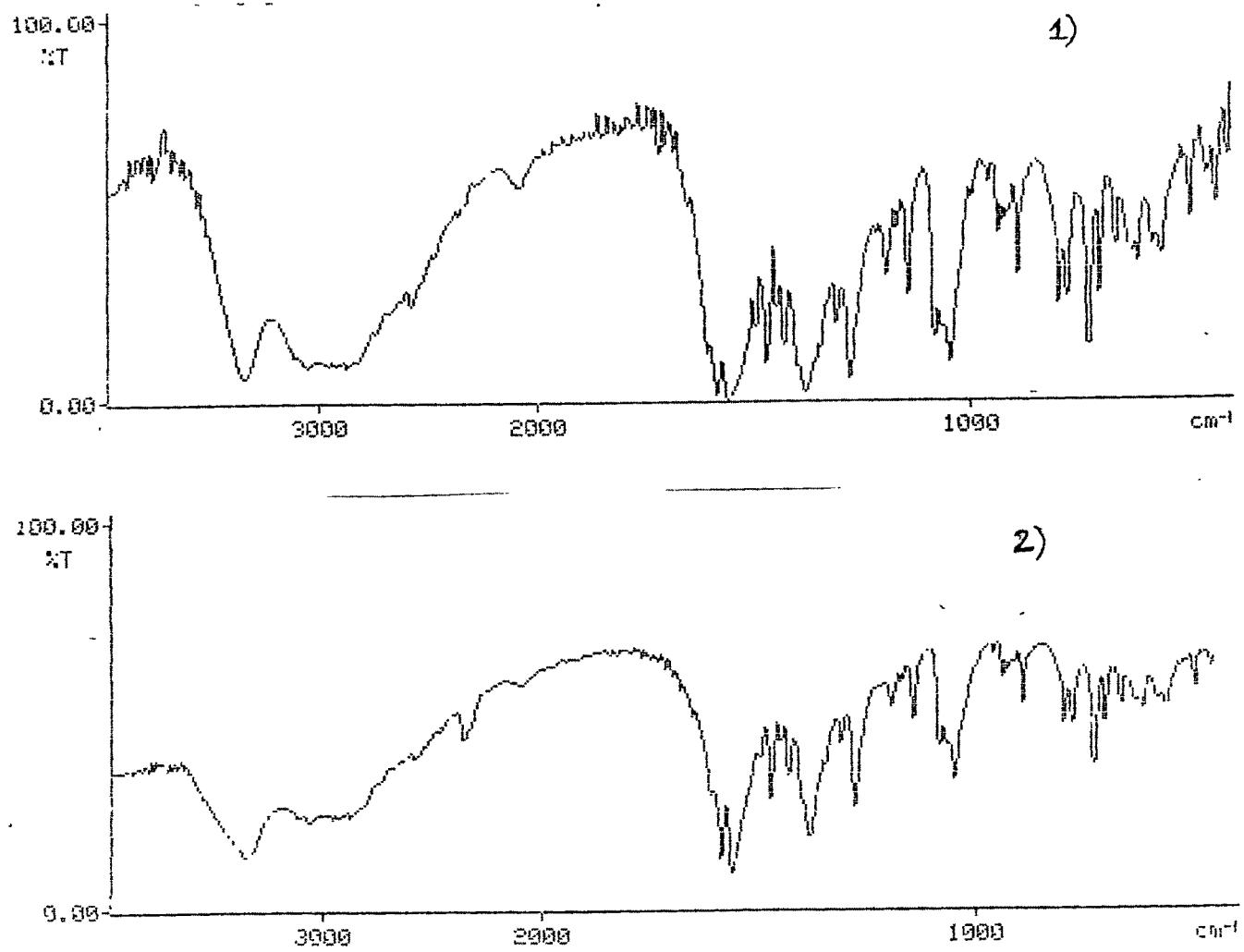


Fig. 3: Infra-red spectrum of Ketorolac tromethamine
1) USP Reference standard 2) drug sample used in the study

2.3.1.2 STANDARDIZATION OF BENZALKONIUM CHLORIDE:

Antimicrobial preservatives are important ingredients in ophthalmic multiple dose containers. Benzalkonium chloride, the most widely used preservative, might have trace amount of impurities which are toxic to the eye. Therefore it was necessary to standardize the preservative.

EXPERIMENTAL:

Benzalkonium chloride 50 % w/v solution was evaluated as per official guidelines given by Indian Pharmacopoeia 1985. The various tests carried out included identification, presence of ammonia compounds & foreign amines, alcohol by gas chromatography and assay by iodimetric titration.

RESULTS & DISCUSSION:

The results of standardization of benzalkonium chloride as per IP are shown in Table-2. The results reveal that benzalkonium chloride conformed to the IP specifications.

TABLE-2. RESULTS OF THE STANDARDIZATION OF BENZALKONIUM CHLORIDE

TEST	SPECIFICATION	RESULT
1. Identification		
(A) Reaction with nitric acid and $HgCl_2$ solution	Precipitate forms which is insoluble in alcohol	Complies
(B) Colour reaction with β-naphthol	Formation of an orange-red coloured complex	Complies
(C) Reaction to litmus and foaming ability	Neutral or slightly alkaline, foams strongly	Complies
(D) Presence of Cl-	To comply	Complies
2. Ammonia compounds	To be absent	Absent
3. Foreign amines	To be absent	Absent
4. Alcohol	NMT 16%v/v	4.8%v/v
5. Assay	49-51%w/v	49.88%w/w

2.3.1.3 STANDARDIZATION OF OTHER ADDITIVES:

The polymers used in the study are official in USP-22/NF17. Hence the quality of the polymers and additives were ensured by carrying out some official tests, and also by the quality control reports provided by their respective manufacturers. Cremophor EL was also standardized by carrying out few official tests as per USP/NF17.

The tests conducted for standardizing HPMC E4M included loss on drying and apparent viscosity. Carbopol 940 was checked for loss on drying and carboxylic acid content. Poloxamer-407 was evaluated only for pH of a 1 in 40 solution. Cremophor EL was standardized by carrying out identification, specific gravity and water content.

The results of the standardization of HPMC E4M, Poloxamer-407, Carbopol 940 are given in Table-3. All the polymers used in the study were found to comply with their respective specifications. Cremophor-EL and other additives used in the formulation also conform to their respective official specifications.

TABLE-3. RESULTS OF STANDARDIZATION OF ADDITIVES

Additive	Test	Specifications	Result
HPMC	Viscosity	3000-5600 cps	3800 cps
	LOD	NMT 5.0% w/w	3.78%
Poloxamer	pH of solution	5.0 - 7.5	6.14
Carbopol	LOD	NMT 2.0 %	1.25%
	Carboxylic acid content	56.0-68.0 %	65.19%

2.3.2 DEVELOPMENT OF ANALYTICAL METHODS :

To have quantitative data on various studies such as purity evaluation of the drug, compatibility studies, stability studies, *in-vitro* release studies etc, it is essential to develop analytical method/s which are precise, specific and accurate. Therefore, the following analytical methods were developed and validated for ketorolac tromethamine as well as for the preservative.

2.3.2.1 A STABILITY-INDICATING HPLC METHOD FOR KETOROLAC TROMETHAMINE:

It is necessary to develop and validate, a stability-indicating method for a drug during preformulation stage. This will help in evaluating the product stability, not only in terms of drug content but also its degradation profile during stability and compatibility studies. KT is sensitive to light[223] and also undergoes degradation in aqueous solution[233]. A few stability-indicating analytical methods for KT have been published, but none of these has been validated to analyze the drug in presence of its degradation products from ophthalmic formulation. Therefore, we developed a stability-indicating method using a liquid chromatographic technique. This method was developed before KT became official in USP-23.

EXPERIMENTAL:

i) Chromatographic condition:

HPLC was performed with Jasco (Jasco Spectroscopic Inc., Japan) instrument equipped with a reciprocating pump 880-PU, a variable wavelength detector 875 UV, a 7125 Rheodyne injector, 20µl fixed loop, C_{18}, Corasil$^{(R)}$ 35-45 µm, 3 cm length guard column and an

analytical column C_8, Finepak$^{(R)}$ 250mmX4.6mm I.D. The mobile phase consisted of acetonitrile, methanol and 0.01M KH_2PO_4 buffer (pH adjusted to 4.2) in the ratio 25:25:50 v/v. The mobile phase was pumped at a rate of 1.0 ml/minute and the eluents were detected at 319 nm.

ii) Standard solutions:

Stock solution having 500 µg/ml of KT in DDW was suitably diluted to prepare calibration curves in the range of 20-70 µg/ml. Each concentration was injected five times and 'Within day' and 'Between days' variations were calculated for six days.

iii) Recovery studies:

To study the interference, of commonly used ophthalmic additives, in analysis of the drug, recovery studies were carried out. The following additives were selected for the study. Benzalkonium chloride (BKC) 10 µg/ml, Di-NaEDTA 100 µg/ml, sodium chloride 70 µg/ml, glycerine 100 µg/ml, propylene glycol 100 µg/ml and HPMC 500 µg/ml. The study was carried out as follows;

a) To 50 µg/ml of pure KT in DDW the above additives were added separately to see individual interference and also mixture of all the additives, and analysed by the HPLC method.

b) Three different levels of preanalysed drug solution corresponding to 80 %, 100 % and 110 % w/v of the label claim was added to the additives and analyzed for the drug recovery. Each level was repeated five times.

c) Analysis of the drug in presence of its degradation products:

1) Authentic degradation products were injected onto the column for identification.

ii) The drug (0.5 % w/v) and additives in DDW was kept at 60°C for 3 months. A 100 µl of the sample was diluted to 10 ml with DDW, and 20 µl of this was injected onto the column.

RESULTS AND DISCUSSION:

The chromatographic purity of KT was verified, by changing mobile phases, mobile phase ratios and its pH. The chromatogram of pure KT is shown Fig. 4. The calibration curve of KT showed linearity in the selected range of 20-70 µg/ml and the equation of the regressed line was Y = 61477.3 X + 8516.7 with r= 0.9996 and coefficient of variation (C.V) less than 2.5 % (n=6). The reproducibility of the calibration curve is given in Table-4.

The additives and degradation products of the drug did not interfere in the analysis of ketorolac tromethamine. The degradation products of KT were well resolved (Fig. 4). The results of recovery studies carried out at different concentrations of KT in presence of additives are given in Table-5. The results suggested that the method is precise, accurate, and stability-indicating.

TABLE-4. RESULTS OF REPRODUCIBILITY OF CALIBRATION CURVE OF KETOROLAC TROMETHAMINE

Conc. (µg/ml)	'Within Day' % C.V (n=4)	'Between Days' % C.V (n=8)
40	1.16	0.41
50	1.56	1.97
60	1.25	0.54

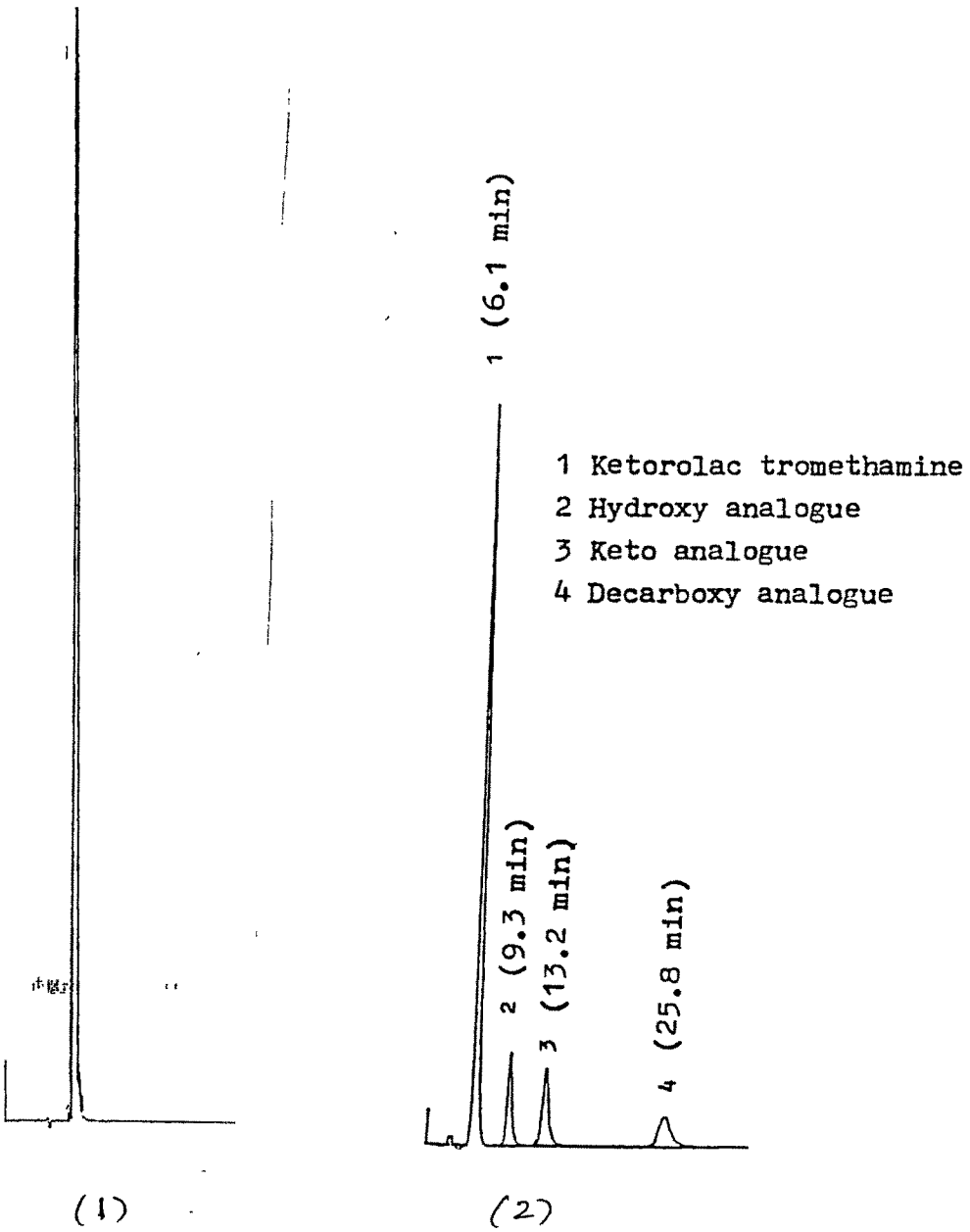


Fig. 4: HPLC chromatogram showing
(1) Purity of ketorolac tromethamine
(2) Separation of KT and its degradation products

TABLE-5. RESULTS OF RECOVERY OF KETOROLAC TROMETHAMINE FROM OPHTHALMIC ADDITIVES

Amount of KT added (µg/ml)	% Mean Recovery	% C.V (n=5)
40	100.28	1.65
50	98.80	0.90
60	100.67	1.07

2.3.2.2 A STABILITY-INDICATING HPTLC METHOD FOR KETOROLAC TROMETHAMINE :

Even though the developed HPLC method was stability indicating, it suffered from long sample run times and consumed large volume of expensive organic solvents. Moreover, the polymers which are to be used in the formulation might affect the column performance. Since HPTLC is rapid, economical, sensitive and multi-sample application is possible with the precoated plates, we developed a method, which could quantitatively analyze the drug and its degradation products and/or the impurities. Moreover, HPTLC method is not reported for analysis of ketorolac tromethamine from any dosage form.

EXPERIMENTAL:

Standard Solutions:

i) *ketorolac Tromethamine* : 1 mg/ml solution of KT in DDW was prepared, and used to plot calibration curve in the range 5-15µg.

ii) *Degradation Products*: The solutions of authentic degradation products of the drug viz. 1-hydroxy analogue (D1), 1-keto analogue (D2) and decarboxy analogue (D3), were prepared in DDW separately. The concentrations of each of these were 1 µg/ml. The standard plots were made in the range of 10-50 ng.

Standard Curve: The calibration curve was made by preparing mixture of drug with the degradation products. The aqueous solution of the drug with the degradation products, were dried under vacuum at or below 60°C after the addition of diclofenac sodium (internal standard). After drying, it was reconstituted with methanol to get 5-50 µg/10 µl of KT, and 5-50ng/10µl of each of the degradation products. The concentration of the internal standard (IS) was 0.5 µg/10 µl at each level of standards. 10µl of solution was applied onto the plates.

TLC System:

Precoated HPTLC plates (Kieselgel 60F254) were used for the analysis. The application of solution on the plates were made with the help of a Linomat IV. The spraying speed was 10 µl/sec. The chromatogram was developed in a saturated chamber in two sequential runs, first to separate the degradation products and the second for KT and IS. The mobile phase consisted of chloroform for the first run and a mixture of toluene, methanol, acetonitrile, triethylamine and ammonia, in the ratio 10:3:2:0.4:0.4 v/v for the second run. The length of the solvent front was 70 mm. The plates were air dried and scanned at 323 nm and 280nm to analyse degradation products and KT & IS respectively, on CAMAG TLC scanner3 using a Cats4.01 software.

Recovery Studies:

i) Conventional and Viscous Solutions of KT: The formulations to be kept for final stability studies, were prepared without adding the drug. To 100µl of the dummy formulations, IS and three different levels of KT were added, and dried at 60°C under vacuum. After complete drying, reconstitution was done with 0.5 ml of methanol. 10 µl of it was applied on to the plates. The amount of IS added for samples and standards was 0.5µg/10µl.

ii) **Ophthalmic Gels:** Dummy gels were made using all the additives except the drug. To 100µg of the gel, IS and 3 different amounts of the drug were added, and dried under vacuum at 60°C, reconstituted with methanol, and applied on to plates.

iii) **Ophthalmic Ointments:** Ointment to be kept for stability studies was prepared without addition of KT. About 200 mg of the ointment was weighed directly in a stopped test tube. After adding the drug and IS, the ointment was warmed to 40°C in a water bath. The drug and IS were extracted by adding 1 ml of methanol. The tube was vortexed while it was warm. 10 µl of the supernatent was applied onto the plates and analysed.

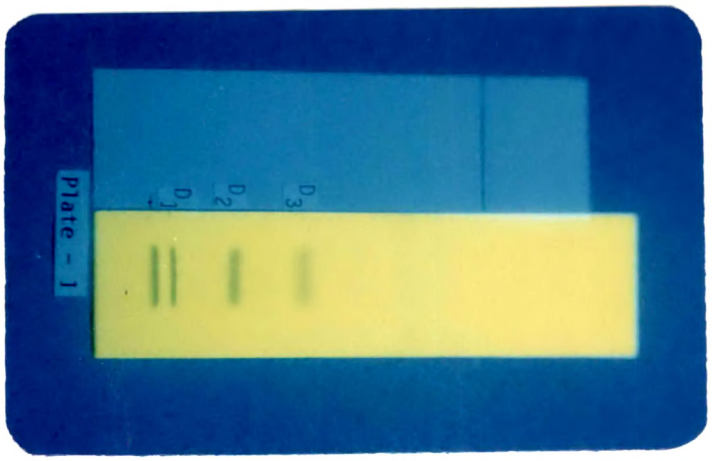
RESULTS & DISCUSSION:

The standard plot was linear in the selected range of 5-50 µg for KT and 8-50 ng for the degradation products. The results of reproducibility of calibration curve of KT is shown in Table-6. The degradation products were well separated with chloroform during the first run of TLC development (Fig. 5). The drug and IS were separated during the second run (Fig. 6).

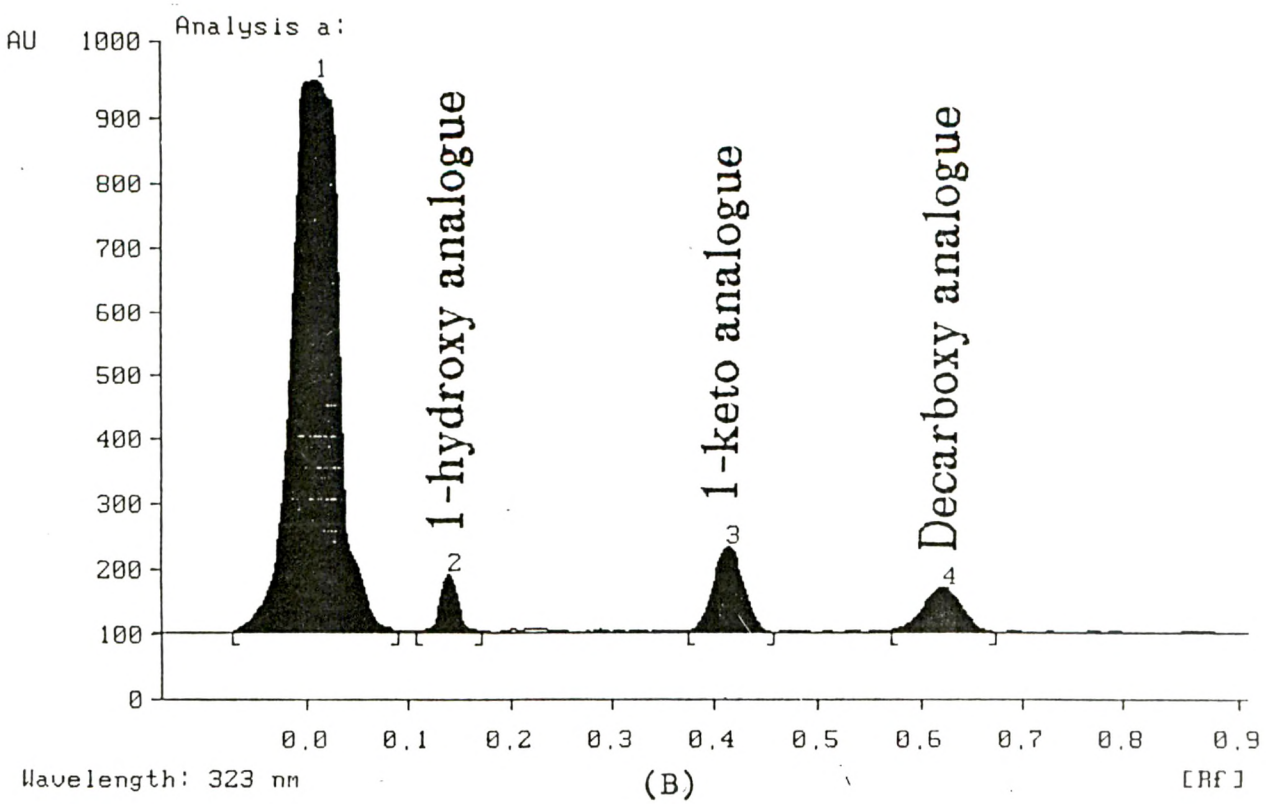
The limit of quantification for KT and the degradation products were as follows:

Ketorolac tromethamine	10 ng
1-Hydroxy analogue	8 ng
1-Keto analogue	10 ng
Decarboxy analogue	6 ng

The results of the recovery of KT and its degradation products in presence of additives used in the formulations are shown in Tables-7 to 11. The additives did not interfere in the analysis of ketorolac tromethamine and its degradation products.



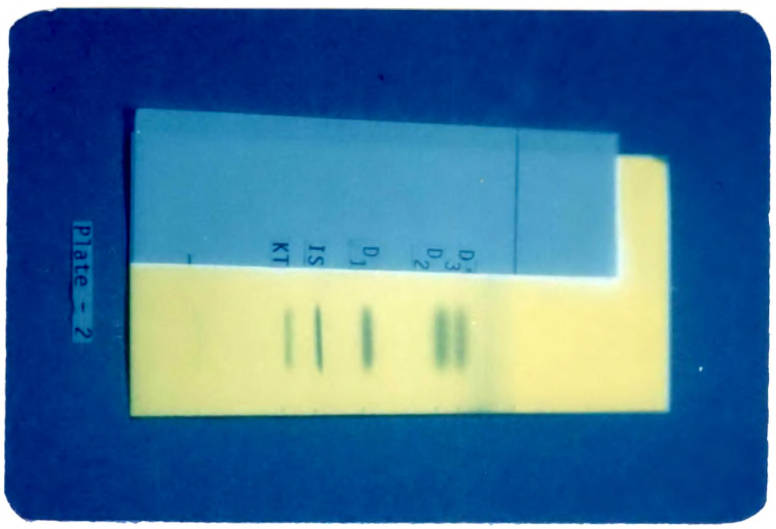
(A)



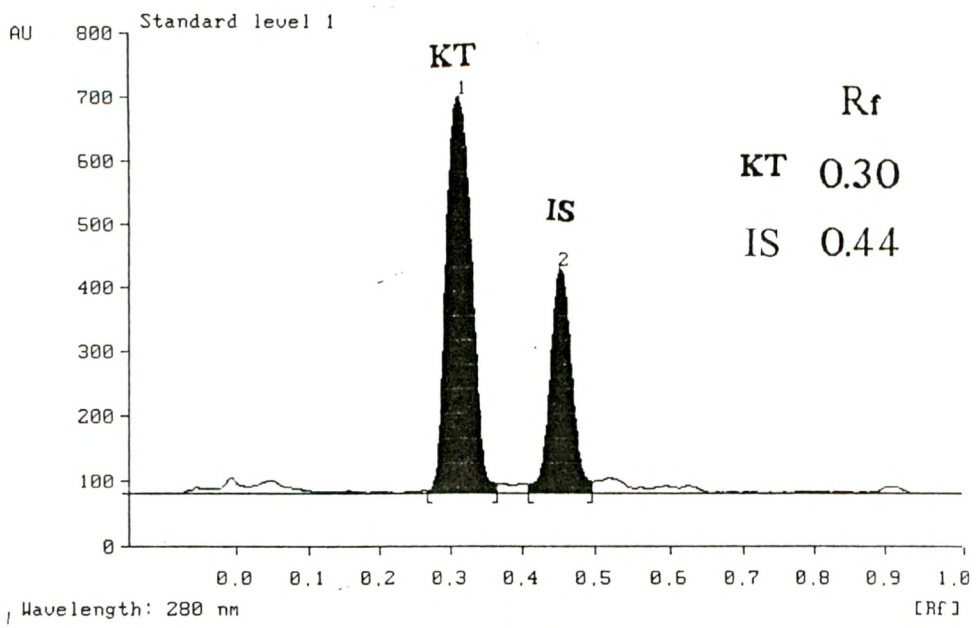
(B)

Fig. 5: (A) Photograph of HPTLC plate showing separation of degradation products of ketorolac tromethamine (First run)

(B) Typical densitogram, showing separation of degradation products (10ng each) of ketorolac tromethamine at 323 nm (First run)



(A)



(B)

Fig. 6: A) Photograph of HPTLC plate showing separation of ketorolac tromethamine and internal standard (Second run)

B) Typical densitogram, showing separation of ketorolac tromethamine and internal standard at 280 nm (Second run)

TABLE-6. RESULTS OF REPRODUCIBILITY OF CALIBRATION CURVE OF KETOROLAC TROMETHAMINE AND ITS DEGRADATION PRODUCTS

Amount of KT (µg)	D1 --	D2 ng	D3 --	'Between days' (% C.V) n=6 KT	D1	D2	D3	'Within day' (%C.V) n=6 KT	D1	D2	D3
8		10		2.08	2.68	2.93	1.83	1.32	1.84	2.02	1.02
10		20		2.42	1.93	2.48	2.08	1.23	1.03	1.48	0.98
12		50		2.00	2.05	1.98	2.52	1.10	0.98	1.21	0.87

TABLE-7. RECOVERY OF KETOROLAC TROMETHAMINE AND ITS DEGRADATION PRODUCTS FROM CONVENTIONAL OPHTHALMIC SOLUTION[a]:

Amount spiked KT (µg)	D1 --	D2 ng	D3 --	KT-Cremophor EL (FFS-1) % Recovery (% C.V) KT	D1	D2	D3	KT-HPβCD (FFS-2) % Recovery (%C.V) KT	D1	D2	D3
8		10		99.32 (0.77)	100.60 (2.09)	99.00 (2.40)	98.45 (1.53)	98.62 (1.30)	101.30 (2.02)	98.82 (1.82)	101.60 (0.88)
10		20		102.08 (1.13)	98.90 (1.21)	99.40 (1.64)	99.08 (2.06)	101.82 (0.98)	99.20 (1.32)	99.02 (2.03)	98.42 (1.87)
12		50		99.50 (1.47)	100.45 (0.99)	101.48 (1.59)	98.66 (1.09)	99.32 (1.61)	101.50 (0.98)	98.82 (1.23)	99.82 (0.83)

TABLE-8. RECOVERY OF KETOROLAC TROMETHAMINE AND ITS DEGRADATION PRODUCTS FROM VISCOUS OPHTHALMIC SOLUTION[a]:

Amount spiked KT (µg)	D1 --	D2 ng	D3 --	KT-Cremophor EL (FFV-1) % Recovery (% C.V) KT	D1	D2	D3	KT-HPβCD (FFV-2) % Recovery (%C.V) KT	D1	D2	D3
8		10		98.32 (0.89)	99.60 (1.02)	100.71 (1.41)	99.45 (0.53)	99.45 (1.08)	100.39 (2.23)	99.82 (1.23)	101.34 (0.78)
10		20		101.08 (1.36	99.90 (1.21)	98.34 (0.98)	99.12 (0.87)	100.12 (1.02)	98.80 (1.24)	99.54 (1.87)	100.11 (0.76)
12		50		99.45 (1.23)	101.05 (1.02)	100.78 (0.98)	98.43 (1.09)	98.92 (1.21)	100.80 (0.67)	99.12 (1.09)	101.02 (0.87)

[a] The values are expressed as mean perecentage of recovery.

