Review Article

Method Development and Validation for Ophthalmic Formulations Containing Antibiotics: A Comprehensive Review

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Bacterial conjunctivitis commonly referred to as pink eye, having an inflammation of the conjunctiva either due to infection or an allergic condition. The outer lining of the white part of our eye gets reddish or pink in color. Ophthalmic antibiotics are instilled for curing this condition. Many ophthalmic formulations are available in the market for curing this condition. Most of them contain an antibiotic along with a steroidal moiety. Method development plays a pivotal role in drug testing, manufacturing operations, stability studies and the long term performance of drugs. International Council for Harmonisation provides guidelines for validating analytical methods. With minimal use of time and money scientists all over the world are developing new methods for the same. Several studies have already been done for the method development of such ophthalmic solutions but still, some antibiotics remain untouched. This review article emphasizes detailed information regarding the methods developed for ophthalmic formulations and their validation as well.

Key words: Antibiotic, conjunctivitis, method development, pink eye, validation

Conjunctivitis is an eye condition in which there's inflammation of the conjunctiva. It can be due to viral infection, bacterial infection, allergies, eye cancer, immune-mediated or some toxicity. It is an inflammation of the conjunctiva, which is the outer lining of the eyeball. Ocular infections are considered to be minor but they can be "vision-threatening". Bacterial conjunctivitis is a self-restricting disease[1,2]. The most common causative agents for infectious disease are *Staphylococcus epidermis* (39 % of cases), *Staphylococcus aureus* (22 % of cases) and *Streptococcus pneumonia* (6 % of cases) as shown in fig. 1. The most common Gram-negative microorganism found in acute conjunctivitis is *Haemophilus influenzae* (9 % of cases). In contact lens wearers, the trend is reversed and more Gram-negative strains are found[3,4].

It was found that in neonatal cases causative agents for bacterial conjunctivitis are *Chlamydia trachomatis*, *Neisseria gonorrhoeae*. In most cases in children, *Moraxella catarrhalis*, *Staphylococcus epidermis*, *Streptococcus viridians* are the causative agents. It represents one of the most common ocular diseases in childhood, affecting 1 in 10 children each year. It can occur at any age, most frequently in school-going children[5]. Most of the registered medical practitioners prescribe topical antibiotics for minimizing the complication and re-occurring of infection[6]. Risk factors for bacterial conjunctivitis have been mentioned in fig. 2.

Adenoviral conjunctivitis is a major cause of acute infectious conjunctivitis among adults. Bacterial conjunctivitis, is also a major cause for so many cases among children[7]. There are other causes for eye infections like foreign objects in the eye, trauma to the eye, chemical substances entering the eye. Acute bacterial conjunctivitis resolves within 7-10 d but a broad-spectrum antibiotic can decrease its severity, transmission and complications[8,9].

Allergic conjunctivitis is typically treated with antihistamines and mast cell stabilizers, but if symptoms persist, therapy is supplemented with

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topical steroids[10]. According to the American Academy of Ophthalmology, the most prominent symptom of pink eye is a greenish discharge that lasts all the day. Redness and itching occur along with it. Corticosteroids are used to treat inflammatory conditions of the eye. A combination of antibiotics along with corticosteroids helps in relieving inflammation and infection of the eye[11,12]. Common symptoms of this disease are itchy and watery eyes or red and swollen eyes with mucous discharges. In the case of severe pain in the eyes, one should seek immediate medical advice from a registered medical practitioner[13]. Other measures which can be taken along with like washing hands properly, not rubbing their eyes, not sharing things, eye glasses and handkerchiefs with others and to wear eye glasses to protect your eyes from sunlight, wind and dust particles and to keep clothing and personal belongings clean[14,15]. Steps for prevention of red eyes are shown in fig. 3. In this study, High Performance Liquid Chromatography (HPLC) method development and validation for ophthalmic formulations has been briefed.

