Development of a cysteamine in situ gelling system for the treatment of corneal crystals in cystinosis

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List of Acronyms

Acronym	Description
AMQ	6-aminoquinoline
AQC or ACQ	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
BAC	Benzalkonium Chloride
BOB	Benzododecinium Bromide
CMQT	2-Chloro-1-Methylquinolinium Tetrafluoroborate
CRN	Cystinosis Research Network
CT Scanning	Computerised Tomography scanning
CTNS	Cystinosis Nephropathic
CYS	Cysteine
EDTA	Disodium edetate
GC	Gas Chromatography
GG	Gellan Gum
HPLC	High Performance Liquid Chromatography
LC/MS/MS	Liquid Chromatography Mass Spectrometry Mass Spectrometry
LC	Liquid Chromatography
MRI	Magnetic Resonance Imaging
MS/MS	Tandem Mass Spectrometry
NHS	N-hydroxysuccinimde
NMR	Nuclear Magnetic Resonance
SH-group	Sulfhydryl group
STF	Simulated Tear Fluid
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
UV	Ultra Violet

Abstract

Cystinosis is a rare autosomal recessive condition, characterised by excessive cystine accumulation within the lysosomes of various cells in the body, such as the kidneys and eyes. The ocular symptoms include photophobia and blepharospasm, if left untreated blindness can occur in patients.

The main drug treatment of choice is cysteamine, a cystine-depleting agent. The topical cysteamine eye drops in use in the UK is prescribed for administration four to six times a day but in practice, it can be administered almost each waking hour.

The overall aim of this project is the formulation and *in vitro* / *in vivo* evaluation of a Cysteamine ophthalmic preparation in a polymeric vehicle. The formulation will be evaluated *in vitro*, *in vivo* in rabbits and finally clinically. The rationale is that increased ocular contact time of the drug by the using an *in situ* gelling and mucoadhesive polymer will reduce lacrimal drainage and thereby increase bioavailability. This should lead to decreased frequency of administration that would improve compliance greatly and decrease morbidity linked to actual treatment.

In order to achieve our aims, the initial objectives focused on formulation of a new cysteamine ophthalmic preparation and developing a robust stability indicating analytical method to assay cysteamine within the formulation

The ideal ophthalmic preparation is one that can be instilled into the eye(s) as a drop, with no blurring effects and a frequency of administration not exceeding twice a day. Preliminary experiments suggested that ionic strength dependant polymer Gellan gum exhibited the best in situ gelling profile. Presently the influence of different necessary excipients such as: tonicity-adjusting agents, antimicrobial preservative, pH buffering agent and an anti-oxidant is being investigated on rheological behaviour in the presence or absence of simulated tear fluid with or without mucin.

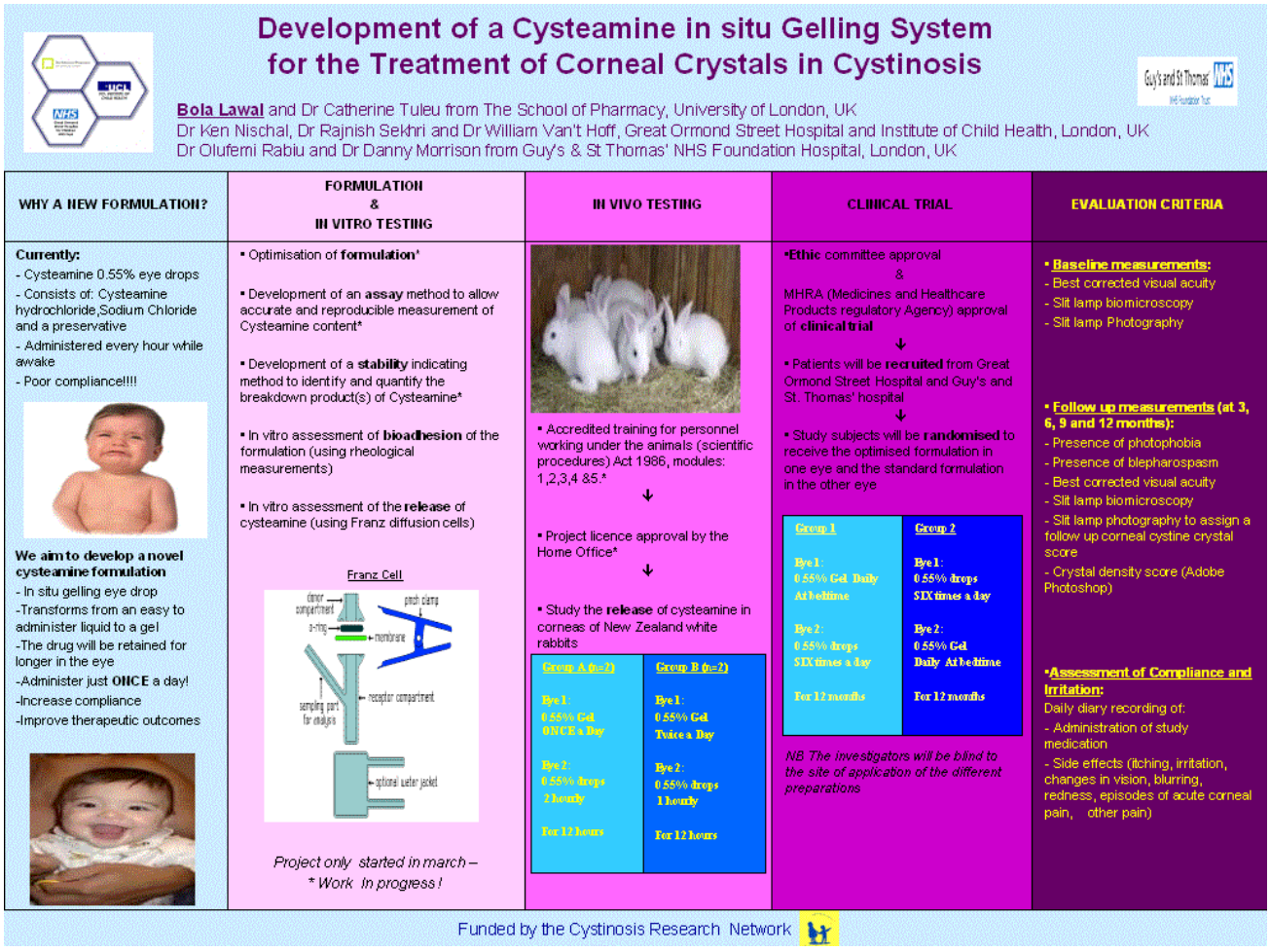
It was found that the drug itself Cysteamine hydrochloride 0.55% caused Gellan gum 0.6% based formulations to form very stiff gels, while Benzalkonium chloride was the only other main excipient that also increased the viscosity of the formulation due to the formation of weak gels. A reduced concentration of Gellan gum as well as mixing with other polymers such as carbopol, alginates and chitosan are under investigation.

Cysteamine lacks a chromophore. It has to be altered in a way that enables the entire new moiety to transmit signals that can be received and quantified by a detector. Two different pre-column derivatization methods were tested: Waters AccQTag method and CMQT pre-column derivatization. Despite the Waters method being a commercial kit, the results were inconclusive and the CMQT derivatization with HPLC UV detection is under further and promising investigation.

The animal project licence application have been prepared and submitted to the ethics committee for approval. An overview is included in this report.

Introduction

The overall objective of this project is to develop and test a new formulation of ocular topical cysteamine, to produce a once or twice daily delivery for cystinotic patients. The rationale is that increased ocular contact time of the drug using an in situ gelling and mucoadhesive polymer will reduce lachrymal drainage and thereby increase bioavailabilty with ease, dose accuracy and reproducibility during application. This should lead to decreased frequency of administration and potentially improved compliance. The aims are to formulate a cysteamine ophthalmic preparation in a polymeric vehicle to achieve the aforementioned goals. It will be tested *in vitro* (characterisation and drug release), *ex vivo* and then *in vivo* in rabbits prior to clinical testing in patients with cystinosis. The poster presented at the CRN conference 2007 illustrates the overall project.



During the 1st year of this project, 3 aspects of the work were undertaken in parallel and these are:

- Pre-formulation and formulation work of the new cysteamine ophthalmic preparation.
- Evaluation of analytical methods of cysteamine and its main degradant cystamine.
- Preparation for the animal experimentation: training (modules 1,2,3,4,5), project licence and personal licence applications.

1. Cystinosis

1.1 Background

Cystinosis is a rare autosomal recessive condition. It is characterised by excessive cystine accumulation within the lysosomes of various cells in the body (Kaiser-Kupfer, Gazzo et al. 1990). Cystine is the disulfide of the amino acid Cysteine as seen in Figure 1.

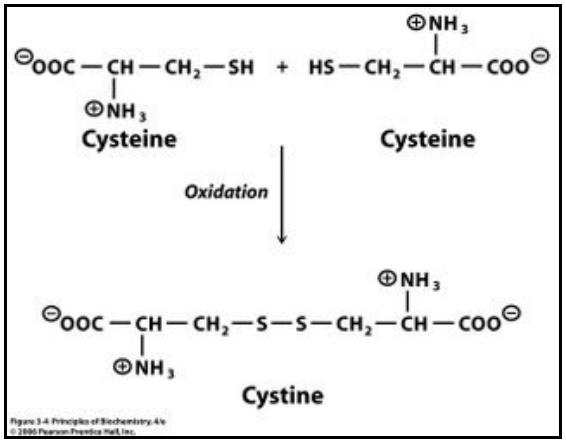
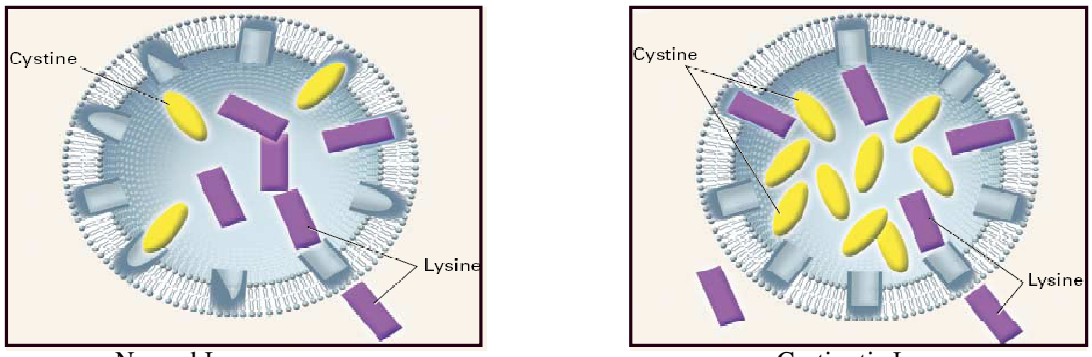


Figure 1: Chemical formation of cystine from cysteine molecules

(Sandwalk.blogspot.com)

The free (non-protein bound) cystine is poorly soluble, reaches very high concentrations and crystallizes within the lysosomes of cells in various tissues, including the kidneys, bone marrow, intestine, liver, spleen, pancreas, thyroid, lymph nodes, retinal pigment epithelium, conjunctiva and corneas (Kaiser-Kupfer, Fujikawa et al. 1987).

The disorder is due to mutations in the CTNS gene that encodes cystinosin, the lysosomal cystine transporter. Defective or absent cystinosin function leads to an accumulation of cystine within lysosomes as shown in figure 2. This accumulation is due to non-transportation of cystine across the lysosomal membrane into the cytoplasm (Bellinda, Huang et al. 2003).



Normal Lysosome Cystinotic Lysosome

Figure 2: Membrane activity within a normal and cystinotic lysosome (Gahl, Thoene et al. 2002)

There are three clinical forms of cystinosis, based on the age at onset, severity of symptoms and level of cystine accumulation at diagnosis: infantile (nephropathic), intermediate (adolescent, late-onset), and adult (benign). Nephropathic cystinosis is a progressive disease and is the most common and most severe, it has been estimated to affect one of every 100,000 to 200,000 children (Elenberg 2006).

Without treatment patients develop chronic renal failure and multisystem damage. Although the first symptom of cystine accumulation is manifest in the kidney, the eyes are involved early in the course of the disease.

1.2 Genetics

Cystinosis is transmitted as an autosomal recessive trait. Multiple observations indicate that the same gene is involved in all forms of the disease (Devonald, Karet 2004). Both the infantile and adolescent forms can occur in a given family. It appears that there is no genetic heterogeneity and that the differences in the clinical manifestations result from mutations occurring in a single gene (allelic mutation). The gene for nephropathic cystinosis has been mapped to chromosome 17p13 and identified (Van't Hoff, Town 1999). The gene, CTNS, encodes for a 367 amino acid lysosomal membrane protein, named cystinosin (Haq, Kalatzis et al. 2002). A large number of mutations have been described in patients with cystinosis, with the clinical phenotype segregating with specific defects.

In infantile cystinosis, large deletions, as well as other mutations that would result in miss sense or in-frame deletions, are associated with infantile cystinosis, the most severe phenotype (Attard, Jean et al. 1999). Among those with such defects in both alleles, these abnormalities may result in the loss of adequate protein function, including no protein expression with some mutations (Forestier, Jean et al. 1999). A 65 kb deletion is the most frequent mutation found in the homozygous state, being observed in nearly one-third of patients with cystinosis. This deletion is present in either the homozygous or the heterozygous state in approximately 75 percent of patients of European origin. By comparison, a 9.5 to 16 kb deletion has only been infrequently observed.

Intermediate cystinosis is more indolent than the other forms of cystinosis. This appears to be due to the inheritance of a mutation known to cause infantile disease in one allele and a relatively less clinically severe mutation in the other, or the inheritance of a relatively less severe mutation in both alleles (Anikster, Shotelersuk et al. 1999).

Adult or benign (or ocular non nephropathic) cystinosis, which is characterized by the presence of corneal crystals and photophobia but no renal disease, has been associated with additional mutations. Similar to intermediate cystinosis, this form may be due to the inheritance of different abnormal alleles, including the presence of a severe plus a mild mutation, the latter of which does not adversely affect kidney function (Forestier, Jean et al. 1999).

1.3 Clinical Features of Cystinosis

1.3.1 Nephropathic/Infantile Cystinosis

Cystine crystals formed in the proximal tubule cells interferes with the normal function of the renal tubular cells, this in turn manifests as clinical symptoms (renal Fanconi syndrome) (Kalatzis, Antignac 2003).

Fanconi syndrome develops between 6 and 18 months of age and is characterized by failure to re-absorb water, sodium, potassium, bicarbonate, glucose, phosphate, calcium, magnesium, amino acids, carnitine and other small molecules (Kaiser-Kupfer, Fujikawa et al. 1987). This causes normal anion gap hyperchloremic acidosis (Elenberg 2006), dehydration, electrolyte imbalance, failure to grow/thrive (growth retardation), and hypophosphatemic rickets.

The disease is fatal if left untreated; end stage renal failure ensues by 7-10 years of age and death occurring between the twenties and thirties. If treatment is started as soon as diagnosis is confirmed, the progression of the disease can be markedly reduced, however if there is already damage to the renal tubular cells, it is irreversible and there might be a need for renal transplant in the future (Cairns, Anderson et al. 2002).

The eyes are involved early in the course of the disease. Cystine deposition occurs in all tissues of the eye, including the cornea and conjunctiva. This can be seen on a slit-lamp examination as refractile crystals (Gahl, Kuehl et al. 2000). Deposition of cystine crystals in the iris, ciliary body, ciliary processes and sclera using high frequency ultrasound has also been shown (Mungan, Nischal et al. 2000).

Cystine crystals begin to deposit in the superficial layers of the cornea progressing towards the endothelium and also in the periphery of the cornea progressing towards the center. The density of these crystals is minimal at 6-12 months of age, increasing to a moderate level between 2-4 years of age, reaching a maximum level at 6-10 years. These deposits are responsible for epithelial surface breakdown leading to photophobia, watering, and blepharospasm, which are universal features after the first decade of life. Irregular and peripheral depigmentation of the retina is also an early finding and may lead to retinopathy resulting in visual morbidity (Gahl, Kuehl et al. 2000). The advent of renal transplantation has resulted in better control of these patients' systemic condition which has resulted in further ocular complications becoming apparent such as glaucoma, a mechanism for which has been postulated based on cystine deposition in the ciliary body and iris root (Mungan, Nischal et al. 2000).

1.3.2 Intermediate/Adolescent Cystinosis

Intermediate/adolescent cystinotic patients present later with renal symptoms, at 8-12 years of age. The onset of the symptoms of Fanconi syndrome is much slower and end stage renal failure transpires after age 15 (Elenberg 2006). The patients do not

suffer from severe tubulopathy or growth retardation (Kalatzis, Antignac 2003). Ocular symptoms are similar to that which presents in the infantile form, but often with less severity.