TABLE-9. RECOVERY OF KETOROLAC TROMETHAMINE AND ITS DEGRADATION PRODUCTS FROM OPHTHALMIC OINTMENT[a]

Amount spiked KT (µg)	D1 --	D2 ng	D3 --	% Recovery (% C.V) KT	D1	D2	D3
8		10		98.78 (1.09)	101.10 (1.12)	100.70 (2.23)	99.45 (1.24)
10		20		101.12 (1.45)	99.50 (1.67)	98.56 (1.53)	100.08 (0.98)
12		50		98.98 (0.87)	100.76 (0.88)	101.10 (1.69)	99.06 (1.23)

TABLE-10. RECOVERY OF KETOROLAC TROMETHAMINE AND ITS DEGRADATION PRODUCTS FROM POLOXAMER BASED OPHTHALMIC GEL[a]

Amount spiked KT (µg)	D1 --	D2 ng	D3 --	KT-Cremophor EL (FFP-1) % Recovery (% C.V) KT	D1	D2	D3	KT-HPβCD (FFP-2) % Recovery (%C.V) KT	D1	D2	D3
8		10		100.10 (0.98)	98.98 (0.76)	101.01 (1.24)	100.45 (0.98)	99.65 (0.98)	100.67 (2.01)	100.52 (1.02)	99.87 (0.66)
10		20		100.38 (0.96)	99.11 (1.43)	99.34 (1.02)	99.65 (1.27)	100.34 (1.12)	98.99 (0.98)	100.54 (0.78)	100.43 (1.60)
12		50		99.89 (0.98)	100.05 (1.02)	99.71 (0.34)	100.21 (0.59)	99.92 (1.34)	101.00 (0.87)	98.72 (0.79)	100.52 (0.87)

TABLE-11. RECOVERY OF KETOROLAC TROMETHAMINE AND ITS DEGRADATION PRODUCTS FROM CARBOPOL BASED OPHTHALMIC GEL[a]

Amount spiked KT (µg)	D1 --	D2 ng	D3 --	% Recovery (% C.V) KT	D1	D2	D3
8		10		98.52 (1.30)	100.60 (2.12)	99.00 (2.23)	101.60 (1.24)
10		20		101.12 (0.98)	99.20 (1.32)	99.02 (2.03)	98.42 (1.87)
12		50		99.32 (1.61)	101.50 (0.99)	98.82 (1.23)	99.82 (0.83)

[a] The values are expressed as mean percentage recovery.

2.3.2.3 A HPLC METHOD FOR DETERMINATION OF BENZALKONIUM CHLORIDE IN KETOROLAC TROMETHAMINE OPHTHALMIC FORMULATIONS:

The preservative concentration used in an ophthalmic formulation, should be monitored during its stability studies. It is a regulatory requirement that the preservative should be quantitatively analysed and be mentioned on the label. Hence, a HPLC method, reported earlier for analysis of BKC in atropine sulphate[269] eye drops, was modified and validated to analyse BKC in ophthalmic formulations of KT.

EXPERIMENTAL:

i) Chromatographic Conditions:

HPLC was performed with the same chromatographic system as mentioned in section 2.3.2.1. The mobile phase consisted of acetonitrile, tetrahydrofuran and 0.1 M KH_2PO_4 containing 30mm TBAB buffer (pH adjusted to 3.0) in the ratio 59:1:40 v/v. The mobile phase was pumped at a rate of 1.2 ml/minute and eluents were detected at 260 nm.

ii) Standard Solutions:

A stock solution having 100 µg/ml of BKC in DDW was suitably diluted with mobile phase, to prepare calibration curves in the range of 8-12 µg/ml. Each concentration was injected five times and 'Within day' and 'Between days' variations were calculated for six days.

iii) Recovery studies:

Recovery studies of BKC were carried out in presence of KT and the additives used in the formulations. The formulations were prepared without addition of BKC. One ml of formulation (ophthalmic solution) or one gram in case of viscous solution, gel and ointment, were spiked with BKC solution to get 10µg/ml BKC concentration and 20µl was injected onto the column. Each level was repeated five times.

RESULTS & DISCUSSION:

The standard plot was linear in the selected range of 8-12µg/ml of BKC. The equation of the regressed line was Y = 1192 X - 156.13 with r=0.9996 (n=6). The reproducibility of calibration curve is given in Table-12. The results of the recovery studies of benzalkonium chloride from various formulations are shown in Table-13.

The developed method was found to be precise, accurate and specific.

TABLE-12. RESULTS OF REPRODUCIBILITY OF CALIBRATION CURVE OF BENZALKONIUM CHLORIDE

Concentration of BKC (µg/ml)	'Between days' % C.V (n=6)				'Within day' %C.V (n=6)			
8	2.62	2.28	2.92	2.76	0.91	1.84	1.02	0.87
10	2.42	2.93	1.28	1.78	2.11	1.54	1.48	0.98
12	1.78	1.25	1.38	2.52	0.65	0.89	1.61	0.99

TABLE-13. RECOVERY OF BENZALKONIUM CHLORIDE FROM VARIOUS FORMULATION OF KETOROLAC TROMETHAMINE

Formulation	Mean % recovery ±s.d (n=6)
KT conventional solution KT-Cremophor EL KT-HPβCD	 101.02 ± 1.87 98.87 ± 2.11
KT viscous solution KT-Cremophor EL KT-HPβCD	 99.11 ± 2.34 98.76 ± 2.67
KT ointment	96.23 ± 6.98
KT Carbopol based gel	98.58 ± 2.31
KT Poloxamer based gel KT-Cremophor EL KT-HPβCD	 101.87 ± 2.43 99.19 ± 1.87

2.3.2.4 UV-SPECTROPHOTOMETRIC METHOD FOR KT:

A UV-spectrophotometric method was developed to analyse ketorolac tromethamine from *in-vitro* release medium as well as from ointment for content uniformity.

i) Calibration Curve For Analysis of KT From *In-vitro* Release Medium:

100 mg of KT was weighed into a 100 ml volumetric flask, and was dissolved in sufficient distilled water, and volume was made to 100 ml (A). From (A), 10 ml was pipetted out into a 100 ml volumetric flask and volume was made upto to the mark either with normal saline or phosphate buffered saline of pH 7.4 (B). Standard plots were made in the concentration range 1-14 µg/ml

in normal saline as well as in phosphate buffered saline by making suitable dilutions from solution (B). The absorbance was measured at 319 nm. The interference of additives were studied by scanning the mixture containing the drug and a additive in the range 200-400 nm.

ii) Calibration Curve of KT For Determination of Content Uniformity of Ointment:

A standard solution containing 100 µg/ml of ketorolac tromethamine was prepared in methanol from a stock solution having 1 mg/ml of the drug. Suitable dilution were made from the standard solution to get concentration in the range of 2-14 µg/ml, and the calibration curve was obtained by measuring absorbance at 319 nm.

RESULTS & DISCUSSION:

The absorption maxima for KT was found to be at 319nm and 323nm in methanol and phosphate buffered saline respectively. The standard plot was linear in the range of 2-12 µg/ml of KT in methanol as well as in phosphate buffered saline. The equation of the regressed line was Y= 18.34x + 0.032 with r=1.00 (n=7) and Y= 18.07x + 0.024 with r=1.00 (n=7), in methanol and phosphate buffered saline respectively.

The additives used in the formulations did not show any absorbance at 319 nm, and hence confirmed non-interference in the analysis of KT from *in-vitro* release medium as well as from ointment.

2.3.3 CLEANING AND STERILIZATION OF CLOSURES AND CONTAINERS:

The eye is a delicate organ and hence the formulation to be administered to the eye should be free from foreign particles,

and should be sterile. A zero defect product is possible only with utmost care taken during each step involved in the manufacture of ophthalmic dosage forms. The cleaning of containers, closures and other packaging components should be given utmost priority. The procedures used for cleaning should not affect integrity of any of the materials. The sterilization procedures followed should have the capability to remove bioburden completly.

Therefore, we have selected the following procedures to clean and sterilize, the containers and closures. The containers and closures, after the sterilization process, were evaluated for sterility as per USP guidelines.

2.3.3.1 PROCEDURE FOR CLEANING AND STERILIZATION OF VIALS:

Before using the amber coloured glass vials they were evaluated as per USP guidelines. Tests for alkalinity was carried out using powdered glass test.

Amber glass vials, type-1, that were used for the preformulation studies and for dispensing final formulations, were cleaned and sterilized as follows:

(i) The vials were rinsed with tap water and soaked in 5% v/v nitric acid, for a period of about 10 hours, so as to neutralize the surface alkalinity.

(ii) The vials were then rinsed with tap water and then immersed in 0.5% w/v Teepol^{R} solution for a period of 2 hours. The vials were then scrubbed with a soft brush and rinsed with water.

(iii) The vials were again soaked in 5% v/v nitric acid for 30 minutes so as to remove excess soap from the surface of the vials.

(iv) The vials were rinsed with tap water, distilled water and finally in the laminar flow hood with membrane filtered double

distilled water.

(v) The vials were placed inverted in an enameled tray, covered with an aluminium foil and sterilized in a hot air oven at 160°C for a period of 3 hours.

The glass vials were found to comply with the USP specifications for type-1 glass and hence considered for filling the formulations. The heat sterilized vials when tested for sterility were found to be sterile.

2.3.3.2 PROCEDURE FOR WASHING AND STERILIZATION OF CLOSURES:

The LDPE bungs were received in presterilized condition. However, they were tested for sterility by the direct inoculation method and were found to be sterile. They had been sterilized by the manufacturer by exposing them to gamma-radiation.

The rubber stoppers were cleaned thoroughly with warm distilled water and finally rinsed with membrane filtered DDW. The rubber stoppers were sterilized by autoclaving at 121°C for 20 minutes with 15 psi pressure.

The rubber stoppers were found to be sterile after autoclaving.

2.3.3.3 PROCEDURE FOR WASHING AND STERILIZATION OF LACQUERED ALUMINIUM TUBES AND CAPS:

The lacquered aluminium tubes and polypropylene screw caps used for dispensing the ointment and gels were cleaned and sterilized using the following procedure:

(i) The ointment tubes and caps were rinsed thrice with 70% v/v isopropyl alcohol.

(ii) The rinsed tubes and caps were completely immersed in a 0.1% w/v solution of benzalkonium chloride in 70% v/v isopropyl alcohol for a period of 4 hours.

(iii) The excess benzalkonium chloride was removed by rinsing several times with sterile 70% v/v isopropyl alcohol. The final rinse was given with sterile isopropyl alcohol.

(iv) The tubes and caps were then kept in a tray in the laminar flow hood for drying overnight.

The cleaned and sterilized tubes and caps were evaluated for sterility following direct inoculation method as per USP.

The **lacquered aluminium tubes** along with their polypropylene caps were found to be sterile.

2.3.4 STABILITY AND STERILIZATION OF KETOROLAC TROMETHAMINE AQUEOUS SOLUTION

It has been reported that aqueous solution of ketorolac tromethamine undergoes degradation[233]. However, the degradation rate is less in the pH region 5-8 as compared to either too acidic or too alkaline solution. In ophthalmic formulation of ketorolac tromethamine, 0.5% w/v concentration has been incorporated. Therefore, the following study was carried out to evaluate the optimum pH, for maximum stability of ketorolac tromethamine aqueous solution (0.5 % w/v), and also a suitable method of sterilization for the drug.

EXPERIMENTAL:

0.5% w/v of KT solution was prepared in distilled water. Aqueous solutions of the drug were prepared having pH of 6.0, 6.5, 7.0 and 7.4, adjusted with 0.1 N sodium hydroxide. The solution was filtered through 0.22 µm membrane filter under laminar flow. These solutions were filled into cleaned and dried borosilicate bottles, and sealed. One set of solution in the bottles were autoclaved at 121°C for 20 minutes. The bottles were stored at 60°C and 45°C for 3 months. The samples were analyzed for drug content, clarity and pH every month. The analysis of KT was carried out by utilizing the stability-indicating HPLC method. The clarity was evaluated by observing the bottles against white and black back-ground. The pH of the stability samples were measured by using a pH meter.

RESULTS & DISCUSSION:

Ketorolac tromethamine gets precipitated below 5.5 pH, and hence pH's between 6 and 8 were selected for the study. The stability of the drug solution was studied at 60°C and 45°C, because at room temperature the reaction would be slow. Since no method is reported for sterilization of KT, we evaluated the feasibility of sterilizing the drug by autoclaving and membrane filtration by studying their effect, on the stability of the drug at different pH's and temperatures.

The filtration method was found to be more suitable for sterilizing KT aqueous solution. The major degradation product was found to be 1-keto analogue. The total percentage of degradation, calculated on basis of percentage of peak area as compared to KT peak, was less than 1.2 % after 3 months of storage at 45°C and after 2 months of storage at 60°C at all pH's as shown in Table-14. Discolouration of the solution was found after 3 months of storage at both the temperatures. At pH 6.0 sedimentation was found after 1 month of storage at 45°C, which could be due to decreased solubility of ketorolac tromethamine. The drug content measured during the study is shown in Table-16. There was no change in pH during the study.

The autoclaved drug solution was not stable physically after 1 month of storage at 60°C. Discolouration and sedimentation was also found after 2 months at 45°C. The percentage of degradation was found to be 2.0 % at all pH's studied, after 3 months of storage at 45°C and 1 month of storage at 60°C as given in Table-15. The drug remaining at different time and temperature, during the study is given in Table-17. The pH of all the solutions remained same throughout the study.

The results of stability of KT at different pH's and at different temperatures suggested that, the stability of the drug is similar at pH 6.5, 7.0 and 7.4. Membrane filtration sterilization was found to be more suitable method for sterilizing KT aqueous solution as compared to autoclaving.

TABLE-14. PERCENTAGE OF 1-KETO ANALOGUE FOUND AT DIFFERENT pH AND TEMPERATURE IN AQUEOUS SOLUTIONS OF KETOROLAC TROMETHAMINE (STERILIZED BY FILTRATION)

Temp. (°C)	Duration (months)	Mean % of 1-keto analogue found (s.d)[a]			
		(pH)			
		6.0	6.5	7.0	7.4
45	1	0.28 (0.012)	0.22 (0.021)	0.19 (0.013)	0.20 (0.018)
	2	0.64 (0.028)	0.75 (0.024)	0.79 (0.033)	0.59 (0.029)
	3	1.38 (0.066)	1.26 (0.079)	1.19 (0.059)	1.12 (0.061)
60	1	0.36 (0.022)	0.35 (0.030)	0.38 (0.028)	0.31 (0.029)
	2	0.85 (0.029)	0.90 (0.031)	0.99 (0.033)	0.76 (0.038)
	3	1.17 (0.033)	1.10 (0.043)	1.12 (0.042)	1.11 (0.038)

TABLE-15. PERCENTAGE OF 1-KETO ANALOGUE FOUND AT DIFFERENT pH AND TEMPERATURES IN AQUEOUS SOLUTIONS OF KETOROLAC TROMETHAMINE (STERILIZED BY AUTOCLAVING)

Temp. (°C)	Duration (months)	Mean % of 1-keto analogue found (s.d)			
		(pH)			
		6.0	6.5	7.0	7.4
45	1	0.32 (0.011)	0.37 (0.022)	0.24 (0.015)	0.29 (0.016)
	2	0.94 (0.038)	0.86 (0.034)	0.99 (0.022)	0.82 (0.026)
	3	1.62 (0.078)	1.65 (0.055)	1.59 (0.052)	1.49 (0.051)
60	1	0.52 (0.031)	0.49 (0.029)	0.53 (0.026)	0.50 (0.022)
	2	1.18 (0.031)	1.39 (0.028)	1.27 (0.022)	1.16 (0.028)
	3	1.89 (0.043)	1.78 (0.040)	1.82 (0.039)	1.71 (0.049)

[a] Values are mean ± s.d.

TABLE-16. PERCENTAGE OF KETOROLAC TROMETHAMINE REMAINING IN AQUEOUS SOLUTIONS AT DIFFERENT pH AND TEMPERATURES (STERILIZED BY FILTRATION)

Temp. (°C)	Duration (months)	% of initial concentration of KT remaining[a] (pH) 6.0	6.5	7.0	7.4
45	1	99.1 ± 0.9	100.2 ± 1.0	100.5 ± 0.8	100.2 ± 0.5
	2	98.5 ± 1.3	98.7 ± 0.8	99.7 ± 0.9	98.9 ± 1.2
	3	98.1 ± 0.7	98.2 ± 1.1	98.0 ± 0.5	98.6 ± 0.9
60	1	98.4 ± 0.6	99.1 ± 0.8	99.0 ± 1.1	99.3 ± 0.4
	2	97.6 ± 0.4	98.1 ± 0.9	98.5 ± 0.7	98.7 ± 0.2
	3	97.0 ± 0.3	98.1 ± 0.1	97.3 ± 0.6	98.0 ± 0.4

[a] values are mean ± s.d.

TABLE-17. PERCENTAGE OF KETOROLAC TROMETHAMINE REMAINING IN AQUEOUS SOLUTIONS AT DIFFERENT pH AND TEMPERATURES (STERILIZED BY AUTOCLAVING)

Temp. (°C)	Duration (months)	% of initial concentration of KT remaining[a] (pH) 6.0	6.5	7.0	7.4
45	1	99.7 ± 0.6	99.2 ± 1.0	100.1 ± 0.7	99.9 ± 0.7
	2	98.0 ± 1.0	98.4 ± 0.9	99.2 ± 0.5	98.2 ± 1.0
	3	97.5 ± 0.9	98.0 ± 0.8	98.1 ± 0.9	98.5 ± 1.1
60	1	98.1 ± 0.8	98.9 ± 0.7	98.7 ± 0.8	99.0 ± 0.6
	2	97.6 ± 0.4	98.1 ± 0.9	98.5 ± 0.7	98.7 ± 0.2
	3	97.1 ± 0.7	97.2 ± 0.1	97.1 ± 0.8	97.3 ± 0.6

[a] Values are mean ± s.d.

2.3.5 COMPATIBILITY STUDIES:

The therapeutically inactive ingredients/additives in ophthalmic dosage forms are necessary to perform one or more of the following functions: adjustment of tonicity, adjustment of pH and buffering, stabilize the active ingredient against decomposition, impart viscosity, preservation and also to act as solvent[270]. The additives can affect the stability of the drugs in various ways. Therefore it was felt necessary to carry out the stability of ketorolac tromethamine in presence of suitable additives so as to screen out additives which posses interaction potential.

The choice of a particular additive(s) and concentration(s) was based, not only on physical and chemical compatibility but also on biocompatibility with the sensitive and delicate ocular tissues. Hence, the use of additives is greatly restricted in ophthalmic dosage forms, even more so than in parenteral formulations. Hence the following compatibility studies with limited number of additives and packaging components were studied.

2.3.5.1 COMPATIBILITY STUDIES OF KETOROLAC TROMETHAMINE AQUEOUS SOLUTION WITH VARIOUS ADDITIVES:

EXPERIMENTAL:

The compatibility of ketorolac tromethamine with various additives was carried out by mixing together aqueous solutions of the two. The final concentration of the drug in each case was 0.5 % w/v, and pH was adjusted to 7.4 with 0.1 N NaOH. The solution was filtered through 0.22 µm filter and filled into cleaned and dried borosilicate bottles, and sealed. All the bottles were wrapped with black paper to protect from light. The bottles were stored at 60°C, 45°C and 25°C for 3 months. The

samples were analyzed for drug content, clarity and pH every month. The concentration of drug used for the study was 0.5% w/v, whereas the additive concentration were as follows;

(i) Tonicity Modifiers: Sodium chloride (0.7% w/v), mannitol (5.0% w/v), propylene glycol (2.0% w/v), glycerol (2.5% w/v), dextrose (5.0% w/v), polyethylene glycol 400 (3.0% w/v) and sorbitol (3.0% w/v).

(ii) Antimicrobial Preservatives: Benzalkonium chloride (0.01% w/v), propyl paraben (0.02%) & methyl paraben (0.1%) and thiomersal (0.01% w/v).

(iii) Stabilizers: Sodium metabisulfite (0.1% w/v), ascorbic acid (0.1% w/v), HPβcd (1:1 molar ratio with the drug), Cremophor EL (0.01% w/v), Brij 35 (0.016% w/v) and di-sodium EDTA (0.1% w/v).

RESULTS & DISCUSSION

(i) Tonicity Adjusting Agents:

The results of compatibility studies of KT with tonicity modifiers are given in Table-18. KT (0.5% w/v) aqueous solution exhibited excellent physical and chemical stability with all the tonicifiers evaluated. The stability of the drug solution was similar to that of pure 0.5% w/v KT aqueous solution as discussed in section 2.3.4. The pH of all the solutions remained same during the study. Since sorbitol, glycerol, propylene glycol and dextrose are non-ionic in nature, the possibility of their interaction with the drug is quite remote and the results confirmed this fact.

All the solution showed discolouration at 45°C after 3 months and at 60°C after 2 months, which was similar to that observed without any additives, i.e. only KT aqueous solution (0.5% w/v). This may be attributed to increase in 1-keto analogue of KT in the solution, which was observed in the HPLC chromatogram.

(ii) Antimicrobial Preservatives:

The compatibility studies with the preservatives were carried out only for a month at the accelerated conditions i.e. 25°C, 45°C and 60°C. Among the preservatives evaluated for compatibility with KT solution, BKC and methyl & propyl parabens were found to be physically and chemically compatible at all the accelerated conditions studied. The stability was similar to that of aqueous solution of KT without any additive. The solution containing the drug with thiomersal showed discolouration after 1 month at 45°C and 60°C. The discolouration observed with thiomersal was more intense than with BKC and parabens. This may be because of hydrolysis of thiomersal to ethylmercury chloride, thiosalicylic acid and 2,2-dithiosalicylic acid. Moreover, mercury ions are also formed at higher temperatures, which could have enhanced the degradation of KT. However, no detailed study could be taken up to elucidate the actual mechanism involved in this change.

Drug content was measured for samples containing BKC and parabens (Table-19). In case of thiomersal, drug content was not measured as it was found physically incompatible after 1 month of storage at all the temperatures. The pH (7.4) of the solutions remained same during the study, in BKC and parabens containing samples. No particles were observed after 1 month of storage at all the temperatures studied.

For further studies only BKC was considered, because it has been reported that, BKC forms a complex with KT in aqueous solution at neutral pH, which is more lipophilic, and has a better corneal permeability than the ionized form of KT[258,259].

(iii) Stabilizers:

The results of compatibility of KT with stabilizers in aqueous solution are given in Table-20. It is reported that KT undergoes autoxidation in aqueous solution[233], leading to 1-keto analogue, the major degradation product at neutral pH. In our study also we observed that, 1-keto analogue was formed at higher temperature such as 45°C and 60°C after one month of storage, and this had led to discolouration of the solution.

The ophthalmic formulations are clear dosage forms and therefore the solution has to be clear during its shelf-life, eventhough, the degradation products are within the limits. Hence, various stabilizers having different properties were evaluated to explore the possibility of improving the clarity and/or stability of KT aqueous solution (0.5% w/v) having pH 7.4.

Sodium metabisulfite and **ascorbic acid** are widely used as antioxidants in ophthalmic dosage forms. The results of compatibility studies, suggested that both the antioxidants were incompatible with KT aqueous solution. Discolouration of the solution was observed even at 25°C after 7 days. The reason could be ascorbic acid and sodium metabisulfite might have undergone oxidation by oxygen present in the head space of the bottle. No further studies could be taken up to elucidate the mechanism behind it, and hence drug content was also not measured in both the cases.

The **non-ionic surfactants** are known to improve clarity and stability[271-274] of many formulations, but their use in ophthalmic formulations is restricted, because of their toxicity. However, polyoxy 35 castor oil (Cremophor EL) and polyoxyethyl lauryl ether (Brij 35) were reported to be non-irritating and

safe to the eye upto 1.0% w/v[275]. Moreover, Cremophor EL is being used in diclofenac sodium ophthalmic drops (Voltaren Ophtha^{R}) at 0.1% w/v concentration as a solubilizer, and also as clarity improving agent[176]. The international KT ophthalmic drops, utilizes octoxynol-40 to improve clarity.

In the present study Cremophor EL and Brij 35 at concentration 0.1% and 0.16% w/v respectively were found to improve the clarity of KT aqueous solutions. Discolouration was not observed after 3 months of storage at 45°C. The solutions prepared with surfactants were kept at 55°C and not at 60°C, because cloud point of both the non-ionic surfactants are close to 60°C. The results of drug content and percentage of the degradation product is given in Table-20. The pH of the solutions remained same throughout the study. No particles were observed after 3 months of storage at 45°C. The possible mechanism involved in improving the stability of ketorolac tromethamine solution could be the protection by micelles, because the concentration of surfactants used was above their critical micellar concentration.

Cyclodextrins (CDs), a group of homologous cyclic oligo-saccharides consisting of six, seven or eight glucose units namely α-, β- or gamma-cyclodextrin, have been used to improve drug solubility, stability and absorption for oral and parenteral administration. Only recently CDs have been evaluated as an additive for ophthalmic dosage forms[277]. The most widely studied CD is HPβCD, because it is nontoxic to the eye upto 12.5 %[278]. The HPβCD has been reported to improve stability as well as penetration of anadamide[279] diclofenac sodium[276], pilocarpine[280,281] etc, in rabbit eyes. CDs are known to form complex with the drug and protect it from degradation. The complexation of drug with CD depends upon lipophilicity[282,283].

In the expected ophthalmic formulation of KT only the drug would have more lipophilicity than other additives.

Therefore, in our study we included HPβCD as a possible stabilizer for KT ophthalmic solution. The concentration selected was 1:1 molar ratio with the drug. It is reported that for many drugs, which have molecular weight of around 300-400 and similar structure as of KT, 1:1 molar ratio of HPβCD with the drug is required to improve the stability[277]. Moreover, it is reported that if the concentration of HPβCD exceeds this level for ophthalmic solution, the possibility of low ocular bioavailability increases. The reason being complexed drug would not penetrate the cornea, so less amount of free drug will be available, when more amount of CD is present.

The physical as well as chemical stability of KT aqueous solution was greatly improved in presence of HPβCD at 1:1 molar ratio with the drug. The solutions were found to be clear even after 3 months of storage at 55°C. No discolouration and no change in pH was observed during the study. The degradation product 1-keto analogue was found to be very less at higher temperatures. This improvement in stability of the drug could be attributed to inclusion of KT in the HPβCD cavity and therefore only a limited number of the drug molecules were available for degradation.

Many ophthalmic solutions need to be dispensed in **amber coloured glass vials.** This type of glass is known to leach metal ions, which in turn initiate or accelerate oxidative decomposition of active ingredient or an additive. Therefore certain **chelating agents** are generally used in ophthalmic dosage forms. Among various chelating agents, di-sodium EDTA is the most widely used. It is very well known that di-sodium EDTA enhances the

antimicrobial activity of benzalkonium chloride by chelating essential metals required for microorganisms. Hence, we evaluated di-sodium EDTA for its compatibility with the drug. The results of compatibility suggested that di-sodium EDTA was found to be compatible with KT. However, it did not improve the stability of the solution. The pH and clarity was not affected during the study.

It was observed that, HPβCd improved physical as well as chemical stability of KT aqueous solution. Non-ionic surfactants improved the clarity of KT solutions, and di-sodium EDTA was also found to be physically and chemically compatible with the drug.

