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Chemical Characterisations of Drug Stability

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CHEMICAL CHARACTERISATION OF DRUG STABILITY

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Declaration

I certify that this thesis which I now submit for the award of M.Phil, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

The thesis was prepared according to the regulations for postgraduate studies by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

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Candidate

This work was carried out in the Pharmacy Department in St. James's Hospital, Dublin under the supervision of Professor Kamal Sabra and in the Dublin Institute of Technology, Kevin Street, Dublin 8 under the supervision of Dr. Eilish O' Donoghue

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PUBLICATIONS AND PRESENTATIONS

- July 2001: Paper titled "Stability of Morphine Sulphate in PCAs", Submitted to European Journal of Hospital Pharmacy pending publication at time of writing.

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ABSTRACT

The objective of the initial stages of this project was to develop a method for the separation of morphine sulphate and haloperidol using high performance liquid chromatography (HPLC). The HPLC methods were then validated. The validation parameters investigated were: accuracy, precision, linearity, specificity, system suitability, robustness, range. Method validation was carried out at the method development stage and throughout the project.

Having successfully developed and validated the HPLC method for the separation of morphine sulphate and haloperidol it was then possible to continue to the next objective of the project i.e. to assess the chemical stability of the following:

1. Morphine sulphate in P.C.A. (Patient Controlled Analgesia) silicone balloon infuser
2. Morphine sulphate in polypropylene syringes
3. Morphine sulphate (0.5-12 mg/ml) and haloperidol (0.25-2 mg/l) in combination in polypropylene syringes

Morphine sulphate (2 mg/l and 10 mg/l) in P.C.A infusers retained stability over the 40 days of testing (room temperature (22±2°C) and 4°C). A 28 day shelf life has been allocated to morphine P.C.A.'s as a result of this work, and having taken into account microbiological validation of the compounding unit in St. James's Hospital. P.C.A's are now available in batches in "ready-to-use" form for in-house, outside hospital use and home healthcare use. Morphine sulphate (2 mg/l and 10 mg/l) in polypropylene syringes retained stability over the 30 days of testing (room temperature (22±2°C) and 4°C).

This project shows that it is feasible to conduct small-scale stability studies using HPLC within a hospital Pharmacy department with significant impact on clinical practice.

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CHAPTER 1

DRUG STABILITY OVERVIEW

1.1: Drug Stability Introduction

Drug stability and compatibility are critical elements in the accurate and appropriate delivery of drug therapy to patients and in the handling of drugs. High performance liquid chromatography is the method of choice for many drug stability protocols. Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems [(1)].

The term instability refers to chemical reactions that are incessant and irreversible and result in distinctly different chemical entities called degradation products that can be both therapeutically inactive and possibly exhibit toxicity. Typical degradation reactions involve oxidation, acid hydrolysis, base hydrolysis, photolysis. Solution pH and temperature are also factors that affect drug stability [(2)].

Temperature is an important factor to consider. Significantly different rates of drug decomposition may occur at a high temperature (e.g. 30°C) compared to those occurring at a lower one (15°C).

It must be noted that the terms stability and compatibility are relative concepts. To be meaningful they require qualifiers regarding time and conditions. Simple conclusions stating that two drugs are compatible or not may not be particularly useful. Operating and testing conditions can affect results, for example more than ten percent of a drug may decompose in solution in 24 hours at 25°C. This would fit the conventional concept of incompatibility. Yet the drug may be useful for a shorter period of time. Thus clinical rationale is required.

The term incompatibility refers to physiochemical phenomena such as concentration dependant precipitation, acid base reactions, solubility changes or container interactions. When incompatibilities result in visible changes such as precipitation, haziness , colour changes, changes in viscosity, or the formation of immiscible layers the term physical or visual incompatibility is applied [(2)].