OPHTHALMIC ANTIBIOTIC FORMULATIONS

Antibiotics such as ciprofloxacin, ofloxacin, gentamicin and other medications that are effective against pink eye infection are found in these ophthalmic preparations. The most often used first-line agents include sodium sulfacetamide, chloramphenicol, gentamicin, tobramycin, azithromycin, neomycin, trimethoprim, ciprofloxacin, gatifloxacin and doxycycline[16,17]. Table 1 enlists all antibiotics used in bacterial conjunctivitis. Immediate diagnosis and treatment are important to avoid vision-threatening outcomes, including corneal scarring or perforation. The choice of treatment is predicated on the use of a single antimicrobial or a combination of antimicrobials that provide a broad range of activity against both gram-positive and gram-negative bacteria[18,19]. The advantages of instilling antibiotic eye drops are to preserve visual acuity, prolonged contact and soothing effect. According to Pujalte *et al.*[20] microorganisms which are resistant to a systemic antibiotic are effectively treated by the topical route of administration due to the higher local concentrations induced by this route of administration[21].

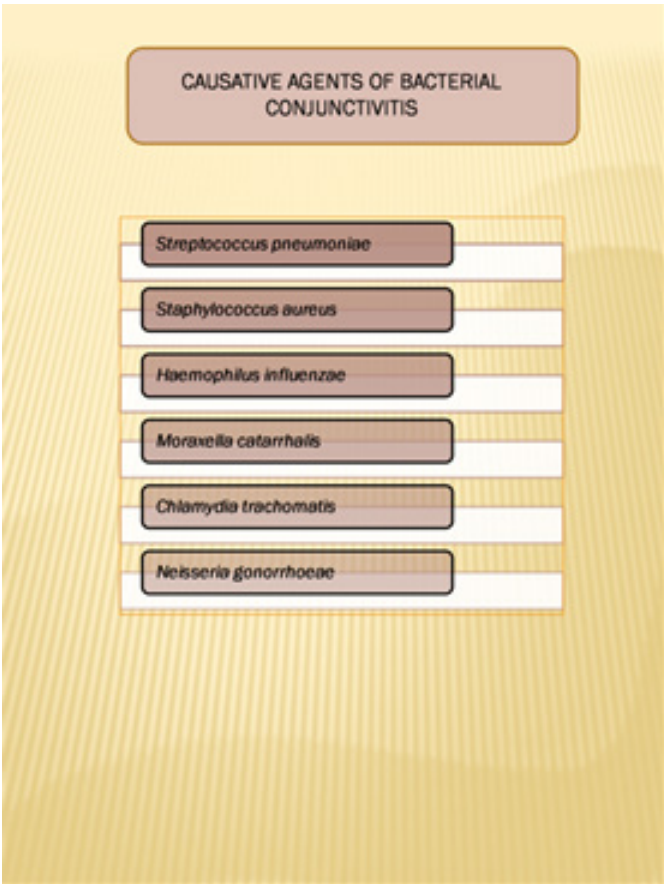

Fig. 1: Causative agents of Bacterial Conjunctivitis

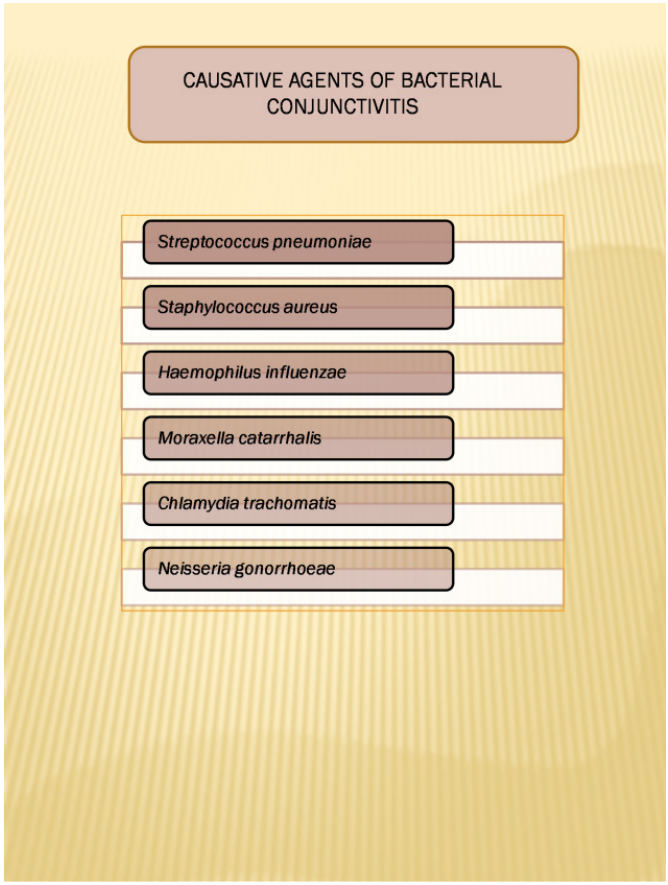

Fig. 2: Risk factors for bacterial conjunctivitis

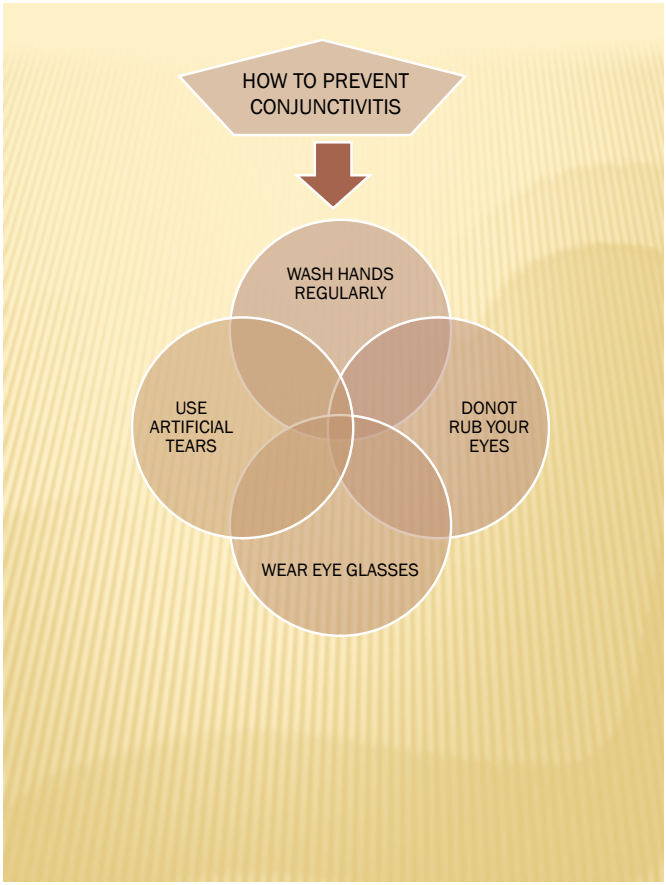

Fig. 3: Steps for preventing red eyes

TABLE 1: THE LIST OF ANTIBIOTICS USED IN OPHTHALMIC FORMULATIONS

Sno	Class of drug	Name of drug	Drug in combinations	Brand names	Mechanism of action	Reference
01	Aminoglycosides	Tobramycin	Tobramycin-loteprednol	Zylet, Loto – T	Prevent growth of bacteria and reduces inflammation of eyes	[7]
		Gentamycin	Gentamycin - prednisolone	Gentapar, Genwell	Inhibits the growth of bacteria	[9]