TABLE-18. MEAN PERCENTAGE OF KETOROLAC TROMETHAMINE REMAINING DURING COMPATIBILITY STUDIES WITH TONICIFIERS STORED AT DIFFERENT TEMPERATURES

Temp	% KT initial concentration remaining ± s.d. (months)		
	1	2	3
(i) Sodium chloride			
25°C	99.89 ± 0.37	100.56 ± 0.42	100.11 ± 0.23
45°C	99.67 ± 0.45	99.12 ± 0.54	98.88 ± 0.66
60°C	99.18 ± 0.44	98.45 ± 0.89	98.21 ± 0.56
(ii) Mannitol			
25°C	100.11 ± 0.65	99.98 ± 0.76	99.89 ± 0.56
45°C	99.72 ± 0.44	99.10 ± 0.54	98.52 ± 0.77
60°C	99.02 ± 0.99	98.62 ± 0.34	97.89 ± 0.56
(iii) propylene glycol			
25°C	100.34 ± 0.23	99.79 ± 0.45	99.98 ± 0.87
45°C	99.56 ± 0.54	98.67 ± 0.48	98.12 ± 0.55
60°C	99.21 ± 0.34	98.82 ± 0.54	98.19 ± 0.43
(iv) Glycerol			
25°C	99.88 ± 0.44	99.34 ± 0.66	99.42 ± 0.87
45°C	99.83 ± 0.76	99.33 ± 0.89	98.67 ± 0.67
60°C	99.43 ± 0.45	99.12 ± 0.69	98.21 ± 0.34
(v) Dextrose			
25°C	100.11 ± 0.65	99.98 ± 0.76	99.89 ± 0.56
45°C	99.72 ± 0.44	99.10 ± 0.54	98.52 ± 0.77
60°C	99.02 ± 0.99	98.62 ± 0.34	97.89 ± 0.56
(vi) Polyethylene glycol 400			
25°C	100.23 ± 0.55	100.11 ± 0.36	99.98 ± 0.46
45°C	99.86 ± 0.54	99.19 ± 0.84	98.91 ± 0.23
60°C	99.55 ± 0.78	99.12 ± 0.45	98.46 ± 0.36
(vii) Sorbitol			
25°C	99.88 ± 0.34	99.67 ± 0.76	99.12 ± 0.88
45°C	99.98 ± 0.67	99.22 ± 0.56	98.78 ± 0.68
60°C	99.45 ± 0.76	99.13 ± 0.49	98.59 ± 0.51

TABLE-19. MEAN PERCENTAGE OF KETOROLAC TROMETHAMINE REMAINING DURING COMPATIBILITY STUDIES WITH PRESERVATIVES STORED AT DIFFERENT TEMPERATURES

Temp	% KT initial concentration remaining ± s.d. (After one month)	
	(i) BKC	(ii) Parabens
25°C	100.18 ± 0.72	100.11 ± 0.23
45°C	99.71 ± 0.75	98.88 ± 0.99
60°C	99.01 ± 0.44	98.33 ± 0.87

TABLE-20. EFFECT OF STABILIZERS ON STABILITY OF KETORLAC TROMETHAMINE AQUEOUS SOLUTION STORED AT DIFFERENT TEMPERATURES

Temp	% KT initial remaining (s.d)			% of 1-keto analogue found(s.d)		
	1	2 Months	3	1	2 Months	3
(i) Cremophor EL						
05°C	100.89	100.76	100.09	0.041	0.051	0.055
	(0.50)	(0.72)	(0.48)	(0.005)	(0.007)	(0.008)
37°C	99.89	99.81	99.45	0.162	0.278	0.545
	(0.78)	(0.89)	(0.87)	(0.014)	(0.01)	(0.021)
45°C	99.67	99.38	99.23	0.212	0.630	0.901
	(0.89)	(0.67)	(0.23)	(0.004)	(0.006)	(0.003)
60°C	99.65	98.89	98.43	0.301	0.681	1.183
	(0.56)	(0.43)	(0.76)	(0.004)	(0.005)	(0.008)
(ii) Brij 35						
05°C	99.96	100.07	99.82	0.052	0.055	0.059
	(0.50)	(0.72)	(0.48)	(0.004)	(0.006)	(0.009)
37°C	99.78	99.56	99.15	0.171	0.252	0.621
	(0.81)	(0.88)	(0.71)	(0.009)	(0.011)	(0.014)
45°C	99.72	99.08	98.83	0.232	0.721	0.954
	(0.97)	(0.67)	(0.23)	(0.006)	(0.005)	(0.009)
60°C	99.34	98.56	98.12	0.323	0.703	1.256
	(0.78)	(0.66)	(0.91)	(0.006)	(0.008)	(0.009)
(iii) Di-sodium EDTA						
05°C	100.02	99.95	99.91	0.062	0.065	0.066
	(0.65)	(0.45)	(0.87)	(0.008)	(0.006)	(0.007)
37°C	99.98	99.68	99.02	0.181	0.272	0.701
	(0.71)	(0.68)	(0.56)	(0.005)	(0.09)	(0.014)
45°C	99.67	99.12	98.64	0.245	0.719	0.895
	(0.89)	(0.78)	(0.54)	(0.001)	(0.012)	(0.019)
60°C	99.54	98.76	98.01	0.319	0.712	1.156
	(0.97)	(0.88)	(0.93)	(0.007)	(0.008)	(0.012)
(iv) HPβCD						
05°C	100.01	100.07	99.98	0.030	0.032	0.033
	(0.40)	(0.92)	(0.58)	(0.007)	(0.006)	(0.004)
37°C	99.96	99.94	99.78	0.033	0.035	0.038
	(0.91)	(0.68)	(0.81)	(0.009)	(0.006)	(0.004)
45°C	99.92	99.93	99.91	0.043	0.057	0.082
	(0.78)	(0.97)	(0.63)	(0.004)	(0.008)	(0.008)
60°C	99.82	98.99	98.82	0.084	0.121	0.163
	(0.56)	(0.96)	(0.88)	(0.006)	(0.004)	(0.007)

2.3.5.2 COMPATIBILITY STUDIES OF KT AQUEOUS SOLUTION WITH POLYMERS:

The purpose of incorporating polymers in ophthalmic dosage form is to prolong residence time, and hence the ocular bioavailability of the administered drug formulation. The most commonly used polymers in ophthalmic formulations such as **HPMC E4M, Carbopol 940 and Carbopol 971P and Poloxamer-407**, were selected for the compatibility studies. These polymers are known to be safe for administration in the eye.

HPMC E4M is being used extensively in ophthalmic dosage forms to increase viscosity of solution, and hence to improve the residence time of dosage form in the eye. The concentration commonly used is 0.25 - 0.5 % w/w. HPMC E4M is non-irritating and recommended for ophthalmic use by the manufacturer. For the compatibility study we selected 0.5 % w/w of HPMC E4M.

Carbopols are acrylic acid polymers widely used in pharmaceutical industries over 25 years. The distinct advantages of these polymers are high viscosities at lower concentrations, compatibility with many additives, bioadhesive properties, and excellent appearance and patient acceptability. Carbopol 940 is known to be non-irritating and has been used in artificial tears, ophthalmic formulations and new drug delivery systems[284,285]. Moreover, it is approved by US-FDA for ophthalmic use.

The most successful ophthalmic gel, Pilopine HS^{R} containing pilocarpine 4% w/w, utilizes Carbopol 940 as gel forming agent[286]. The gel is applied only once a day as compared to 4-5 times administration of pilocarpine conventional solution. Therefore Carbopol 940 was evaluated for compatibility with KT solution.

Poloxamer 407, is one in the series of Poloxamer ABA block copolymers, containing 70-79 % polyoxyethylene with a nominal molecular weight of 12,500. The different types of Poloxamers vary over a wide range of molecular weight and relative proportions of the polyoxyethylene and polyoxypropylene groups. The interest of Poloxamer-407, results from its very low toxicity and its inertia towards mucosa even at higher concentration like 20-30%[130]. Ophthalmic preparations have been evaluated with the aim of prolonging the pharmacological action of drugs. In addition, aqueous solutions of the polymer in concentrations above 20% w/w exhibit the phenomenon of reverse thermal gelation, remaining as solution at refrigerated temperatures and gelling upon warming to ambient levels. Hence, Poloxamer-407 was also evaluated for compatibility with KT aqueous solution.

EXPERIMENTAL:

The samples for compatibility studies were prepared by mixing sterilized (0.22µm membrane filtration) drug solution with sterilized polymer, and pH was adjusted to 7.2 ± 0.5 with 0.1 N NaOH. The HPMC E4M containing samples were filled into amber coloured vials, and sealed with presterilsed LDPE bungs. In case of Poloxamer-407, it was filled both into amber coloured vials as well as lacquered aluminium collapsible tubes. The Carbopol 940 containing samples were filled into lacquered aluminium tubes. The concentration of HPMC E4M, Carbopol 940 and Poloxamer-407 used for the study were 0.5% w/w, 1.5% w/w and 20 % w/w respectively.

The sealed vials and tubes were stored at 45°C, 25°C and at refrigerated conditions for a month. The samples were evaluated for viscosity, pH, drug content and physical changes before and during the study.

RESULTS & DISCUSSION:

Carbopol 971P did not give good consistency even at 6.5% w/w, and hence was not considered for the study. All the polymers were found to be physically as well as chemically compatible with KT aqueous solution at all the temperatures studied. The results of percentage of initial drug remaining, and viscosity measured during the study are shown in Table-21. The pH of the viscous solution and gels remained same (7.0±0.4) at all the temperatures.

Viscosity of the polymers in presence of drug did not change during the study. Autoclaving of the polymer solution did not affect the viscosity or pH. The initial viscosity was 25±5 cps, 75,000±5000 and 12±0.5 lakhs for HPMC E4M (0.5%), Carbopol 940 91.5%) and Poloxamer-407 (20%) respectively.

Since, HPMC E4M and Poloxamer-407 are non-ionic in nature, the interaction with KT could be ruled out. However, Carbopol 940 which is a polyacrylic acid polymer has free carboxyl group, which decreases the pH of the solution. This decrease in pH could precipitate KT as free acid. Therefore, before adding KT to Carbopol 940 solution, the free carboxyl groups were neutralized with NaOH and the pH was adjusted to approximately 7.0. By this method no interaction was observed after 1 month of storage at 45°C.

TABLE-21. RESULTS OF COMPATIBILITY STUDIES OF KETOROLAC TROMETHAMINE WITH VARIOUS POLYMERS AFTER ONE MONTH OF STORAGE AT DIFFERENT TEMPERATURES

Polymer	Temp	% of initial KT remaining ± S.D (n=3)	Viscosity (cps)
HPMC E4M	5°C 25°C 45°C	99.89±0.87 99.97±0.65 99.34±0.56	25.0 26.1 24.5
Cabopol 940	5°C 25°C 45°C	100.09±0.45 99.98±0.89 99.92±0.76	75,700 75,120 75,100
Poloxamer-407	5°C 25°C 45°C	100.12±0.56 99.45±0.89 99.22±0.76	12.4 lakhs 12.3 lakhs 12.4 lakhs

2.3.5.3 COMPATIBILITY STUDIES OF KETOROLAC TROMETHAMINE SOLUTION WITH CONTAINERS:

Faulty packaging of ophthalmic dosage forms can invalidate the most stable formulation. Consequently, it is essential that the choice of container materials for any particular product be made, only after a thorough evaluation has been made of the influence of these materials on the stability of the product and of the effectiveness of the container in protecting the product during extended storage under varying environmental conditions of temperature, humidity and light. The final container should be appropriate for the ophthalmic product and its intended use, and should not interfere with the stability and efficacy of the preparations.

Type I and type II glass vials are still used for dispensing ophthalmic solutions. Flint glass has the disadvantages of being transparent to light rays above 300 mμ. As a result, amber glass, which has the property of shutting out certain portions of the light spectrum, has been used extensively for ophthalmic dosage forms[287].

To decide on the type of vials for dispensing the formulations, two types of containers were selected viz., type I amber coloured and colourless glass vials, and compatibility of KT was carried out as described below.

EXPERIMENTAL:

0.5% w/v of KT aqueous solution was prepared in distilled water and pH was adjusted to 7.4 with 0.1 N NaOH. The solution was filtered through 0.22 µm membrane filter and filled into amber coloured as well as colourless glass vials, and sealed. To one set of solutions in amber coloured vials, di-sodium EDTA (0.1 % w/v) was also added. The vials were kept at 60°C and also on a laboratory window in order to expose to sunlight, for a period of 1 month. After a month, the solutions were evaluated for physical changes and stability of the drug using the HPLC method. The change in colour was evaluated using Nessler's cylinders.

RESULTS & DISCUSSION

The amber coloured vials are useful in protecting degradation of drug, which are photosensitive. However, the disadvantage of amber coloured glass is leaching of Fe^{3+} into the solution, which has the capability of accelerating oxidation-reduction reaction of ingredients. Since KT is sensitive to light amber coloured glass vials were evaluated for compatibility, and di-sodium EDTA was added to study its chelating effect on leaching of ions from amber coloured vials.

The colourless glass vials did not give any protection to KT solution. The solution turned brown within 15 days on exposure to sunlight. The 1-keto analogue of KT was found to be more than 3.0% after one month of storage, which shows that KT has undergone photolysis.

The amber coloured glass vials protected the photolysis of the drug as 1-keto analogue was less than 1.0% and no discolouration of the solution was observed. In case of solution without di-sodium EDTA, sedimentation was found at the bottom of the vial after one month of storage at 60°C.

The results suggested that amber coloured glass vial with di-sodium EDTA is required for dispensing KT aqueous solutions.

2.3.5.4 COMPATIBILITY STUDIES OF KETOROLAC TROMETHAMINE SOLUTION WITH CLOSURES:

The closure is normally the most vulnerable and critical component of a container insofar as stability and compatibility with the product are concerned. The most widely used closures for glass vials are either made of rubber (synthetic or natural) or low-density polyethylene (LDPE). Since the composition of rubber stoppers is complex and manufacturing process involved is complicated, it is common to encounter problems with certain rubber formulas. Hence, LDPE, which are inert plastics have been used in many ophthalmic solutions. However, these LDPE are permeable to oxygen and water vapours[287].

This study was undertaken to evaluate the effect of various closures on stability of ketorolac tromethamine solution. The various stoppers evaluated were grey butyl, grey bromo butyl, natural rubber latex and LDPE.

EXPERIMENTAL:

0.5% w/v KT aqueous solution was prepared in distilled water and pH was adjusted to 7.4 with 0.1 N NaOH. The solution was filtered through 0.22 µm filter under laminar flow. They were filled into amber coloured vials and sealed with the above

mentioned closures. The vials were stored at 60°C, 45°C and refrigerated conditions for a period of 3 months. The stored solutions were evaluated for physical changes and stability of the drug using the HPLC method.

RESULTS & DISCUSSION:

Except LDPE, all other closures showed interaction with KT solution at 60°C and 45°C after one month of storage. The solutions were turned brown and particles were found in case of grey butyl rubber stoppers within 15 days. Therefore grey butyl stoppers were not considered for further studies.

The vials sealed with natural rubber latex or grey bromobutyl rubber (GBB) stoppers showed discolouration after 2 months at 45°C and after one 1 month at 60°C. The reason could be the interaction of rubber stoppers with KT, because in inverted positions, the solutions showed brown colour which was more intense than those kept in upright position.

The LDPE closures showed no interaction with the drug even after 3 months of storage at 45°C. There was no change in pH and volume of solution with LDPE stoppers. The results showing the clarity and stability of the drug after 3 months of storage at different temperatures is shown in Table-22.

TABLE-22. RESULTS OF COMPATIBILITY OF KETOROLAC TROMETHAMINE WITH CLOSURES AFTER 3 MONTHS OF STORAGE AT DIFFERENT TEMPERATURES

Closures	Temp	% of 1-keto analogue found ± S.D (n=2)	Clarity/Colour
LDPE	5°C	0.050 ± 0.006	Clear/colourless
	45°C	0.951 ± 0.012	Clear/colourless
	60°C	1.592 ± 0.016	Clear/colourless
GBB	5°C	0.101 ± 0.005	Clear/Colourless
	45°C	1.212 ± 0.009	Particles/Brown
	60°C	1.925 ± 0.017	Particles/Brown
Latex	5°C	0.071 ± 0.008	Clear/colourless
	45°C	1.179 ± 0.007	Particles/Brown
	60°C	1.762 ± 0.025	Particles/Brown

2.3.6 STERILIZATION OF KETOROLAC TROMETHAMINE POWDER:

Since, we were in need of sterile KT powder for the development of ointment for ophthalmic use, we carried out feasibility of sterilizing the drug by dry-heat under vacuum and by using ethylene oxide gas, as there is no method reported for sterilization of KT.

EXPERIMENTAL:

2.3.6.1 Ethylene Oxide Sterilization (EtO): For this purpose, micronized KT powder was filled in a thin polyethylene bags as thin layer and covered by black polyethylene bags. These were sterilized by 100% EtO by using the following sterilization cycle:

(a) 2 steam pulses: 11 minutes each

(b) Humidification: 98 minutes

(c) Gas pressure: 1.28kg/m^3

(d) Gas exposure time: 361 minutes

(e) Nitrogen purging: 2 pulses, 40 minutes each

After sterilization cycle, KT powder was evaluated for possible physicochemical changes like: stability by HPLC, changes in

melting point, pH of 1% solution or changes in molecular structure, using UV/IR spectroscopy. The sterility of the powder was evaluated using USP direct inoculation method.

2.3.6.2 Dry-heat Sterilization: About 1 gm of KT powder was taken in sterilized shallow borosilicate bottle and subjected to dry-heat. Sterilization was attempted at 140°C for 3 hours in vacuum oven. After sterilization, the powder was evaluated for physical changes, purity of the drug and sterility.

RESULTS & DISCUSSION:

(A) The results of evaluation of EtO sterilized KT powder is given in Table-23. There was no change observed in melting point and pH of 1% w/v of KT solution before and after EtO exposure. The HPLC chromatogram and IR spectrum of KT after sterilization by EtO are given in Fig. 7 and 8. EtO did not affect KT chemical identity. Hence, the drug could be sterilized by EtO.

(B) The dry-heat sterilized drug turned brown within a week, so further evaluation was not carried out.

TABLE-23. RESULTS OF THE EVALUATION OF STERILIZING KETOROLAC TROMETHAMINE BY EXPOSURE TO EtO GAS

METHOD	PARAMETER	DESCRIPTION	
		BEFORE EXPOSURE	AFTER EXPOSURE
VISUAL OBSERVATION	Appearance	Off-white	Off-white
HPLC	Retention time	6.01 min	6.12 min
	% purity	99.89	99.81
IR	Changes in characteristic peaks	Both the spectra were found to be superimposible	
UV	Absorbance maxima	Both the spectra were found to be superimposible.	
STERILITY		----	Sterile

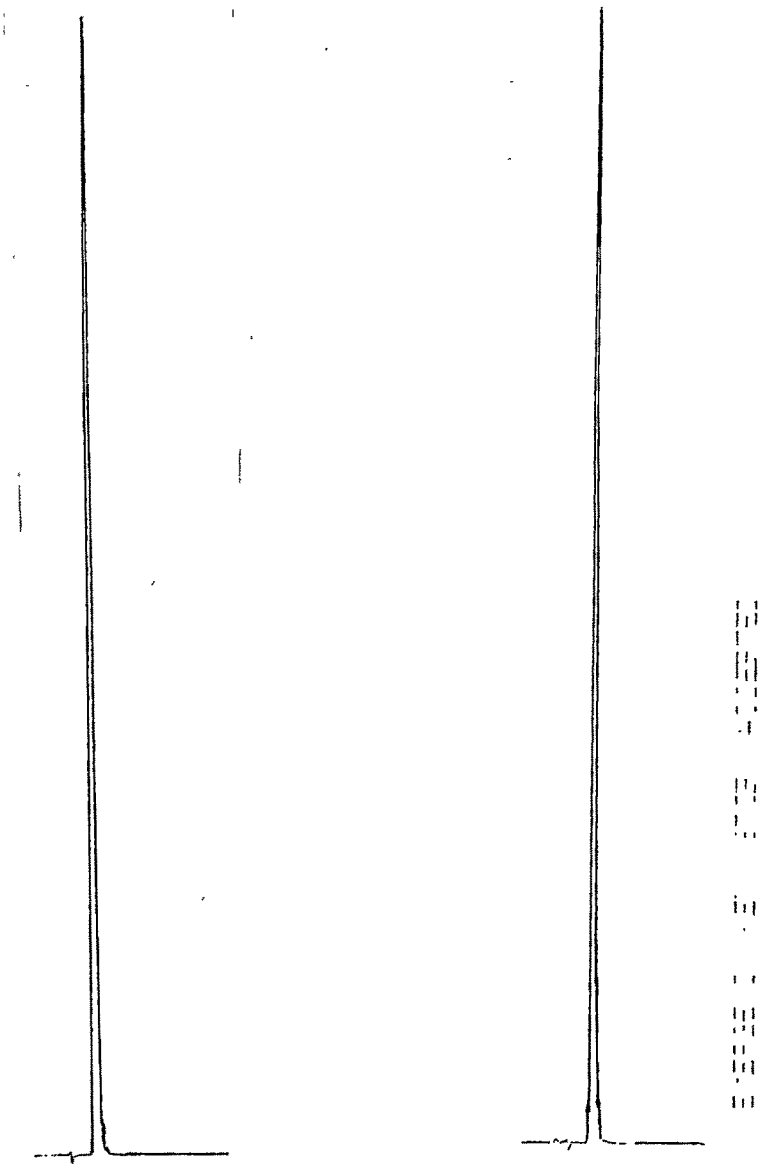


Fig. 7: Chromatogram showing ketorolac tromethamine
1) Before EtO exposure 2) After sterilization by EtO

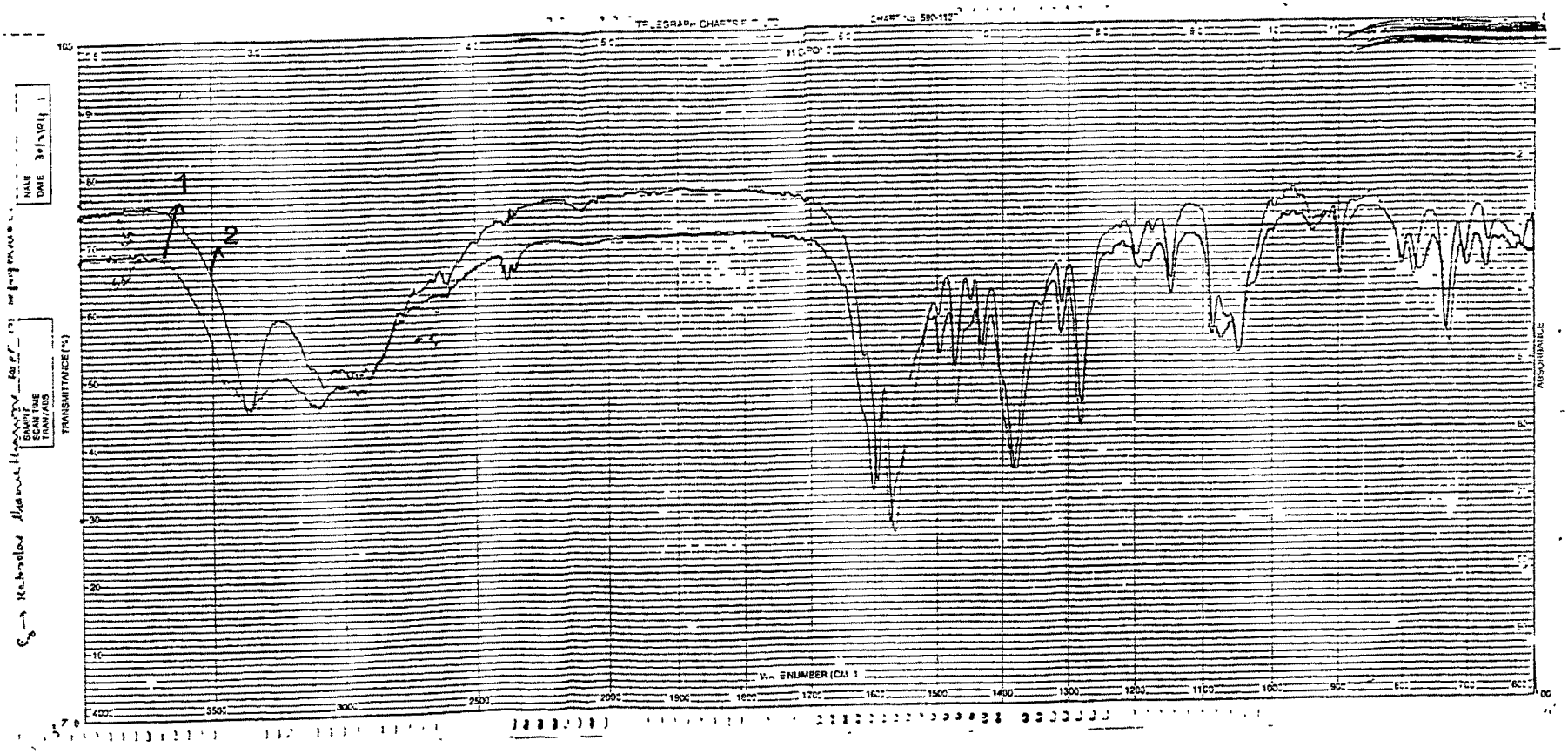


Fig. 8: Infra-red spectrum of Ketorolac tromethamine
1) Before EtO exposure 2) After sterilization by EtO.

2.4 PROTOTYPE FORMULATIONS:

Prototype formulations of ketorolac tromethamine were prepared by utilizing the compatible additives, closures and containers and sterilized by suitable sterilization technique. The different prototype formulations prepared were as follows:

2.4.1 KETOROLAC TROMETHAMINE (0.5% w/v) OPHTHALMIC SOLUTIONS:

Based upon the results of compatibility studies of KT solution with additives, various **prototype formulations (PF)** of aqueous ophthalmic solutions were prepared.

EXPERIMENTAL:

The prototype formulations of KT solutions prepared are shown in Table-24.

TABLE-24. PROTOTYPE FORMULATIONS OF KETOROLAC TROMETHAMINE CONVENTIONAL OPHTHALMIC SOLUTIONS

Ingredients	Prototype formulations PF-1	PF-2	PF-3	PF-4
Ketorolac tromethamine USP	+	+	+	+
Disodium EDTA IP	+	+	+	+
Benzalkonium chloride IP	+	+	+	+
Cremophor EL NF	+	-	-	-
HPβCD	-	-	+	-
Brij 35	-	+	-	-
Sodium chloride IP	+	+	+	+
Distilled water	+	+	+	+

+ indicates presence of the ingredient
- indicates absence of the ingredient

KT solution was sterilized by filtration through 0.22µm membrane filter and all other ingredients were sterilized by autoclaving at 121°C for 20 minutes. After mixing all the ingredients, the pH of each of the formulation was adjusted to 7.4 with 0.1 N NaOH. The formulations were filled into cleaned and auotoclaved amber coloured vials. The vials were stoppered with gamma-radiation sterilized LDPE bungs, and sealed with cleaned aluminium crimps. All the steps were carried out on cleaned laminar flow system.

The prepared formulations were stored at various temperatures such as 60°C, 45°C, 37°C and refrigerated conditions for 3 months. The stability samples were evaluated every month for drug content, clarity and change in pH, before and during the stability studies.

RESULTS & DISCUSSION:

Sodium chloride was selected as tonicity modifier because, it is widely used, and available in pure form at low cost. The water used was freshly distilled and sterilized. The incorporation of di-sodium EDTA into the formulation had two purposes. It could chelate metal ions and protect the drug undergoing oxidation triggered by metal ions and secondly to enhance the antimicrobial activity of benzalkonium chloride. The concentration of each ingredient selected were those that are generally accepted for ophthalmic use.

Since, there was no major difference in the stability of KT solution between pH 6.5 and 7.4, it was decided to adjust the pH of all the formulation to 7.4, the ideal pH for ophthalmic formulations.

The pH of all the prototype conventional formulations 7.4 ± 0.2, at all the temperatures studied, for three months. The PF-4 did not give clear solution and hence was not considered. The PF-1 and PF-2 showed haziness at 60°C after 1 month, which could be because of cloud point of both the surfactants. However, PF-3 showed excellent physical and chemical stability at all the temperatures.

The drug content in all the prototype formulations were above 98.0 %. There was no significant change in drug content. The major degradation product was 1-keto analogue. The percentage degradation of KT from each prototype formulation at different temperatures and at different time period is given in Table-25.

It was observed that prototype formulations prepared with HPβCD and cremophor-EL could be taken for further evaluation, as they were physically and chemically stable.

TABLE-25. RESULTS OF STABILITY STUDIES OF PROTOTYPE FORMULATIONS OF KT OPHTHALMIC SOLUTIONS STORED AT DIFFERENT TEMPERATURES

Temp	% of 1-keto analogue found			Physical appearance		
	1	2 Months	3	1	2 Months	3
(i) PFS-1						
05°C	0.051 (0.008)	0.059 (0.004)	0.062 (0.007)	Clear	Clear	Clear
37°C	0.145 (0.009)	0.248 (0.01)	0.645 (0.017)	Clear	Clear	Clear
45°C	0.221 (0.007)	0.587 (0.01)	0.891 (0.03)	Clear	Clear	Clear
60°C	0.332 (0.009)	0.621 (0.008)	0.912 (0.013)	Clear	Particles	Particles
(ii) PFS-2						
05°C	0.062 (0.008)	0.076 (0.004)	0.09 (0.007)	Clear	Clear	Clear
37°C	0.153 (0.006)	0.232 (0.012)	0.689 (0.014)	Clear	Clear	Clear
45°C	0.331 (0.006)	0.821 (0.009)	0.984 (0.005)	Clear	Clear	off-white
60°C	0.434 (0.012)	0.886 (0.02)	1.346 (0.014)	Clear	Particles	Particles
(iii) PFS-3						
05°C	0.021 (0.012)	0.032 (0.006)	0.038 (0.006)	Clear	Clear	Clear
37°C	0.038 (0.008)	0.035 (0.011)	0.039 (0.009)	Clear	Clear	Clear
45°C	0.045 (0.004)	0.060 (0.008)	0.110 (0.008)	Clear	Clear	Clear
60°C	0.091 (0.008)	0.131 (0.009)	0.178 (0.019)	Clear	Clear	Clear

2.4.1.1 Optimization of Surfactant Concentration:

The actual concentration of surfactant required in the formulation was evaluated by selecting four different concentrations of Cremophor-EL. The different concentrations of Cremophor-EL selected for the study included 0.005, 0.01, 0.05 and 0.1 % w/v. The prototype formulations were prepared by using the ingredients as mentioned for PF-1 and were filled in amber coloured vials and stoppered with LDPE bungs and sealed with aluminium crimps. They were subjected to accelerated stability studies at 55°C, 45°C and refrigerated temperature for three months. The stability samples were evaluated for drug content, pH and clarity every month.