1.3.3 Adult/Benign Cystinosis

Adult/benign cystinotic patients are often asymptomatic. An ophthalmologist investigating a patient presenting with photophobia usually diagnoses the condition. The symptom "photophobia" may not present until middle age and is a very mild form of the disease. Diagnosis is confirmed by slit-lamp photography (Elenberg 2006) of the eye to reveal deposited corneal crystal, presence of cystine crystals in the bone marrow and leukocytes (but not present in the kidney and retina). Again the ocular features tend to be less severe and patients may be asymptomatic from an ocular point of view.

1.3.4 Incidence of Cystinosis

US: Incidence of infantile nephropathic cystinosis is 1 case per 100,000-200,000 live births; there are around 300-400 children presenting with the disease in America (Elenberg 2006).
Europe: The annual incidence rate is 1 case per 115,000-179,000 live births (Cairns, Anderson et al. 2002).
UK: An estimated 200 patients presents with the disease in the United Kingdom(Cairns, Anderson et al. 2002).

1.4 Diagnosis

1.4.1 Laboratory Studies:

- Serum electrolyte measurements: hypokalemia, hyponatremia, hypophosphatemia, and low bicarbonate concentration.
- Blood gases to detect metabolic acidosis.
- Urine testing to reveal hypo-osmolality, glycosouria, and tubular proteinuria.
- Measurements of urine electrolytes to detect the loss of bicarbonate and phosphaturia.
- Confirm diagnosis by measuring the cystine levels in polymorphonuclear leukocytes or cultured fibroblasts (Elenberg 2006).
  Normal leukocytes: ***<0.2 nmol half-cystine/mg cell protein***
  Infantile cystinotic leukocytes: ***5-15 nmol half-cystine/mg cell protein*** (Smolin, Clark et al. 1997)
  Intermediate cystinotic leukocytes: ***3-6 nmol half-cystine/mg cell protein***
- The cystine levels in Chorionic villi or cultured amniotic fluid cells can be measured in a foetus at risk of cystinosis.

1.4.2 Imaging Studies:

- Renal ultrasonography: for patients with elevated urine calcium excretion to exclude nephrocalcinosis (Elenberg 2006).

- Radiography for kidneys, ureters and bladder: to investigate further possible urine tract calcifications in patients with hypercalciuria or as a diagnostic evaluation of severe abdominal pain.
- CT scanning and MRI are used to evaluate adult patients with infantile nephropathic cystinosis with Central nervous system symptoms.

1.4.3 Other Studies

- Slit lamp examination of the eyes: to reveal corneal and conjunctival cystine crystals as early as 1 year of age.
- Eye fundi examination: to reveal the presence of peripheral retinopathy that is more severe on the temporal side than on the nasal side. In some cases, retinopathy can lead to blindness.

1.5 Treatment

This entails the administration of glucose and electrolytes to combat the effects of renal fanconi syndrome, oral cysteamine (Cystagon®) to deplete cystine levels, topical cysteamine eye drops to deplete ocular cystine deposits and renal transplant.

Example of a treatment regime for a typical cystinotic patient (Cairns, Anderson et al. 2002):

- Cysteamine: acts to reduce the intracellular cystine levels.
- Growth hormone: to promote short and long term growth in stunted children with chronic renal failure.
- Glucose: to oppose the imbalance as a result of Fanconi syndrome.
- Electrolytes: to oppose the imbalance as a result of Fanconi syndrome.
- L-Carnitine: to combat the effects of muscle weakness due to urinary losses.
- Indomethacin: a non-steroidal anti-inflammatory drug administered for its ability to retain sodium and potassium.

The main drug treatment of choice for cystinosis is cysteamine. It is an aminothiol that acts as a cystine-depleting agent. The drug is a weak base that enters cystinotic lysosomes, concentrates within the cell, then reacts with cystine to form a mixed disulfide of half-cystine and cysteamine (see figures 3 and 4). This mixed disulfide has a stearic resemblance to the amino acid lysine as shown in figure 3 and it rapidly exits the lysosomes via lysine transporters (Cairns, Anderson et al. 2002).

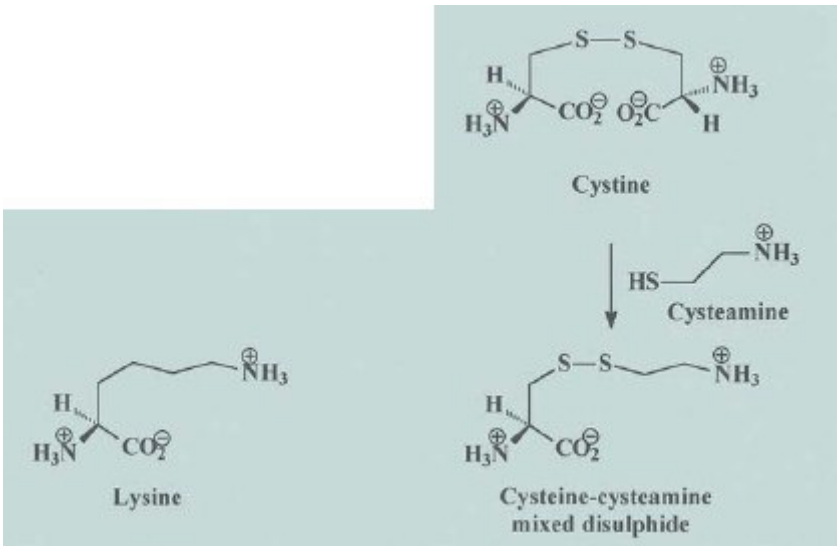
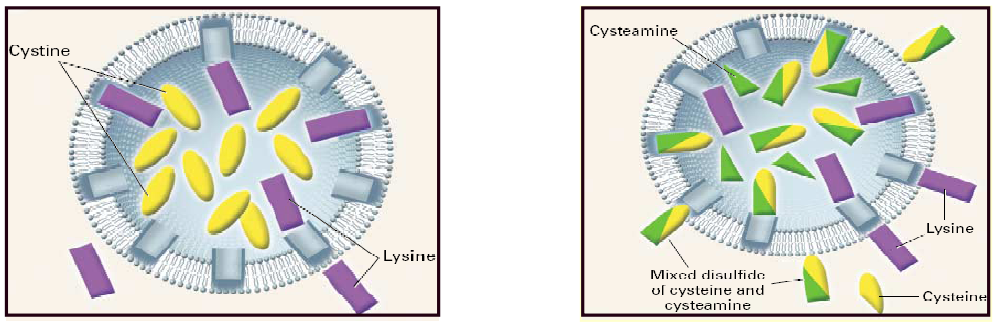


Figure 3: Structural similarity between lysine and cysteine-cysteamine mixed disulfide

(Cairns, Anderson et al. 2002)



Un-treated cystinotic lysosome

Cystinotic lysosome treated with cysteamine

Figure 4: Membrane activity within an un-treated cystinotic lysosome and a cystinotic lysosome treated with cysteamine (Gahl, Thoene et al. 2002)

The oral dose of cysteamine should be progressively increased from 10 to 50 mg/kg per day. The drug is rapidly absorbed and its effect lasts for only six hours. It should therefore be given every six hours to prevent intracellular cystine accumulation. Cysteamine should be started as soon as the diagnosis of cystinosis is confirmed (Gahl, Balog et al. 2007).

Although there is no apparent effect of oral cysteamine on corneal crystals, there is an effect of oral Cysteamine in preventing or retarding the posterior segment changes (pigmentary retinopathy and retinal dystrophy) in infantile cystinosis (Tsilou, Rubin et al. 2006). The most likely explanation of the lack of effect of oral cysteamine on corneal crystals is due to the avascularity of the cornea.

Topical cysteamine drops have been developed and have been in use since 1986. The current formulation in the UK is produced by the pharmacy-manufacturing unit at Guy's & St Thomas' NHS Foundation Hospital. There are two preservative free preparations containing 0.11 and 0.55% of cysteamine and disodium edeteate as an excipient. The products have a shelf life of seven days from the time of opening and it is recommended that they are stored in a fridge.

The recommended frequency of administration is four to six times a day (Iwata, Kuehl et al. 1998) but in practice, it can be administered almost each waking hour as reported by various authors (Hsuan, Harding et al. 1996). The very high frequency of instillation of drops leads to non-compliance. Hourly timers, a device that sets off an alarm every hour to remind the patient when a dose is due had been provided by Gahl et al., which increase the compliance to a certain level (Gahl, Kuehl et al. 2000). The formulation of a once daily delivery formulation is likely to greatly improve compliance and improve the quality of life for these patients.

2. Development of an Analytical Method For Cysteamine and Cystamine

2.1 Background:

The manufacturing unit at Guys and St. Thomas' Hospital currently manufactures the cysteamine eye drops being used in the UK. They use an iodometric titration method for the analysis of the finished ophthalmic preparation. Using the titration method to quantify cysteamine and the degradant (cystamine) at a low level is debatable, hence other methods that can quantify the active drug and especially any degradant(s) present in the formulation had to be explored.

Hence one of the initial aims of the project was to develop an analytical method:

- To allow accurate quantification and reproducible measurements of the active drug (cysteamine) within the formulation.
- To allow accurate quantification and reproducible measurements of the breakdown product (cystamine). This is crucial for stability studies and determination of shelf life of the formulation.

Cysteamine is an aminothiol as shown in figure 5. Other names include: 2-aminoethanethiol, Mercaptamine, β-mercaptoethylamine, 2-aminoethanethiol, 2-mercaptoethylamine, decarboxycysteine, and thioethanolamine.

Figure 5: The chemical Structure of Cysteamine

Cysteamine undergoes oxidation on exposure to air to form cystamine. The free thiol group allows cysteamine to act as a reducing agent itself by becoming oxidized to its disulphide cystamine as seen in figure 6 (Hsuan, Harding et al. 1996).

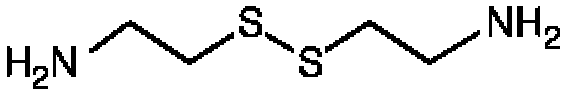


Figure 6: The chemical Structure of Cystamine

The analysis of compounds with thiol groups can be quite difficult, due to the susceptibility to oxidation before or during analysis and a lack of the structural properties (chromophore) required for the generation of signals compatible with common HPLC detectors, including: UV absorbance and fluorescence (Bald, Glowacki 2001).

2.2 Analytical methods

Despite the physiological importance and clinical applications of cysteamine, only a few procedures have been described for its analysis. The procedures that can be employed for the analysis of cysteamine are:

- Enzymatic assay: method is specific for cysteamine, but lacks sensitivity and derivatives are unstable (kataoka, Imamura et al. 1993).

- High-voltage electrophoresis: lacks sensitivity and is time-consuming, usually requires a preliminary sample clean up[1] (kataoka, Tanaka et al. 1994).

- Ion-exchange column chromatography: lacks sensitivity and is time-consuming (kataoka, Imamura et al. 1993).

- HPLC

    - Using fluorometric detection: highly sensitive, but lacks specificity (kataoka, Tanaka et al. 1994)[1].
    - Using electrochemical detection: highly sensitive, but lacks specificity[1].
    - Using UV detection with pre-column derivatization: highly sensitive and specific. It is labour intensive, as a derivatization reagent has to be synthesized initially for the preparation of stable derivatives.
    - Using fluorescence detection with pre-column derivatization: highly sensitive and specific.

- Stable Isotope dilution LC/MS/MS: highly sensitive and specific, automated and saves time, but it is very expensive. The equipment and expertise is accessible through a contact at Guy's Hospital. It is proposed for the analysis of cysteamine within the corneas of rabbits during the *in vivo* stage of the project.

- Gas chromatography

    - Using flame ionisation detection: this method lacks sensitivity and specificity, the preparation of the stable derivatives involves a time-consuming procedure and requires anhydrous conditions.
    - Using flame photometric detection: a number of amines can be analysed as their N-alkoxy-carbonyl derivatives. It is a sensitive and selective method, but requires the preparation of stable derivatives via a time-consuming procedure.

[1] Equipment not available locally.

Amongst the aforementioned techniques, it was decided to explore some of the techniques because of automation and availability of equipment:

Gas Chromatography using flame photometric detection

A number of amines can be analysed by gas chromatography (GC), using pre-column derivatization to form stable N-alkoxy-carbonyl derivatives. The actual derivatization involves alkoxycarbonylation of cysteamine with alkyl chloroformate in an aqueous medium to form stable N,S-diisobutoxycarbonyl derivatives (kataoka, Imamura et al. 1993).

The GC at the School of pharmacy does not have an autosampler, making it impractical and time consuming.

Stable Isotope dilution LC/MS/MS

This might be especially valuable when it comes to measuring cysteamine corneal content, during *in vivo* studies in rabbits. A source of stable isotope labelled cysteamine has been identified, and contacts have been made with an expert in the use of this technique (Dr Neil Dalton, based at the Well Child laboratory, Evelina Children's hospital), to explore this option if necessary. Using it for routine measurement might raise a cost issue though.

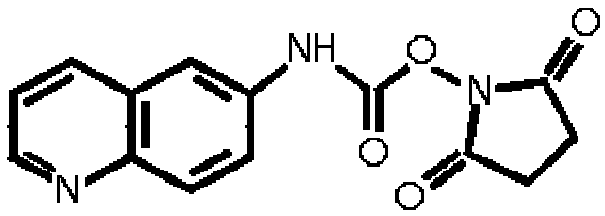
The ones using HPLC either with fluorescent or UV detection

Nevertheless a compound that lacks a chromophore such as cysteamine has to be altered or enhanced in a way, to enable the entire new moiety transmit signals that can be received and quantified by a detector. Furthermore the labile sulfhydryl group has to be blocked to prevent oxidation. Hence the need for pre-column derivatization of cysteamine. Two approaches were taken: a Waters AccQTag and CMQT pre-column derivatization methods.

2.3 Waters® AccQTag Method

In this first method, a commercially available pre-column derivatization kit from Waters® was used (Cohen, Michaud 1993). Routine analysis of compounds that contain amino groups or thiol groups can be time consuming and labour intensive. This method was chosen due to: its commercial availability, accuracy, simplicity/ease of use, sub-picomole sensitivity and automation. Furthermore it can be employed during the *in vivo* stage of the project (Waters Ltd.).

The AccQTag Method is based on a derivatizing reagent developed specifically for amino acid analysis. Waters AccQFluor Reagent (6-aminoquinolyl-N-hydrozysuccinimidyl carbamate, or ACQ) as shown in figure 7 is an N-hydroxysuccinimide-activated heterocyclic carbamate, a new class of amine-derivatizing compounds.



Waters AccQ•Fluor Reagent

Figure 7: Waters pre-column derivatizing agent AccQFlour Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) - www.waters.com

2.3.1 Derivatization procedure

The key to the rapid and simple Waters AccQTag Amino Acid Analysis method is a new derivatizing reagent, ACQ and a simple pre-column derivatization protocol.

The reagent is a highly reactive compound. It converts compounds with amino groups to stable ureas in a matter of seconds (figure 8) and hydrolyses to yield 6-aminoquinoline, a non-interfering by-product. These fluoresce strongly at 395nm. The resulting derivatives are easily separated by reversed phase HPLC, with a run time of about 35 minutes per sample and are stable at room temperature for up to one week (Waters Ltd).

Excess reagent is consumed during the reaction to form aminoquinoline (AMQ). AMQ has significantly different spectral properties from any other derivatized products. This allows programming of the fluorescent detector to the wavelength that can maximize the spectral emission response of the derivatized products, while minimizing the response of the AMQ. This gives a good degree of separation of all peaks on a chromatogram.

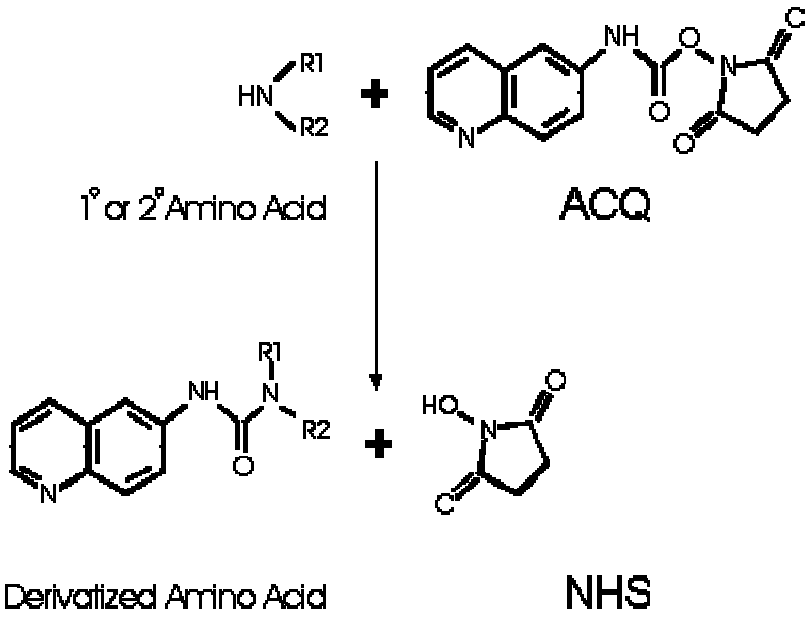


Figure 8: The derivatization process showing the reaction between ACQ and an amino acid to yield a stable urea molecule - www.waters.com

2.3.2 Reagent Hydrolysis

In a slower reaction, excess reagent hydrolyzes to produce 6-aminoquinoline (AMQ), N-hydroxysuccinimide, (NHS) and carbon dioxide (figure 9). The destruction of excess reagent is complete within a minute.