		Neomycin	Neomycin-dexamethasone-polymyxin B	Maxitrol, Polyzen – D	Inhibts growth of bacteria and reduces swelling of eyes	[9]
2	Macrolides	Azithromycin	Azithromycin - dexamethasone	Azidex, Kapiaz-D	Inhibts the growth of bacteria along with decreasing inflammation of eyes	[9,17]
		Erythromycin	Erythromycin-mineral oil-white petroleum	Pertigo, Bausch & Lomb	Inhibts growth of bacteria	[16,23]
3	Quinolones	Ciprofloxacin	Ciprofloxacin - dexamethasone	Cipwell, Ciplox	Inhibts growth of bacteria	[23]
		Moxifloxacin	Moxifloxacin - dexamethasone	Moxicip, Mahaflox	Inhibts growth of bacteria	[23,26]
		Gatifloxacin	Gatifloxacin - dexamethasone	Gatiquin, Zymar	Inhibts growth of bacteria	[23,26]
		Levofloxacin	Levofloxacin - prednisolone	Levokap – P, LV Flox	Inhibts growth of bacteria	[26]
		Lomefloxacin	Lomefloxacin	Lyflox, Lomibaq	Inhibts growth of bacteria	[9,26]
		Norfloxacin	Norfloxacin – benzalkonium chloride	Norflox, Nflox	Inhibts growth of bacteria	[12]
		Ofloxacin	Ofloxacin – benzalkonium chloride	Oflox, Oflacin	Inhibts growth of bacteria	[18]
4	Others	Chloramphenicol	Chloramphenicol - dexamethasone	Clowell-D, Chlorocol	Inhibts growth of bacteria	[12]
		Fusidic acid	Fusidic acid – mannitol-benzalkonium chloride	Fucidin, Fucithalmic	Inhibts growth of bacteria	[15]
		Rifamycin	Oral	Myrifa 200, Rifwell 300	Inhibits bacterial infection if any blood	[23]
5	Tetracyclines	Chlortetracycline	Chlortetracycline – benzalkonium chloride	Chlotracare, Chlorcol H	Inhibts growth of bacteria	[23]
		Tetracycline	Tetracycline – benzalkonium chloride	Kaytet, Terramycin	Inhibts growth of bacteria	[9,23]

Uncomplicated cases can be treated with a topical antibiotic such as tobramycin, trimethoprim/polymyxin B, a fluoroquinolone or chloramphenicol four times daily for 5-7 d to accelerate recovery and under the observation of a physician every 2-3 d until signs and symptoms are resolved[22]. Aminoglycosides act by inhibiting