Results & Discussion

The haziness of the formulation was more in case of 0.05 % and 0.1% of Cremophor-EL at 55°C after one month. At 0.005% the solution turned off-white at 45°C after two months of storage. Therefore 0.01% was found to be ideal for improving the clarity of ketorolac tromethamine ophthalmic solution.

2.4.2 KETOROLAC TROMETHAMINE (0.5% w/v) VISCOUS SOLUTIONS:

The viscous solutions of KT were prepared by incorporating HPMC E4M, the most commonly used polymer to impart viscosity in ophthalmic formulations.

EXPERIMENTAL:

A study was carried out to select the concentration of HPMC E4M required in the formulation. The Different concentrations of HPMC E4M such as 0.25%, 0.5%, 0.75% and 1.0% w/w were dispersed in distilled water and sterilized by autoclaving. The pH was

adjusted to 7.2 ± 0.2. The final solution was filtered through sterile 0.22 µm membrane filter and filled into amber coloured vials and sealed with LDPE bungs, and then with aluminium crimps. The vials were stored at 45°C, 37°C and refrigerated conditions for one month, and observed for any change in viscosity, pH and clarity.

Various Prototype formulations were prepared by incorporating 0.5% w/w of HPMC E4M (Table-26).

TABLE-26. PROTOTYPE FORMULATIONS OF KETOROLAC TROMETHAMINE VISCOUS OPHTHALMIC SOLUTIONS

Ingredients	Prototype formulation PFV-1	PFV-2
Ketorolac tromethamine USP	+	+
Disodium EDTA IP	+	+
Benzalkonium chloride IP	+	+
Cremophor EL NF	+	-
HPβCD	-	+
HPMC E4M USP	+	+
Sodium chloride IP	+	+
Distilled water	+	+

+ indicates presence of the ingredient

- indicates absence of the ingredient

The HPMC E4M solution with other ingredients were sterilized by autoclaving and after cooling to room temperature, KT solution was added and volume was made up with sterile distilled water. The final solution was filtered through sterile 0.22µm membrane filter, and filled into amber coloured vials, and sealed with LDPE bungs, and then with aluminium crimps. The stability studies of the prepared formulations were carried out in a similar manner as that of conventional formulation.

RESULTS & DISCUSSION

Among the different concentration of HPMC E4M evaluated, 0.5 % showed very good clarity and an ideal viscosity. During the stability study at 45°C for 3 months, viscosity was not decreased. The viscosity obtained with 0.5 % w/w of HPMC E4M was 24 cps.. The other concentrations of HPMC, like 0.75 % and 1.0 % w/w were too viscous to administer from the vial, whereas 0.25% w/w had only 12 cps viscosity. Therefore 0.5% w/w of HPMC E4M was selected for preparing the prototype formulations.

The prototype viscous formulations of KT prepared with HPβCD were physically as well as chemically stable throughout the stability study. The major degradation product was found to be 1-keto analogue, which was less than 0.2 % after 3 months of storage at 45°C. The other prototype formulation was also found to be physically as well as chemically stable with less than 1.0 % of degradation product at 45°C after 3 months of storage. The good stability observed with viscous solution could be attributed to the viscosity where intermolecular interaction would be less as compared simple solution.

2.4.3 OPHTHALMIC CARBOPOL GEL OF KETOROLAC TROMETHAMINE:

Carbopol 940 was selected based upon its compatibility with the drug, for preparing long acting gel. It has been reported that sodium chloride and sodium borate decrease the viscosity of the Carbopol gels, because of negative effect of the ions on the rheological property of Carbopols[284]. Hence mannitol, propylene glycol, glycerine and sorbitol were evaluated as tonicity modifiers for Carbopol 940 gel.

EXPERIMENTAL:

The various prototype formulations prepared using Carbopol 940 are given in Table-27.

TABLE-27. PROTOTYPE FORMULATIONS OF KETOROLAC TROMETHAMINE LONG ACTING CARBOPOL GELS

Ingredients	Prototype formulation PFC-1	PFC-2	PFC-3	PFC-4
Ketorolac tromethamine USP	+	+	+	+
Disodium EDTA IP	+	+	+	+
Benzalkonium chloride IP	+	+	+	+
Propylene glycol IP	+	-	-	-
Sorbitol IP	+	+	-	+
Mannitol IP	-	-	+	-
Glycerine IP	-	+	-	-
Carbopol 940 NF	+	+	+	+
Sodium hydroxide IP	+	+	+	+
Distilled water	+	+	+	+

+ indicates presence of the ingredient

- indicates absence of the ingredient

The pH of all the prototype formulations were adjusted to 7.2 ± 0.4. The method of preparation of the gel is described under section 2.5.3.

The prepared gels were filled into cleaned and sterilized lacquered aluminium tubes, and sealed. These were stored at 55°C, 45°C and refrigerated conditions for one month and observed for physical changes such as viscosity, consistency, clarity and pH.

RESULTS & DISCUSSION

From the compatibility studies of Carbopol 940 with KT, it was evident that degradation of the drug was less in gel form than in

solution form. Therefore only physical changes were expected to occur in the prototype formulations. The tonicity modifiers selected for the gel were non-ionic in nature and were known to be compatible with the polymer[284]. These tonicifiers are being used in many ophthalmic gels such as Pilopine HS^{R} in presence of BKC.

The prototype gel formulations were physically stable at all the temperatures after 3 months. The viscosity and pH did not change during the study. However, PFC-1 gave very good consistency, spreadability and clarity compared to other prototypes. The one more advantage of PFC-1 was, drying out phenomenon was not observed at the tip of lacquered aluminium tubes, whereas other prototypes showed a crust at the tip. Sorbitol is a good humectant and more hygroscopic at lower concentration (~ 3.0%) compared to other tonicifiers studied. Sorbitol is inert chemically and compatible with most additives. It is stable in air. It does not darken or decompose at elevated temperature or in the presence of amines. It can be autoclaved and can be stored in glass, plastic and aluminium container. Therefore PFC-1 was taken-up for further evaluation.

2.4.4 OPHTHALMIC POLOXAMER GELS OF KETOROLAC TROMETHAMINE:

Gels prepared with Poloxamer-407 have a unique property of thermoreversibility. Therefore the main rationale for selecting Poloxamer-407 in ophthalmic formulation is that it can be dispensed as a drop form which gels in the eye and provides long activity of the instilled drug, which means accurate drug administration is possible with this type of dosage forms. In case of ointments and preformed gels it is difficult to administer exact amount.

2.4.4.1. Selection of Poloxamer-407 Concentration:

In order to determine the optimum polymer concentration to be used in the formulation, different concentrations of Poloxamer-407 was added to KT conventional solution. The resulting solution was studied for liquefaction-gelling behavior between refrigerated condition and at 37°C. The results are listed in Table-28.

Table-28. Results of the liquefaction and gelling behaviour of KT gels prepared with various concentrations of Poloxamer:

CONCENTRATION OF POLOXAMER-407	NATURE OF SOLUTION	LIQUEFICATION BEHAVIOUR	GELLING BEHAVIOUR
15 % w/w	Viscous solution	----	---
18 % w/w	Gel	Fast	Incomplete, fails to gel at times
20 % w/w	Gel	Fast	Gels completely
22 % w/w	Gel	Slower	Gels completely
25 % w/w	Gel	Very slow, Fails to liquefy at times	Gels completely

Based on the above results, it was decided to use 20% w/w Poloxamer-407, in the prototype formulations.

2.4.4.2 Prototype Formulations of Poloxamer Based KT Gel

The various prototype formulations of Poloxamer based KT gels are given Table-29.

TABLE-29. PROTOTYPE FORMULATIONS OF KETORLAC TROMETHAMINE LONG ACTING POLOXAMER GELS

Ingredients	Prototype formulation PFP-1	PFP-2	PFP-3
Ketorolac tromethamine USP	+	+	+
Disodium EDTA IP	+	+	+
Benzalkonium chloride IP	+	+	+
Poloxamer 407 NF	+	+	+
HPβCD	-	+	-
Cremophor EL NF	-	-	+
Sodium chloride IP	+	+	+
Distilled water	+	+	+

+ indicates presence of the ingredient

- indicates absence of the ingredient

Poloxamer gels can be prepared by the cold as well as the hot process. The cold process is however the more preferred method, and is also recommended by the manufacturers, since there is a problem of air entrapment and severe foaming with the hot process. Therefore the cold process was utilized for preparing the long-acting gel. The detailed method of preparation of Poloxamer gel is described under section 2.5.4. The pH of all prototype formulation were adjusted to 7.2 ± 0.2.

The prepared formulations were filled into cleaned and sterilized amber coloured vials as well as in lacquered aluminium tubes, and were kept for stability at 45°C, 37°C and refrigerated conditions for 3 months. The stability samples were evaluated for pH, clarity, colour, viscosity and drug content at 1 and 3 months.

RESULTS & DISCUSSION:

The polyhydroxy compounds such as mannitol, glycerol, sorbitol, polyethylene glycols etc. are reported to decrease the gelation temperature of Poloxamers-407 in aqueous solution, hence sodium chloride was used as the tonicity adjusting agent[288]. The preservative and the chelating agent used in the formulation were known to be compatible with polymer.

In the compatibility studies of the drug with Poloxamer-407, it was observed that the KT content was decreased to 99.0 % whereas, 1-keto analogue was increased to 0.9 % at 45°C after 3 months of storage. Therefore it was necessary to evaluate HPβCD or cremophor EL as stabilizer in Poloxamer based KT gel. The pH of all prototype formulation were between 7.2 ± 0.4, during the study at all temperatures, in vials as well as in tubes. The prototype formulation, PFP-1 showed discolouration at 45°C after 3 months. Whereas, PFP-2 and PFP-3 were clear without any discolouration throughout the study. The drug content in all the formulations were above 98.0 % compared to that of original content, which shows that Poloxamer-407 did not interact with other ingredients used in the formulation.

Single point viscosity of all prototype Poloxamer gels, measured at 25±0.5°C, using helipath stand and T-F spindle at 0.6 r.p.m were between 12-12.5 lakhs cps at all temperatures during the stability study. This shows that temperature and the drug did not affect the viscosity of the Poloxamer gel. Based on the above results PFP-1 and PFP-2 were considered for the final evaluation.

2.4.5. KETOROLAC TROMETHAMINE (0.5% w/v) OINTMENT:

Ophthalmic ointments are very important products which are used in the treatment of a variety of ocular diseases. These products must be sterile as prescribed for all ophthalmic products. At present, KT ointment is not available in India or abroad. Therefore, it was decided to develop and evaluate ophthalmic ointment containing ketorolac tromethamine.

EXPERIMENTAL:

2.4.5.1 Particle Size Determination of KT:

Prior to the preparation of prototype formulations of KT ointment, the particle size distribution studies of the drug to be incorporated into the ointment was carried out by using an eye-piece micrometer along with a light microscope. The drug powder was suspended in light mineral oil and a drop of this suspension was placed on a slide. The length of 300 particles were measured.

2.4.5.2 Prototype Formulations of KT Ointment:

The various prototype formulations prepared are given in Table-30.

TABLE-30. PROTOTYPE FORMULATIONS OF KETOROLAC TROMETHAMINE OINTMENT

Ingredients	Prototype formulation PFO-1	PFO-2
Ketorolac tromethamine USP	+	+
Benzalkonium chloride IP	+	+
α-Tocopherol NF	+	+
White soft paraffin IP	+	+
Cholesterol NF	-	+

+ indicates presence of the ingredient
- indicates absence of the ingredient

To incorporate the drug in powder form in ointment base, KT was sterilized by ethylene oxide, as dry-heat method of sterilizing the powder was not suitable. BKC was dried in vacuum oven and passed through a ASTM 400 #. White soft paraffin was heated to about 90°C and sterilized by membrane filtration. The drug and BKC were dispersed in the sterile white soft paraffin using a sterile tile, and a sterile spatula under laminar flow bench. After mixing, the drug content was analysed by UV-spectrophotometric method, by warming ointment to about 45°C and extracting the drug with methanol on a vortex mixer. The detailed procedure for preparation and analysis is described in section 2.5.5.

The prepared ointments were filled into cleaned and sterile lacquered aluminium collapsible tubes. The tubes were stored at 45°C, 37°C and 5°C as well as at 37°C with 70% R.H. for 3 months. The stability samples were evaluated for physical changes and for the drug content. The stable formulation was tested for sterility following filtration method as per USP guidelines. A pilot eye-irritation studies were also carried out on both the prototype ointments before going into further studies.

RESULTS & DISCUSSION;

The average particle size (300 particles) of ketorolac tromethamine powder was found to be 4.18 ± 1.67 (mean ± s.d).

Components of ophthalmic ointments are usually white petrolatum, mineral oil, lanolin derivatives, and in some instances a surfactant. The majority of available ointments utilizes white soft paraffin as a principle ingredient of base with other base modifiers. The base modifiers are added to serve specific purposes e.g. liquid paraffin is added to petrolatum to lower its

fusion point, but its addition introduces a problem of separation upon storage and therefore was not used[270]. Woolfat and beeswax are reported to be incompatible with BKC, as they contain fatty acids, which are anionic in nature. We therefore, chose to use white soft paraffin alone, stabilized with 10 ppm of α-tocopherol. The other additive selected was cholesterol, to make the hydrophobic base hydrophilic.

White soft paraffin can be sterilized by either dry-heat, gamma-radiation as well as by membrane filtration. In some instances, dry-heat sterilization at 190°C, has been reported to cause oxidation and discolouration, on the other hand gamma-irradiation has also been reported to cause discolouration and swelling. The only other method left was membrane filtration. Since the petrolatum is intended to be used for ophthalmic products, it has to be free from particulate matter and would have to undergo filtration in any case. Thus it was decided to sterilize it by membrane filtration.

The content uniformity of prototype ointment was found to be between 99.5-100.5% with C.V less than 2.0%. This shows the mixing procedure followed for the preparation is suitable. However, it needs confirmation on large scale batches.

No discoloration of the product was observed during the stability study at all temperature and humidity. The drug was found to be more stable in ointment form than solution or gel, even without any stabilizer. The reason could be the drug was in solid dispersed form and moisture & air was not available for the drug to interact and undergo degradation. Therefore the HPLC chromatogram of stability samples of ointment did not show increase in 1-keto analogue.

The prototype formulations were found to be sterile before and after stability studies. The PFO-2 was found to be irritating in the rabbit eye during the pilot eye-irritation studies. The conjunctival redness and blinking rate was more compared to the other formulation. Hence, PFO-2 was not considered for further evaluation.

2.5 PREPARATION OF SELECTED KT OPHTHALMIC FORMULATIONS:

Based on the results obtained from evaluation of various prototype formulations, the following formulations of ketorolac tromethamine were prepared. The prototype formulations selected for the following study were physically and chemically stable for at least 3 months at 45°C.

For the preparation of aqueous formulations, freshly distilled and sterile filtered water was used. The drug solution was sterilized by membrane filtration (0.22 µm) and other additives by autoclaving at 121°C for 20 minutes. In case of ointment, the drug powder was sterilized by exposing to EtO gas. All the formulations were prepared under aseptic conditions. The procedure used for cleaning and sterilizing containers and closures is described under section (2.3.3). The concentration of KT used in all the formulations was 0.5% w/v or w/w.

The various formulations prepared were:

i) KT conventional ophthalmic solutions

ii) KT ophthalmic viscous solutions

iii) Long acting KT ophthalmic Carbopol gel

iv) Long acting KT ophthalmic Poloxamer gel

v) KT ophthalmic ointment

2.5.1. KT (0.5% W/V) CONVENTIONAL OPHTHALMIC SOLUTIONS:

The evaluation of results of prototype formulations suggested that formulation containing either Cremophor-EL or HPβCD were stable and suitable.

EXPERIMENTAL:

The formulations described in Table-31 were selected and a one litre batch was prepared.

TABLE-31. KETOROLAC TROMETHAMINE CONVENTIONAL OPHTHALMIC SOLUTIONS

Ingredients	Formulations FFS-1	FFS-2
Ketorolac tromethamine USP	+	+
Disodium EDTA IP	+	+
Benzalkonium chloride IP	+	+
Cremophor EL NF	+	-
HPβCD	-	+
Sodium chloride IP	+	+
Distilled water	+	+

+ indicates presence of the ingredient
- indicates absence of the ingredient

In about 500 ml of distilled water disodium EDTA, sodium chloride, Cremophor-EL and BKC were dissolved and sterilized by autoclaving at 121°C for 20 minutes. To the autoclaved solution, ketorolac tromethamine was added and mixed gently using a overhead stirrer. The volume was made up to 1000 ml after adjusting the pH to 7.2 ± 0.2. The formulation was finally sterilized by passing through sterile 0.22µm membrane filter. The solution was filled in the cleaned and sterile vials under laminar flow and the vials were stoppered with presterilized LDPE plugs. Sterile type-1 amber glass vials were used. The vials were then sealed with aluminium crimps.

RESULTS & DISCUSSION:

Being a simple solution, it was prepared by simply mixing together all the sterile solutions of additives and resterilizing the final solution by membrane filtration. All the additives had

to be presterilized so as to reduce the microbial burden. BKC was used as the preservative, as it was found to be compatible with the drug. It is the most widely used preservative in ophthalmic preparations because of its potency, broad spectrum of activity against both bacteria and fungi, and effectiveness over a broad range of pH.

The pH, selected was similar to that of tear which is also suitable for stability of the drug. Cremophor-EL was used to improve the clarity of the formulation and the concentration selected was found to be optimum. HPβCD was selected as it improved the stability of the aqueous solution of KT at neutral pH. The amber coloured glass vials used were type-1 and found to be compatible with KT aqueous solutions. The LDPE stoppers which are very inert plastics and are available in presterilized, ready to use form, were also found to be compatible with the drug, and hence were used in the formulations.

2.5.2 KT (0.5% W/W) OPHTHALMIC VISCOUS SOLUTIONS:

In the preparation of viscous solution the ingredients used were similar to that of conventional solution. The additional ingredient was HPMC E4M. The results of evaluation of prototype viscous formulations, suggested that, formulations containing either Cremophor-EL or HPβCD were found to be physically as well as chemically stable.

EXPERIMENTAL:

The viscous formulations prepared for final evaluation are given in Table-32.

TABLE-32. KETOROLAC TROMETHAMINE OPHTHALMIC VISCOUS SOLUTIONS

Ingredients	Formulations FFV-1	FFV-2
Ketorolac tromethamine USP	+	+
Disodium EDTA IP	+	+
Benzalkonium chloride IP	+	+
HPMC E4M USP	+	+
Cremophor EL NF	+	-
HPβCD	-	+
Sodium chloride IP	+	+
Distilled water	+	+

+ indicates presence of the ingredient

- indicates absence of the ingredient

To about 700 ml of hot (80-90°C) distilled water, HPMC E4M was added and dispersed by using a overhead stirrer. After complete dispersion, the solution was cooled to about 15°C and disodium EDTA, sodium chloride, Cremophor-EL or HPβCD and benzalkonium chloride were added. The resulting solution was sterilized by autoclaving at 121°C for 20 minutes. To the autoclaved solution, KT was added and mixed gently using a overhead stirrer under aseptic conditions. The volume was made up to 1000 ml with freshly distilled sterile water, after adjusting the pH to 7.2±0.2. The formulation was finally sterilized by passing through sterile 0.22µm membrane filter.

Type-1 amber glass vials of 10 mL capacity were used for dispensing the viscous solutions. The solutions were filled in the cleaned and sterile vials under laminar flow and were capped with presterilized LDPE plugs. The vials were then sealed with aluminium crimps.

RESULTS AND DISCUSSION:

The method followed for the hydrating HPMC E4 M was prescribed by the manufacturer, and is the standard method of hydrating the polymer. The HPMC E4M solution was passed through 0.45µm filter to remove fibers associated with the polymer. The HPMC E4M solution along with other additives were autoclaved in order to remove bio-burden, if any. However, KT solution was sterilized by membrane filtration, as it was sensitive to heat.

The concentration of HPMC E4M used gave ideal clarity and viscosity, as well as pourability from the vial and compatibility with KT.

2.5.3 LONG ACTING KT OPHTHALMIC CARBOPOL GEL

EXPERIMENTAL:

Only one formulation was prepared based on evaluation of results obtained from prototype formulations. The formula and mode of preparation is described below. The quantity of gel prepared was 750 gms.

Formulation No.1 (FFC-1):

Sr.No.	Ingredient
1	Ketorolac tromethamine USP
2	Disodium EDTA IP
3	Benzalkonium chloride IP
4	Propylene glycol IP
5	Sorbitol IP
6	Carbopol 940 NF
7	Distilled water

Carbopol was dispersed in 500 ml of distilled water at 1200-1400 rpm using an overhead stirrer. After complete addition and dispersion, di-sodium EDTA, propylene glycol, sorbitol and benzalkonium chloride were added and mixed thoroughly. The gel

was autoclaved at 121°C for 20 minutes. Sterile KT solution was added to the resulting gel, and pH was adjusted to 7.2 ± 0.4. The desired weight was achieved by adding sufficient sterile distilled water and mixed thoroughly well with the help of an overhead stirrer. The prepared gel was filled aseptically into cleaned and sterile lacquered aluminium tubes, capped and sealed.

RESULTS AND DISCUSSION:

The results of compatibility study of KT with Carbopol showed that the amount of 1-keto analogue formed at 45°C was less as compared to pure aqueous solution of the drug. Furthermore, no discolouration was observed. This shows the drug is stable in the gel form. The prototype formulation prepared by using the ingredients mentioned, was stable throughout the study and hence only one formulation was prepared.

The method followed for dispersing Carbopol 940 was as prescribed by the manufacturer. Autoclaving of Carbopol along with other additives was done to remove any bio-burden. It has been reported that autoclaving does not affect the viscosity of Carbopol gels. It was observed that the autoclaved gel had better optical clarity than the unautoclaved one.

2.5.4 THERMOREVERSIBLE OPHTHALMIC GEL OF KT:

EXPERIMENTAL:

Based on the results obtained in the prototype formulations containing Poloxamer-407, the following formulations were prepared as given Table-33 for final evaluation. A 1000 gm of the gel was prepared and the procedure is described below.

TABLE-33. OPHTHALMIC POLOXAMER GELS OF KETOROLAC TROMETHAMINE:

Ingredients	Formulations FFP-1	FFP-2
Ketorolac tromethamine USP	+	+
Disodium EDTA IP	+	+
Benzalkonium chloride IP	+	+
Poloxamer 407 NF	+	+
Cremophor EL NF	+	-
HPβCD	-	+
Sodium chloride IP	+	+
Distilled water	+	+

+ indicates presence of the ingredient
- indicates absence of the ingredient

The 'cold process' was used for hydrating Poloxamer in water. About 500 mL of freshly distilled membrane filtered water was poured in a tared 2 lit capacity glass beaker and this was placed in an ice-bath. 200 gm of Poloxamer 407 was added in parts to the cold water (<10°C) under stirring (500-600 rpm). Stirring was continued after all the polymer was added, till a clear solution was formed.

In about 200 mL of distilled water, disodium EDTA, sodium chloride and benzalkonium chloride were dissolved and this solution was added to the polymer solution under stirring. The resulting solution was filtered and autoclaved at 121°C for 20 minutes. The gel was cooled to less than 10°C and sterile ketorolac tromethamine containing either Cremophor or HPβCD solution was added and volume was made upto 1000 gm by adding sufficient sterile distilled water under laminar flow. The gel was mixed thoroughly for 5-10 minutes under cold condition, after adjusting the pH to 7.2±0.2.

The gel was filled into sterile type-1 glass amber vials. The vials were capped with presterilized LDPE stoppers and sealed with aluminium crimps. The gel was also filled into sterile lacquered aluminium tubes, capped and sealed.

RESULTS AND DISCUSSION:

Since, the 'cold process' is preferred over 'hot process', the gel was prepared by using 'cold process'. The manufacturer also recommends cold method wherever possible. The hydration of Poloxamer-407 occurs slowly if the temperature goes above 10°C, hence the temperature was maintained below 10°C during hydration.

If the stirring speed increases above 500 rpm, chances of air entrapment also increases, therefore speed was limited to 500 rpm. No change in the clarity was observed after autoclaving the Poloxamer-407 with additives.

2.5.5 KT (0.5% w/w) OPHTHALMIC OINTMENT

Among the two prototype ointment formulations evaluated, the cholesterol containing formulation showed significant eye irritation in pilot eye-irritation studies in rabbits. Therefore only one formulation was prepared for final evaluation.

EXPERIMENTAL:

The formula and procedure involved in the preparation of 250 gm of ointment is as below.

Formulation-1 (FFO-1)

Sr. No.	Ingredient
1	Ketorolac tromethamine USP
2	Benzalkonium chloride IP
3	α-tocopherol NF
4	White soft paraffin IP

The entire procedure was carried out in a laminar flow hood. A membrane filter assembly was sterilized in an autoclave at 121°C for 20 minutes. After sterilization with steam, the assembly was dried at 105°C in a hot air oven for 1 hour. About 200gm of white soft paraffin was heated to 90°C, and was sterilized by membrane filtration. 100gm of this was separated on a sterile mixing tile. BKC was micronized by passing it through a ASTM 400# sieve. The EtO sterilized drug was weighed and transferred onto the sterile tile, and mixed with BKC using a sterile spatula. The sterile white soft paraffin was mixed with the drug powder in arithmetic proportions, and mixing was done by the technique of levigation, till all the paraffin was incorporated.

Before filling the ointment into the tubes, 6 samples of approximately 0.2 gm were sampled from different locations and assayed, after weighing accurately, to ensure content uniformity. After ensuring content uniformity, the ointment was filled into the sterile tubes with the help of a spatula and was then capped and sealed.

RESULTS AND DISCUSSION:

The reasons for selecting the base and its sterilization procedure for the preparation is given under section 2.4.5. Since ophthalmic ointments are sterile dosage forms, utmost care had to be taken during their preparation.

The mixing was done using a sterile tile and spatula as it gave excellent content uniformity with C.V less than 3.0%, which is the official acceptable range. The prepared ointment had excellent consistency, appearance and no lumps were found.

2.6 EVALUATION OF THE PREPARED FORMULATIONS:

To ensure that the product is of high quality, stable, sterile, safe and effective during its shelf life, the prepared formulations were evaluated for:

i) Physicochemical characterization such as clarity, drug content, stability, pH, viscosity and *in-vitro* release profile

ii) Microbiological evaluation included sterility and efficacy of antimicrobial preservative

iii) Biological evaluation which included eye-irritation, anti-inflammatory activity of the ophthalmic solution and determination of aqueous humor drug concentration-time profile of all the formulations in rabbits.

2.6.1 PHYSICOCHEMICAL CHARACTERIZATION:

The following physicochemical parameters were studied immediately after preparation of the formulations.

2.6.1.1 ANALYSIS OF INITIAL DRUG CONTENT IN ALL THE PREPARED FORMULATIONS:

EXPERIMENTAL :

The drug content of the formulations were determined by using the developed stability-indicating HPTLC method. Six samples were analyzed for each of the formulations. The assay of the samples were carried out as follows:

(i) Ketorolac tromethamine conventional ophthalmic solution:

100 µL of the formulation was pipetted out using a micropipette into test tubes and diclofenac sodium (0.5µg/10µl) was added as internal standard. The solution was evaporated to dryness *in vacuo* at 60°C. The residue was reconstituted in methanol prior to application on HPTLC plates. The injected amount was 10µg of KT in duplicates.

(ii) Ketorolac tromethamine ophthalmic viscous solution:
100 mg of the formulation was weighed accurately on a analytical balance in tared test tubes, and diclofenac sodium solution was added. The solution was evaporated to dryness *in vacuo* at 60°C. The residue was reconstituted in methanol prior to application on HPTLC plates.

(iii) Ketorolac tromethamine ophthalmic gel (Carbopol):
200 mg of the formulation was weighed directly in tared test tubes and diclofenac sodium was added. The solution was evaporated to dryness *in vacuo* at 60°C. The residue was reconstituted with 1 ml of methanol by vortexing for a minute prior to application on HPTLC plates.

(iv) Ketorolac tromethamine ophthalmic thermoreversible gel: The procedure of sample preparation was same as described for Carbopol based gel.

(v) Ketorolac tromethamine ophthalmic ointment:
200 mg of the ointment was directly weighed in a tared 15 mL stoppered conical tubes and diclofenac sodium solution was added. Ointment was warmed to about 45°C and the drug was extracted by adding 1 ml of methanol. The tube was vortexed for 5 minutes in warm condition. 10 µl of the supernatent layer was applied directly onto HPTLC plates.

RESULTS & DISCUSSION:
The results of the drug content, in each of the formulation, analysed by the HPTLC method before keeping for stability are shown in Table-34. The results showed that the drug content was close to the label claim with very less variation. This also suggested that uniform mixing was done during the preparation.