The major hydrolysis product, AMQ, fluoresces weakly at 395nm. AMQ produces a small peak that is easily resolved chromatographically. NHS and carbon dioxide does not interfere with the analysis.

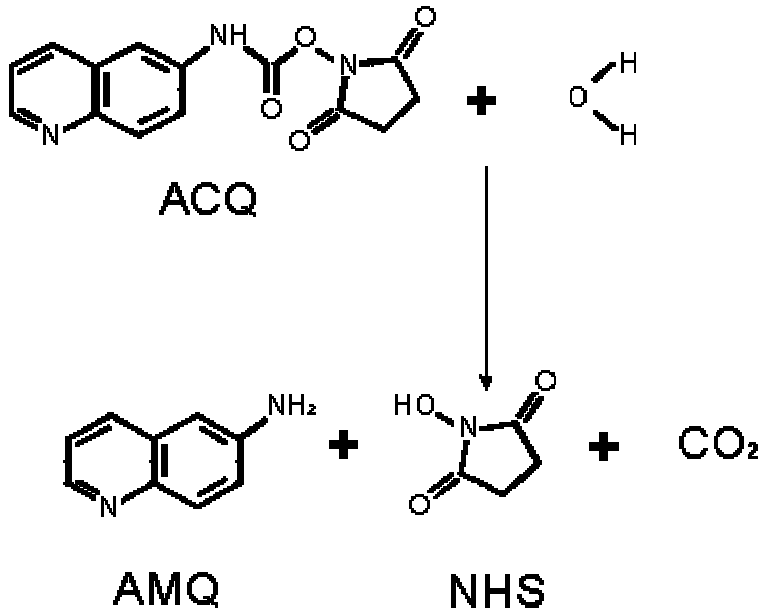


Figure 9: Excess reagent (ACQ) being consumed by a hydrolysis reaction, to form AMQ, NHS and CO2 - www.waters.com

2.3.3 Application of the Waters AccQTag method

This method is sold for analysis of compounds with an amino group. We wanted to see if it works with cysteamine and its degradants in a specific and sensitive manner because the amino acid cysteine (Cys) has close resemblance to the drug cysteamine, furthermore the technique has been used to successfully analyse cysteine.

2.3.3.1 Material and Methods

Samples of Cysteamine (from Sigma Aldrich) and cystamine (from Sigma Aldrich) were prepared (1µg/mL to 500µg/mL) in 0.1M HCL, AccQFluor reagent borate buffer and ACQ reagent (from Waters) were reconstituted as directed in the AccQTag chemistry manual.

A water bath was preheated to 55ºC, 10µL of sample was placed in an eppendorf, 60µL of AccQFluor borate buffer was added to the sample in the eppendorf and vortex-mixed. 200µL of ACQ was added to the resulting mixture and vortex-mixed.

After waiting for 1 minute (to allow for excess reagent (ACQ) to be consumed, to terminate the derivatization reaction), the contents of the eppendorf were transferred to an autosampler vial and placed in the water bath for 10 minutes at 55ºC, then 10µL was injected into the HPLC system.

Chromatographic analysis was performed using Agilent LC system equipped with a quaternary pump, and an autosampler. Waters 470 scanning fluorescent detector was used and analytes were separated on a 5-µm particle, 250mm x 4.6mm, Sun fire C-18 column (from Waters).

A gradient system was adopted, using: an aqueous buffer supplied by Waters and HPLC grade Acetonitrile (from Sigma Aldrich). A flow rate of 1ml/min was maintained, fluorescent detector excitation wavelength was set at 350nm and emission wavelength was set at 395nm. The column was thermostated at 37ºC and the injection volume was 10µL.

2.3.3.2 Results and discussion

The results from the analysis performed at The School of Pharmacy were not conclusive, because compounds being eluted from the column were changing over time with the formation of new peaks and existing peaks were increasing in area over time without any relation to the theoretical concentration of the sample

This analysis was repeated several times over 3 months to exclude possible experimental, operator, inter and intra-day errors. Going through the whole cycle of repeating experiments precisely, it was postulated that perhaps the old fluorescence detector or the lamp was faulty. Unfortunately, it was not possible to invest in a new fluorescent lamp at a cost of about £3,000 or a whole new detector priced at £10,000.

To shed some light on the reason why the results were inconclusive for months, the experiments were also repeated at the analytical offices at Waters in Borehamwood (UK), using their state of the art equipments.

A sample of the results obtained at Waters laboratories are represented in figures 11 and 12 of cysteamine and cystamine 0.5mM respectively.

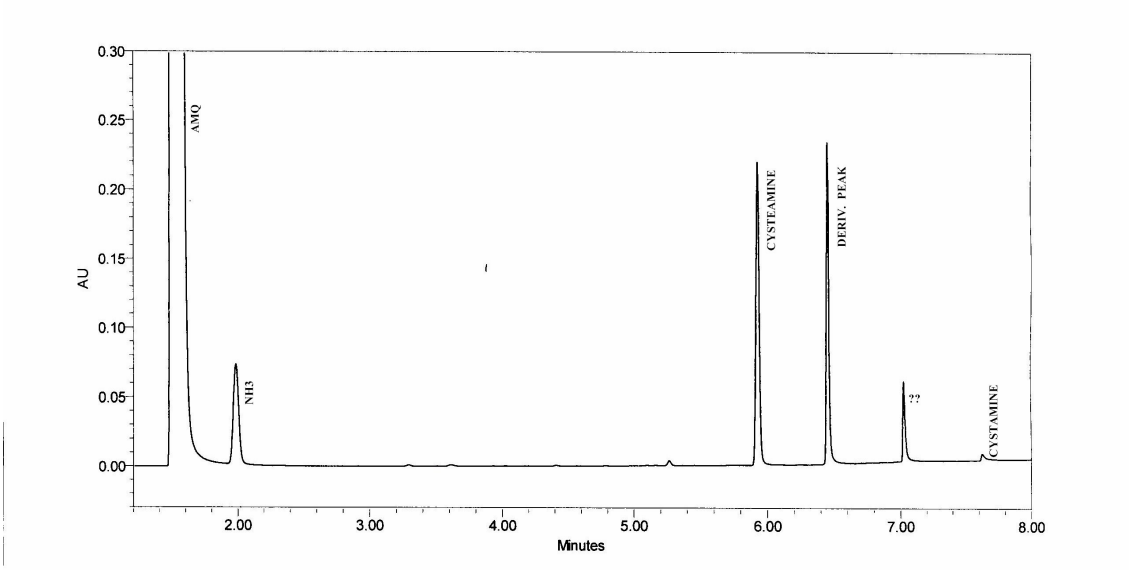


Figure 11: HPLC analysis of cysteamine 0.5mM

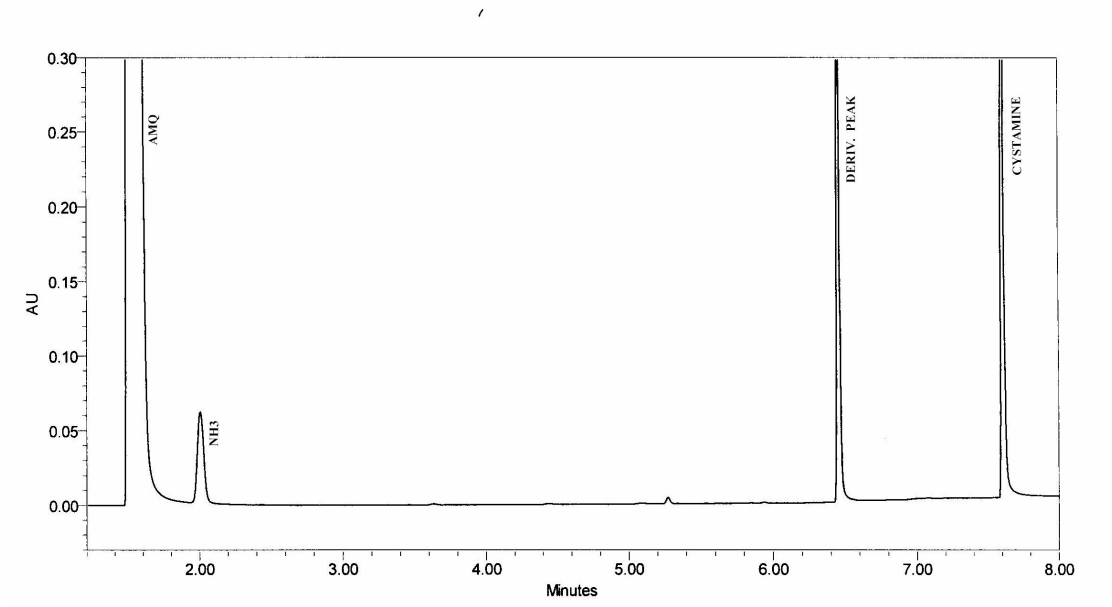


Figure 12: HPLC analysis of cystamine 0.5mM

At 1.5 minutes AMQ is assumed to be eluted. AMQ is only produced after excess reagent is consumed post-derivatization. Its presence provides a good indication that complete derivatization of cysteamine and cystamine has occurred.

Figure 11 shows six main peaks. The peak at 5.9 minutes is assumed to be the active ingredient cysteamine because it is absent on the chromatogram showing the analysis of cystamine as seen in figure 12. At 6.5 minutes it is assumed that the derivatization agent ACQ is being eluted because this peak is common to figure 11 and 12. At 7 minutes an unknown compound, perhaps some impurity is assumed to be eluted and at 7.5 minutes it is assumed that the breakdown product cystamine is being eluted

because this peak is absent on the chromatogram showing the analysis of cysteamine as seen in figure 11.

In figure 13 and 14, the chromatographs show analysis of cysteamine 5mM, whereby figure 14 represents the latter analysis of the same HPLC vial repeated 11 minutes after the one showed in figure 13. It could clearly be seen that all the peaks in figure 14 have changed (increased).

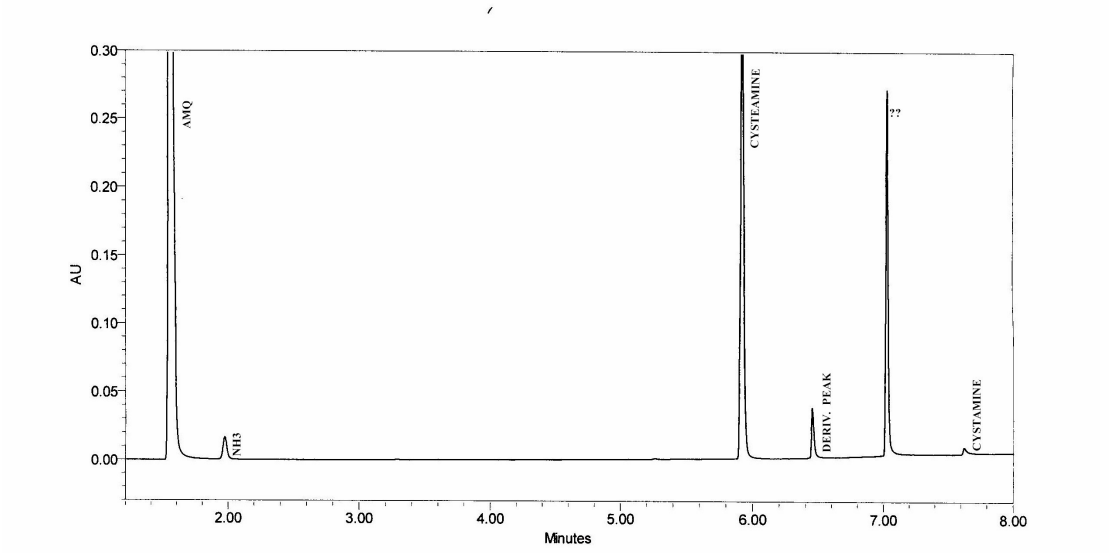


Figure 13: HPLC analysis of cysteamine 5mM

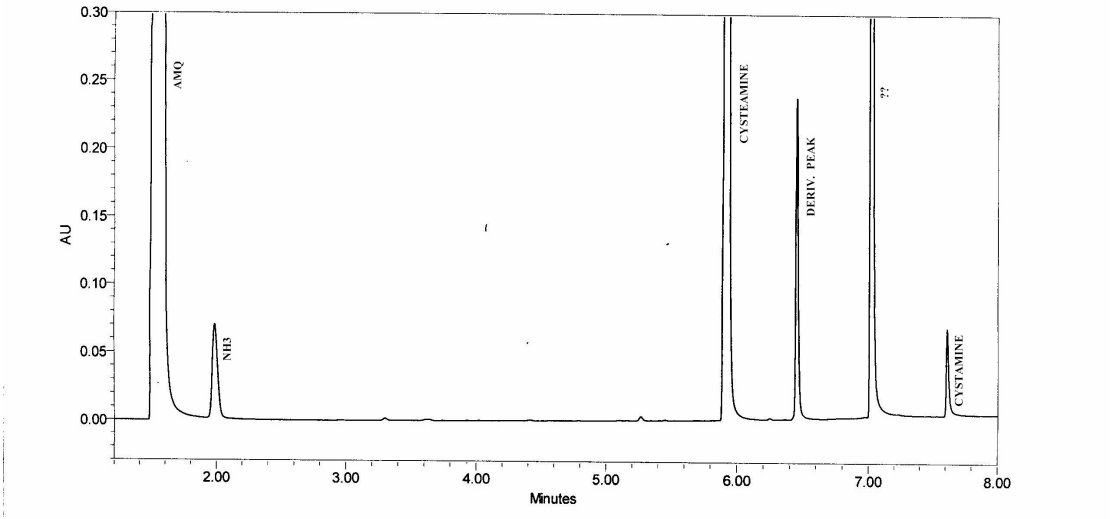


Figure 14: HPLC analysis of cysteamine 5mM carried out 11 minutes after the first HPLC run of the same sample

Unfortunately the results obtained at waters laboratories were similar to those that had been obtained repeatedly for months. Waters was unable to fully explain why the results were inconsistent. At this point and in order not to delay the work any further,

another possible analytical method was investigated with a different type of HPLC pre-column derivatization technique with UV detection.

2.4 An Analytical method using CMQT pre-column derivatization

2.4.1 Introduction

Analysing thiols can be quite difficult due to their susceptibility to oxidation which can occur before or during analysis and cannot be detected and quantified by spectrophotometry because they lack a suitable chromophore, hence an analyst must seek to derivatize to block the labile sulfhydryl groups and improve the signals for ultra violet detection (Kusmierek, Bald 2007).

Bald and colleagues have developed and carried out extensive work on the pre-column derivatization of thiols and has successfully determined cysteamine content and total mercaptamine in urine and plasma (Bald, Chwatko et al. 2004). Hence the use of their derivatization agent 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) was explored. CMQT reacts rapidly with thiols by nucleophilic displacement. The chloride is substituted by the sulphur from the –SH group on the thiol leading to the formation of a stable thioether linkage in a 2-S-quinolinium derivative. CMQT also performs well as a thiol specific tagging agent in terms of derivatization reaction velocity, stability, and chromatographic properties (UV-detectable S-quinolinium derivatives) of the derivatives (Bald, Glowacki 2001).

The method is based on the derivatization of cysteamine via the –SH group with CMQT, to form stable and UV-detectable S-quinolinium derivatives. To account for cystamine, which is oxidized cysteamine within a given sample, total cysteamine should be determined by initially reducing cystamine to cysteamine, before the derivatization step with CMQT.

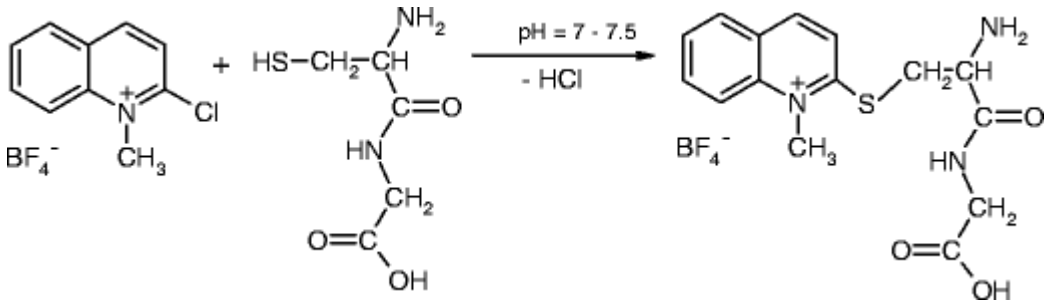


Figure 15: Chemical equation for derivatization of urinary thiols, represented by cysteinylglycine, with 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT)

2.4.2 Materials and Methods

A commercial kit is not available. The reagent (CMQT) has to be synthesized freshly each week before using it for the derivatization procedure, which is more labour intensive and time consuming.