protein synthesis by binding to both the 16S (in the 30S subunit) and 23S (in the 50S subunit) rRNA molecules of the bacterial ribosome[10,23]. Fluoroquinolones/quinolones prevent bacterial DNA from unwinding and duplicating. Macrolides bind to bacterial ribosomes to inhibit protein synthesis. Chloramphenicol is bacteriostatic in nature[24,25]. It inhibits protein synthesis by deactivating the functioning of peptidyl transferase[26,27]. Tetracyclines are protein synthesis

inhibitors that bind to the 30S ribosomal subunit. Rifamycins inhibit DNA-dependent RNA synthesis. Fusidic acid is a bacteriostatic antibiotic[28,29]. Fig. 4 shows chemical structures of these drugs.

METHOD DEVELOPMENT AND VALIDATION FOR OPHTHALMIC FORMULATIONS

Analytical method development and validation play a vital role in drug discovery, development and production of prescribed drugs[30]. It is the process of proving that the method developed is appropriate for its use in the development, production, and testing of pharmaceutical drugs. As outlined by United States Pharmacopeia (USP), method validation provides an assurance of reliability during regular use. It is generally defined as the process of providing documented evidence that the method does what it is intended to do[31,32]. Validation helps in meeting all desired characteristics of the analytical method. International Council for Harmonisation (ICH) (Q2-R1) guidelines are available for validating an analytical method. It assures a great impact on quality assurance and cost reduction[33,34]. Validation is required in the situations when the process is totally new, while installing and using new equipment, any process or piece of equipment that is subject to changes in usage as a result of altered conditions[29,35] and when process in which the test of the end product is not good, gives an indicator of poor quality.