TABLE-34. INITIAL DRUG CONTENT OF THE PREPARED FORMULATIONS

Formulation	Mean drug content[a] (n=6)
KT solution	
i) FFS-1	99.52 ± 0.45
ii) FFS-2	100.17 ± 0.56
KT viscous solution	
i) FFV-1	100.24 ± 0.32
ii) FFV-2	100.11 ± 0.25
KT ointment	102.78 ± 0.67
KT Carbopol gel	99.69 ± 0.88
KT Poloxamer gel	
i) FFP-1	100.33 ± 0.42
ii) FFP-2	99.97 ± 0.52

[a] Drug content is expressed as Mean % ± S.D.

2.6.1.2 ANALYSIS OF INITIAL PRESERVATIVE CONTENT IN ALL THE PREPARED FORMULATIONS:

EXPERIMENTAL:

The concentration of benzalkonium chloride was determined by the standardized HPLC method. In case of the conventional solution 100 µL of the formulation was pipetted out using a micropipette, and was diluted to 1 mL with DDW, and 20µl was injected onto the column. In case of the viscous solution and gels 100 mg of the sample was weighed and dissolved in DDW, volume was made upto 1 ml and then 20 µl was injected onto the column. In case of ointment, 100 mg of the ointment was weighed in a stoppered conical flask and dissolved in 1mL of chloroform and this was extracted with 1 ml of 0.1M phosphoric acid. Further, extraction was carried out by vortexing the tubes for a period of 1 minute and then was shaken manually over a 5 minute period. 20µL of this solution was injected onto the column.

RESULTS & DISCUSSION:

The maximum amount of BKC that can be used in ophthalmic formulation has been restricted to 0.01 % w/v by Indian FDA in 1995 because of its ocular toxicity at higher concentrations.

The preservative content needs to be determined during stability studies, along with proving its efficacy against certain bacteria and fungi, by carrying out preservative efficacy test. Therefore initial preservative drug content was determined.

The results of initial content of BKC from various formulations are tabulated in Table-35. The BKC content was found to be close to the label claim. The variation within the formulation was found to be less, proving proper mixing in all the formulations.

TABLE-35 INITIAL ASSAY OF BKC FROM VARIOUS FORMULATIONS

Formulation	Mean % BKC content ± s.d (n=6)
KT solution i) FFS-1 ii) FFS-2	 99.42 ± 0.85 98.19 ± 0.96
KT viscous solution i) FFV-1 ii) FFV-2	 99.24 ± 0.99 99.12 ± 0.89
KT ointment	98.65 ± 1.67
KT Carbopol gel	99.34 ± 0.58
KT Poloxamer gel i) FFP-1 ii) FFP-2	 100.56 ± 0.32 99.17 ± 0.62

2.6.1.3 CLARITY/DISCOLOURATION OF FORMULATIONS:

EXPERIMENTAL:

Clarity of the conventional and viscous solutions were checked by visual inspection of the filled vials against black and white background, under fluorescent white light. The clarity of Carbopol and Poloxamer based gel were evaluated by spreading the gel on a clean transparent glass slide, and inspecting visually against white and black background, under fluorescent light. To detect fine particles or fibers if any, a drop of the solution/gel was placed on a coverslip which was then inverted on a cavity slide, for observation under a microscope at a magnification of 450X.

RESULTS & DISCUSSION:

Change in clarity of formulation was the first indication of incompatibility or drug undergoing degradation, and it was observed consistently during compatibility studies and also while evaluating prototype formulations. Therefore clarity assessment was carried out, by visual observation as well as by microscopy.

Since the solutions were filled in amber coloured vials the clarity was evaluated by transferring into cleaned and dried colourless vials for visual examination. The prepared formulations namely solutions, viscous solutions and gels were free from particulate matter. The results of clarity also suggested that the manufacturing area and filtration assembly were free from any particulate matter, thus ensuring compliance to cGMP requirements.

2.6.1.4 DETERMINATION OF INITIAL pH OF THE FORMULATIONS:

EXPERIMENTAL:

The pH of the prepared formulations was measured using a calibrated pH meter immediately after the preparation of formulation and during the stability studies after 3 months and 6 months. Two vials in case of conventional solution, viscous solution & Poloxamer gel and four tubes in case of Carbopol and Poloxamer based gel were randomly sampled and used to measure pH each time.

RESULTS & DISCUSSION:

The results of determination of pH of various formulations of KT are given Table-36. The results suggested that the pH of all the formulations were close to the expected value of 7.2 ± 0.2.

TABLE-36 INITIAL pH OF THE PREPARED FORMULATIONS:

Formulation	pH
KT solution	
i) FFS-1	7.34
ii) FFS-2	7.46
KT viscous solution	
i) FFV-1	7.45
ii) FFV-2	7.38
KT Carbopol gel	7.30
KT Poloxamer gel	
i) FFP-1	7.35
ii) FFP-2	7.42

2.6.1.5 DETERMINATION OF INITIAL VISCOSITY OF THE FORMULATIONS:

EXPERIMENTAL:

The viscosity of the prepared formulations was measured using a Brookefield viscometer at 25 ± 0.3°C, immediately after the preparation and at the end of stability studies.

The viscosity of KT viscous solution was measured using a spindle S-18 at various speeds using 8 ml of the formulation. At each speed the spindle was allowed to rotate for 15 minutes to examine any changes in viscosity. The viscosity of ointment and Carbopol & Poloxamer based gels were measured with the help of a helipath stand using T-F bar.

RESULTS & DISCUSSION:

The results of viscosity measurement of various formulations are given in Table-37to39. The viscosity of Carbopol and Poloxamer based gels had to be measured by using helipath stand with T-bars, because the viscosity value was not within the range of other spindles.

All the gel formulations showed non-Newtonian behaviour. When speed of the spindle was taken from lower to higher values, the

viscosity decreases and when brought back to lower speeds, the readings fell upon themselves. This was in agreement with the reported rheological behaviour of Carbopol 940[125] and Poloxamer-407[133] gels. In case of ointment viscosity was measured only at a single point i.e. 12 rpm. The initial viscosity was found to be 40,000 cps.

TABLE-37 INITIAL VISCOSITY OF KT VISCOUS SOLUTIONS

Spindle speed (rpm)	Measured viscosity (cps) FFV-1	FFV-2
12.0	32.2	32.6
30.0	26.6	26.9
60.0	25.0	25.2
100.0	24.4	24.5

TABLE-38 INITIAL VISCOSITY OF CARBOPOL BASED KT GEL

Spindle speed (rpm)	Measured viscosity (centipoise)
0.6	959300
1.5	430000
3.0	240000
6.0	132500
12.0	75700

TABLE-39 INITIAL VISCOSITY OF POLOXAMER BASED KT GEL

Spindle speed (rpm)	Measured viscosity (cps) FORMULATION FFP-1	FFP-2
0.3	14,10500	14,04321
0.6	12,24000	12,10342

2.6.1.6 IN-VITRO *DRUG RELEASE STUDIES:*

The *in-vitro* drug release was studied for the viscous solutions, ointment and gels using a USP type 5 dissolution test apparatus, as was reported by Rozier *et al* with slight modification[144,289]. The *in-vitro* release studies were done immediately after the preparation and at end of the stability studies.

EXPERIMENTAL:

One gm of the formulation was placed in a 50 mm diameter glass petriplate. The viscous solution and Poloxamer based gel were placed evenly, whereas ointment and Carbopol gel had to be kept in the form of cylindrical segments. The petri plates were covered with a 30 # nylon mesh. Phosphate buffered saline IP (400mL), maintained at 37°C was used as the dissolution medium. The paddle speed was 30 rpm. After placing the petriplate in the dissolution medium, 5mL aliquots were withdrawn at intervals of 2.5, 5, 10, 15, 20, 30, 45, 60, 75 and 90 minutes. After each aliquot was withdrawn, it was replaced with an equal volume of fresh dissolution medium maintained at 37°C. The concentrations of ketorolac tromethamine in the aliquots were then estimated by measuring their absorbance at 319 nm using a spectrophotometer. Each experiment was performed in triplicate.

In-vitro release studies were also carried out for gels prepared with different polymer concentrations, viz. 20, 22 and 25% w/w in case of Poloxamer and 1.0, 1.5 and 2.0 w/w for Carbopol based KT gel.

RESULTS AND DISCUSSION:

There is no official guideline to evaluate the *in-vitro* release profiles of ophthalmic gels and ointments. However, the literature survey on such studies suggested that method reported

by Rozier et al[144]. is most suitable for ophthalmic semisolids. This method has been used to study the *in-vitro* release of timolol maleate from ophthalmic gel made of gellan gum.

The drug release was rapid in case of conventional solution. The viscous solutions released the drug within 2.5 minutes. So no plot could be made for both of these formulations. There was no drug release from ointment even after keeping for 6 hours in the dissolution system. This could be due to insufficient shearing in the dissolution system. The hydrophobicity of the ointment base could have acted as a barrier for the entry of the dissolution medium into the ointment.

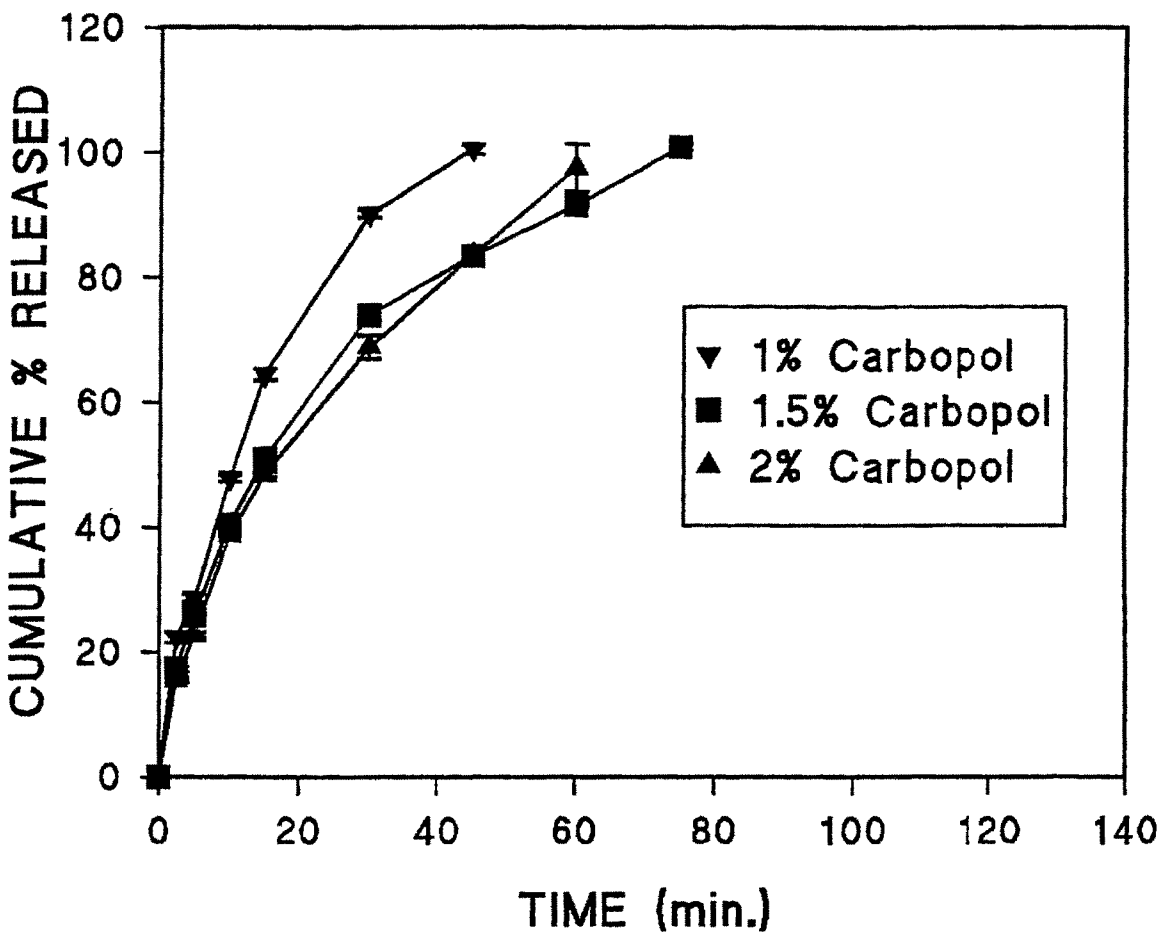
The plot of cumulative percent drug release versus time from Carbopol based gel is shown in Fig. 9. The drug release data different concentration of Carbopol (1.0, 1.5 and 2.0 % w/w) was well fitted into Higuchi kinetics as there was a linearity in the Q vs $\sqrt{t}$ plot, which indicated that the drug release was by diffusion. Similar release kinetics were reported for other nonsteroidal anti-inflammatory drugs[290]. The correlation coefficient values obtained from Q vs $\sqrt{t}$ plot are given in Table-40.

To determine the nature of diffusion pattern of the drug from the gel, release data was fitted into following Krosmayer and Peppas equation.

$$\frac{M_t}{M_\infty} = Kt^n$$

M_t/M_∞ = Fraction amount of drug released at time t
K= Constant
t= Time
n= Diffusion coefficient

FIG. 9 In-vitro release of KT from Carbopol gels: Effect of polymer concentration on release profile



Each Point represents Mean ± S.E.M (n=3)

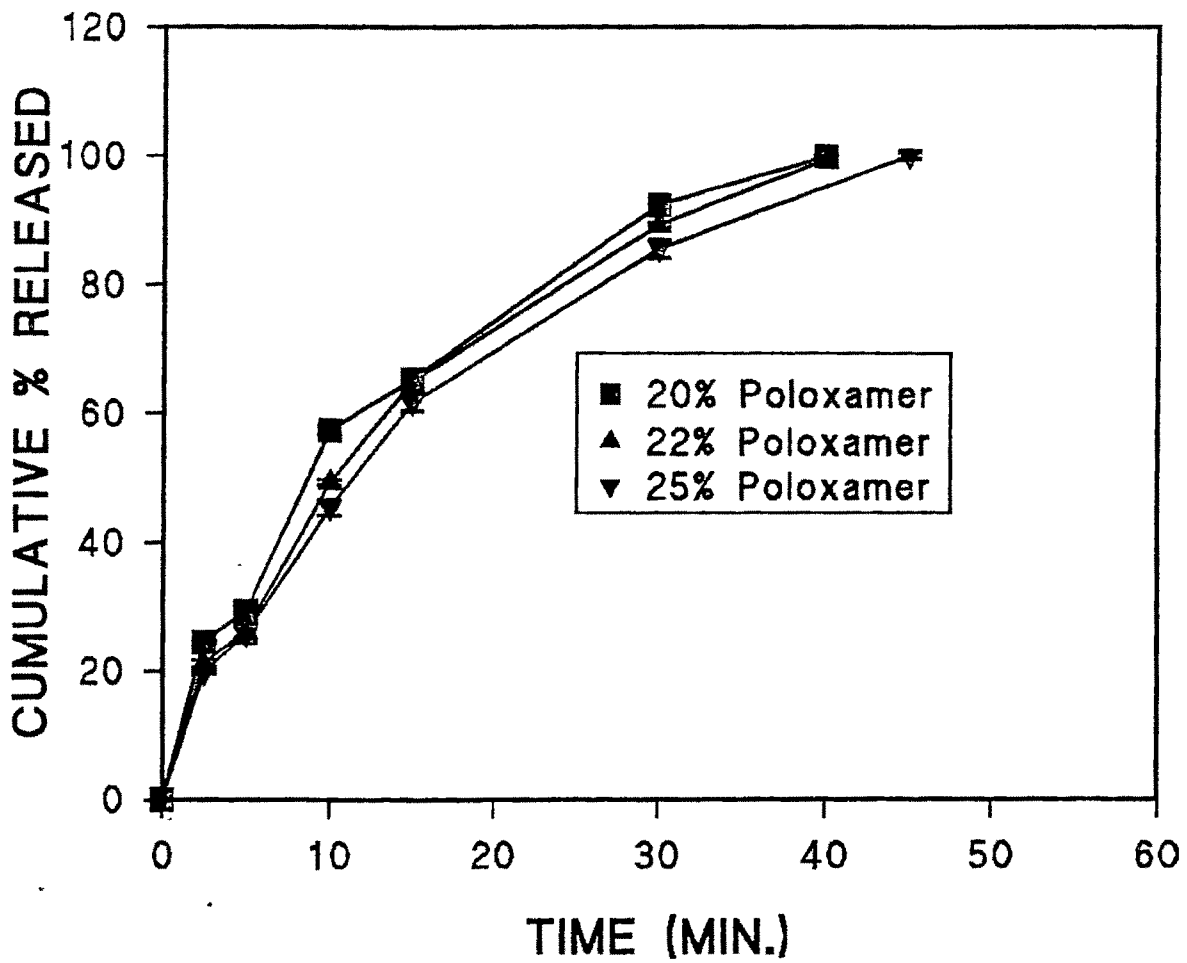
The 'n' values obtained from different concentration of Carbopol based KT gel were very close to 0.5, which means the release of drug follows non-Fickian diffusion pattern. The values of 'n' and t_{90} (time taken to release 90% of the drug from the gel) are given in Table-40.

The plot of cumulative percent drug released versus time from **Poloxamer based gel** is shown in Fig. 10. The Poloxamer based gel, at 20, 22, and 25 % w/w concentration, also showed diffusion controlled mechanism as there was good correlation with Q --> $\sqrt{t}$ plot. To understand the type of diffusion mechanism the release data was fitted into Krosmeyer-Peppas equation and it was found that 'n' values were close to 0.5. Hence the release of KT from Poloxamer based gel follows non-Fickian diffusion pattern. The values of correlation coefficient (r) and t_{90} and 'n' are shown in Table-40.

TABLE-40 THE KINETIC ASSESSMENT OF *IN-VITRO* RELEASE DATA OF CARBOPOL AND POLOXAMER BASED KT GELS

Polymer concentration	'n'	Correlation coefficient	t_{90} (minutes)
i) Poloxamer 407			
20% w/w	0.5750	0.9978	30.75
22% w/w	0.5610	0.9986	31.05
25% w/w	0.6018	0.9955	32.83
ii) Carbopol 940			
1.0% w/w	0.5890	0.9918	32.97
1.5% w/w	0.5223	0.9989	54.06
2.0% w/w	0.5575	0.9934	56.01

FIG.10 In-vitro release of KT from Poloxamer gels: Effect of polymer concentration on release profile



Each point represents Mean ± S.E.M (n=3)

2.6.2. ACCELERATED STABILITY STUDIES:

EXPERIMENTAL:

The prepared formulations were subjected to accelerated stability studies at 5°C, 25°C, 37°C and 45°C for 6 months. Ointment was subjected to humidity stress conditions at 37°C/75% RH also. Light stability studies were also carried out at ambient temperature by keeping the formulations on laboratory window where direct sunlight would be incident on them. All the stability studies were carried out in the final pack for a period of 6 months.

The stability samples were evaluated for physicochemical characteristics such as clarity, pH and drug content at 1, 2, 3, 5 and 6 months. Preservative content was measured at the end of stability period. Viscosity and *in-vitro* release rates were also determined, for viscous solution, gels and ointment, at the end of the stability period.

The drug content was measured by the stability-indicating HPTLC method. For the purpose of assaying the drug, a 3-point standard curve corresponding to 90, 100 and 110 % of original content was applied in duplicate on each plate along with stability samples. Assay of the preservative was carried out at the beginning and end of the accelerated stability studies using the HPLC method, by making a 3-point standard plot corresponding to 80, 100 and 110 % of original content.

RESULTS AND DISCUSSION:

The new guidelines for accelerated stability studies as per ICH, are to store the product at 15°C above the mean kinetic room temperature for a minimum of 6 months[291,292]. The mean

temperature for India would be 31-32°C. Therefore samples were kept at 45°C for six months. The other temperatures studied would help in calculating shelf life of the product if, there is any significant change in drug content or the degradation products.

There is no official guideline available for conducting the light stability studies. However, from the literature survey, it was revealed that, light stability studies by exposing to direct sunlight in the final package keeping it in laboratory window could be an appropriate method.
The various physicochemical parameters evaluated during stability studies are described below:

2.6.2.1 Discolouration:

EXPERIMENTAL:

Discolouration of the prepared formulations, on being subjected to accelerated conditions of temperature and light, was assessed at the end of 1, 3 and 6 months. In case of conventional, viscous and Poloxamer gel, the test was done by pouring out the contents of two vials into a Nessler's cylinder, and comparing it visually with a freshly prepared respective formulation.

In case of Carbopol gel, it was filled into colourless tubes, carefully taking precaution to avoid air entrapment using a syringe. It was compared with freshly prepared gel.

RESULTS & DISCUSSION:

Since the formulations were filled in amber coloured vials, it was difficult to perceive any difference in colour. Hence, the contents of the vials were emptied into Nessler's cylinders and were observed against a white background.

None of the formulations showed discolouration, except Poloxamer based gel containing Cremophor, which was filled in ointment tubes. The discolouration was seen at the tip of the ointment tubes after 4 months of storage at 45°C.

2.6.2.2 Drug Content During Stability Studies:

The measured drug content of various formulation kept for stability studies are shown in Table 41-50.

(i) KT Conventional Solution (FFS-1):

The content of the drug was found to remain constant throughout the stability studies period (Table-41). The percentage of 1-keto analogue was 1.17 % at 45°C after 6 months. KT ophthalmic formulation is not official in any pharmacopoeia. However parenteral injection of KT is official in USP-23, but no limits are given for 1-keto analogue in the product. As per new stability guidelines of ICH, significant change in drug content means more than 5 % of the label claim. Since there was no significant change in the drug content during the stability study, this product could be given a shelf life of 18 months.

TABLE-41. AMOUNT OF KT REMAINING IN CONVENTIONAL SOLUTION (FFS-1) DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] Sampling time in months				
	1	2	3	5	6
5°C	99.78 ±0.23	100.19 ±0.91	100.67 ±0.45	98.89 ±0.78	98.88 ±0.45
25°C	99.89 ±1.03	99.77 ±0.43	100.12 ±0.65	98.86 ±0.78	98.18 ±0.57
37°C	99.58 ±0.52	100.19 ±1.16	99.44 ±0.67	98.79 ±0.98	98.40 ±0.21
45°C	99.77 ±1.02	99.33 ±0.85	99.37 ±0.49	98.01 ±0.99	97.87 ±0.44
light	99.76 ±0.69	--	--	--	98.12 ±1.09

[a] The values are mean ± s.d

(ii) KT Conventional Solution (FFS-2):

The content of ketorolac tromethamine was found to remain constant throughout the period of stability studies (Table-42). The percentage of 1-keto analogue was less than 0.5 % at 45°C after 6 months. The product was extremely stable physically as well as chemically, so a shelf life of 18 months could be assigned.

TABLE-42. AMOUNT OF KT REMAINING IN CONVENTIONAL SOLUTION (FFS-2) DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] Sampling time in months				
	1	2	3	5	6
5°C	99.89 ±0.43	99.93 ±0.66	100.12 ±0.78	99.49 ±0.81	99.88 ±0.56
25°C	99.65 ±0.73	100.06 ±1.23	99.82 ±0.45	99.96 ±0.78	98.85 ±0.78
37°C	100.09 ±0.34	100.04 ±0.96	99.57 ±1.07	99.88 ±1.18	99.20 ±0.61
45°C	99.81 ±0.92	99.65 ±0.67	99.51 ±0.45	98.89 ±0.78	98.67 ±0.55
light	99.67 ±1.04	--	--	--	98.89 ±0.91

[a] The values are mean ± s.d

(iii) KT Viscous Solution (FFV-1):

The results of drug content measured at various time points are given in Table-43. The content of ketorolac tromethamine was found to remain constant throughout the stability studies. The percentage of 1-keto analogue was 1.03 % at 45°C after 6 months. The 1-keto analogue found in viscous solution was less compared to the conventional solution. Since there was no significant change in ketorolac tromethamine content during the stability study, this product could be given a shelf life of 18 months.

TABLE-43. AMOUNT OF KT REMAINING IN VISCOUS SOLUTION (FFV-1) DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] Sampling time in months				
	1	2	3	5	6
5°C	99.34 ±0.53	100.23 ±0.94	99.57 ±1.03	99.09 ±0.66	98.33 ±0.45
25°C	99.55 ±1.13	99.87 ±0.38	99.12 ±0.32	98.89 ±0.91	99.18 ±0.77
37°C	100.18 ±0.26	100.09 ±1.22	99.88 ±0.88	98.92 ±0.78	98.60 ±1.01
45°C	99.65 ±0.72	99.32 ±0.85	99.07 ±0.76	98.77 ±0.34	98.27 ±0.23
light	99.88 ±0.43	--	--	--	98.76 ±0.89

[a] The values are mean ± s.d

(iv) KT Viscous Solution (FFV-2):

The results of the drug content measured at various time points during stability studies are given in Table-44. The content of ketorolac tromethamine was found to remain constant throughout the period of stability studies. The little variation found might be, in part, because of variation in the analysis. The percentage of 1-keto analogue was 0.46 % at 45°C after 6 months, which shows excellent stability of KT in presence of HPβCD.

The product was extremely stable physically as well as chemically, so a shelf life of 18 months could be assigned for this formulation.

TABLE-44. AMOUNT OF KT REMAINING IN VISCOUS SOLUTION (FFV-2) DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] Sampling time in months				
	1	2	3	5	6
5°C	99.69 ±1.03	99.88 ±0.54	100.03 ±0.78	98.99 ±0.56	99.33 ±0.33
25°C	100.35 ±1.03	99.46 ±0.23	99.82 ±0.65	99.66 ±0.89	99.05 ±0.23
37°C	100.19 ±0.65	99.89 ±0.34	99.43 ±1.12	98.98 ±0.18	99.80 ±0.31
45°C	99.78 ±0.67	99.15 ±0.88	100.21 ±0.33	99.89 ±0.78	98.67 ±0.56
light	100.23 ±0.98	--	--	--	99.09 ±0.76

[a] The values are mean ± s.d

(v) Long Acting Carbopol Gel of KT:

The results of the drug content measured at various time points during stability studies are given in Table-45.

The KT content in the gel during the stability remained fairly constant. The 1-keto analogue of the drug did not increase from the initial value, which suggests that the drug is more stable in the gel form than in the aqueous solution. The light stability results suggested that the packaging material protected the product from light.

Since, the Carbopol based KT gel exhibited excellent physical and chemical stability, and no significant change in the content was observed during the stability studies, the gel could be given a shelf life of 18 months.

TABLE-45. AMOUNT OF KT REMAINING IN CARBOPOL BASED GEL DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] — Sampling time in months				
	1	2	3	5	6
5°C	100.34 ±0.83	100.56 ±1.04	99.33 ±0.93	99.89 ±0.67	99.13 ±0.85
25°C	100.15 ±0.89	99.33 ±0.76	99.85 ±0.45	99.39 ±0.76	99.21 ±0.43
37°C	99.18 ±0.76	100.09 ±0.27	99.33 ±0.32	98.96 ±0.43	98.78 ±1.12
45°C	99.88 ±0.44	99.34 ±0.87	99.40 ±0.56	98.89 ±0.14	98.17 ±0.83
light	99.32 ±0.56	--	--	--	98.76 ±0.96

[a] The values are mean ± s.d

(vi) Poloxamer Based Gel:

Ketorolac tromethamine content in the gel during the stability studies, remained fairly constant in vial as well as in tubes (Table-46 to 49). The gel containing HPβCD did not show any degradation, whereas Cremophor containing gel showed 1% of 1-keto analogue at 45°C after 6 months.

The results of the light stability studies suggested that the packaging material protected the product from light. The gel containing HPβCD maintained its integrity throughout the stability studies. The gel stored in vials had an excellent appearance and clarity after completion of the stability studies at all temperatures, but the gel stored in tubes showed discolouration at the tip of the tube after 3 months of storage at 45°C. Hence the gel stored in tubes were not taken for further studies.

The percentage decrease in viscosity after 6 months was found to be less than 5.0% in both the formulations. Since, the gel stored in vial showed excellent physical and chemical stability and no significant change in the ketorolac tromethamine content was observed during the stability studies, the gel could be given a shelf life of 18 months.