Synthesis of CMQT

2-chloroquinoline, nitromethane, trimethyloxonium tetrafluoroborate, diethyl ether, diethyl ether and phosphorous pentoxide (were all purchased from Sigma Aldrich). The procedure was carried out in a fume cupboard.

1g (3.112mmol) of 2-Chloroquinoline, 1.2mL Nitromethane and 1g (6.760mmol) of trimethyloxonium tetrafluoroborate were transferred into a 25mL beaker and mixed using a magnetic stirrer. 4mL of Diethyl ether was added to the reaction mixture and continuously mixed until a white precipitate was formed. Mixing was stopped when the evolution of dimethyl ether ceased. The white precipitate was filtered off using a Pore size 5-glass filter in a Buchner flask. It was dried in a dessicator for two days. CMQT was prepared weekly

NMR Sample preparation and analysis

CMQT was analysed by NMR to confirm the success of the synthesis. 10mg of sample was placed in an eppendorf and dissolved in 0.6mL Deuterium oxide. The content of the eppendorf was transferred into an NMR sample tube for analysis.

NMR spectra of samples were obtained on BRUKER AVANCE 400 spectrometer equipped with a multinuclear probehead with z-gradient. The Topsin v1.3 software was used for spectra acquisition and Spinworks v3.0 was used for processing. The spectra were recorded at 300K and the chemical shift calibration was carried out on residual solvent peak. The size of all 1D spectra was 65K and the number of transients was 64.

Quantification of Cysteamine and Cystamine

A range of samples of cysteamine and cystamine 0.1mg/mL to 0.7mg/mL was prepared by precise dilution in 0.1mM EDTA in amber volumetric flasks.

Materials

CMQT (prepared in the lab), $Na_2HPO_4.7H_2O$ (Sodium Hydrogen heptahydrate), $NaH_2PO_4.2H_2O$ (Sodium dihydrogen phosphate dihydrate), TCA (Trichloroacetic acid), $LiOH.H_2O$ (Lithium Hydroxide Monohydrate for pH adjustment of TCA buffer), HPLC grade Acetonitrile and TCEP(Tris(2-carboxyethyl)phosphine hydrochloride (all reagents were purchased from Sigma Aldrich).

Preparation of Phosphate buffer (pH 7.5, 0.1mol/L)

13.404g of $Na_2HPO_4.7H_2O$ (Sodium Hydrogen heptahydrate) was placed in a 500mL volumetric flask and made upto volume with water (0.1mol/L), and then 7.8g of $NaH_2PO_4.2H_2O$ (Sodium dihydrogen phosphate dihydrate) was placed in a 500mL volumetric flask and made upto volume (0.1mol/L). The solution made of

$NaH_2PO_4.2H_2O$ (Sodium dihydrogen phosphate dihydrate) was titrated into Sodium Hydrogen heptahydrate solution to attain a pH of 7.5.

Preparation of TCA buffer
8.2mLs of TCA 6.1N solution was placed in a 1L volumetric flask and made upto volume with water (0.05mol/L), then 2.1g of $LiOH.H_2O$ was placed in a 1L volumetric and made upto volume (0.05mol/L). The Lithium hydroxide solution was titrated into the TCA solution to attain a pH of 3.15.

- Determination of cysteamine:
To 100µL of sample in an eppendorf, 200µL of Phosphate buffer (pH 7.5, 0.1mol/L) and 10µL of 0.1mol/L CMQT were added, vortex-mixed and left for 5 minutes to ensure complete derivatization. The contents of the eppendorf were transferred into an HPLC vial and 20µL was injected into the HPLC column. A blank sample was obtained by mixing 200µL of Phosphate buffer (pH 7.5, 0.1mol/L) and 10µL of 0.1mol/L CMQT in an eppendorf, vortex-mixed and left for 5 minutes.

- Determination of cystamine:
To accurately quantify the cystamine content within a sample, cystamine should initially be converted to cysteamine by a reduction process using a reducing agent such as: Tris(2-carboxyethyl)phosphine hydrochloride (TCEP).

To 100µL of sample in an eppendorf, 200µL of phosphate buffer (pH 7.5, 0.1mol/L) and 10µL of TCEP (0.25mol/L) were added and left to incubate at room temperature for 15 minutes. 20µL CMQT was added to the mixture, vortex-mixed and left for 5 minutes to ensure complete derivatization of the thiols to form stable S-quinolinium derivatives. The content of the eppendorf was transferred to an HPLC vial and 20µL was injected into the HPLC system. A blank sample was obtained by mixing 200µL of Phosphate buffer (pH 7.5, 0.1mol/L) and 10µL of TCEP (0.25mol/L) in an eppendorf, vortex-mixed and left to incubate for 15minutes at room temperature. 20µL CMQT was added to the mixture, vortex-mixed and left for 5 minutes.

Chromatographic analysis was performed using Agilent 1200 series LC system equipped with a quaternary pump, diode array detector and autosampler. Analytes were separated on a 5-µm particle, 150mm x 4.6mm, Zorbax C-18 column (Agilent). A gradient system was adopted, using: Trichloroacetic acid buffer and Acetonitrile. A flow rate of 1mL/min was maintained, UV detector wavelength was set at 355nm, the column was thermostated at 40ºC and 20µL was injected into the column.

Table 1: HPLC gradient elution for the CMQT derivatization method

Time (minutes)	% TCA buffer (A)	% Acetonitrile (B)
0	85	15
30	75	25
30-31	85	15
35	85	15

2.4.3 Results and discussion

- NMR

The successful synthesis of CMQT was confirmed by the NMR spectrum (figure 16). Six peaks were identified on the spectrum: 9.2(doublet), 8.54(doublet), 8.44(doublet), 8.38(triplet), 8.25(doublet), 8.12(triplet) and at 4.79(singlet 3H, CH_3).

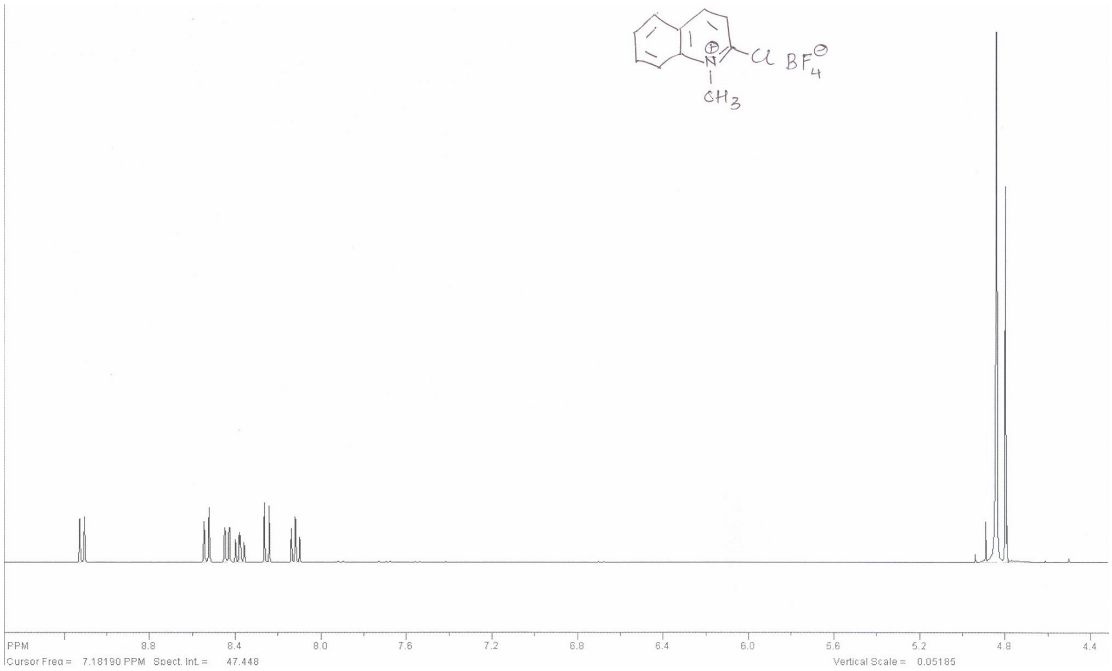


Figure 16: NMR spectrum of CMQT 10mg/0.6mL (1H NMR, D2O)

- HPLC results

Chromatographs were obtained for a calibration curve set up for Cysteamine and cysteamine 0.1mg/mL to 0.7mg/mL. Figure 17 shows the analysis of a blank sample. It could be assumed from it that CMQT was eluted at 12.75 minutes because that was the only main peak present.

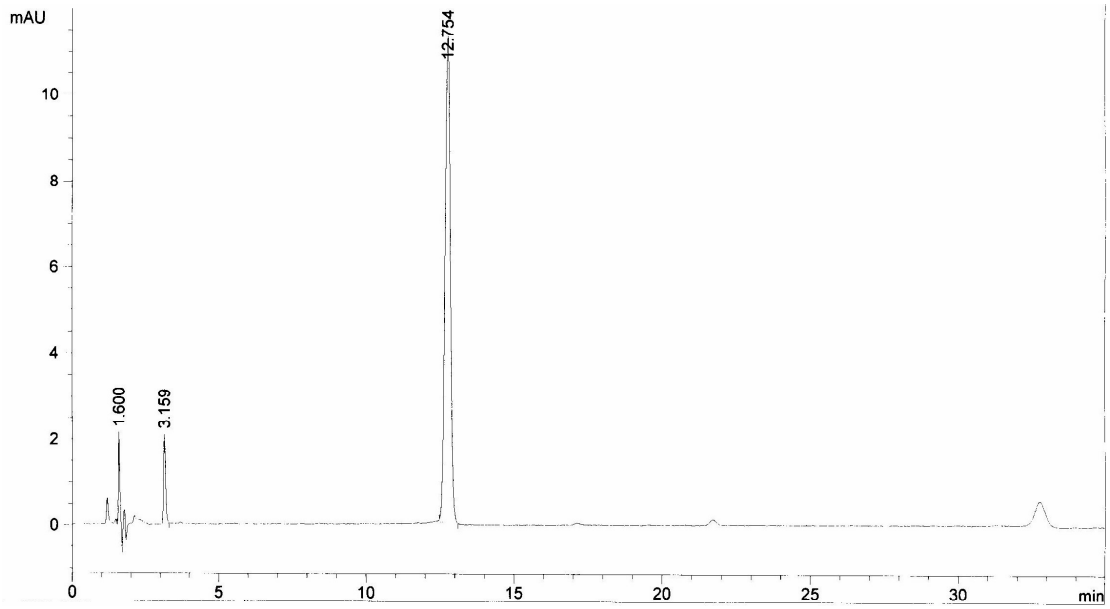


Figure 17: HPLC analysis of a blank sample

Figure 18 shows the analysis of cysteamine 0.3mg/mL for which two compounds were identified: one at 12.7 minutes (assumed to be CMQT) and the other at 16.6 minutes (assumed to be cysteamine).

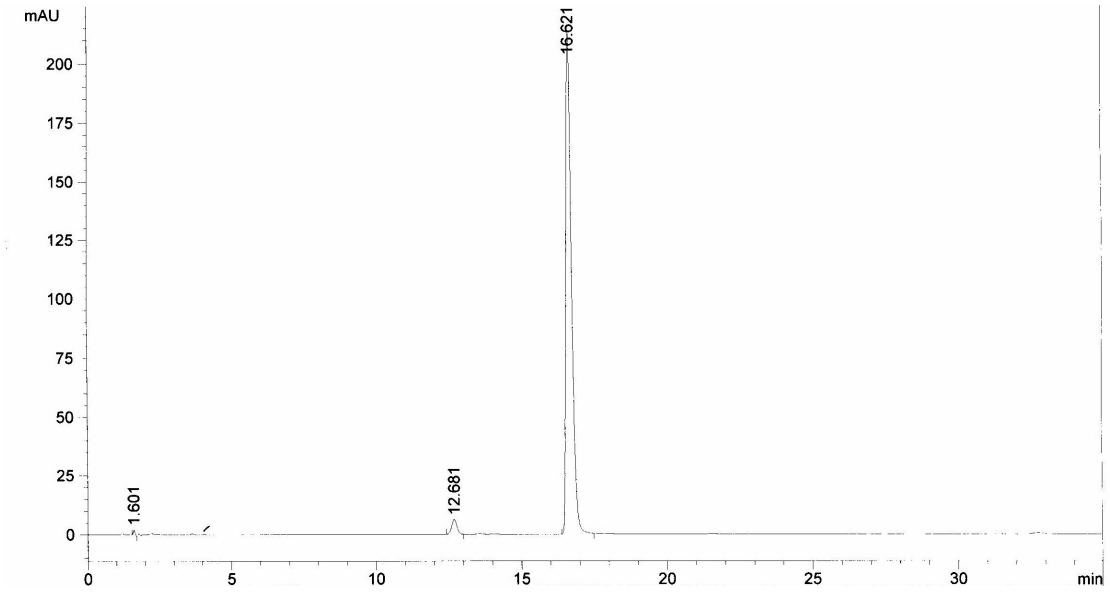


Figure 18: HPLC analysis of cysteamine 0.3mg/mL

Figure 19 shows the analysis of cystamine 0.3mg/mL reduced with TCEP for which four peaks were observed: at 12.7 minutes (CMQT), at 13.9 minutes (an unknown compound), at 16.6 minutes (reduced cysteamine) and at 19 minutes (an unknown compound).

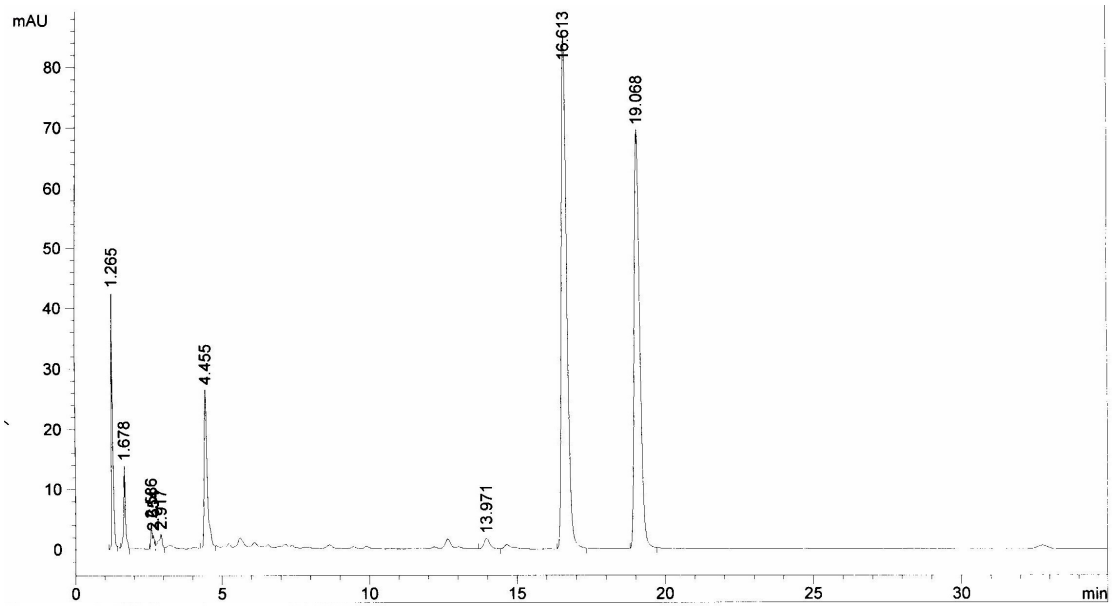


Figure 19: HPLC analysis of cystamine 0.3mg/mL (CMQT derivatization method)

A calibration curve was prepared for 0.3mg/mL to 0.5mg/mL of cysteamine. Run 1 was the first analysis carried out, followed by run 2 carried out 3 hours after run 1 and finally run 3 carried out 6 hours after run 1. Figure 20, 21 and 22 represent the Area under the curve (respectively for run 1, 2 and 3) of the various peaks as a function of cysteamine concentration.

In the first run (Figure 20), there were only 3 compounds being eluted; at 12.75 minutes the compound being eluted was assumed to be the CMQT, while the peak at 16.63mins was assumed to be the cysteamine peak. The last peak at 14.02mins was thought to be degradants or impurities.