For the analysis of ophthalmic formulations, inwardly developed or significantly modified methods are sometimes used[36,37]. Method development provides critical information about a drug's potency, bioavailability, stability and effects. There is a lack of regulatory guidance on *in vitro* release testing methods. Method development can also be used to check the compatibility of the active drug with the formulation matrix. Optimization based on a trial and error approach is very time-consuming. Nowadays, software-assisted and automated method development can speed up the process of validation[38]. The development of methods for drug analysis in ophthalmic formulations is required when a new drug combination is developed that is not available in the Pharmacopoeia, and for existing drugs, less reliable methods for their analysis are available based on information provided by a review of literature. When formulation excipients such as benzalkonium chloride, hydroxyethylcellulose and polyvinlyalcohol create interference, no analytical technique has yet been established[39]. So yet, no analytical technique for quantifying analytes in biological fluids has been established. The existing analytical procedures involve the use of costly reagents and solvents. Development of method is also needed for routine analysis of drugs in ophthalmic formulations, to analyze the total content of formulations and to perform an assay of ophthalmic formulations[40]. There are no methods developed for checking the permeability of drugs into the cornea in the case of poorly soluble drugs. The same can also check for impurities and excipients interfering[41]. Disposable systems utilized while manufacturing of ophthalmic formulations also need validation. The method developed is validated for various concentrations of active drugs so that any changes in formulation or concentration do not require additional validation. There are some steps for developing HPLC methods[42,43] for ophthalmic formulation as shown in the fig. 5.

As per guidelines of ICH[44-47], the developed methods will be validated for following parameters like accuracy, precision, specificity, limits of detection, linearity and range and robustness. Accuracy is defined as the closeness between true value and reference value. Specificity means to assess analyte in presence of other components. Precision is the most important parameter which defines closeness between different values obtained. Repeatability means to do the same experimental work with complete precision under same operating conditions. Reproducibility should be achieved while working in different laboratories. The experimental work done should be able to produce results of various tests done.

The lowest amount of sample which can be obtained quantitatively is known as Quantitation limit. The measure of capacity of method to remain unaffected is known as robustness of that method. Table 2 shows various validation parameters according to ICH and USP guidelines. Validation comprises of minimum five steps including planning and performing the tests; statistical evaluation of results; report on the validation parameters; application of all information; full validation processes along with their explanations. Analytical method validation has an important role in having best quality and safety of the final product.