TABLE-46. AMOUNT OF KT REMAINING IN POLOXAMER BASED GEL (FFP-1) STORED IN VIALS DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] — Sampling time in months				
	1	2	3	5	6
5°C	99.87 ±0.54	100.32 ±0.46	99.88 ±0.63	99.91 ±0.46	98.34 ±0.65
25°C	99.59 ±0.76	99.67 ±0.43	99.88 ±0.64	99.59 ±0.80	99.28 ±0.33
37°C	100.28 ±0.64	99.49 ±1.02	99.32 ±0.48	98.12 ±0.76	98.09 ±1.11
45°C	99.87 ±0.47	99.72 ±0.97	99.37 ±1.06	98.38 ±0.98	97.61 ±1.03
light	99.32 ±0.54	--	--	--	98.03 ±0.79

[a] The values are mean ± s.d

TABLE-47. AMOUNT OF KT REMAINING IN POLOXAMER BASED GEL (FFP-2) STORED IN VIALS DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] — Sampling time in months				
	1	2	3	5	6
5°C	99.44 ±1.53	100.23 ±1.14	99.87 ±0.77	100.19 ±0.46	99.33 ±0.45
25°C	99.85 ±1.12	99.65 ±1.48	100.12 ±0.87	99.39 ±0.93	99.78 ±1.12
37°C	100.23 ±0.56	100.19 ±0.52	99.78 ±0.43	99.52 ±0.78	98.89 ±0.34
45°C	99.73 ±0.92	99.23 ±0.89	99.19 ±0.65	98.87 ±0.77	98.74 ±0.55
light	99.78 ±0.76	--	--	--	98.06 ±0.69

[a] The values are mean ± s.d

TABLE-48. AMOUNT OF KT REMAINING IN POLOXAMER BASED GEL (FFP-1) STORED IN TUBES DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] — Sampling time in months				
	1	2	3	5	6
5°C	99.56 ±0.88	99.23 ±1.06	99.84 ±1.63	99.71 ±0.55	99.12 ±0.45
25°C	99.77 ±0.88	99.69 ±1.23	99.39 ±1.43	98.68 ±0.59	98.21 ±0.93
37°C	100.18 ±1.12	99.69 ±0.89	98.13 ±0.67	98.09 ±0.97	97.09 ±1.21
45°C	99.57 ±0.57	99.12 ±1.07	98.37 ±0.77	97.41 ±0.98	96.45 ±0.63
light	98.98 ±0.84	--	--	--	97.13 ±0.55

TABLE-49. AMOUNT OF KT REMAINING IN POLOXAMER BASED GEL (FFP-2) STORED IN TUBES DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] — Sampling time in months				
	1	2	3	5	6
5°C	99.65 ±1.76	100.33 ±1.34	100.11 ±0.98	99.59 ±1.02	99.78 ±0.67
25°C	100.23 ±1.72	99.78 ±0.41	100.61 ±0.98	99.29 ±0.67	99.66 ±0.65
37°C	99.84 ±0.63	99.67 ±0.43	99.56 ±0.34	98.88 ±0.23	98.43 ±0.77
45°C	99.91 ±0.67	99.34 ±0.45	99.09 ±0.78	98.47 ±0.43	98.18 ±0.89
light	101.02 ±0.96	--	--	--	99.32 ±1.09

[a] The values are mean ± s.d

(vii) Ketorolac Tromethamine Ophthalmic Ointment:

The results of the drug content measured at various time points from the ointment during stability studies are given in Table-50. The prepared ointment showed excellent physical as well chemical stability through out the stability studies. The consistency of the ointment remained constant, and no lumps were found, at any temperatures till the end of the stability studies.

1-keto analogue, the major degradation product of ketorolac tromethamine, too did not increase. However, the samples stored in humidity cabinets showed slight increase in the 1-keto analogue which was less than 0.61 % at 45°C after 6 months of storage, which could be attributed to moisture pick-up by the ointment and making a way for the drug to undergo degradation.

The good stability observed in KT ointment as compared to gels and solutions, (with out HPβCd) could be because, the drug is dispersed in solid form, and ketorolac tromethamine is known to be more stable in solid form than in solution.

No significant change observed was in the drug content at any of the accelerated conditions during and at the end of accelerated stability studies. Therefore a shelf life of 18 months can be proposed for this preparation.

TABLE-50. AMOUNT OF KT REMAINING IN OINTMENT STORED IN TUBES DURING STABILITY STUDIES: (n=2)

Condition	Mean % of initial KT remaining[a] Sampling time in months				
	1	2	3	5	6
5°C	102.65 ±1.99	101.23 ±1.67	99.55 ±1.99	99.78 ±2.01	99.69 ±2.44
25°C	99.88 ±1.45	100.91 ±1.89	99.24 ±2.18	99.76 ±2.22	99.87 ±1.75
37°C	101.33 ±1.87	99.61 ±1.42	98.73 ±2.96	99.59 ±2.28	99.89 ±1.88
45°C	99.39 ±1.45	100.76 ±1.79	99.96 ±1.33	99.12 ±1.90	98.62 ±2.25
37°C/ 75% RH	99.72 ±2.14	100.22 ±1.64	99.21 ±2.98	98.82 ±1.68	98.27 ±0.93
light	101.22 ±1.78	--	--	--	98.92 ±1.22

[a] The values are mean ± s.d

2.6.2.3 Preservative Content:

In addition to antimicrobial preservative effectiveness test, it is necessary to measure the changes in preservative content in the sterile multiple-unit dosage forms, before and after the stability studies.

The content of benzalkonium chloride in the prepared formulations, measured after 6 months of storage at 5°C and 45°C is given in Table-51.

TABLE-51. ASSAY OF BENZALKONIUM CHLORIDE FROM VARIOUS KT FORMULATIONS AT THE END OF 6 MONTHS OF ACCELERATED STABILITY STUDIES: (n=2)

Formulation	Mean % of BKC remaining ± s.d Storage condition 5°C	45°C
KT solution		
i) FFS-1	97.23 ± 3.76	95.08 ± 2.19
ii) FFS-2	98.18 ± 2.09	96.56 ± 1.67
KT viscous solution		
i) FFV-1	98.89 ± 1.28	96.34 ± 1.92
ii) FFV-2	97.19 ± 1.92	96.66 ± 1.55
KT ointment	95.12 ± 4.17	93.55 ± 5.87
KT Carbopol gel	98.18 ± 1.22	96.38 ± 1.74
KT Poloxamer gel		
i) FFP-1 (Vial)	97.34 ± 2.86	95.43 ± 1.97
ii) FFP-2 (vial)	98.56 ± 1.56	96.53 ± 2.45
iii) FFP-1 (Tube)	95.65 ± 4.32	95.23 ± 3.43
iv) FFP-2 (Tube)	97.78 ± 3.02	97.23 ± 2.23

The content of benzalkonium chloride was found to be only slightly reduced in the solutions and gels. However, in case of the ointment and Poloxamer gel (FFP-1) stored in tube, a slightly more reduction in assay was observed at both 5°C as well as 45°C. In case of the ointment a moderate reduction would not be considered as significant, as chances of contamination are lesser as compared to the solutions. Since there is no guideline available at present for the limits of preservative concentration

during the stability studies, the antimicrobial effectiveness along with the concentration should be considered.

By looking at the results of preservative content as well as the antimicrobial effectiveness of the preservative in the prepared formulations, it could be concluded that the benzalkonium chloride added in the formulation maintained its integrity throughout the stability studies.

2.6.2.4 pH of the Formulation During Stability Studies:

The results of pH, measured at 1, 3 and 6 months during the stability studies of ketorolac tromethamine ophthalmic solutions, viscous solutions and gels, stored at 45°C are given in Table-52. The pH of all the formulations remained fairly constant during the stability studies.

Table 52. **pH of the KT preparations at different times during accelerated stability studies:**

Formulation	pH		
	1 Month	3 Months	6 Months
KT solution			
i) FFS-1	7.33	7.30	7.42
ii) FFS-2	7.44	7.38	7.41
KT viscous solution			
i) FFV-1	7.27	7.46	7.23
ii) FFV-2	7.37	7.42	7.34
KT Carbopol gel	7.34	7.45	7.52
KT Poloxamer gel			
i) FFP-1 (Vials)	7.35	7.51	7.37
ii) FFP-2 (Vials)	7.37	7.43	7.35
iii) FFP-1 (Tubes)	7.44	7.63	7.60
iv) FFP-2 (Tubes)	7.38	7.43	7.47

2.6.2.5 Viscosity Measurements:

The viscosity of the prepared formulations were measured before and after conducting the stability studies. The viscosity would give an added proof, to changes, in *in-vitro* release of the drug from the gels. Eventhough the polymers used in the formulations are known to be stable for long storage periods, but in presence

of drug and additives the behaviour might change. It has been reported that Carbopol 940 looses its viscosity slightly in presence of room light after 6 weeks of storage at room temperatures[284]. Because of these reasons the viscosity measurements were carried out on stable formulations at the end of the stability studies. The results of viscosity measurements of various formulations are shown below.

TABLE-53. COMPARISON OF VISCOSITY OF VISCOUS SOLUTIONS OF KT BEFORE AND AFTER THE COMPLETION OF 6 MONTHS OF ACCELERATED STABILITY STUDIES:

Spindle speed		Viscosity in cps Storage condition			
(rpm)	Initial	5°C	25°C	37°C	45°C
i) FFV-1					
12.0	32.2	31.0	28.6	29.5	27.5
30.0	26.6	26.2	27.0	24.9	24.1
60.0	25.0	25.2	25.0	23.8	23.2
100.0	24.4	24.1	23.9	23.1	22.6
ii) FFV-2					
12.0	32.6	31.7	29.3	29.9	28.4
30.0	26.9	26.6	26.9	27.3	26.1
60.0	25.2	25.4	24.9	24.2	24.0
100.0	24.5	24.1	23.4	24.8	23.6

TABLE-54. COMPARISON OF VISCOSITY OF CARBOPOL BASED KT GEL BEFORE AND AFTER THE COMPLETION OF 6 MONTHS OF ACCELERATED STABILITY STUDIES:

Spindle speed		Viscosity in cps Storage condition			
(rpm)	Initial	5°C	25°C	37°C	45°C
0.6	959300	959216	956780	948520	944550
1.5	430000	432500	428550	422500	417900
3.0	240000	239820	237650	235400	233250
6.0	132500	133690	131750	129900	127900
12.0	75700	75778	74790	73980	73750

TABLE-55. COMPARISON OF VISCOSITY OF POLOXAMER BASED KT GEL BEFORE AND AFTER THE COMPLETION OF 6 MONTHS OF ACCELERATED STABILITY STUDIES:

Spindle speed	Viscosity in cps Storage condition				
(rpm)	Initial	5°C	25°C	37°C	45°C
i) FFP-1					
0.3	14,10500	14,61890	13,85609	14,04120	13,67810
0.6	12,24000	12,54890	10,73450	11,96000	11,01000
ii) FFP-2					
0.3	14,10500	14,29120	14,05600	13,78200	13,56770
0.6	12,24000	11,89000	12,55890	11,15400	10,91900

The viscosities of viscous solutions, gels and ointment, measured after 6 months of storage at different temperatures showed similar values as that of the initial values. This suggested that the rheological behaviour of the formulation was not affected on long storage, and also additives and the drug did not affect the polymer property. Therefore, the *in-vitro* release of drug, from stability samples should give comparable result as that of the initial release.

2.6.2.6 In-vitro *Release Studies:*

In vitro drug release studies were carried out for the stable Carbopol based and Poloxamer based gel at the end of stability studies.

The results of *in-vitro* drug release from gels measured after 6 months of storage at 5°C, 25°C, 37°C, 45°C and under accelerated conditions of light are given in Table-56 to 57. The results suggested that, there appeared to be no significant change in the drug release profile, before and after storage at accelerated conditions of temperature as well as light for a period of 6 months.

TABLE-56. MEAN PERCENT OF KT RELEASED VERSUS TIME FROM CARBOPOL BASED GEL BEFORE AND AFTER 6 MONTHS OF ACCELERATED STABILITY STUDIES:

Time in min.	Mean % released ± S.E.M.					
	Storage condition					
	Initial	5°C	25°C	37°C	45°C	Light
2.5	17.29 ±0.41	17.09 ±1.01	17.82 ±0.87	18.12 ±0.91	18.45 ±0.88	17.22 ±1.22
5.0	25.92 ±0.26	22.55 ±0.45	26.10 ±1.05	26.98 ±1.09	27.56 ±0.89	24.66 ±0.67
10.0	40.49 ±0.67	39.89 ±0.91	40.20 ±1.49	41.44 ±1.04	41.93 ±1.19	40.51 ±0.88
15.0	51.27 ±0.61	50.04 ±1.22	49.13 ±0.98	52.03 ±1.23	53.67 ±0.36	50.98 ±0.67
30.0	73.95 ±0.55	72.00 ±0.89	71.54 ±1.09	74.12 ±1.13	74.88 ±0.99	74.00 ±1.98
45.0	83.47 ±1.44	83.02 ±3.62	82.63 ±0.98	83.20 ±1.12	84.20 ±1.36	83.06 ±1.97
60.0	91.03 ±0.88	91.68 ±1.19	92.38 ±1.15	93.07 ±0.88	93.59 ±1.34	92.12 ±0.51
75.0	99.92 ±0.57	100.10 ±1.22	99.89 ±0.89	100.79 ±1.51	100.41 ±0.77	99.77 ±1.01

TABLE-57. MEAN PERCENT OF KT RELEASED VERSUS TIME FROM POLOXAMER BASED GEL (FFP-2) STORED IN VIALS BEFORE AND AFTER 6 MONTHS OF ACCELERATED STABILITY STUDIES:

Time in min.	Mean % released ± S.E.M					
	Storage condition					
	Initial	5°C	25°C	37°C	45°C	Light
2.5	24.08 ±1.22	23.87 ±0.65	24.22 ±0.99	22.89 ±0.77	25.66 ±0.34	23.98 ±1.12
5.0	29.92 ±0.65	28.85 ±0.98	29.11 ±1.22	29.81 ±1.15	30.56 ±0.77	29.66 ±0.98
10.0	56.18 ±0.49	56.44 ±0.92	57.02 ±1.33	57.53 ±1.22	58.40 ±1.09	56.88 ±0.66
15.0	64.04 ±0.56	65.14 ±1.05	64.89 ±0.98	66.01 ±0.87	66.35 ±1.09	64.78 ±1.22
30.0	91.84 ±0.78	90.56 ±1.11	91.76 ±1.09	92.12 ±0.67	93.08 ±0.78	92.22 ±1.27
40.0	99.68 ±1.87	100.22 ±1.62	99.81 ±0.67	101.12 ±0.62	100.06 ±0.36	99.87 ±1.17

The profile of the *in-vitro* drug release from Carbopol and Poloxamer based ketorolac tromethamine gel, showed that, the properties of the gel remained same during the stability studies at different temperatures. At all the time points, the release data from the stability sample was comparable with that of the initial profile. These findings were in good agreement with that of viscosity, as there was no considerable change in viscosity during the stability study.

The release data suggested that, the prepared gels will retain their properties at different temperatures and light conditions during shelf life.

2.6.3 MICROBIOLOGICAL EVALUATION OF THE FORMULATIONS:

2.6.3.1 TEST FOR STERILITY:

Ophthalmic formulations are sterile preparations. Therefore it is regulatory requirement that all ophthalmic formulations should be sterile immediately after their preparations and during their storage till the end of their shelf-life in unopened container. Sterility of the prepared formulations were evaluated as per USP guidelines by using filtration method[293]. The USP specifies filtration method whenever a formulation has an antibacterial agent. The test is based on the principle that, if microbes are present in the formulation, they would multiply and show turbidity when product is inoculated into nutrient media. The different media used are fluid thioglycollate and soyabean casein digest, the former supports anaerobic as well as aerobic bacteria, whereas the latter supports the growth of yeast and fungi.

EXPERIMENTAL:

The brief procedure for test for sterility of various prepared formulations is described below.

(i) **Ketorolac tromethamine ophthalmic conventional and viscous solutions:**
Two vials were taken for each of the formulations, and the contents were emptied onto the sterile filter, and were filtered with the aid of suction. The filter was washed thrice with a 10 mL portions of 0.1% w/v sterile solution of peptone (Fluid A) in water.

(ii) Ketorolac tromethamine ophthalmic gels: In case of the Carbopol and Poloxamer based gel, 1.0 gm of the gel was transferred directly into 50 mL of sterile saline, and then aseptically filtered through a sterile membrane filter. The filter was then washed thrice with 10mL portions of Fluid A.

iii) **Ketorolac tromethamine ophthalmic ointment:** About 1 gm of the ointment from each of the two tubes were combined and dissolved in 100 mL of sterile isopropyl myristate (IPM). The IPM had been rendered sterile by means of membrane filtration. This oily mixture was aseptically transferred to membrane filter funnels and filtered with the aid of suction. Following filtration of the sample, the membrane was washed thrice with 100 mL portions of sterile Fluid K and then with 100 mL of Fluid A.

All the membranes were cut into two halves aseptically using a sterile forceps and a pair of scissors. One half of it was aseptically transferred to 100 mL of sterile fluid thioglycollate medium and the other half to 100 mL of sterile soyabean casein digest medium. During the experiment negative and positive controls were also used. For the negative controls, a mock

inoculation was performed and in the case of positive controls, live microbes (*S. aureus* and *A. niger*) were inoculated into the medium. All the flasks containing soyabean casein digest medium were incubated at 25°C and fluid thioglycollate medium at 32°C for 14 days. At the end of 7 and 14 days the flasks were observed for presence of turbidity.

RESULTS & DISCUSSION:

The sterility test was carried out using filtration method, as this method is prescribed for ketorolac tromethamine parenteral injections in USP-23. Moreover, the prepared formulations contained a preservative. All the ophthalmic formulations of ketorolac tromethamine evaluated for sterility, were found be sterile.

2.6.3.2 ANTIMICROBIAL PRESERVATIVE EFFECTIVENESS TEST:

Antimicrobial preservatives are usually added in multiple dose container to inhibit the growth of microorganisms that may be introduced inadvertently during use. It is regulatory requirement that any formulation where antimicrobial preservative is used, it should be evaluated for the effectiveness against possible microorganisms which are known to contaminate the product during its use. The tests and standards apply only to the product in original unopened container in which it was dispensed[294].

EXPERIMENTAL:

The test involves challenging the formulation with atleast 5 test microorganisms, namely, *Aspergillus niger (ATCC 16404), Candida albicans (ATCC 10231), Escherichia coli (ATCC 8739), Pseudomonas aeruginosa (ATCC 9027) and Staphylococcus aureus (ATCC 6538P).* The microorganisms were grown on suitable medium and the cells or

spores were harvested with sterile saline and adjusted to a concentration of ~10^8/mL. This suspension was inoculated into the product container such that the formulation contained between 10^5-10^6 microorganisms/mL. The formulations were incubated at 25°C for fungi and 32°C for bacteria, and periodically observed at 7, 14, 21 and 28 days for any changes in appearance was observed. Additionally, the number of viable microorganisms remaining at the end of each period was determined by the plate count method. The percentage change in the concentration of each microorganism during the test was determined.

RESULTS & DISCUSSION:

Eventhough the preservative concentration was analysed before and during the stability studies, it is a regulatory requirement that, both concentration as well as efficacy studies of a preservative against the microbes which are known to contaminate the product during its use after opening the container are carried out. Therefore, the preservative effectiveness test was carried out following the guidelines given in the USP. This test is official in USP, BP and also included in IP in 1996.

The results of the study are summarized in Table-58:

TABLE-58. RESULTS OF PRESERVATIVE EFFECTIVENESS TEST OF VARIOUS PREPARED FORMULATIONS:

Formulation	Microbial count				
	C. A.	A. N.	S. A.	E.C.	P.A.
KT Solution (FFS-1)					
C.f.u.per mL Initial	$3.13X10^5$	$2.18X10^5$	$3.34X10^5$	$2.93X10^5$	$3.15X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00
KT solution (FFS-2)					
C.f.u. per mL Initial	$3.13X10^5$	$2.18X10^5$	$3.34X10^5$	$2.93X10^5$	$3.15X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00

Contd...

Contd. 58

Formulation	Microbial count				
	C. A.	A. N.	S. A.	E.C.	P.A.
KT-Poloxamer gel (FFP-1) stored in vials					
C.f.u/gm Initial	$4.8X10^5$	$2.23X10^5$	$4.15X10^5$	$3.93X10^5$	$3.65X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00
KT-Poloxamer gel (FFP-2) stored in vials					
C.f.u/gm Initial	$4.8X10^5$	$2.23X10^5$	$4.15X10^5$	$3.93X10^5$	$3.65X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00
KT Carbopol gel					
C.f.u/gm Initial	$4.8X10^5$	$2.23X10^5$	$4.15X10^5$	$3.93X10^5$	$3.65X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00

Table 58. contd....

KT viscous solution (FFV-1)					
C.f.u/gm Initial	$3.13X10^5$	$2.18X10^5$	$3.34X10^5$	$2.93X10^5$	$3.15X10^5$
Microbial count as % of initial					
Day 7	0.00	0.000	0.00	0.00	0.00
Day 14	0.00	0.000	0.00	0.00	0.00
Day 21	0.00	0.000	0.00	0.00	0.00
Day 28	0.00	0.000	0.00	0.00	0.00
KT viscous solution (FFV-2)					
C.f.u/gm Initial	$3.13X10^5$	$2.18X10^5$	$3.34X10^5$	$2.93X10^5$	$3.15X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00
KT ointment					
C.f.u/gm Initial	$4.8X10^5$	$2.23X10^5$	$4.15X10^5$	$3.93X10^5$	$3.65X10^5$
Microbial count as % of initial					
Day 7	0.32	0.10	0.00	0.00	0.00
Day 14	0.04	0.03	0.00	0.00	0.00
Day 21	0.00	0.02	0.00	0.00	0.00
Day 28	0.00	0.01	0.00	0.00	0.00

C.A. = *Candida albicans* A.N. = *Aspergillus niger*
S.A. = *Staphyloccus aureus* E.C. = *Escherichia coli*
P.A. = *Pseudomonas aeruginosa*

According to the USP specifications, the fungal count in the formulation should remain at or below the initial concentrations during the first 14 days, the bacterial counts should be reduced to NMT 0.1% of the initial concentration by the 14th day. During the remaining period of test, the concentration of each test microorganism should remain at or below these designated levels.

In the present study bacterial as well as fungal counts, in the aqueous solutions and gels came down to zero at the end of the first week itself and thus meets the requirements of the USP.

Similar results were obtained before the stability studies. In case of the ointment, a few fungal and yeast cells were observed during the first 14 days, however their counts were well within the official limits.

Therefore it could be concluded from the results that, all the formulations passed the official antimicrobial preservative effectiveness test before and after completion of the accelerated stability studies.

2.6.4 BIOLOGICAL EVALUATION:

The preclinical safety and efficacy studies are of great use to a formulator in deciding the formulation for clinical evaluation. Based on type of dosage form, suitable animal model/s are usually evaluated. For certain dosage forms official guidelines are also available. We have studied the eye-irritation, anti-inflammatory efficacy of conventional ketorolac tromethamine drops and aqueous humor drug-concentration in rabbits for all the prepared formulations.

2.6.4.1 THE EYE-IRRITATION TEST:

The eye-irritation studies were carried out for all the stable formulations following the guidelines of Draize[295] test, the official test developed in 1944.

EXPERIMENTAL:

New Zealand white rabbits of either sex weighing between 2.0-3.0kg were used in the study. The study design was as follows: Four rabbits were used for each formulation. The test formulation was applied to one eye of each rabbit and the contralateral eye served as a control in which a non-irritating

sterile normal saline was instilled at the same time and same dose as that of the test formulations. After instillation, the eyes were observed for any immediate reaction and subsequently were observed on the morning of the next day.

The various tissue response parameters evaluated included:
i) Blinking rate, ii) pupil diameter, iii) corneal redness, iv) tear flow, v) conjunctival redness, vi) corneal opacity and viii) discharge. Depending on the severity of these responses, grades were allocated, the least severe reaction received the lowest grade.
The dosing schedules for the various formulations were as follows:
In case of conventional and viscous solutions, 50µl of the formulation was administered every 2 hours with a total 6 doses per day for a period of one week.

The gels and ointments were dosed every 4 hours with a maximum of three doses per day for a period of one week. The formulation was filled in microlitre syringes and 50µl dose was administered.

RESULTS AND DISCUSSION:
KT ophthalmic formulations tested for eye-irritation were found to be essentially non-irritating in rabbits. The Parameters listed by Draize to evaluate the eye-irritation potential included light reflex, corneal damage (area of cornea affected), intensity of corneal damage, flare and hyperaemia of the iris, tear flow, conjunctival redness, conjunctival discharge. All of these parameters are rated on a scale of 1-3 or 1-4, depending upon the severity of the reaction. None of these parameters were observed after administration of the various KT formulations during the study. The reason could be due to the neutral pH used

in all the formulations. However, blinking rate was found to increase when the conventional drops prepared with Cremophor was administered, as compared to normal saline, but for a 1-2 minutes. The ointment prepared with cholesterol showed conjunctival redness and increased blink rate as well as tear flow.

The gels, on the other hand, did not show any irritation potential because, the instilled gel, released the drug over a period of time. Carbopol 940 has been used in a commercial Betaxolol (Betoptic S^R) to decrease the severe burning effect and ocular pain induced by the drug[296].

Corneal and iris injury are considered more relevant to the overall irritation potential and it was proposed by Bayard *et al* to weigh the daily scores by using a multiplier of 15 for corneal damage score, a multiplier of 5 for iris scores and a multiplier of 2 for the conjunctival scores. The total score is simply the sum of the scores of all the days on which the preparations were instilled or applied. The total scores were evaluated using the following scale:

Severely irritant: 326-550 Strong irritant: 201-325

Moderately irritant: 66-200 Marginally irritant: 65

In our study, the total scores was never above 20, which is much below 65, the score of a marginally irritant substance.

Therefore from the eye-irritation study it could be concluded that all the final formulations evaluated, were found to be non-irritating in the rabbit eye, when tested for a period of one week in rabbit eyes.

2.6.4.2 EFFICACY STUDY: ANTI-INFLAMMATORY ACTIVITY OF KETOROLAC TROMETHAMINE CONVENTIONAL OPHTHALMIC SOLUTION (FFS-1):

The efficacy study was carried out only for conventional solution containing Cremophor EL (FFS-1). The other formulations were evaluated by carrying out pharmacokineic studies in rabbits eyes.

The anti-inflammatory activity of the conventional formulation was evaluated by comparing it with the innovator's product (Acular^{R}). An alkali induced inflammation model in rabbits was used[205]. This model is being used to evaluate anti-inflammatory agents[297].

EXPERIMENTAL:

The procedure for inducing inflammation with an alkali in rabbit eyes is as follows:

New Zealand white rabbits weighing between 2.0-3.0 kg of either sex with normal ocular anatomy were selected for the study. The rabbits were held in restrainers and were anaesthetized with an intramuscular injection of Ketamine (5 mg/kg) and Xylazine (25 mg/kg). The eye was anaesthetized locally using 4% xylocaine solution. A 7 mm filter paper disc (Whatman # 42) was soaked in 1 N NaOH for 30 seconds and excess amount was removed by keeping it on a filter paper. The disc was placed on the centre of the cornea of anaesthetized rabbit. After one minute, the disc was removed, and the eye was washed with 15 ml of sterile normal saline. The cornea was stained with methylene blue (1.0 % w/v) to measure the de-epithelialized area, and also to take the photographs. The eyes were washed with sterile saline.

The dosing schedules were as follows: Within 10 minutes of induction of inflammation, 50µl of formulation under test was administered to one eye, and the other eye received Acular^{R}.

Four eyes were used as controls, which received sterile normal saline at the same time as that of formulations. A total of 5 doses with an interval of 3 hours between dose were given every day, for a period of 14 days.

The various parameters evaluated during the study included:

i) Polymorphonuclear leukocytes (PMNs) in corneal washes

ii) PMNs in aqueous humor

iii) Protein concentration in aqueous humor

iv) Vascularization of the cornea and

v) Corneal re-epithelialization.

In brief, the procedure used to measure the above mentioned parameters is as follows:

i) Determination of polymorphonuclear leukocytes in corneal washes:

The method reported by Srinivasan et.al[298,299] was used for collecting the corneal washes from the inflamed eye. 50µl of sterile normal saline, at predetermined intervals, was administered into the inflamed rabbit eye, and about 25µl of tear was withdrawn using a micropipette at 5, 8, 12, 24, 36 and 48 hours post-inflammation. The tear sample was placed on an improved Neubauer chamber and cells were counted using a microscope at 100X. The cells/µl were calculated by using the formula:

$$\text{Cells}/\mu l = \frac{\text{Number of cells counted X Dilution}}{\text{Area counted X Depth}}$$

ii) Determination of PMNs in aqueous humor:

About 200µl of aqueous humor was aspirated using a sterile 30 gauge needle at 6 hr, 12 hr, 24hr, 48 hr, 72 hr, day 6, day 10 and day 14 of post-inflammation. About 50µl of the sample was used for measurement of PMN's. The procedure for counting PMNs and calculation were same as described for corneal washes.

iii) **Measurement of protein concentration in aqueous humor:**
The remaining part of aqueous humor, that was left after measuring PMNs, was utilized for determination of protein at the same time points as described above (ii). The protein concentration was measured by using Lowry's colorimetric method[300]. The standard plots were made using pure albumin in the concentration range of 30-300 mg/ml. 100µl of aqueous humor was used at all time points for protein estimation from treated and control eyes.

iv) **Evaluation of vascularization of the inflamed cornea:**
After inducing inflammation, the eyes were observed for 14 days for development of blood vessels towards the inflammatory site. Photographs of vascularization were taken.

v) **Evaluation of re-epithelialization of inflamed cornea:**
The de-epithelized area of inflamed cornea was measured using a centimeter ruler, after staining with either methylene blue or sodium fluorescein. Staining was done on each day before giving the first dose and area was measured & photographs were taken to monitor the re-epithelialization process.