Thus if a drug is unstable or a drug combination is incompatible after a period of time shelf life restrictions must be imposed. Typically the period of time for which a minimum of 90% of the drug remains intact and available for delivery is the standard used for quantifying stability [2,3]. This term though assumes that the initial drug concentration is at one hundred percent. If initial drug concentration is compared with a reference standard then the time zero drug concentration value may be higher or lower than one hundred percent.

Thus for the morphine stability study in PCA (Patient Controlled Analgesia) infusers (see section 1.5) and in polypropylene syringes, and for the combination study of morphine and haloperidol in polypropylene syringes, drug concentration values will be declared as indicating stability if the concentration does not decrease by more than 10% of the initial time zero concentration.

One of the objectives of this project having carried out the development and validation of a suitable HPLC method was to analyse the stability of morphine sulphate in PCAs, and in syringes and to assess the stability of morphine sulphate and haloperidol in combination in syringe drivers.

In St. James's Hospital, morphine sulphate is the opioid used for the treatment of severe pain. Nausea and vomiting affect a percentage of those patients on strong opioids and it is often necessary to use drug combinations to counteract some of these side effects where possible. In St. James's Hospital and various hospices in both Ireland and the United Kingdom, clinical experience and anecdotal information support the use of drug combinations. Scientific evidence of stability or compatibility is rare. Much of the early work on drug stability relied on the presence or absence of physical degradation such as precipitation or colour change as a measure of drug compatibility [4], which quite obviously is not a truly accurate representation of stability or compatibility.

One drug combination that has been prioritised for further research in St. James's Hospital is morphine sulphate and haloperidol. Haloperidol is a butyrophenone. It is a powerful anti-emetic, anti-psychotic and an anxiolytic. The compatibility of

morphine sulphate and haloperidol can be examined at a number of concentrations. Table 1.1 below shows, from clinical practice there is a large range of concentrations used for each drug .

Drug	Clinical Use	Concentration (mg/24hr)	Reference
Morphine	Analgesia	30-5000	Zachrisson & Furst, 1998[5]
	Analgesia	4-160	SJH, Murray, 1997[6]
Haloperidol	Anti-emetic	1-10*	Murray 1997 [6], McQuillan 1996 [7]
	Anxiolytic	5-30**	Murray 1997 [6], McQuillan 1996 [7]

*most common dose in SJH is 5mg/24hr

**Max used in Our Ladies Hospice is 15mg/24hr

Table 1.1: Concentration ranges of morphine sulphate and haloperidol

A literature search found no assessment of chemical stability and compatibility of morphine sulphate and haloperidol (as Serenace®) by HPLC. LeBelle (1995) [8] assessed morphine sulphate and haloperidol base (Haldol®) for chemical stability. Cloudiness was immediately evident when the morphine and haloperidol solutions were mixed. HPLC and GC-MS analysis showed the precipitation to be haloperidol and methyl and propylparaben. Methylparaben and propylparaben are preservatives used in the Haldol preparation and there are no such preservatives used in the Serenace preparation used for this stability study.

Schriver (1998) [9] assessed varying concentrations of morphine hydrochloride with a fixed concentration of haloperidol using UV spectrophotometry. The combinations prepared were found to be apparently stable but a more specific method of analysis such as HPLC was necessary.

Chandler (1996) [10] assessed the stability of morphine sulphate and haloperidol lactate using visual examination and a Tyndall beam. A turbidimeter was used for cloudiness analysis. The combinations tested were found to be stable.

The literature survey revealed several references that dealt specifically with the stability of morphine and table 1.2 below summarises several of these references.