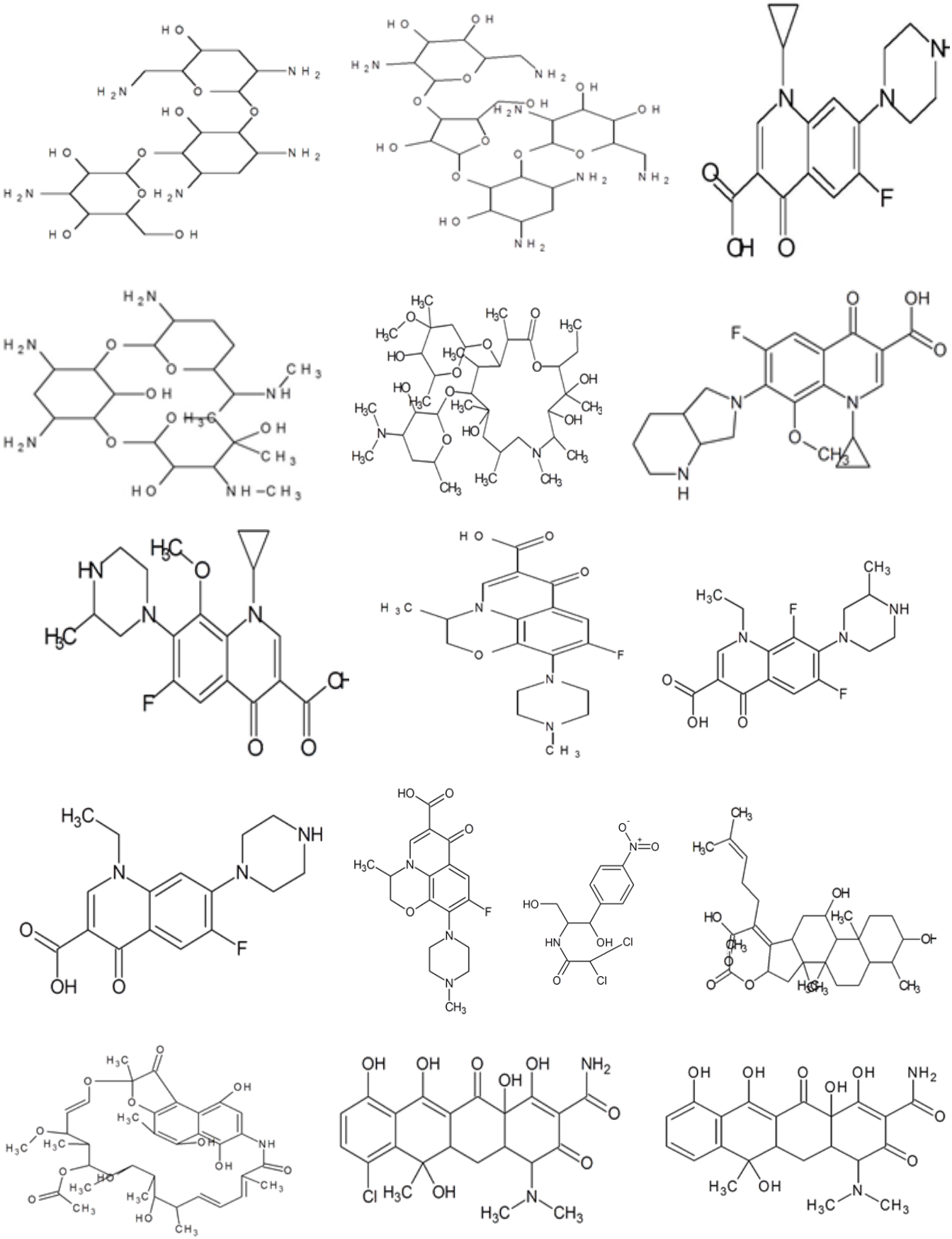

Fig. 4: Chemical structures of antibiotics used in ophthalmic formulations
Note: (a): Tobramycin; (b): Neomycin; (c): Ciprofloxacin; (d): Gentamycin; (e): Azithromycin; (f): Moxifloxacin; (g): Gatifloxacin; (h): Levofloxacin; (i): Lomefloxacin; (j): Norfloxacin; (k): Ofloxacin; (l): Chloramphenicol; (m): Fusidic acid; (n): Rifamycin; (o): Chlortetracycline; (p): Tetracycline

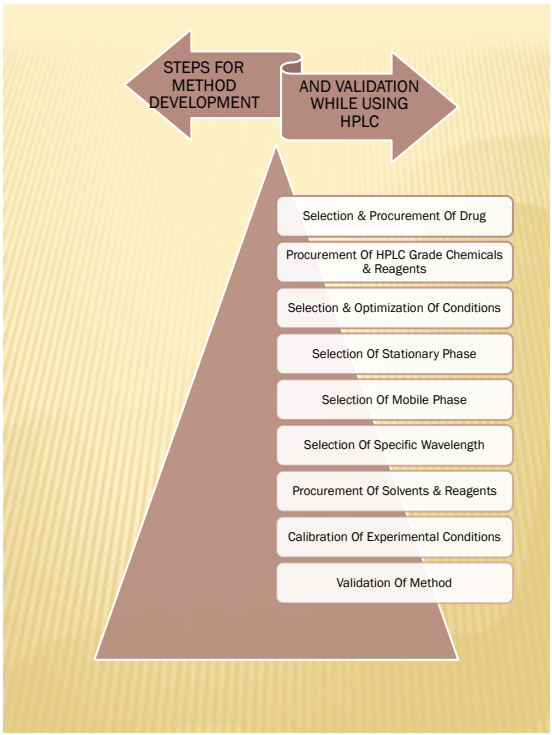

Fig. 5: Steps for HPLC method development

TABLE 2: VALIDATION PARAMETERS

ICH	USP
Specificity	Specificity
Linearity	Linearity and range
Range	Accuracy
Accuracy	Precision
Precision	Limit of detection
Limit of detection	Limit of quantification
Limit of quantification	Ruggedness
---	Robustness

METHOD DEVELOPED FOR OPHTHALMIC PREPARATIONS

Typically, ophthalmic formulations are subjected to forced degradation, stability, and drug determination studies[48-54]. Many drugs used for ophthalmic formulations have undergone such testing. Antibiotics are the core heart of ophthalmology. Following are the highlights of the HPLC methods already developed for the ophthalmic formulations which are used for bacterial conjunctivitis.