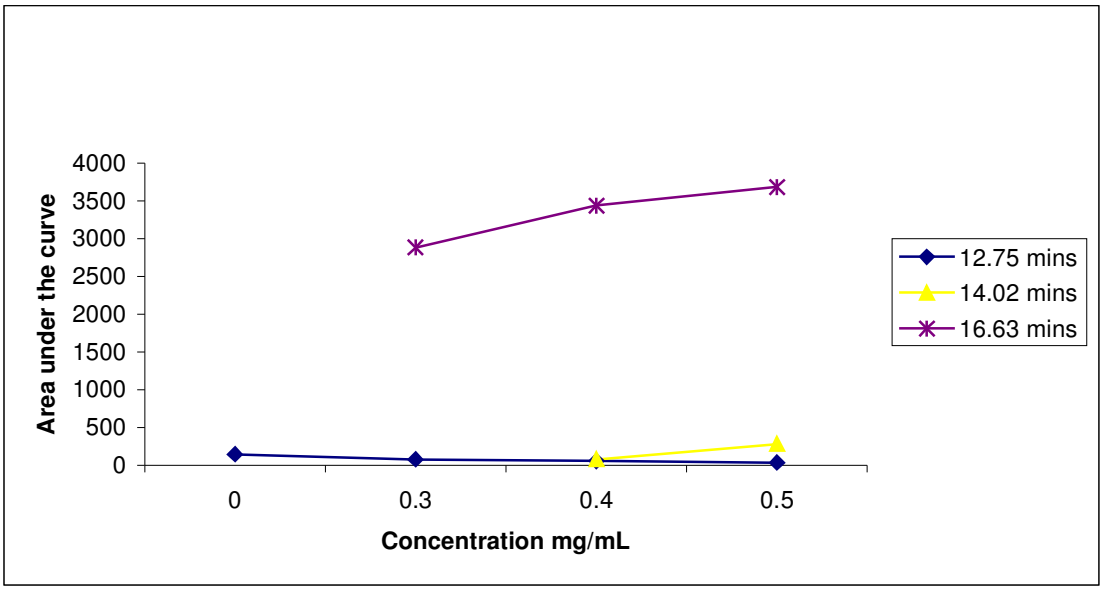


Figure 20: Area under the curve (Run 1) of various peaks as a function of cysteamine concentration. (CMQT derivatization method)

In the second run (figure 21), a new compound is being eluted at retention time 19.14 minutes. The peak at 12.75mins, presumably CMQT increased steadily over the 3 HPLC runs; the peak at 14.02mins, presumed degradants also increased steadily over the 3 runs, the peak at 16.63mins, presumed cysteamine reduced steadily over the 3 runs and the new peak formed during run 2 and 3 (figure 22) increased steadily too.

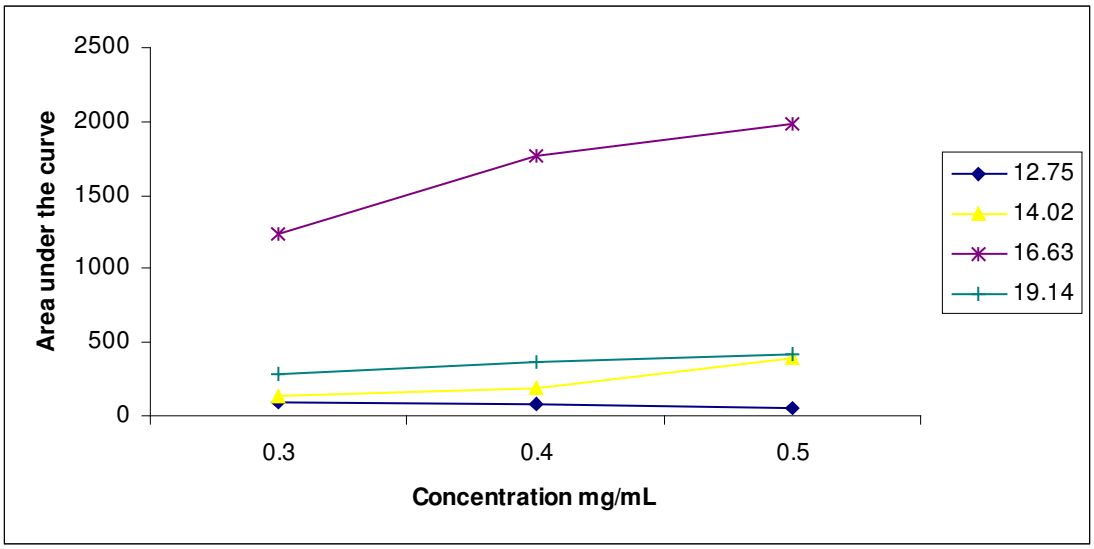


Figure 21: Area under the curve (Run 2) of various peaks as a function of cysteamine concentration. (CMQT derivatization method)

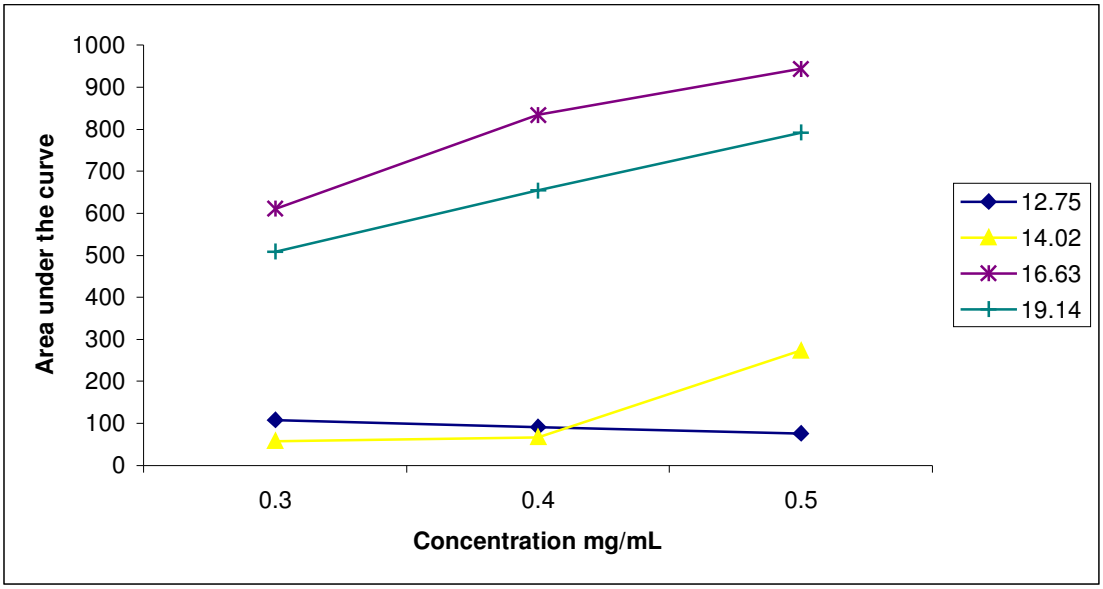


Figure 22: Area under the curve (Run 3) of various peaks as a function of cysteamine concentration. (CMQT derivatization method)

The compound(s) being eluted from the column over time were not in correlation with the initial content of the cysteamine sample. There was formation of new peaks, and existing peaks were increasing in area over time. It was concluded that there was possibly other reactions occurring apart from the proposed derivatization, hence the changes in the peaks on the chromatographs. It is also important to note that the ratio of CMQT to Cysteamine was 2:1. This is to ensure that CMQT was in excess to consume all the cysteamine available to achieve complete derivatization.

This analysis was repeated over 2 weeks, with the preparation of a fresh sample of CMQT to exclude possible experimental, operator, inter and intra-day errors. To shed some light on the reason why the results are inconclusive, Professor Edward Bald who originally developed the method was involved.

2.4.4 Modification of CMQT pre-column derivatization procedure

Professor Edward Bald invited us over to their laboratory in Poland to investigate further why the results from the CMQT pre-column derivatization of cysteamine were inconclusive. During the visit, the following reasons were identified.

- **Post-derivatization acidification of sample**
  The journal articles described an acidification process after the derivatization of physiological samples such as urine or blood with CMQT. This acidification step with 3M perchloric acid was for the precipitation of proteins. The samples being analysed were non-physiological samples, such as the cysteamine eye drops, hence there was no need for this extra step.

  Surprisingly, the acidification step carried out post-derivatization serves two purposes: to precipitate the proteins in the sample and also to reduce the pH of

the sample from pH 7.5 to about pH 2. Apparently the CMQT derivatization method is very sensitive to pH, if this critical acidification step is omitted then an unknown substance is formed and keeps reacting within the reaction vessel.

It explains why previously the peaks that were obtained on the chromatographs were unstable as the peaks formed kept changing between HPLC runs.

- **Molar excess of CMQT**
  The derivatization step with CMQT was performed using a ratio of 2 moles of CMQT to 1 mole of cysteamine. Since the reaction is 1 mole of cysteamine with 1 mole of derivatization agent, it was assumed that using a ratio of 2:1 would create molar excess anyways and would be sufficient for the procedure.

  Unpublished data (personal communication, Bald et al, 2008) suggests that at least a seven fold molar excess of CMQT is required for complete derivatization of 1 mole of cysteamine. The data was traced back to a thesis written by one of the colleagues a few years back.

- **pH of mobile phase**
  The journal articles described using a TCA buffer adjusted to pH 3.2 with lithium hydroxide, which was employed during previous CMQT pre-column derivatization. It was suggested during the visit to adjust the pH of the mobile phase to a value not greater than pH 2.5. As mentioned previously this procedure is highly sensitive to pH, even this slight change from a pH of 3.2 to 2.5 can have a huge impact on the separation of peaks on the chromatogram.

Quantification of cysteamine and cystamine 2.5µmol/L to 7.5µmol/L
On return to the UK, the CMQT pre-column derivatization procedure was modified to accommodate the new changes.

Materials
All the materials used in the modified procedure were the same as used previously.

- Determination of cysteamine:
To a known amount of sample in a 5mL volumetric flask, 500µL of Phosphate buffer (pH 7.5, 0.1mol/L) and 20µL of 0.1mol/L CMQT were added, mixed and left for 5 minutes to ensure complete derivatization. Then 50µL of 3M HCL was added to terminate the derivatization reaction and made up to final volume with water. The contents were transferred into an HPLC vial and 20µL was injected into the HPLC column. A blank sample was obtained by mixing 500µL of Phosphate buffer (pH 7.5, 0.1mol/L) and 20µL of 0.1mol/L CMQT in a 5mL volumetric flask, mixed and left for 5 minutes, then acidified with 50µL of 3M of HCL and made up to final volume with water.

- Determination of cystamine:
To accurately quantify the cystamine content within a sample, cystamine should be initially converted to cysteamine by a reduction process using a reducing agent such as: Tris(2-carboxyethyl) phosphine hydrochloride (TCEP).

To a known amount of sample in a 5mL volumetric flask, 500µL of phosphate buffer (pH 7.5, 0.1mol/L) and 10µL of TCEP (0.25mol/L) were added and left to incubate at room temperature for 15 minutes. 20µL CMQT was added to the mixture mixed and left for 5 minutes to ensure complete derivatization of the thiols to form stable S-quinolinium derivatives and 50µL of 3M HCL was added to terminate the reaction. The contents were transferred to an HPLC vial and 20µL was injected into the HPLC system. A blank sample was obtained by mixing 500µL of Phosphate buffer (pH 7.5, 0.1mol/L) and 10µL of TCEP (0.25mol/L) in a 5mL volumetric flask, mixed and left to incubate for 15minutes at room temperature. 20µL of CMQT was added to the mixture, mixed and left for 5 minutes, and then 50µL of 3M HCL was added to terminate the reaction and finally made up to final volume with water.

Chromatographic analysis was performed using Agilent 1200 series LC system equipped with a quaternary pump, diode array detector and autosampler. Analytes were separated on a 5-µm particle, 150mm x 4.6mm, Zorbax C-18 column (Agilent). An isocratic elution was employed, using: Trichloroacetic acid buffer pH 2.5 and Acetonitrile (ratio 75%: 25%). A flow rate of 1mL/min was maintained, UV detector wavelength was set at 355nm, the column was thermostated at 40ºC and 20µL was injected into the column.

Results and discussion

Modifications of the pre-column derivatization procedure were a great success. The use of an isocratic elution simplified the procedure even further and the run time was shortened to 10 minutes per sample compared to the previous 35 minutes.

Figures 23 and 24 show the chromatogram of a blank CMQT sample and cysteamine pre-column derivatization with CMQT respectively. The solvent front is observed around 2 minutes; CMQT is eluted around 5.4 minutes and cysteamine around 3.9 minutes.

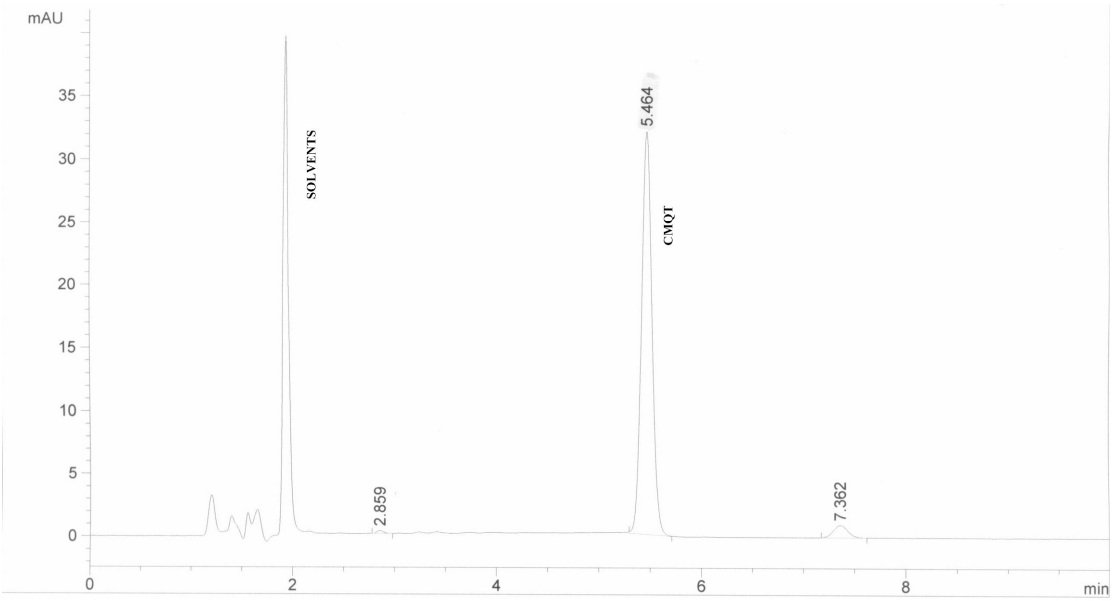


Figure 23: HPLC analysis of Blank CMQT pre-column derivatization

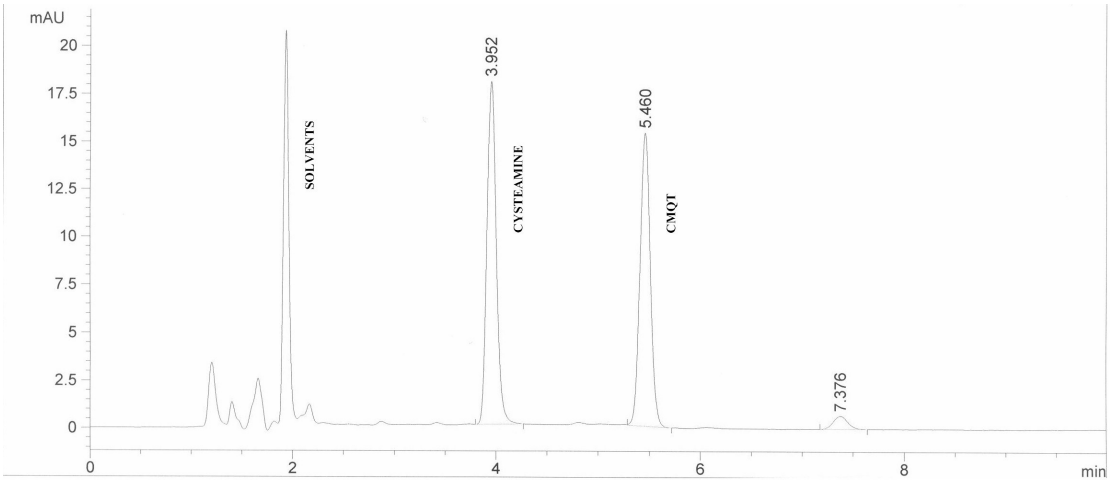


Figure 24: HPLC analysis of cysteamine 5µmol/L using CMQT pre-column derivatization

The small peaks around 2.8 minutes and 7.4 minutes in figure 23 are presumed to be impurities. Furthermore the s-quinolinium derivatives formed post-derivatization with CMQT was found to be stable for at least 24 hours.

Figures 25 and 26 show the chromatogram of a blank sample containing TCEP with CMQT and cystamine initially reduced with TCEP prior to the pre-column derivatization with CMQT respectively. The solvent front is observed around 2 minutes; CMQT is eluted around 5.4 minutes and reduced cystamine (cysteamine) around 3.9 minutes.