RESULTS AND DISCUSSION:
Corneal inflammation, ulceration and vascularization are often coincident in corneal disease. Several experimental models have been developed to study these individual events. Although their pathophysiology are interrelated, many factors involved in the pathogenesis of these disease states may differ among models. However, a standard model of corneal injury using alkali-immersed filter paper discs has been reported, which exhibits many symptoms of corneal inflammation. Moreover, this model has been reported to be standardized for concentrations of alkali and their response on cornea[205]. This model also mimics clinical

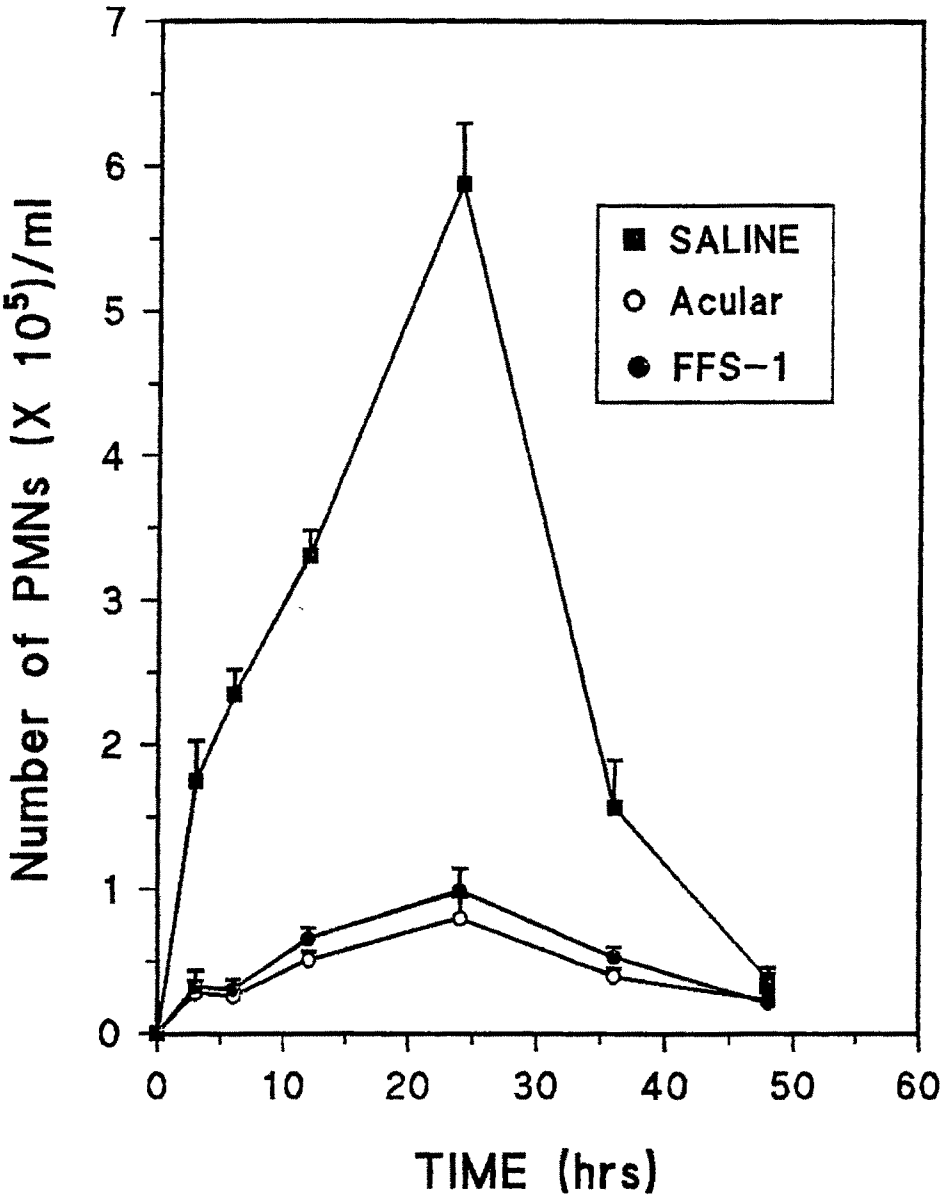
situation of alkali burn of the cornea, which is a severe clinical problem, since vision may be reduced due to corneal opacification. The above reasons were taken into consideration in selection of animal model for evaluation of prepared ketorolac tromethamine formulations.

The alkali burn causes infiltration of PMNs, which plays a central role not only in the initial inflammatory response but also in the process which leads to ulceration, vascularization and sometimes perforation[301]. The breakdown of aqueous humor-barrier increases the protein concentration in the humor. Therefore in our study evaluation included measurement of PMNs in corneal washes & aqueous humor, protein estimation in aqueous humor and vascularization.

The efficacy data was statistically evaluated by applying a split-plot design and Tukey's multiple comparison tests.

The results of infiltration of PMNs in the cornea following the alkali burn is shown in fig. 11. The results suggested that, the PMNs in the treated eye, either with Acular^R or prepared conventional solution was significantly ($p<0.05$) less at all time points except at 48 hour as compared to 0.9 % saline. There was no significant difference ($P>0.05$) between Acular^R and FFS-1 at any time point. This showed that the prepared formulation was equieffective as that of innovator's product in inhibiting the infiltration of PMNs into the cornea. The reason for inhibition of PMNs by ketorolac tromethamine could be attributed to its activity against cycloxygenase enzyme and also might be inhibition of lipoxygenase at higher concentration. However, it is not possible to conclude any actual mechanism involved at this stage.

Fig. 11. Time course of appearance of PMNs in corneal washes after an alkali burn



Each point represents Mean ± S.E.M (n=4).

The number of PMNs in aqueous humor in KT treated eyes were significantly less ($P<0.05$) after 6 hour, as compared to saline treated eyes. The time course of PMNs movement into the aqueous humor was biphasic (Fig.12). The first phase was acute, which was similar to that seen in ocular tissues in response to a variety of diverse inflammatory stimuli. The second phase of PMNs, building up after 7 days after the alkali burn, is of particular interest because it coincides with manifestation of corneal ulceration. Therefore corneal ulceration was observed in case of saline treated eyes and not in KT treated eyes. The number of PMNs observed after treatment with either Acular^{R} or prepared formulation were found to be similar and insignificant statistically ($P>0.05$).

The concentration of proteins after the alkali burn was found to increase significantly ($p<0.05$) in saline treated eyes, till the 14th day as compared to its base line value (Fig.13), whereas in KT treated eyes, the concentration of proteins was not statistically significant ($p>0.05$) from the base value. The protein concentration were found to be similar in eyes, treated either with Acular^{R} or FFS-1. From this it can be concluded that the blood-aqueous barrier was damaged to a lesser extent in case of KT treated group than the saline treated group. It has been reported that KT 0.5% w/v prevents post-surgical blood-aqueous barrier breakdown[264].

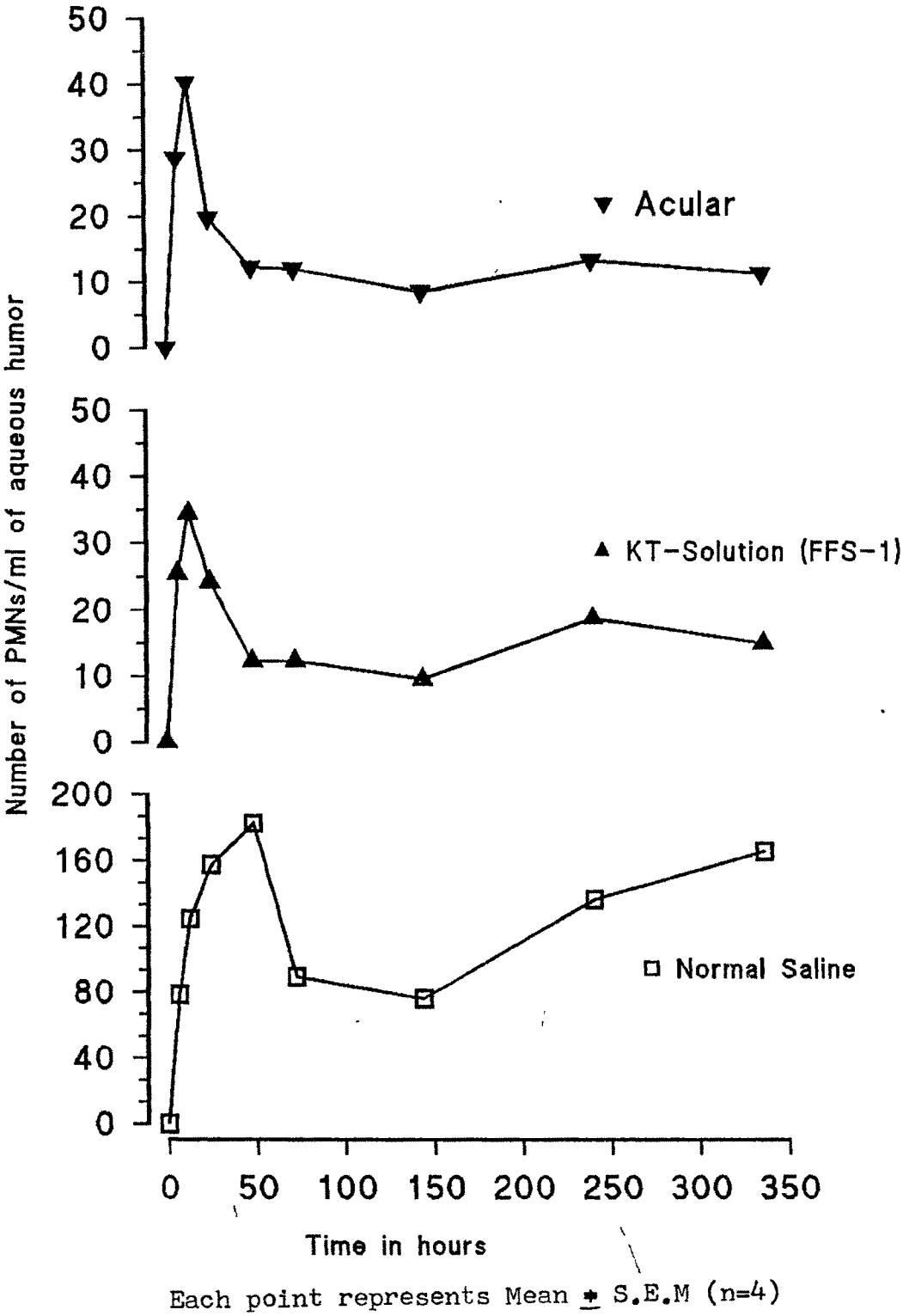
The re-epithelialization of the cornea, after the alkali burn was monitored by taking photographs, after staining the eye either with 0.5 % methylene blue or with 0.2% sodium flourescein. The staining method followed has been reported for similar type of studies[302,303]. This method was used to evaluate qualitatively,

the effect of the prepared formulation on the re-epithelialization process of the inflamed cornea, as compared to saline, and the innovator's product. The results suggested that, the epithelial closure was not affected by the prepared formulation. The re-epithelialization was complete in 3 days for slaine as well as for Acular^{R} and the formulation under test. There was no difference in de-epithelized area between control and KT treated eyes. The epithelial closure pattern is shown in fig.14 to 15. These results suggested that, KT does not interfere in the epithelial wound healing, and the prepared formulation was as good as innovator's product.

Corneal vascularization was observed only in the saline treated eyes and not in the KT treated groups. The vascularization was appeared on day 4 or 5 and kept on increasing day by day in saline treated group (Fig.16). These could be related to increase in PMNs in saline treated group, after one week of alkali burn. Since, in KT treated group, the PMNs did not increase after 48 hours and no ulceration was found, and hence vascularization could be ruled out.

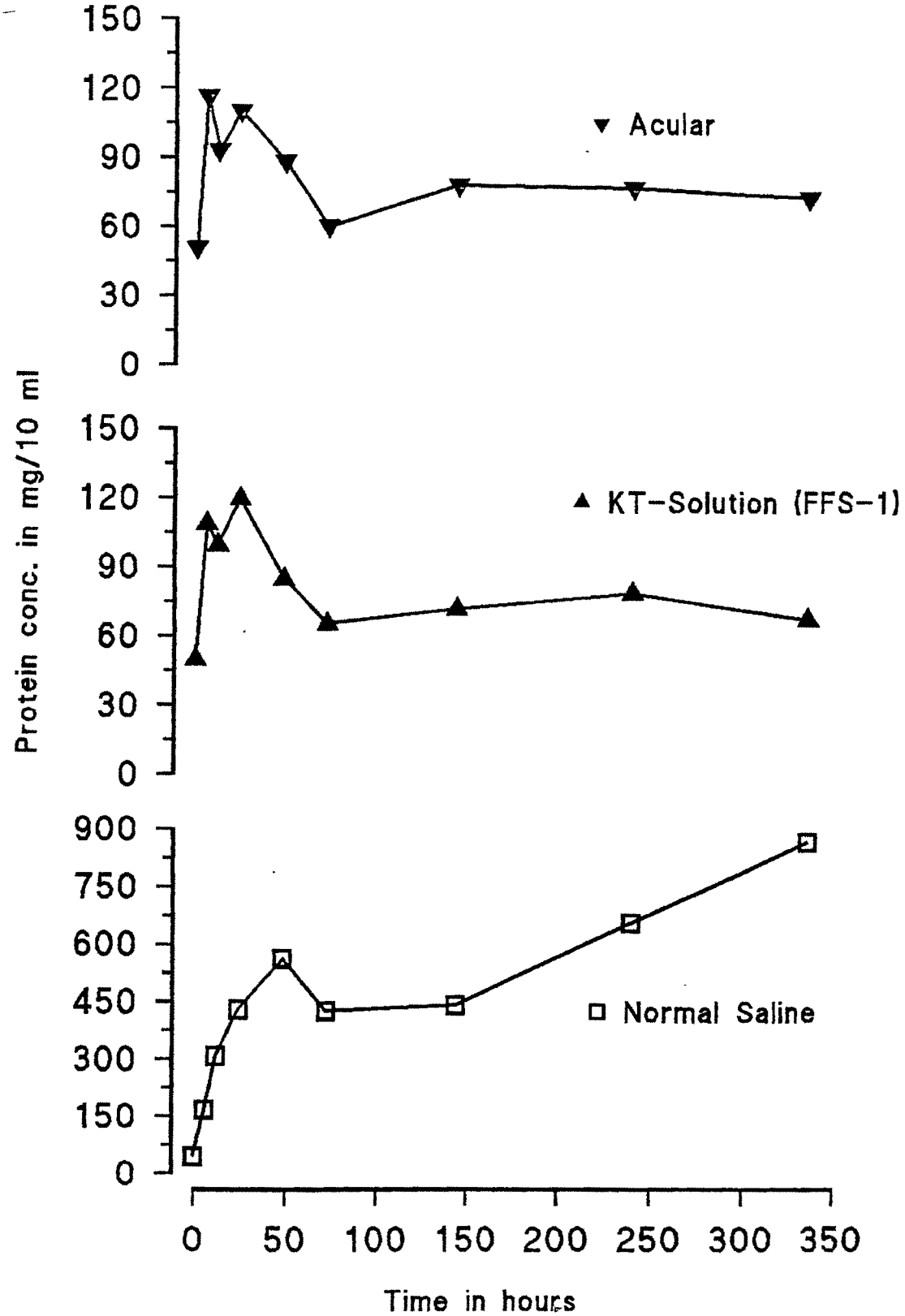
The *in-vivo* efficacy study provided many factors to evaluate the prepared formulation in comparison with the innovator's product. It can be concluded that the prepared formulation was as effective as that of Acular^{R}.

FIG. 12 Effect of KT treatment on infiltration of PMNs in aqueous humor after an alkali burn

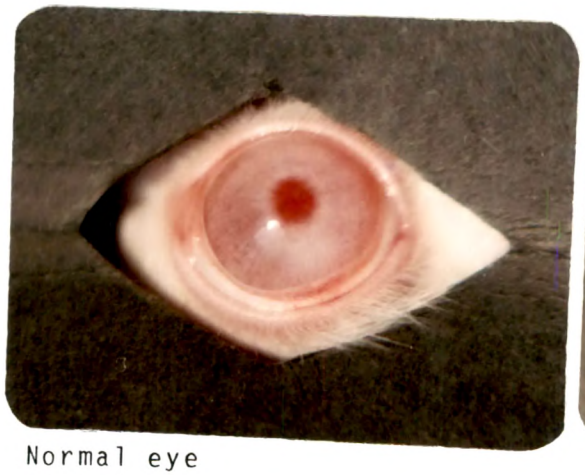


Each point represents Mean ± S.E.M (n=4)

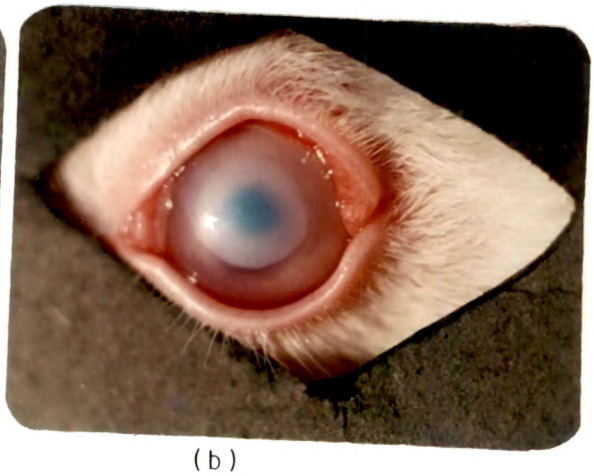
FIG. 13 Effect of KT treatment on the protein concentration in aqueous humor after an alkali burn



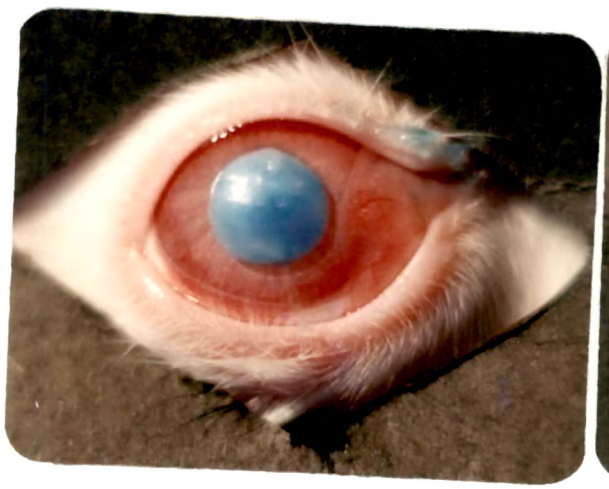
Each point represents Mean ± S.E.M (n=4)



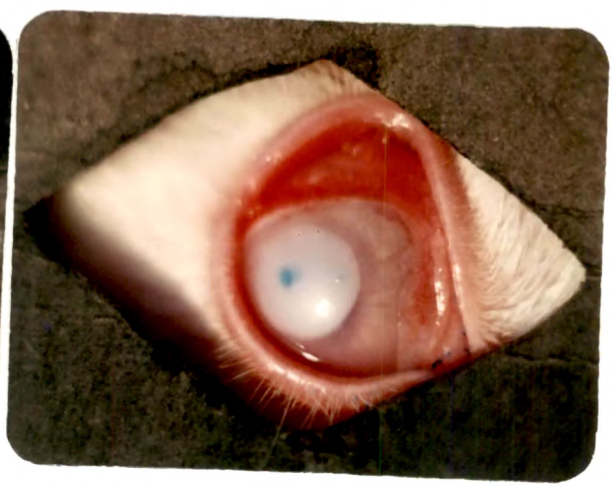
Normal eye



(b)



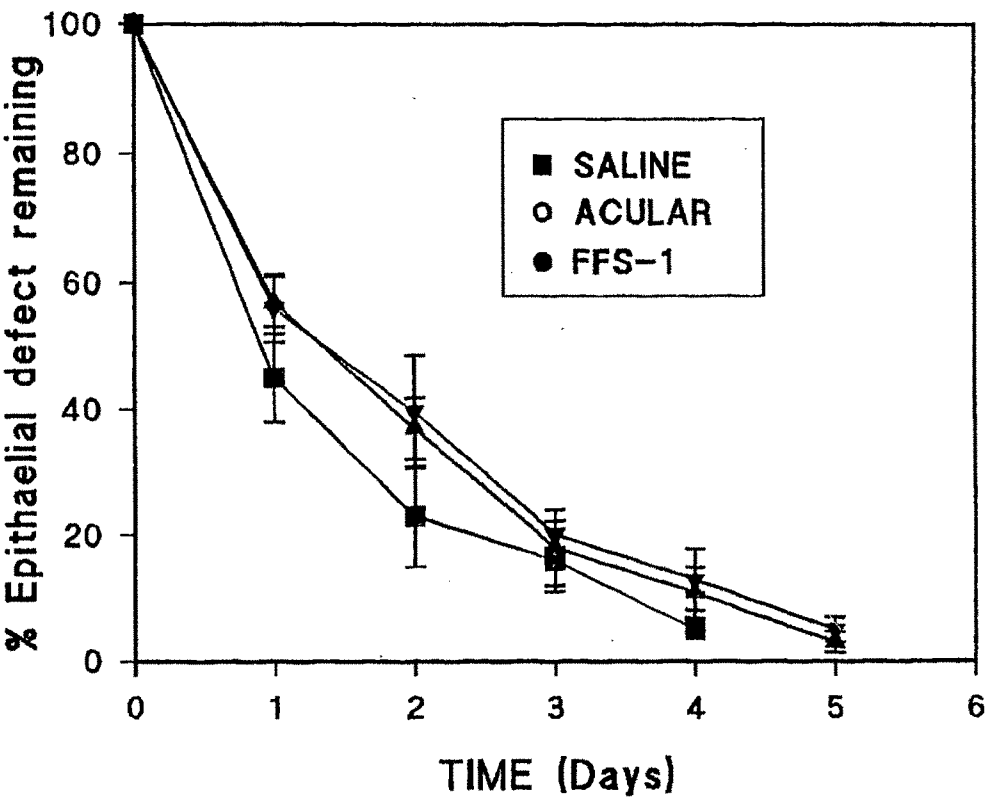
(a)



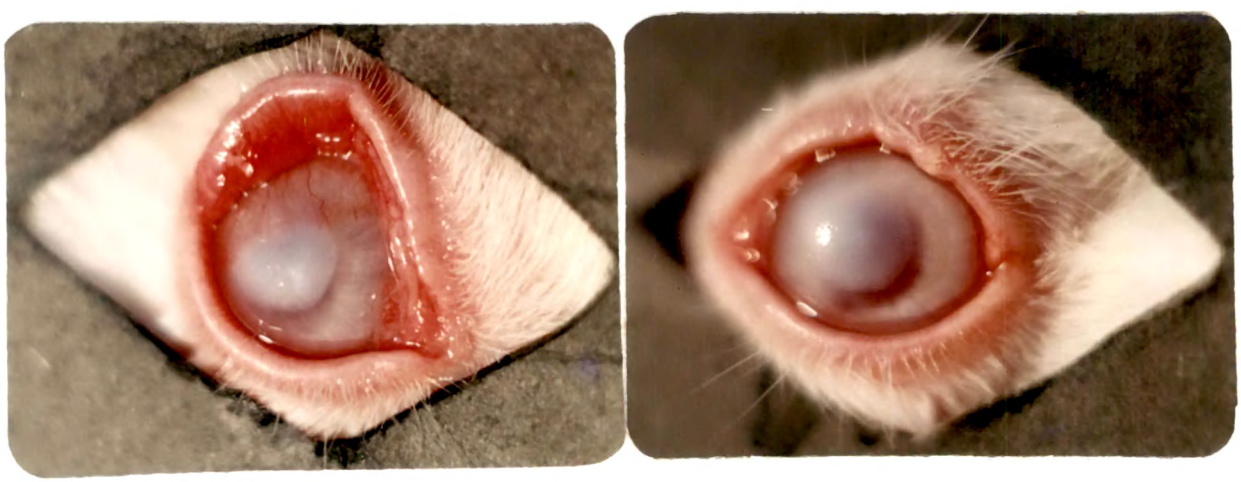
(c)

Fig.14: Photographs of the rabbit eyes showing corneal re-epithelialization pattern following alkali burn: (a) day 1 (b) day 2 (c) day 3

Fig. 14 Corneal re-epithelialization pattern after an alkali burn: Effect of KT formulations and saline



Each point represents Mean ± S.E.M (n=4)



(A) (B)

Fig. 16: Effect of Ketorolac tromethamine on Corneal neovascularization in rabbit eyes after an alkali burn

A) Normal saline B)KT solution (FFS-1)

2.6.4.3 DETERMINATION OF THE AQUEOUS HUMOUR DRUG CONCENTRATION TIME PROFILE

The efficacy study was time consuming, required large number of animals and variation in the results was more. Once the efficacy of KT conventional solution was established, it was decided to compare the concentration of KT in aqueous humor at different time intervals following topical administration of the prepared formulations in New Zealand rabbit eyes.

EXPERIMENTAL:

The experimental design used for the study is as given below:

(i) **First part** of the study included comparison of single dose administration of prepared conventional solution such as FFS-1 and FFS-2 with Acular^{R}.

(ii) **The second part** included comparison of aqueous humor drug concentrations, after single dose administration of conventional solution (FFS-1) with viscous solution (FFV-2), Poloxamer based gel (FFP-2), Carbopol based gel and the ointment.

(iii) The gels were prepared with an objective of minimizing the frequency of administration as compared to conventional solution. Therefore, a single dose of gel was compared with multiple dose of conventional solution. The dosing frequency used for solution was similar to that used for efficacy studies.

The selection of animals, number of animals, dosing and sampling procedure was same for (i) and (ii) studies. Therefore procedure is given in detail for the first study.

2.6.4.3.1 Determination and comparison of the aqueous humor drug concentration-time profile, following topical application of single dose of KT solution and single dose of Acular^R:

New Zealand albino rabbits, weighing between 2.0-3.0kg, of either sex and having normal ocular anatomy were selected for the study. These animals were divided into 5 groups of 3 animals each. One group of animals was sampled at only one time point (sparse sampling) and samples were analysed separately. In order to dose the animals, they were placed in restrainers, and the lower eyelid was pulled away to form the cul-de-sac. 50µL of either formulation was carefully instilled into the cul-de-sac with the help of a microsyringe and then the eyelids were closed for a period of 30 seconds. The dosing and sampling protocol was as described in Table-59.

TABLE-59. DOSING AND SAMPLING SCHEDULE FOR DETERMINING THE AQUEOUS HUMOR DRUG-CONCENTRATION TIME PROFILE FOR COMPARISON OF KT CONVENTIONAL SOLUTIONS WITH ACULAR^R

Animal No.	Group	Left eye	Right eye	Sampled at
1, 2, 3	I	KT solution	Acular^R	0.5 hr.
4, 5, 6	II	KT solution	Acular^R	1.0 hr.
7, 8, 9	III	KT solution	Acular^R	2.0 hr.
10, 11, 12	IV	KT solution	Acular^R	3.0 hr.
13, 14, 15	V	KT solution	Acular^R	4.0 hr.

The sampling was done under anaesthesia by giving intramuscular injection of ketamine (5 mg/kg) and xylazine (25 mg/kg). Before sampling, the eye was washed thoroughly with normal saline, and blotted dry with a tissue paper. About 250µl of

aqueous humor was aspirated with a sterile 30 gauge needle. The sample was transferred to small glass conical tubes, and sealed with parafilm, and frozen at -20°C till the day of analysis. The samples were analyzed by the HPTLC method.

The internal standard was added to the aqueous humor sample and dried at 60°C under vacuum. It was reconstituted with methanol and 10µl was applied, in duplicate on HPTLC plate.

2.6.4.3.2 Determination and comparison of the aqueous humor drug concentration-time profile following topical application of single dose of KT solution with single dose of viscous solution (FFV-2), Carbopol gel, Poloxamer gel (FFP-2) and ointment:

The number of animals and sampling procedure for the study was same as mentioned in section (2.6.4.3.1). In case of gels and ointment the formulation was filled into microsyringe carefully and exactly 50µl was dosed. The dosing and sampling protocol used for the individual study are given in the Table-60. The right eye received KT solution, in all the groups, whereas left eye received the formulation under test. The sampling time was same for KT solution and the formulation under test.

TABLE-60. DOSING AND SAMPLING SCHEDULE FOR DETERMINING THE AQUEOUS HUMOR DRUG-CONCENTRATION TIME PROFILE FOR COMPARISON OF KT CONVENTIONAL SOLUTIONS (FFS-1) WITH VARIOUS FORMULATIONS

Animal No.	Group[a]	Formulation sampled at			
		FFV-2	FFP-2	Ointment	Carbopol gel
1, 2, 3	I	1.0 hr	1.0 hr	1.0 hr	1.0 hr
4, 5, 6	II	2.0 hr	2.0 hr	2.0 hr	2.0 hr
7, 8, 9	III	3.0 hr	3.0 hr	4.0 hr	3.0 hr
10, 11, 12	IV	4.0 hr	4.0 hr	6.0 hr	4.0 hr
13, 14, 15	V	6.0 hr	6.0 hr	8.0 hr	8.0 hr

[a] Right eye: KT solution
Left eye : Formulation under test.

2.6.4.3.3 Determination and comparison of the aqueous humor drug concentration-time profile following topical application of multiple dose of KT solution (FFS-1) and single dose of Carbopol based gel and Poloxamer based gel:

The number of rabbits, selection criteria, procedure for sampling and analysis of samples were same as mentioned for single dose studies in section (2.6.4.3.1). The dosing and sampling schedule was as shown in Table-61.