HPLC methods:

Khalil *et al.*[55] developed a Reverse Phase-HPLC (RP-HPLC) technique for chloramphenicol and its hydrolytic derivatives. In this study, eight commercial batches of ophthalmic solutions were used. All of these batches were kept at room temperature. A Waters 600E system controller, a Waters 715 ultra WISP sample processor, a Waters 991 photodiode array detector, and a PDA integrator were utilized. A reverse phase C-18 5u (30 mm×4.6 mm i.d.) column was used. Water, methanol and ammonia make up the mobile phase (58:40:2). Using glacial acetic acid, the pH was adjusted to 7. The flow rate was 1.5 ml/min and the wavelength was 278 nm. Chloramphenicol was acid hydrolyzed to produce the hydrolytic product (2-amino-l-(4-nitrophenyl)-propane-l-diol hydrochloride). There was no influence from preservatives or contaminants. The procedure devised was found to be more precise and accurate.

Muralidharan *et al.*[56] devised a new RP-HPLC technique for determining ketotifen fumarate. Thermo C18 (250 mm×4.6 mm id) was employed in this isocratic RP-HPLC technique. The mobile phase was made up of methanol and 10 mM ammonium acetate (30:70 % v/v, pH=3.5). The flow rate was 1

ml/min, and the effluent was measured at 298 nm. The average retention time was 5 min. For accuracy, specificity, linearity, robustness and precision, the devised method was validated.

Erk *et al.*[57] developed an HPLC technique for determining dorzolamide hydrochloride and timolol maleate in eyedrops at the same time. A diode array detector was employed in this approach at two fixed wavelengths, 250 nm for dorzolamide and 300 nm for timolol maleate. The chromatographic separation was performed on an RP-YMC pack ODS A-132 C18 (5 micron, 15 cm×6.0 mm) column with an acetonitrile:phosphate buffer (pH 2.5):methanol (5:85:10 v/v/v) mix with a flow rate of 1.2 ml min-1.

Razzaq *et al.*[48] developed and validated a new RP-HPLC technique for the simultaneous detection of gatifloxacin and ketorolac tromethamine in combination dose form. For detection at 270 nm, a Shimadzu LC-20A system was used in conjunction with an SPD-M20A Ultra-violet (UV) detector. For this chromatographic separation, a BDS Hypersil C8 column (250 mm×4.6mm) was utilised. At a flow rate of 1.5 ml/min, the mobile phase was composed of methanol:phosphate buffer (55:45 v/v). The technique was linear at concentrations of 30-90 g/ml (for gatifloxacin) and 50-110 g/ml (for ketorolac tromethamine). Both analytes separated well, with adequate tailing and resolution. This created approach may be used on a regular basis for analysis.