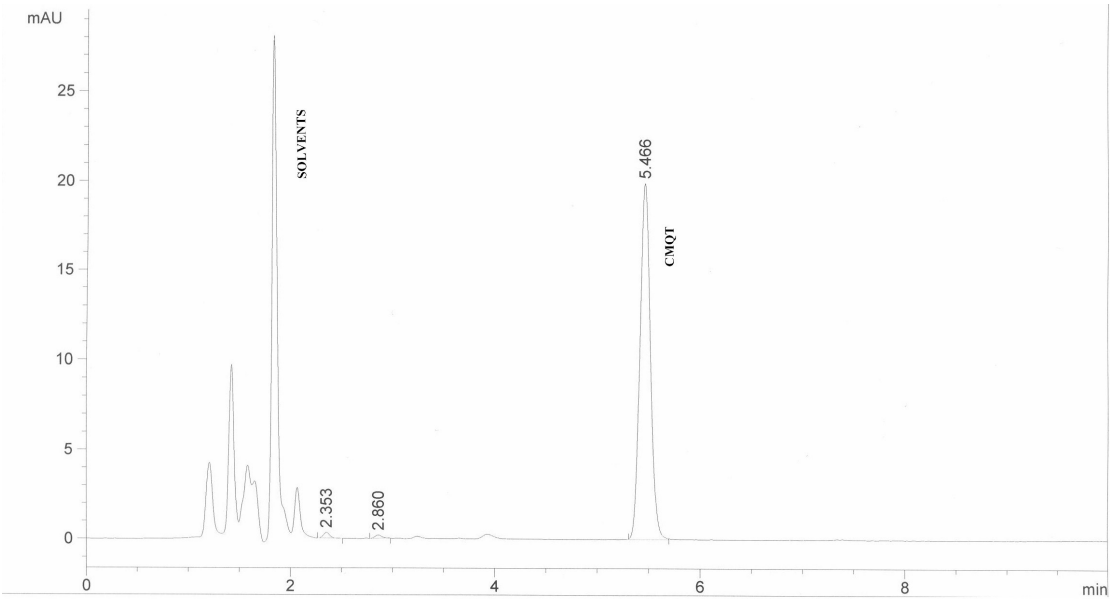


Figure 25: HPLC analysis of blank reduction with TCEP

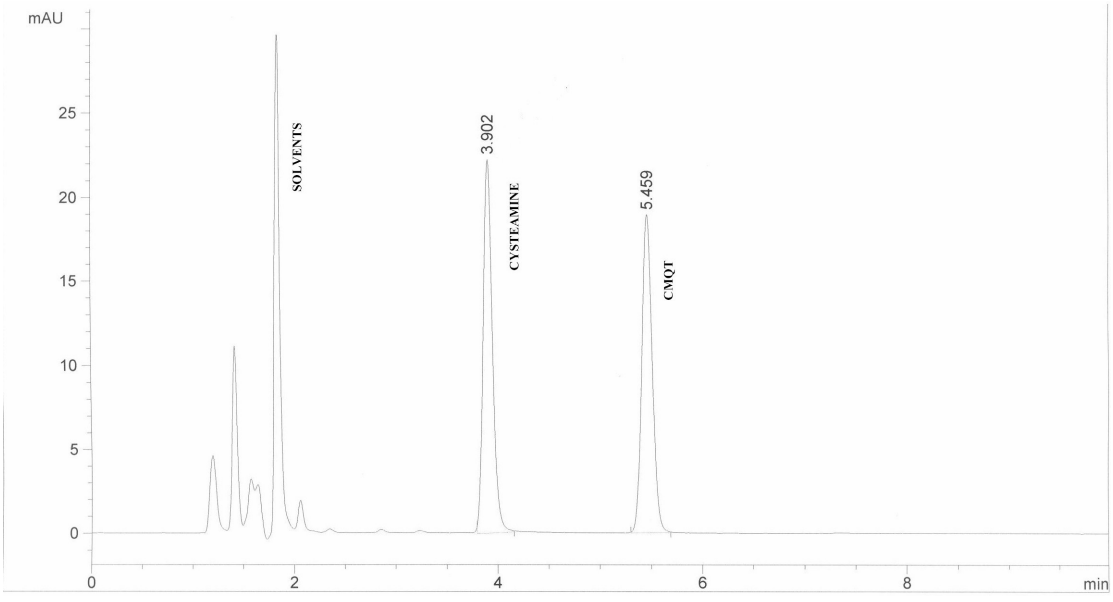


Figure 26: HPLC analysis of cystamine 5µmol/L with initial TCEP reduction and CMQT pre-column derivatization

The small peaks around 2.4 minutes and 2.9 minutes in figure 25 are presumed to be impurities. Furthermore the s-quinolinium derivatives formed post-derivatization with CMQT was found to be stable for at least 24 hours.

A linear calibration curve was obtained from 2.5µmol/L to 7.5µmol/L using cysteamine and cystamine standards.

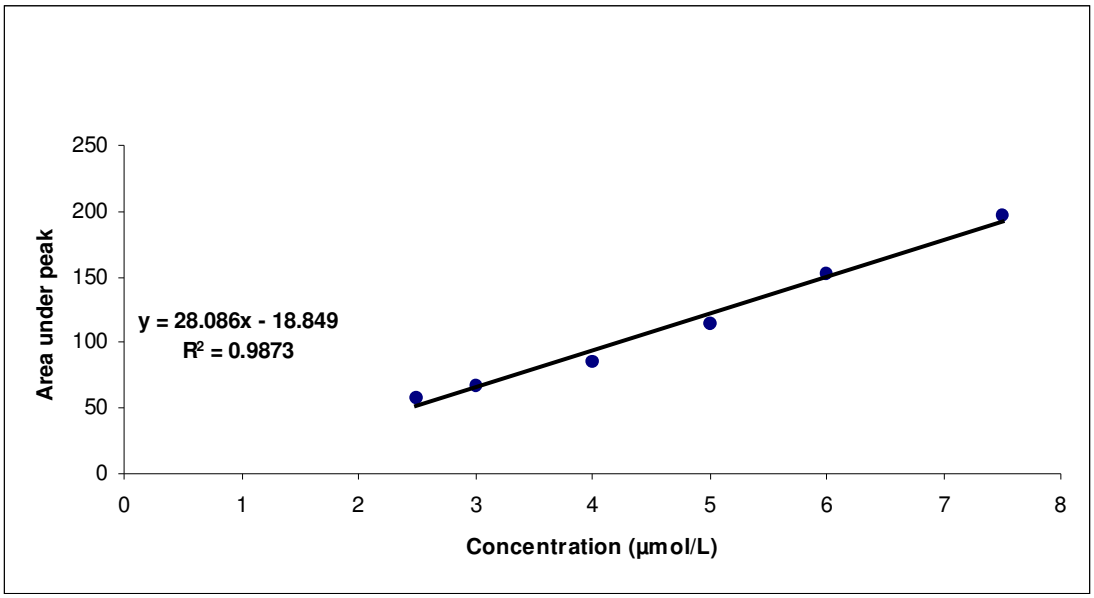


Figure 27: Calibration curve for Cysteamine using CMQT pre-column derivatization

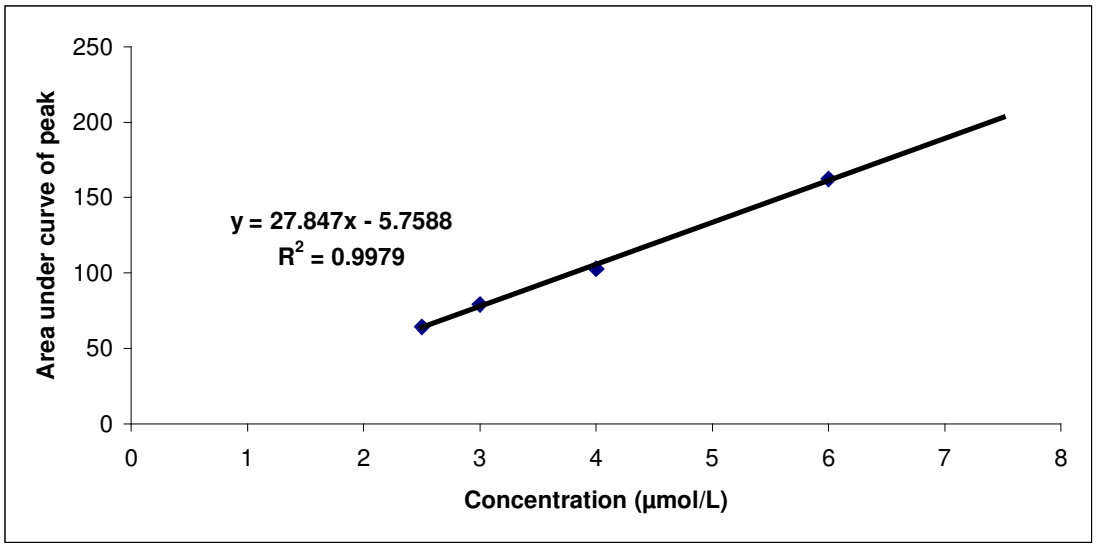


Figure 28: A Calibration curve for Cystamine with initial TCEP reduction and CMQT pre-column derivatization

The results from the modifications to the procedure were indeed encouraging. The next and final step in the development of the analytical method will be the validation of the method.

3. Formulation Development

3.1 Background

3.1.1 Structure of the eye

Drug delivery into the eye is challenging, owing to the anatomy, physiology and biochemistry of the eye, which renders it impervious to foreign substances. The aim of this project is to develop an ophthalmic delivery system that can circumvent the protective eye barriers and mechanisms without causing damage to the tissues.

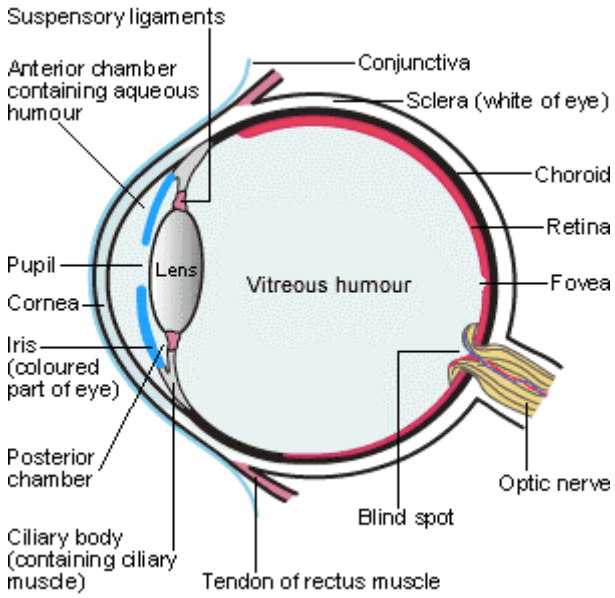


Figure 29: The anatomy of the Eye

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The cornea covers only one-sixth of the total surface area of the eyeball, and is considered to be the main route of drug absorption into the eyes. The cornea is made up of three layers: epithelium, endothelium and stroma. The epithelial and endothelial cells are rich in lipids and highly permeable to lipophilic substances, while the stroma is hydrophilic and highly permeable to water soluble substances.

The conjunctiva consists of a dense mucus-covering layer. This mucus layer serves best as a site for mucoadhesion (the attachment of a drug carrier to mucin). The theory of mucoadhesion is one to be explored in this project. Mucin is an anionic biopolymer, which is composed of glycoproteins that are hydrophilic. Most ophthalmic preparations are instilled into the cul-de-sac of the eyes by: grasping and pulling back on the lower eyelid to create a pocket. If a mucoadhesive polymer is incorporated within an ophthalmic formulation, it will therefore be in direct contact with the mucus found on the conjunctiva, permitting adhesion, and increasing contact time of the drug with its absorptive surface.

Tear fluid: consists of water, mucin, lipids, lysozyme and ions such as calcium, potassium and sodium. It continuously bathes the corneal epithelium, conjunctiva and walls of the conjunctival cul-de-sac. Tear fluid plays a vital role in the maintenance of the integrity of the ocular surface; provides nutrition for the cornea, offers protection against bacterial infection and the removal of cellular debris or foreign substances. It also acts as a lubricant to facilitate the movements of the eyelids over the eye globe.

3.1.2 Barriers to Ophthalmic drug delivery:

Drug administration via the oral route has very little or no effect on ocular uptake because of the avasularity of the eyes, thus the only way to achieve ocular drug permeation is by administering the therapeutic agent locally as a topical preparation. The eye has various protective barriers and mechanisms, shielding it from foreign substances, this includes: cellular and environmental debris. These protective barriers and mechanisms also act as a barrier against any ophthalmic preparation administered into the eyes.

The main barriers include:

- Nasolacrimal drainage: as soon as an ophthalmic solution is administered into the eyes, most of the solution will be lost through the nasolacrimal duct. This causes the drug to be absorbed systemically via the nasal mucosa and gastrointestinal tract.
- Volume: conventional eye drops deliver volumes of about 25-50µL, while the conjunctival sac can hold volumes of ~7µL (Carlfors, Edsman et al. 1998). After instillation of an ophthalmic preparation into the lower cul-de-sac, the solution is rapidly cleared by spillage and nasolacrimal drainage to restore to the normal conjuctival residence volume of 7µL.
- Lacrimation: this is a normal eye mechanism to maintain the health and integrity of the ocular surface. However this same lacrimation reflex is produced when an ophthalmic preparation is administered into the eyes. The role of increased lacrimation is to cleanse the eye surface of any foreign materials such as cellular debris or environmental contaminants. Unfortunately increased lacrimation causes further dilution of the drug administered.
- Tear dilution: increased lacrimation causes dilution of the administered drug, rendering it less potent and less therapeutically favourable.
- Blinking: this is a normal protective eye mechanism to prevent damage due to cellular debris or environmental contaminants. Blinking serves to dislodge and remove any foreign entities in the eye by the rapid movements of the upper eyelids over the eye globe. This reflex action is increased when an ophthalmic solution is administered into the eyes, causing a large portion to be lost by spillage and nasolacrimal drainage.

These ophthalmic drug delivery barriers accounts for a significant loss in administered ophthalmic preparations; the contact time of the drug with the ocular tissue is very short (about 1 to 2 minutes) (Gokulgandhi, Modi et al. 2007) with only 1% or less of administered solutions being absorbed via the cornea.

3.1.3 Ophthalmic dosage forms

Traditional ophthalmic formulations are available as: solutions, ointments, suspensions, inserts and gels. Early attempts to enhance the ocular bioavailability of topically instilled drugs have involved the use of ointments and suspensions; to ensure a higher bioavailability by increasing the contact time with the eye, reduce dilution by tears and nasolacrimal drainage. Unfortunately they cause blurring of vision and crusting of the eyelids. Furthermore there is a huge variability in the efficiency of suspensions mainly due to patients not adequately shaking the suspension prior to administration.

The more recent attempts to enhance ocular bioavailability of topically administered drugs include: systems that provide controlled and continuous delivery and systems that increase corneal absorption and decrease pre-corneal loss.

Implantable systems such as ocuserts and collagen shields can provide the controlled and continuous delivery of drugs, but the disadvantages include the need of surgery for insertion and difficulty in patient self-administration.

Nanoparticles and microspheres can be used to increase the residence time of the drug; unfortunately it has been observed that poly (alkyl cyanoacrylate) a major component can damage the corneal epithelium by altering the cell membrane.

Liposomes are unstable due to the potential hydrolysis of phospholipids, and they have limited drug-loading capacity.

3.1.4 In situ forming hydrogels

The ideal dosage form is a system that can be instilled into the eye(s) in a droppable/reproducible form, with no blurring effects but with sustained release of drug to the eye in order to achieve a low frequency of administration (not exceeding twice a day).

In situ forming hydrogels are liquids that can undergo a phase transition from a liquid to a gel (or solid phase), upon instillation into the lower cul-de-sac of the eye. It transforms from an easy to instill liquid to a transparent or translucent gel that is retained longer in the eye by reduced lachrymal drainage, prolonged release of the drug, and increased bioavailability, thus reducing the need of frequent administration.

There are various factors that predispose this phase change, this includes: temperature gradient, pH gradient and change in ionic strength.

Main physiological mechanisms of phase transition:

- pH dependent: certain polymeric solutions can undergo a phase transition from a liquid to a gel when the native pH of the polymer solution (between pH 4-6) is raised to pH 7.4 by the tear fluid when instilled into the eye (Charoo, Kohli et al. 2003). Examples of some polymers that undergo this transition include: carbopol, cellulose acetate phthalate and polyacrylic acid.
- Temperature dependent: certain polymeric solutions can undergo a phase transition from a liquid to a gel when the native temperature of the polymer

solution (between 4º-15ºC) is raised to 34ºC when instilled into the eye. Examples of some polymers that undergo this transition include: poloxamers and pluronics.

- Ionic strength dependant: certain polymeric solutions can undergo a phase transition from a liquid to a gel when in contact with the monovalent and divalent cations found in tear fluid, namely: Na^+, Ca^{2+}, K^+ and Mg^{2+} (Masteikova, Chalupova et al. 2003). Examples of some polymers that undergo this transition include: Gellan gum and Sodium alginate.

3.2 Earlier Work

The polymer of choice to be incorporated into the ophthalmic preparation was chosen during earlier experiments performed in the lab (Orubu 2004), which screened few in situ gelling polymers as shown in table 2.