TABLE-61. DOSING AND SAMPLING PROTOCOL FOR DETERMINATION OF THE AQUEOUS HUMOR DRUG CONCENTRATION-TIME PROFILE FOLLOWING MULTIPLE DOSE OF SOLUTION AND SINGLE DOSE OF EITHER CARBOPOL OR POLOXAMER GEL

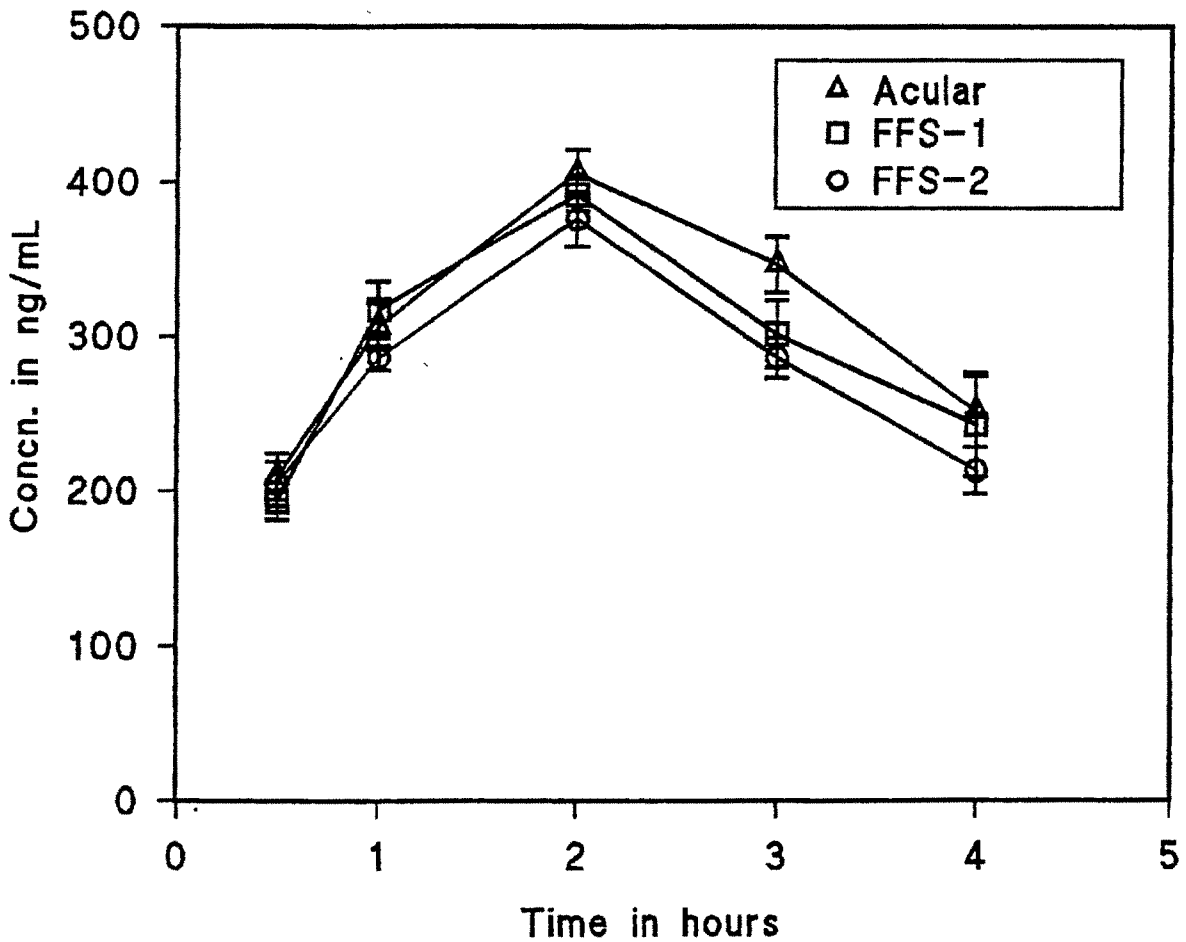
Animal No.	Group	Left eye (KT solution)		Right eye (KT gel)	
		Dosed at	Sampled at	Dosed at	Sampled at
1, 2, 3	I	0 hr	3 hr	0 hr	3 hr
4, 5, 6	II	0, 3 hr	6 hr	0 hr	6 hr
7, 8, 9	III	0, 3, 6 hr	9 hr	0 hr	9 hr
10, 11, 12	IV	0, 3, 6, 9 hr.	12 hr	0 hr	12 hr
13, 14, 15	V	0, 3, 6 9,12 hr	24 hr	0 hr	24 hr

RESULTS & DISCUSSION:

The various parameters compared for evaluating the prepared formulations included area under the curve (AUC) of aqueous humor drug concentration-time plot, Cmax and tmax.

The results of the aqueous-humor drug concentration time profile, after administration of KT conventional solutions and Acular^{R} are shown in Fig.17. The drug concentrations in aqueous humor were found to be comparable, after single dose administration of FFS-1 or FFS-2 with Acular^{R}. The AUC was calculated by applying trapezoidal rule. Since the study was based on sparse sampling, the Bailer's method was used for comparison of the AUCs by calculating variance AUC for each of the formulations[304-306].

FIG. 17 Aqueous Humour Concentration-Time Profile of KT Following Topical Administration of Conventional Solutions



Each point represents Mean ± S.E.M (n=3)

The calculated AUCs and variance AUCs for KT solution and Acular^R is given below.

Formulation	AUC_{0-t} ng.hr/ml	Variance AUC (s^2AUC)
KT Solution (FFS-1)	1175.8	576.56
KT Solution (FFS-2)	1146.6	974.56
Acular^R	1209.4	820.24

To test the null hypothesis Ho: AUC1=AUC2 Vs H1:AUC1 $\neq$AUC2, the following test statistic was used.

$$Z_{observed} = \frac{AUC_1 - AUC_2}{\{(s^2AUC_1) + (s^2AUC_2)\}^{\frac{1}{2}}}$$

Ho would be rejected if |Zobserved| $\geq$ Zcritical, where Zcritical is the critical value of standard normal distribution. This test is an asymptotic two-tailed hypothesis test with significance level α. The results suggested that both FFS-1 and FFS-2 do not significantly differ from Acular^R at the 5% level of significance.

The measured pharmacokinetic parameters after single dose administration of each of the prepared formulation is tabulated in Table-62.

TABLE-62. THE PHARMACOKINETIC PARAMETERS OBTAINED AFTER SINGLE DOSE ADMINISTRATION OF PREPARED FORMULATIONS:

Formulation	C_{max} (ng/ml)	T_{max} (hour)	AUC_{0-t} (ng.hr/ml)
KT solution (FFS-1)	408.2	2.00	1175.8
KT solution (FFS-2)	391.5	2.00	1146.6
Acular^{R}	405.7	2.00	1209.4
KT viscous solution	532.5	2.00	1901.3
KT ointment	386.3	4.00	1429.5
KT-Poloxamer gel	975.2	4.00	4184.2
KT-Carbopol gel	2368.4	6.00	16981.2

The results of single dose of KT **viscous solution** showed that, the increase in viscosity of the formulation improves the bioavailability. The aqueous humor drug concentration, after administration of viscous solution and conventional solution is shown in Fig.18. Viscous solution made of HPMC E4M have been reported to increase the contact time of the formulation in the eye and hence improved availability of drug. It is known that increase in viscosity from 1-100 cps causes a decrease in drainage and hence, increases corneal contact time of the dosage form. KT concentration in the aqueous humor was detectable for 6 hours, as compared to 4 hours in case of conventinal solution.

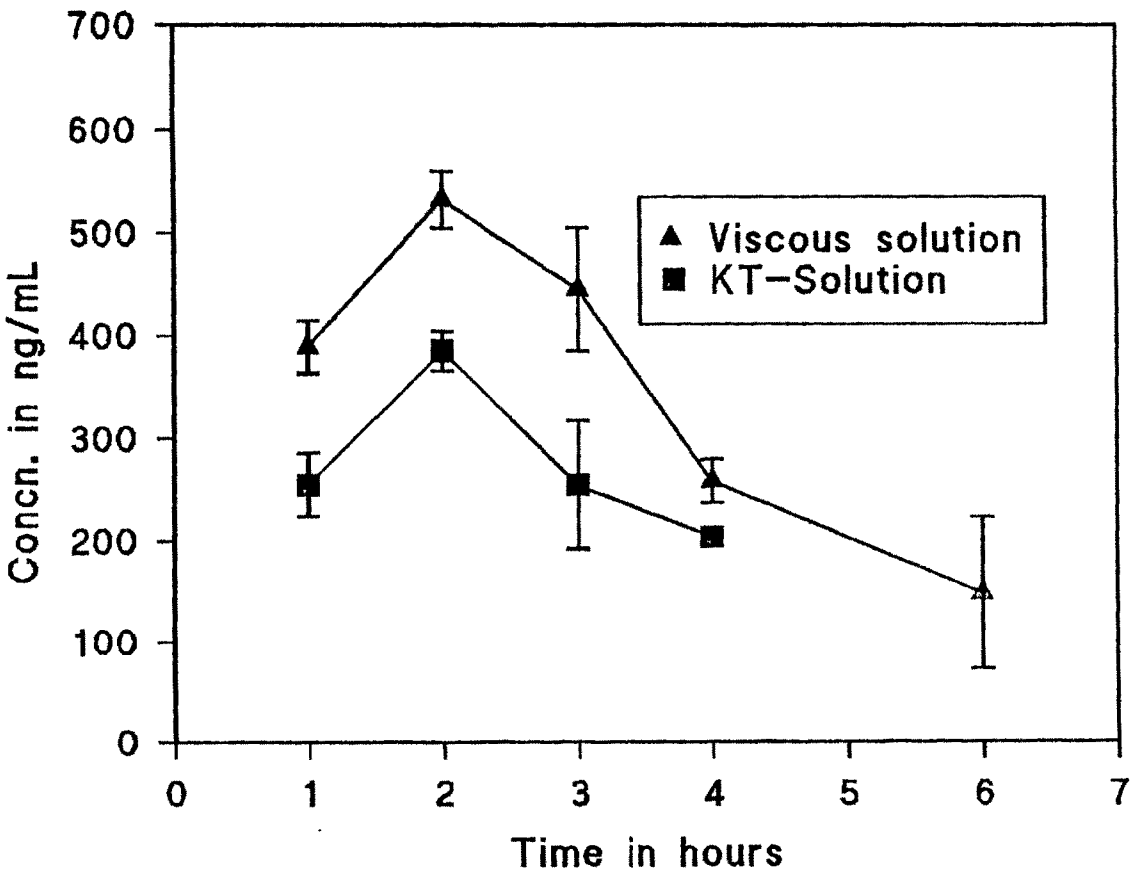
The **ointment** released the drug very slowly which is reflected in the Cmax and tmax values. The concentrations obtained were erratic and had large variations (Fig.19). This could be attributed to improper mixing of ointment in the rabbit eye, because the blinking rate of rabbit is much less than the man and also expulsion of ointment from the eye after dosing was observed. However, ointment showed higher AUC as compared to

outward diffusion of drug into the tear pool. Many drugs such as Sodium cromoglycate[113] and flouromethalone[307] when administered as ointments, have shown to improve the ocular bioavailability as compared to the conventional solution. Therefore, ointment can be used during night, while other dosage forms in the morning times.

The results of single dose administration of gels suggested that Carbopol based KT gel gave more prolonged release of drug in the eye, as compared to Poloxamer based gel (Fig. 20 and 21). It has been reported that the viscosity of the vehicle increases the retention time of the product in the eye and hence improves its bioavailability[308]. In our study Poloxamer gel was more viscous than Carbopol gel. But, Poloxamer was retained in the eye for 45-60 minutes. The reason could be, Poloxamer is not mucoadhesive and loses its gelling structure below 18 %.

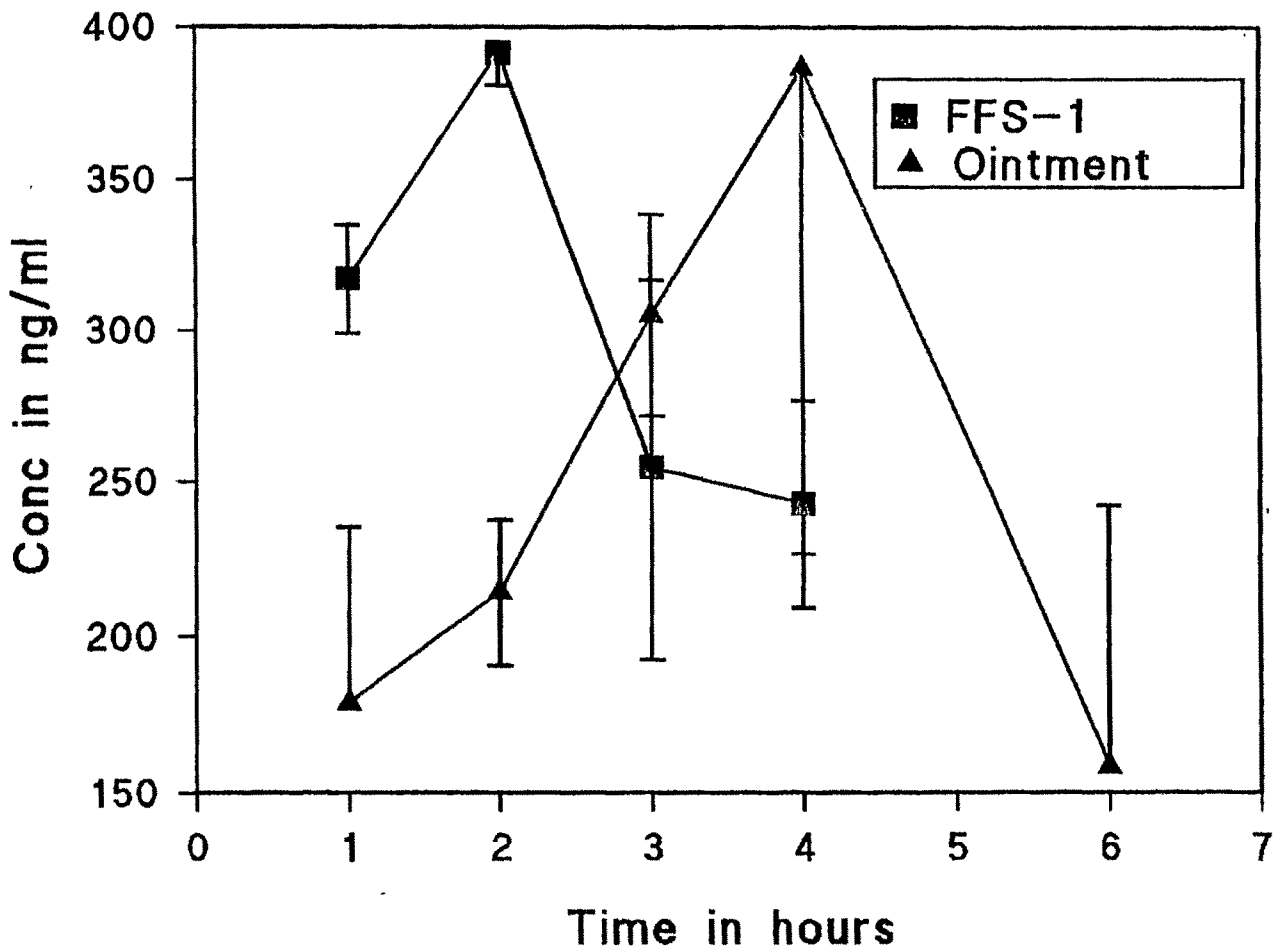
Prolonged ocular retention of an aqueous poly(acrylic acid) formulation, as measured by lacrimal dacryoscintigraphy, and the resulting prolonged miotic response to pilocarpine have recently been reported by Davies et.al[120]. Although the ocular retention of the polycarbophil formulations was not evaluated quantitatively in our study, the Carbopol gel was clearly visible in the cul-de-sac even after 4 hours of topical administration. Similar retention time for Carbopol gel, containing gentamicin and pilocarpine, in the eye has been reported by Lee.et.al[308] and Schoenwald et.al[123]. The long retention time of the Carbopol based KT gel could be attributed to its high yield stress value, which allows it to withstand the *in-vivo* shearing of eyelid and eye ball movements. Moreover, Carbopol 940 has a mucoadhesive property, which could have increased the contact time in the eye.

Fig.18 Aqueous humour drug concentration–time profile of KT following conventional and viscous solution



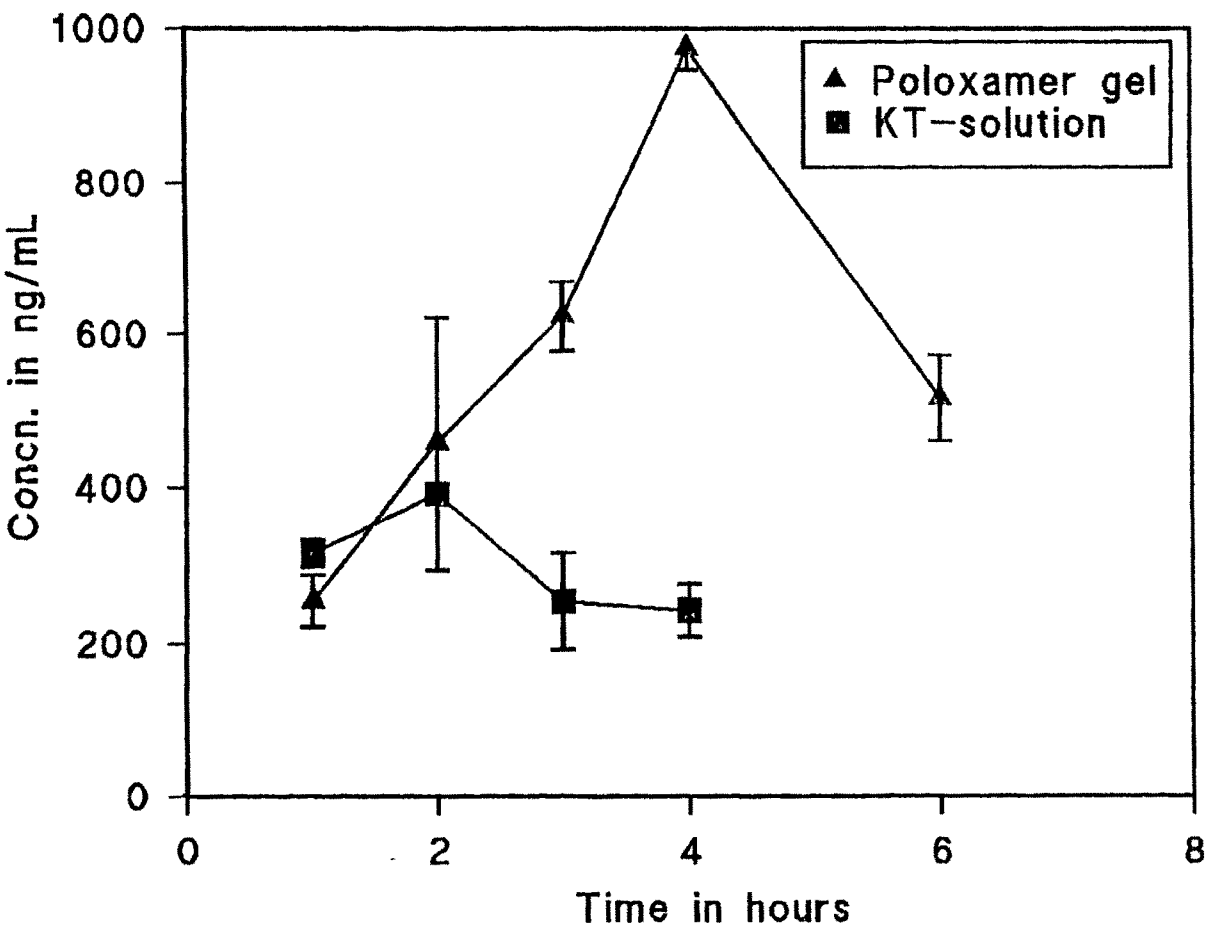
Each point represents Mean ± S.E.M (n=3)

Fig. 19 Aqueous humour drug concentration–time profile of KT after single dose administration of conventional solution & ointment



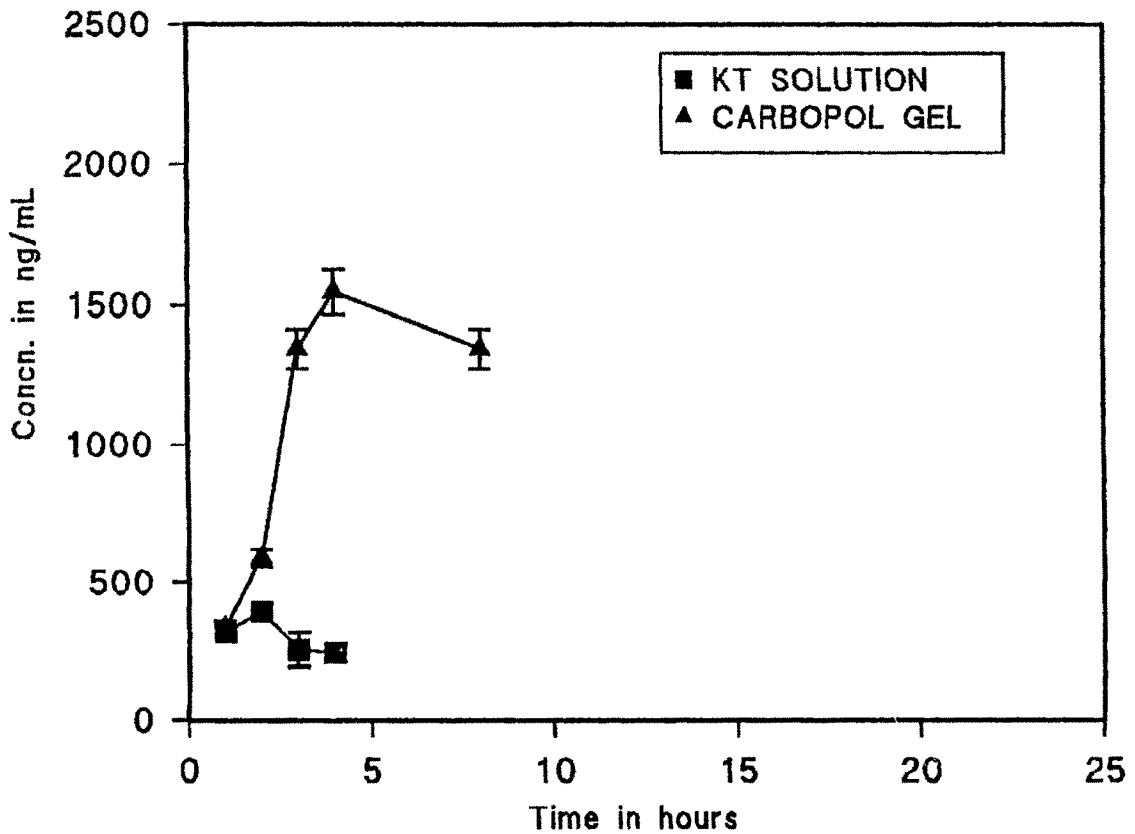
Each point represents Mean ± S.E.M (n=3)

Fig. 20 Aqueous humour drug concentration–time profiles of KT following single dose administration of KT solution and Poloxamer gel



Each point represents Mean ± S.E.M (n=4)

Fig. 21 Aqueous humour drug concentration–time profile of KT following single dose administration of conventional solution and carbopol gel



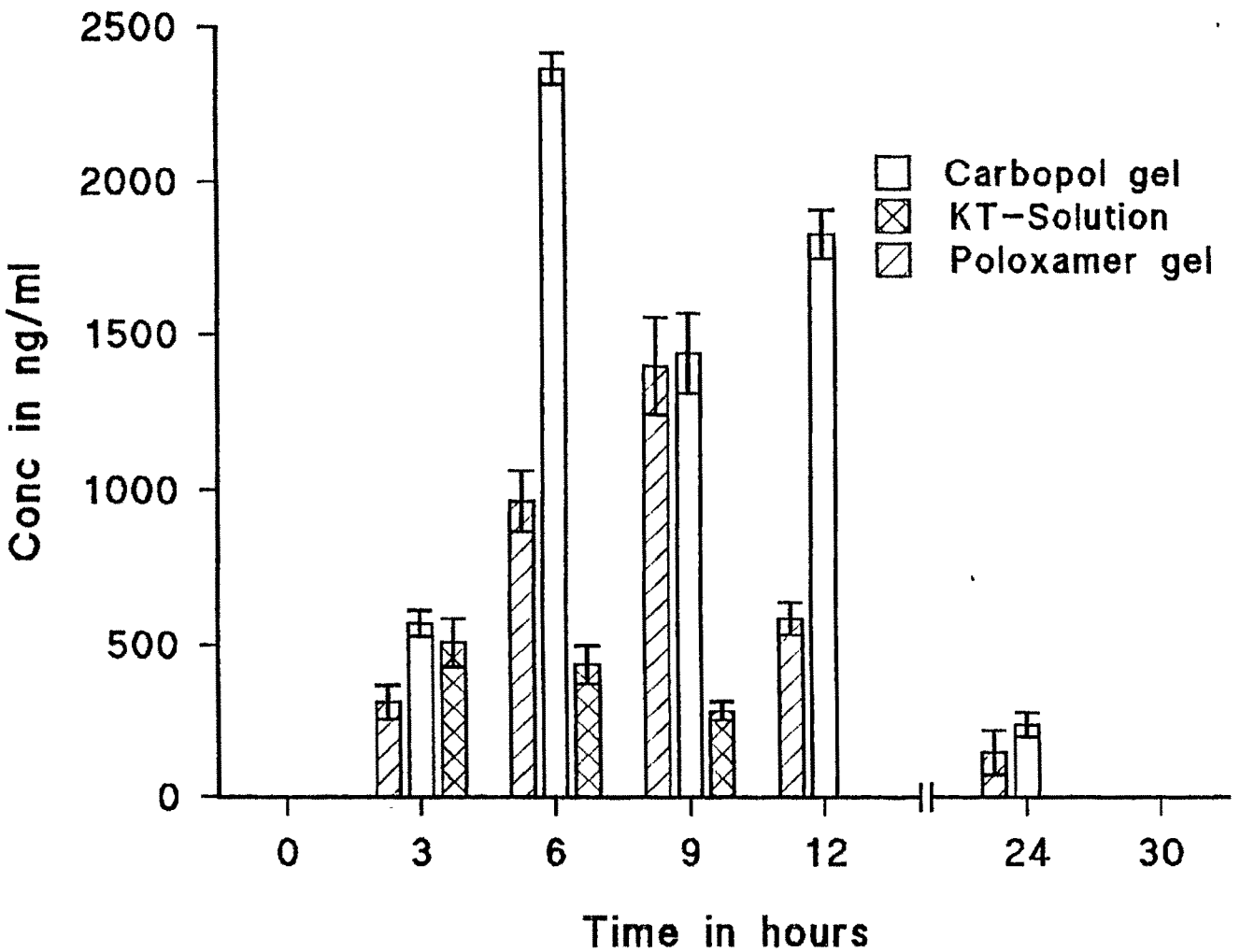
Each point represents Mean ± S.E.M (n=3)

The AUC obtained after single dose administration of Carbopol based KT gel was approximately 14 times greater than the conventional solution, whereas with Poloxamer based KT gel, 4 time increase in AUC was observed. Schoenwald et.al have shown that, the Carbopol gel increased the area under the aqueous humor curves for prednisolone sodium phosphate by 10 times, as compared to the aqueous solution preparation[125].

Since ketorolac tromethamine is known to penetrate the cornea in both ionized and unionized form, in case of gel, more amount of drug can have access into cornea till the saturation of the layers in the cornea occurs. Later on, the cornea acts as reservoir for the drug and releases the drug into the aqueous humor slowly. This hypothesis is evident from our gel systems as drug levels were seen in the aqueous humor even after 24 hours in case of Carbopol gel. Since ketorolac tromethamine is a water soluble drug, any formulation that increases its contact time in the eye should increase the drug concentration in aqueous humor theoretically.

After multiple dosing of conventional solution, the concentration of drug in the aqueous humor was comparable to that of single dose of the Carbopol based KT gel till 24 hours (Fig.22). On dosing the conventional solution repeatedly, a steady increase in the aqueous humor drug concentration was observed, which justifies the frequency of instillation of the drops. However, with multiple dosing of the conventional solution, levels rise at a slow rate, but with a single dose of the Carbopol gel, high concentrations were attained much faster. The high Cmax values obtained with single dose of the Carbopol gel was hit by the conventional solution after the fourth dose. This shows that the Cmax observed after administration of Carbopol gel is safe.

Fig. 22 Comparison of Aqueous Humor Concentration of KT Following Single Dose of the Gel and multiple dose of conventional solution



Each point represents Mean ± S.E.M (n=4)

In case of Poloxamer based gel the aqueous humor drug concentrations were detectable till 9 hours and the C_{max} obtained was less as compared to multiple dose of KT solution (Fig.22).

From the above results it could be concluded that with Carbopol and Poloxamer based ketorolac tromethamine gel, the dosing frequency can be greatly reduced or the amount of dose can be decreased. Moreover, these dosage forms are absolutely non-irritating and thereby improves patient compliance.