Ruckman K *et al.*[58] assessed ophthalmic formulations containing Tobramycin using RP-HPLC with a UV detector set at 210 nm. It was discovered to be simple, precise, accurate and quick in nature. The error rate was 0.80 percent, while the Relative Standard Deviation (RSD) was less than 2.0 percent. The mobile phase was buffered with 0.05 M diammonium hydrogen phosphate and pH was adjusted to 10.0 using tetramethyl ammonium hydroxide. Purosphere RP-8e column (250 mm×4.6 mm, 5 m) was utilised. At 1.0 ml/min, isocratic elution was performed. Excipients and mobile phase did not cause any interference. This technique may be utilised for routine ophthalmic analysis and quality control. For the measurement of ciprofloxacin in serum, aqueous humour, and ocular drops, a bioanalytical technique for RP-HPLC was devised by Khan A *et al.* [59] the mobile phase consisted of acetonitrile and 0.25 M phosphoric acid (60:40 v/v) in a Perkin Elmer series 200 HPLC fitted with a UV-visible detector. The wavelength of the UV-visible detector was set to 275 nm. The flow rate was 1 ml per min. The calibration curves linearity was tested from 5-75 ng/ml. The r2 values for mobile phase, serum and aqueous humour were determined to be 0.999, 0.997 and 0.998 respectively. Endogenous compounds did not cause any interference. A simple, selective and isocratic RP-HPLC technique for analysing moxifloxacin hydrochloride in the presence of its degradation products was devised and validated by Dewani *et al.*[60]. It makes use of a thermo separation product system linked to a quaternary pump system 4000. A UV detector with a wavelength of 294 nm was utilised. For chromatographic separation, a Grace C18 column (250 mm×4.6 mm) was utilized. At a flow rate of 1 ml/min, the mobile phase is composed of 10 mM sodium phosphate buffer and methanol (60:40, v/v) with a pH of 4.4. The regression coefficient was determined to be 0.999. This method's system adaptability, linearity, precision and accuracy were all confirmed[60].

For the examination of natamycin 5 % w/v eye drops, a RP-HPLC technique was devised by Chaudhari *et al.*[61]. For chromatographic separation, Perkin Elmer and a series 200 UV-Visible detector were employed. C18 column (100×4.6 mm, 5 m) was used, and sample was injected using a Rheodyne injector valve with a 20 l sample loop. The mobile phase is made up of two solvents, solvent A (methanol) and solvent B (pH 3.5 buffer). 1.36 gm potassium di-hydrogen phosphate and o-phosphoric acid were used to make the buffer (HPLC grade). UV detection was performed at a wavelength of 304 nm (λ_{max}). The retention time was discovered to be 8.96 min. Natamycin was exposed to a forced degradation procedure.

An HPLC technique for Besifloxacin hydrochloride was developed by Costa *et al.*[62] using an Agilent liquid chromatograph (model Q), 1311A quaternary pump, ALS-G1329 auto sampler, TCC-G1316A column oven, and G1315B photodiode array detector. It was an Agilent Eclipse plus C18 column (150 mm×4.6 mm, id 5 m) that was utilised. The mobile phase is composed of 0.5 % triethylamine solution and acetonitrile (74:26, v/v), with the pH adjusted to 3.0 using 10 % phosphoric acid. The injection volume was 20 l and the flow rate was 1.0 ml/min. UV detection was performed using a photodiode array at a wavelength of 295nm (λ_{max}). This approach was compared to the drug's microbiological test method. There was no statistical difference between the two approaches. Both techniques were quite

durable. The linearity, specificity, precision and accuracy of the HPLC technique were all confirmed. For the simultaneous measurement of Moxifloxacin hydrochloride and dexamethasone, a simple and sensitive RP-HPLC technique was devised by Razzaq *et al.*[48] . Shimadzu LC-20A system with LC-20AT pump and SPD-M20A diode array detector at 254 nm comprised the HPLC system. The stationary phase was a BDS Hypersil C8 column (250×4.6 mm, id 5 m) with a mobile phase consisting of phosphate buffer (20 mM) containing 0.1 % (v/v) triethylamine, pH 2.8, adjusted with dilute phosphoric acid and methanol (38:5:61.5 v/v) at a flow rate of 1.5 ml/min. This technique was verified for specificity, linearity, accuracy, and precision in accordance with ICH standards.

CONCLUSION

The foundation of ophthalmology is ophthalmic formulations. Analytical investigations have made use of a variety of methods, as well as method development. Almost majority instances of acute bacterial conjunctivitis are self-limiting and will resolve within 10 d with topical antibiotic eye drop treatment. As a result, illness transmission is reduced and disease occurrences are lower in the community. Method development and validation is a crucial stage in analyzing a pharmaceutical formulation component and conducting stability tests, and it is suggested for all newer medication investigations.

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Conflict of interest:

The authors report no conflicts of interest.

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