Table 2: Four different in situ gelling polymers screened in an earlier study (Orubu, 2004)

Mechanisms of phase transition	Chemical name	Trade name
pH triggered system	Carbomer	Carbopol® 974 PNF
Temperature dependent system	Poloxamer 407	Lutrol® F127
Ionic strength systems	Sodium alginate Gellan Gum E418	Manugel® DMB Kelcogel®

Figure 30 shows the viscosity of the polymers presented in table 2 used at various concentrations before and after dilution in STF in a ration of 1:1. Timoptol LA® was used as the standard for comparing the polymers, because it is the only in situ gelling ophthalmic preparation on the market.

Among four common ophthalmic in situ gelling polymers (Table 2), Gellan gum was found to be the most suitable as it gelled significantly when in contact with the ions contained in simulated tears and did not loose its rheology parameters upon dilution with simulated tear fluid. It exhibited a better gellation profile compared to sodium alginate. Moreover a pH dependant polymeric solution could be quite acidic (sometime as low as pH 4) and could cause stinging on instillation. This will cause pain and discomfort in the target treatment group (cystinotic children). A temperature dependant polymeric solution (transition sol to gel at 34°C) would require a cold chain throughout manufacture, storage and once dispensed to the patients, which could be impractical.

It can be seen that gellan gum 0.6% is the only polymer that exhibited a gellation profile comparable to Timoptol LA®. The gelling force of gellan gum 0.6% when in contact with the ions present in STF overcomes the dilution effect observed in some other polymers such as carbopol 1% in STF.

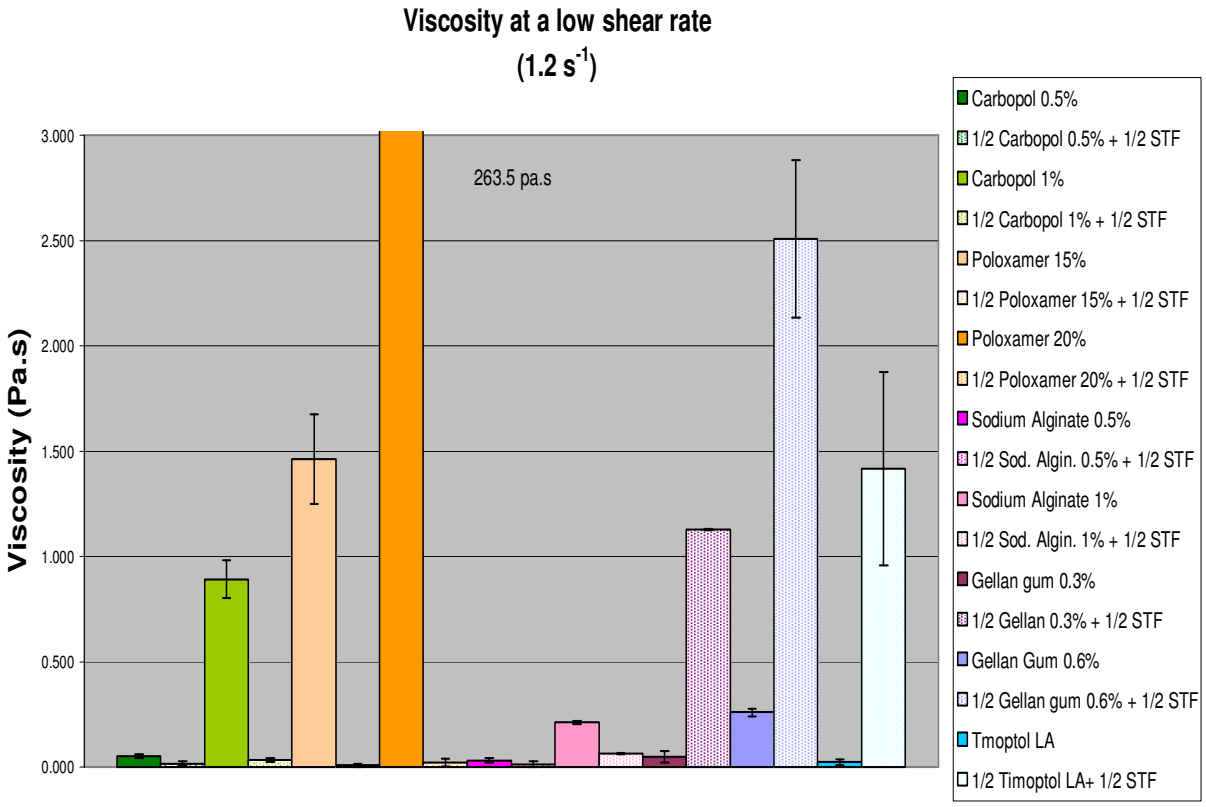


Figure 30: Viscosity (Pa.s) at low shear rate (1.2 s-1) in the presence and absence of simulated tear fluid (STF) of four polymers tested at various concentrations. (Orubu, 2004)

Moreover the final ophthalmic formulation needs to be terminally sterilised. Earlier work showed that terminal sterilisation by autoclaving at 121°C for 15 minutes can affect the formulation's rheological behaviour and can cause drug loss (Shah 2005).

3.3 Compatibility of Gellan Gum with individual excipients by rheological assessment

Cysteamine is rapidly oxidised to the disulphide cystamine, which has not been shown to be effective in topical therapy (Iwata, Kuehl et al. 1998). Therefore and essential excipients such as an antioxidant (e.g. EDTA) has to be added and its content optimised.

Commonly used preservatives for eye drops such as ionic preservatives compounds (benzalkonium chloride) or non-ionic compounds (chlorbutanol) (Rabiu, Forsey et al. 2004) may also be incorporated into the preparation to prevent microbial contamination but could affect the formulation (change in gelling capacity of the polymer).

The aim of these experiments was to screen the influence of each of the excipients on the rheological behaviour of the formulation, consisting of cysteamine hydrochloride as the active ingredient and gellan gum as the in situ gelling polymer.

3.3.1 Materials and Methods

Materials
Chemicals purchased from Sigma-Aldrich Ltd included: Gelrite® (Gellan gum), trizma pre-set crystals (pH 7.4), cysteamine hydrochloride, Disodium edetate, benzalkonium chloride, mannitol, mucin (type 2 from porcine stomach), sodium chloride, sodium hydrogen carbonate, calcium chloride, potassium chloride. Timoptol LA® 0.5% was purchased from AAH and benzododecinium bromide was purchased from Chemos.

Preparation of samples

- Gellan gum solution
To prepare Gellan gum solution, the required amount of polymer powder (%, w/w) was weighed and dispersed in trizma solution (pH 7.4). The dispersions were stirred at 100ºC for 20 minutes under nitrogen bubbling to protect it from oxidation, in the final sterilizable amber bottle (from Duran) to protect from light degradation. They were subjected to terminal sterilization by autoclaving at 121ºC for 15 minutes. All the formulations were always prepared in duplicate. The in situ gels were stored in the fridge but left to equilibrate at room temperature before any measurement.

- Simulated tear fluid (STF)
It is an electrolyte solution consisting of 6.8g NaCl, 2.2g $NaHC0_3$, 0.084g $CaCl_2.2H_20$ and 1.4g KCl in 1L of water (Paulsson, Hagerstrom et al. 1999).

- Mucin dispersions
The simulated tear fluid (STF) was used as a vehicle to prepare the mucin dispersions. After dispersing the required amount of mucin powder (%, w/w) in STF, the resulting dispersion was sonicated for 30 minutes to allow complete hydration.

pH measurements
A calibrated pH meter by Hanna instruments (PH 211) was used to carry out the pH assessments of all prepared formulation.

Osmolarity measurements
A freezing point Roebling micro osmometer, Type 5R was used to carry out the osmolarity assessments of all prepared formulation. Measurements were carried out in duplicates.

Rheological assessments

The rheological assessments were carried out using a Carri Med CSL^2 500 Rheometer (TA instruments Ltd.), at 34ºC, which represents corneal temperature. The geometry used was a cone and plate 4cm, 2degree truncation (from TA instruments Ltd.)

Flow measurements were used to determine the viscosity of the formulations 24 hours after autoclaving, where a shear rate ramp was applied. The shear rate was increased from $10s^{-1}$ to $1200s^{-1}$ over 25 minutes. The viscosity at shear rates $12.71s^{-1}$ and $743.5s^{-1}$ are used as an evaluation parameter, to assess the effects of low blinking which corresponds to low shear rates and high blinking which corresponds to high shear rates respectively (Srividya, Cardoza et al. 2001).

Viscosities of samples were measured in duplicate before and after triggering in situ gellation, with STF or STF/Mucin. To trigger in situ gellation, the formulation was mixed with STF or STF/Mucin dispersions in the ratio of 25:7 (Rozier, Mazuel et al. 1989) owing to conventional eye dropper volume of 25µL and resident ocular tear fluid volume of 7µL.

Timoptol LA® was used as a standard to compare the in situ gels formed by the different formulations being screened. It contains gelling gum as the in situ gelling polymer. Rheological tests were carried out on different formulations. The formulations were prepared in duplicates, while the rheological evaluations were carried twice; hence n=4 data sets per formulation tested.

3.3.2 Results and Discussion

The measurements taken during this assessment were all from samples terminally sterilized by autoclaving, as it was discovered in an earlier study (Orubu 2004) that autoclaving could influence the rheological behaviour of the polymeric ophthalmic preparations.

Figures 31 and 32 show the viscosity of different placebo formulations all containing 0.6% Gellan gum with various excipients present, at low shear rates and high shear rates respectively. In this assessment the effects of low shear rate, which corresponds to low blinking, and high shear rate, which corresponds to high blinking, were evaluated because these shear forces can cause shear thinning of the in situ gels.

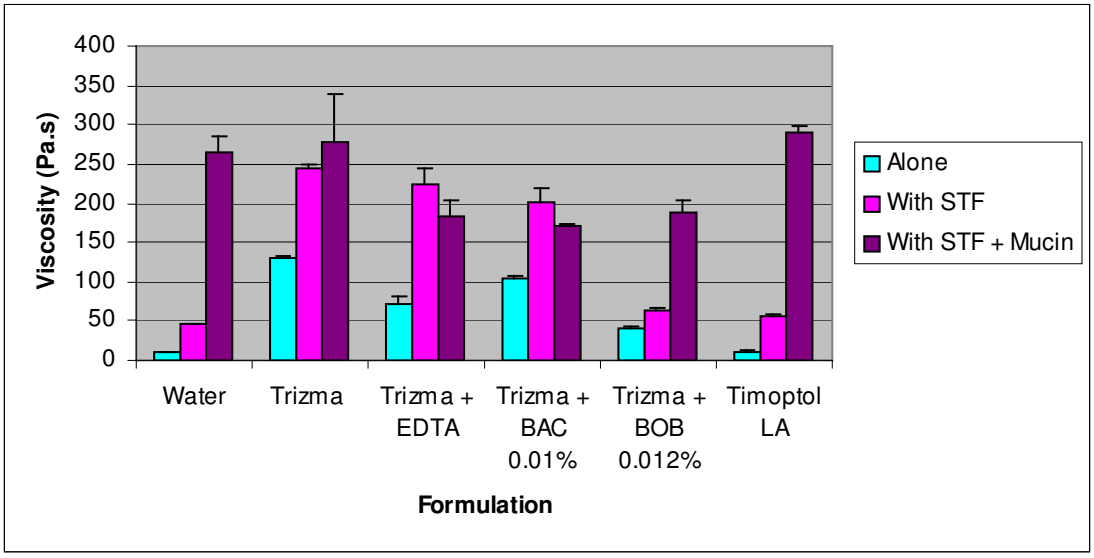


Figure 31: Viscosity (Pa.s) of various Gellan Gum 0.6% formulations at low shear (12.71 s-1)

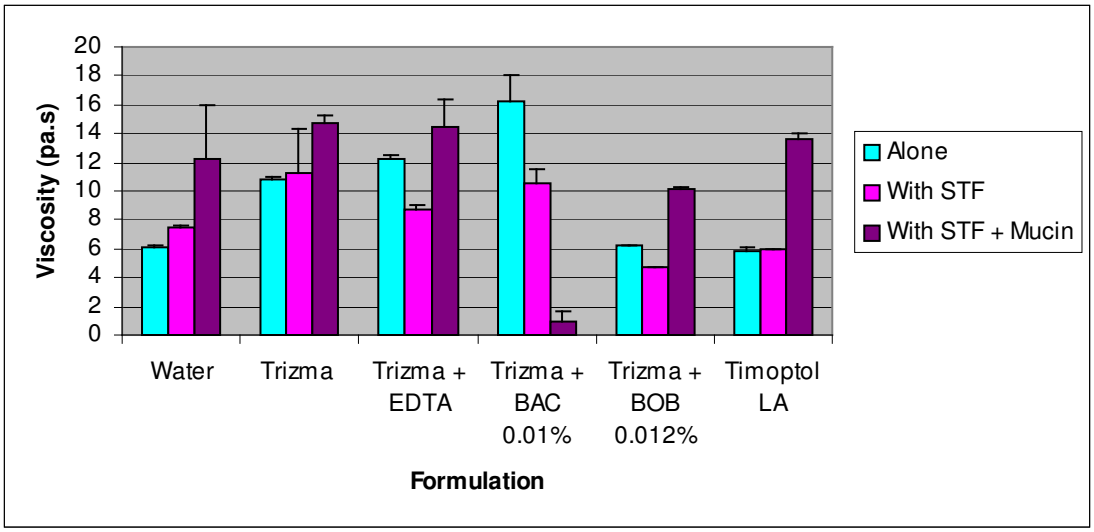


Figure 32: Viscosity (Pa.s) of various Gellan Gum 0.6% formulations at high shear (743.50 s-1)

- Gellan gum in Water
Gellan gum 0.6% in water exhibited a good gellation profile. The viscosity of the gel was very high, 265 Pa.s in the presence of STF containing mucins. The gellation profile of this formulation was similar to that which is formed by the standard (Timoptol LA®) both at low and high shear rates, the only in situ gelling ophthalmic preparation on the market in the UK.

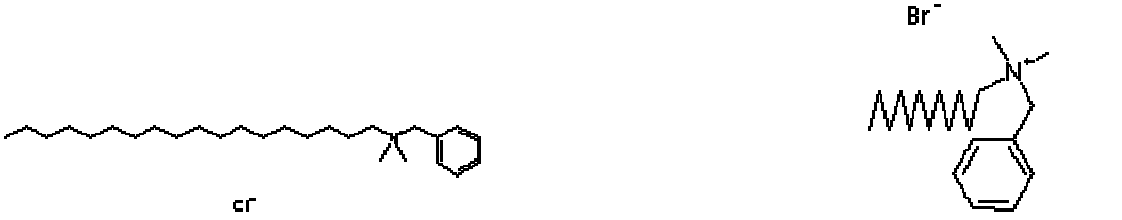
- Influence of Buffer
The buffer system maintains the pH of the formulation in the region of 7.4. The formulation containing Gellan Gum 0.6% in Trizma was slightly more viscous (128.7 Pa.s) at low shear rate. The consistency was similar to that of a shower gel, due to the formation of weak gel networks, even in the absence of STF and STF+Mucin.

Nevertheless it was still instillable as a drop. At high shear rates, the viscosity of the gels with STF+Mucin was still comparable to the standard. It is important to maintain the pH of the formulation within the physiological range; hence it is vital to incorporate a buffer system such as trizma for patient comfort.

- Influence of Antioxidant
The formulation containing Gellan Gum 0.6% in Trizma + 0.01% disodium edetate, the anti-oxidant had a low viscosity (70.79 Pa.s) and a consistency similar to baby oil, due to the formation of weak gel networks even in the absence of STF and STF+Mucin. It was instillable as a drop. The viscosity measurement showed a slight increase in viscosity compared to the formulation in water at high shear rate in presence of STF (+/- mucins).

- Influence of Preservatives
The addition of 0.01% Benzalkonium chloride preservative further induced gellation especially at high shear rate where it lost its in situ gelling capacity when mixed with STF+/-Mucin. When a reduction in gellation occurs, such as with the addition of STF+Mucin (it was speculated that this could be due to a dilution effect of the gels formed, as slight gellation (105.3 Pa.s) was already induced by the formulation alone. This preservative did not seem to be suitable for the formulation process. Instead 0.012% Benzododecinium Bromide was tested.

Benzododecinium bromide is a quaternary ammonium compound similar to benzalkonium chloride (see figure 33). It is the preservative used in the only in situ gelling ophthalmic preparation Timoptol LA®. This formulation showed a better and more acceptable gellation profile compared to the former. This preservative seems to be better choice.



Benzalkonium Chloride **Benzododecinium Bromide**

Figure 33: Chemical structure of the preservatives Benzalkonium Chloride and Benzododecinium Bromide. (www.chemexper.com)

- Influence of Cysteamine

The rheological assessment of Gellan Gum 0.6% in Trizma with 0.55% Cysteamine hydrochloride could not be carried out because the formulation formed a stiff gel (cuttable gel). Cysteamine itself is the main agent that causes gellan gum to completely gel; other options for consideration include reducing the concentration of the polymer within the formulation. This may reduce or inhibit gelation before instillation or perhaps combine gellan gum with another polymer that can undergo in situ gellation, such as a temperature, pH dependant or even another ionic strength dependant polymer.