Ref.	λ (nm)	Flow Rate	Retention Time	Mobile Phase	Column	Conc range
11	280	1.0 mls/min	2.6 mins	25% acetonitrile : 75% 0.05M phosphate buffer (pH 7.5)	Novapak C18 4u 150 x 3.9mm	80-550 µg/ml
12	254	0.5mls/min	4 mins	12% methanol : 88% 0.05M phosphate buffer (pH 7.5)	Novapak C18 5u 3.9 x 15 cm	10-100 µg/ml
13	280	1.5mls/min	7 mins	35% methanol : 65% K_3PO_4 buffer	LC-18DB C18 5u 250 x 4.6mm	70-1510 µg/ml
14	300	1.0 mls/min	3.7 mins	20% acetonitrile : 80% 0.1M sodium phosphate dihydrate (pH 4.2)	Cyano reverse phase 5u 250 x .6mm	N/A
15	284	2.0 mls/min	0.62 mins	0.01M sodium pentene-sulphonate/acetonitrile/ ortho phosphoric acid	RP; Hypersil ODS 5um 100 x 5mm ID	100-2000 µg/ml
16	280	1.0 ml/min	5.4 mins	Methanol/ammonium phosphate (40:60, v/v), pH 6.22 with TFA	u-Bondapak C18, 300 x 3.9mm ID	N/A
17	216	1.5 mls/min	6.7 mins	50% acetonitrile in 0.02 M potassium phosphate pH 5.4	Spherisorb cyano column 300 x 4.6mm ID, 5um particle size	100-150 µg/ml
18	280	2.0 mls/min	2.5 mins	7% acetonitrile and 93% 0.01M sodium phosphate	Hamilton PRP-1 rigid copolymer column	N/A

Table 1.2: Morphine Sulphate methodologies

Other methodologies were found for morphine hydrochloride [19] and morphine tartrate [20]. The literature search also brought to attention a small number of haloperidol studies which are presented in table1.3 below:

Ref	λ (nm)	Flow Rate	Retention Time	Mobile Phase	Column	Conc range
8	254	1ml/min	21.5 mins	65%triethylamine, 25% acetonitrile and 10% terahydorfuran, pH7	Supelcosil LC-8, 5um	N/A
21	220	1ml/min	11.8 mins	7% 0.02M Sodium dihydrogen phosphate and 29%tetrahydrofuran	Symmetry C18 3.9 x 150mm	2-20ng/ml
22	196	2mls/min	6.7 mins	55% Acetonitrile and 45% 100mM KH_2PO_4 pH 3.8-4.0	Reverse Phased octadecyl 10um C18 250 x 4.6mm ID	N/A
23	250	0.5 ml/min	12.5 mins	63% methanol and 37% water with ammonium acetate	Nucleosil C18 5um 250 x 4 mm Internal diameter	N/A
24	EC	N/A	N/A	90% Acetonitrile, 5% methanol, 5% 0.1M ammonium acetate	Utrasphere cyano 5um	> 50pg/mls

Table 1.3: Haloperidol Methodologies

1.2: Morphine Sulphate Chemistry

Opiates and their synthetic counterparts (opioids) are used in modern medicine to relieve acute pain suffered as a result of disease, surgery or injury, in the treatment of some forms of acute heart failure. They are not the desired treatment for the relief of chronic pain, because long-term and repeated use can lead to drug dependence and side effects. They are however, of particular use in control of pain in the later stages of chronic illness where the possibility of dependence is not a significant issue [(25)].

Opium contains a mixture of almost twenty-five alkaloids. The principle alkaloid in the mixture, and the one responsible for analgesic activity, is morphine, named after the ancient Greek god of dreams, Morpheus. Only morphine and codeine are still in widespread clinical use today . Pure morphine was isolated in 1803 but was not produced on a commercial scale until 1833 [(26)]. Macfarlane and Co. (now Macfarlane and Smith) isolated and purified it on a commercial scale.

However since morphine was poorly absorbed in an oral dosage form, it was little used in medicine until the hypodermic syringe was invented in 1853, allowing doctors for the first time to inject morphine directly into the blood stream [(27)].

Morphine was then found to be an effective analgesic and sedative and was far more effective than crude opium. By 1881 the functional groups of morphine were identified but it took many years to establish the full structure.