Alam (2008) carried out further investigation into the effect of polymer concentration reduction. Formulations containing Gellan gum 0.15, 0.3 and 0.45% were assessed for in situ gellation. It was found that the current formulation consisting of: pH buffer (trizma), drug (cysteamine hydrochloride) and antioxidant (EDTA) with Gellan gum 0.45% was able to induce in situ gellation when mixed with STF (569 Pa.s), but had already formed firm gel networks prior to addition of STF (421 Pa.s). The gellation profile of the formulation containing 0.3% and 0.15% when mixed with STF were similar (respectively 51 and 71 Pa.s) and both were still instillable as a drop.

- Polymer combination

In order to avoid this self-gelation due to the drug itself, polymer combinations were tried out. The combination with a pH dependant polymer (Carbopol) was attempted. Moreover Carbopol has excellent mucoadhesive characteristics. The results from the evaluation of Gellan gum 0.15% with Carbopol 0.1% showed the gellation profile of Gellan gum 0.15% alone (71 Pa.s) was similar to that which was combined with Carbopol (53 Pa.s), while Carbopol 0.1% on its own (4 Pa.s) did not induce any gel formation when mixed with STF.

In order to validate this combination, mucoadhesion experiments should be carried out. It was interesting to note that the pH of the formulation was only reduced to 6.5 by 0.1% Carbopol, compared to 7.3 (pH of formulation without Carbopol) which was deemed as still acceptable in term of patients tolerance (Alam 2008).

Osmolarity Measurements

The osmolarity values of different Gellan gum formulations are displayed in figure 28. Formulations with individual excipients, 0.6% gellan gum and no cysteamine were hypo-osmolar, while the complete formulations with all excipients combined had osmolarity values ranging between 255 to 264 mosm/L. This value is quite close to the physiological range of 280-300mosm/L.

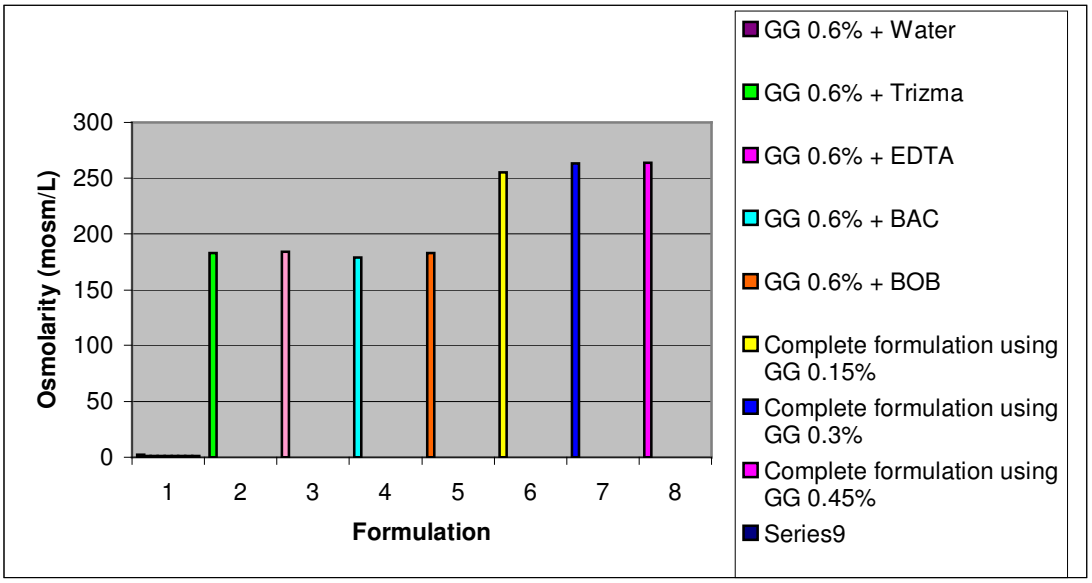


Figure 34: Osmolarity (mosm/L) of preparations containing Gellan Gum. (Complete formulation consists of: Trizma, EDTA, and BOB)

4. In vivo studies in rabbits

Modules 1,2,3,4 and 5 of the accredited training for personnel working under the animals (scientific procedures) Act 198 have been successfully completed.

A project licence application could then be submitted to the ethics committee at the School of Pharmacy for approval. The initial application was not accepted, as there were questions and further comments that needed clarification. All the questions and comments have been actioned and has been re-submitted. Below the future ex vivo and in vivo work is summarized.

The objective of the ex vivo/ in vivo experiments in the study is to assess the corneal absorption of cysteamine from an optimised formulation of cysteamine in situ gel eye drops.

Animals have to be used, as we need to establish that the active drug cysteamine will be absorbed into the cornea to offer the desired therapeutic effect, and to confirm pharmacological activities in vivo. Furthermore data from the animal study will be needed for the scheduled clinical trials in cystinotic children.

Rabbits are commonly used in ophthalmic *in vivo* work (Jain, Kuwabara et al. 1988). This animal model was chosen, because the procedure should not produce any suffering to the animal due to ocular application of a non-irritant formulation of cysteamine in situ gel eye drops. The formulation will maintain a physiological pH and an osmolarity within a range of 280-300mOsmol.

After the optimisation of the formulation of a Cysteamine 0.55% in situ gel eye drops (pH near to physiological pH, iso-osmolarity and a system that will undergo the phase transition from a liquid to a gel when in contact of the eye), various stages are involved

1. Ex vivo permeation study: this will be carried out using whole eyeballs of rabbits to be procured from a slaughterhouse. The eyeballs should be immersed and kept in a triggered gel (triggered gel: optimised formulation mixed with simulated tear fluid and mucin in a ratio of 25:7 to form a gel) (Carlfors, Edsman et al. 1998). After 12 hours the corneas should be excised and assayed for Cysteamine content.

2. Pilot study in vivo: this will be carried out in two New Zealand white rabbits, to determine:
   - The dosing interval of the optimised formulation in the actual *in vivo* release studies (x)
   - The dosing duration in the actual in *vivo* release studies (y)
   - The timing of the sacrifice of the animals (z)

3. Study of the release of Cysteamine in vivo: this stage of the project will be carried out in New Zealand white rabbits. The optimised formulation will be administered twice a day x hours apart (x will be determined from the pilot

study) and will be compared to the conventional Cysteamine eye drops administered every hour.

An overview of the general plan of work is provided below in the flow diagram. It shows the sequence of or links between experiments anticipated, decision points, and where work fits in with other approaches.

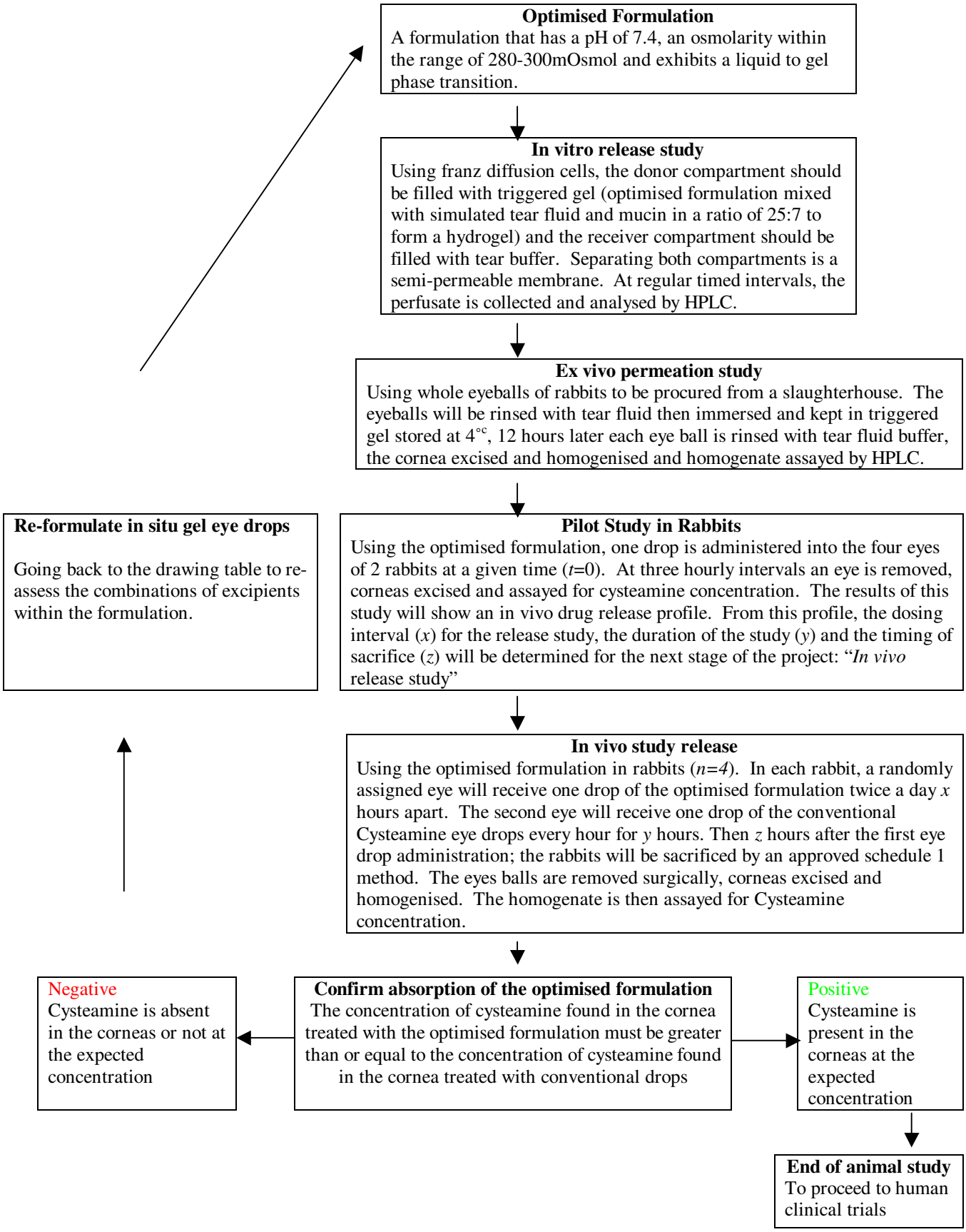


Figure 35: Overview of the proposed ex and in vivo study in rabbits

4.1 Pilot Study

Using the optimised formulation, one drop of the in situ gel eye drops is administered into the lower eyelids of the four eyes of two New Zealand White rabbits at a given time (*t=0*).

At *t=3* (three hours post dose), general anaesthesia without recovery is administered to rabbit (1). A randomly selected eye of rabbit (1) is removed and the upper and lower eyelids are sutured. Immediately the excised eyeball is rinsed with saline to ensure all gels are removed from the eye surface. The cornea is then removed and prepared for homogenisation and assay for Cysteamine content.

At *t=6* (six hours post dose), the second eyeball of anaesthetised rabbit (1) is removed and immediately rinsed with saline to ensure all gels are removed from the eye surface. The cornea is then removed and prepared for homogenisation and assay for Cysteamine content. Rabbit (1) is then sacrificed by an approved schedule 1 method.

At *t=9* (nine hours post dose), general anaesthesia without recovery is administered to rabbit (2). A randomly selected eye of rabbit (2) is removed and the upper and lower eyelids are sutured. Immediately the excised eyeball is rinsed with saline to ensure all gels are removed from the eye surface. The cornea is then removed and prepared for homogenisation and assay for Cysteamine content.

At *t=12* (twelve hours post dose), the second eyeball of anaesthetised rabbit (2) is removed and immediately rinsed with saline to ensure all gels are removed from the eye surface. The cornea is then removed and prepared for homogenisation and assay for Cysteamine content. Rabbit (2) is then sacrificed by an approved schedule 1 method.

How to prepare the excised cornea for an assay (Frucht-Pery, Mechoulam et al. 2004)

- Within five minutes of sacrificing the animals, the surface of the eye should be rinsed with 10mLs of saline to ensure that any remaining gel on the eye surface is removed.
- Excise the cornea and rinse again with 5mLs of saline.
- Weigh the cornea and mince with a surgical blade and place in a microcentrifuge tube.
- To each tube, add 0.5mL 0.01M phosphate buffered saline (pH 7.2) (spiked with a known concentration of Cysteamine).
- Incubate the tubes for 18 hours in a water bath heated to 37˚C, shaking at 100 oscillations per minute.
- Thereafter, centrifuge the tubes at 2000rpm for 10 minutes
- Recover from each tube 425µL of the supernatant and assay for cysteamine concentration.

- Calculation: (Frucht-Pery, Mechoulam et al. 2004)

$$[\text{conc. of cysteamine in the cornea}] = \frac{[\text{cornea weight + buffer volume}] \times [\text{conc. of cysteamine in buffer}]}{[\text{cornea weight}]}$$

4.2 Study of the release of Cysteamine in vivo

The study of the release of cysteamine from the in situ gel into the cornea will be carried out in New Zealand White rabbits (*n=2*).

To rabbit (1) and (2), a randomly selected eye will receive into the gap between the lower eye lids one drop of the in situ gel eye drops twice a day *x* hours apart. To the second eye, one drop of the conventional Cysteamine eye drops is administered into the gap between the lower eyelids every hour for *y* hours.

The animals will be killed by an approved schedule 1 method, *z* hours after the first administration of the eye drops. After sacrifice, the eyeballs are removed, the corneas excised and prepared for homogenisation and assay.

The conventional cysteamine eye drops administered every hour will serve as the control for the study to compare the concentration of the drug (cysteamine) released into the corneas from the optimised in situ gel eye drops. The homogenised rabbit corneas will be assayed for cysteamine concentration. The concentration of Cysteamine found in the corneas treated with the optimised formulation must be greater than or equal to the concentration of Cysteamine found in the corneas treated with the conventional eye drops.

This study will be carried out twice. This ensures there are 4 sets of data points for each variable to make the results statistically significant. Hence in total **n=4 rabbits** (eight eyes and four sets of data per variable).

4.3 Minimisation of animal numbers

Prior to the phase of the project that involves in vivo studies, some in vitro work will be carried out, such as:

- In vitro release study (using franz diffusion cells)
- In vitro permeation study, and
- Pilot study using two New Zealand White rabbits. The aim of the pilot study is to establish:

    ⇒ Dosing interval (*x)* of the in situ gel eye drops
    ⇒ Duration of study (*y)* and
    ⇒ Time of sacrifice (*z*)

The three results (*x, y and z*) obtained from the pilot study mentioned above will reduce the need to repeat the in vivo release study. This can occur due to the non-

release of the drug from the formulation, hence having to re-formulate and optimise and re-test or due to blindly choosing a dosing interval, time of sacrifice and duration of study.

The rabbits will be provided with a fitted neck collar to prevent them form rubbing their eyes (dislodging or removal of the gel in the eyes). The rabbits will be supervised and left to freely roam in a study area with direct access to food and water.

5. Work in progress and future work

Analytical work

The CMQT pre-column derivatization method for thiols is being further explored at the moment with the help of Professor Edward Bald.

The pre-column derivatization method will be used for the analysis of cysteamine in formulation for assay, stability and release studies. The *in vivo* studies that require the determination of cysteamine within rabbit corneas may require a more sensitive method. If a very low limit of detection using the CMQT pre-column derivatization is achieved, then that will be sufficient for the *in vivo* analysis; otherwise the stable isotope LC/MS/MS method may be explored.

Formulation work

Following the screening of excipients, formulation development will progress by evaluating other polymer combinations using gellan gum, sodium alginate and chitosan with the chosen excipients. Furthermore oscillatory measurements in the rheological evaluation to determine and compare the strength of the gels formed by the various formulations will be performed.

In vitro drug release studies

It will be carried out using dialysis bags or Franz diffusion cells. The cell consists of a receptor solution reservoir with a side-arm sampling port. The system mimics the behaviour of the in situ gel in the eyes. Sampling the stirred receptor chamber solution at regular timed intervals monitors the release of the active drug, "cysteamine" from the *in situ* gel. The result is plotted against time to give the release profile. The proposed analytical method will be utilised to determine the concentration of cysteamine in samples collected from the sampling port of the franz cells.

Stability studies and scale up

Once an optimised formulation is designed, stability studies will be carried out to determine the optimal storage conditions and shelf life of the product. In parallel will be the scale up manufacturing process at Guys and St. Thomas'.

In vitro permeation study

This will be carried out using whole eyeballs of rabbits to be procured from a slaughterhouse.

In vivo pilot study: this will be carried out in two New Zealand white rabbits, to determine:

In vivo study of the release of cysteamine

This stage of the project will be carried out in New Zealand white rabbits.

Clinical Trial

This final stage of the project will be carried out in cystinotic children for a period of twelve months.

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