Sir Robert Robinson proposed the first correct structure of morphine in 1925, and the full synthesis of morphine was achieved in 1952, but it was not until 1968 that the structure proposed by Robinson was finally established when it was studied by X-ray crystallography [27].

The development of narcotic analgesics is a good example of the traditional approach to medicinal chemistry and provides a good example of the various stages which can be employed in drug development. Several stages can be identified in the development of morphine as shown in table 1.4 below [27].

Stage	Process
Stage 1	Recognition that a natural plant or herb (opium from the poppy) has a pharmacological action
Stage 2	Extraction and identification of the active principle (morphine)
Stage 3	Synthetic Studies (Full and partial synthesis)
Stage 4	Structure-activity relationships-synthesis of analogues to see which parts of the molecule are important to biological activity.
Stage 5	Drug development-synthesis of analogues to try and improve activity or reduce side effects.

Table 1.4: Development of morphine

1.3: Structure and Properties of Morphine Sulphate

Structures of Morphine Sulphate

Figure 1.1: Structure of morphine [$C_{17}H_{19}N0_3$]

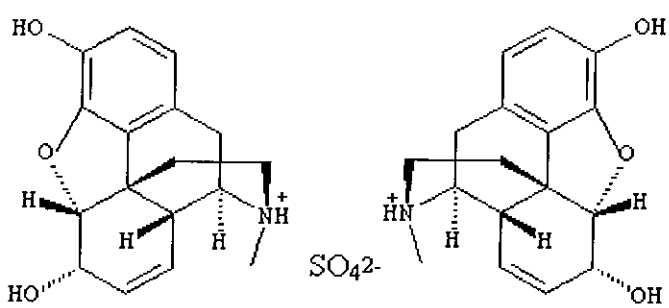

Figure 1.2: Structure of morphine sulphate [$(C_{17}H_{19}NO_3)_2.H_2SO_4$]

Morphine Sulphate Characteristics

Compound:	Morphine Sulphate
Chemical Name:	(5α, 6α)-7,8-Didehydro-4,5-epoxy-17-methyl-morphinan -3,6-diol
Molecular formula:	$(C_{17}H_{19}NO_3)_2.H_2SO_4$
Molecular Weight:	758.8
Composition of morphine:	C 71.6% H 6.7% N 4.9% O 16.8%
Characteristics:	White feathery, silky crystals, cubical masses of crystals or a white, crystalline powder.
Storage:	Morphine sulphate should be stored in a well closed container and protected from light
Actions and Use:	Narcotic Analgesic
Solubility	Soluble in water and ethanol, insoluble in ether
Light Absorption	λmax = 298nm in aqueous solution
Melting point:	254°C to 256°C (decomposition)

A saturated solution of morphine is basic to litmus. pKa values are 9.51 at 25°C for the phenolic group, pKa = 8.31 at 25°C for the amino group. Although morphine contains many functional groups that undergo reaction under certain conditions the functional group primarily responsible for its instability in aqueous solution is the phenolic moiety. This group readily undergoes oxidation reactions the major products derived are pseudomorphine (fig 1.3) and morphine N-oxide (fig 1.4) [28].

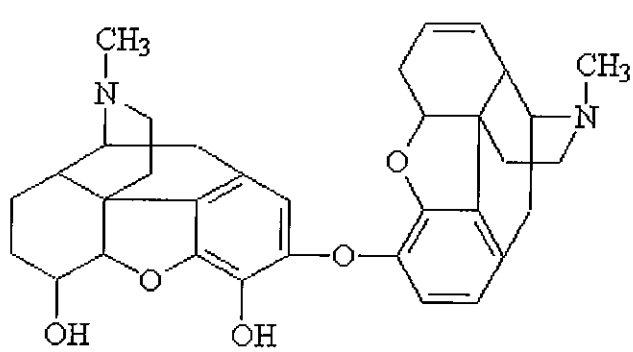

Figure 1.3: Pseudo Morphine

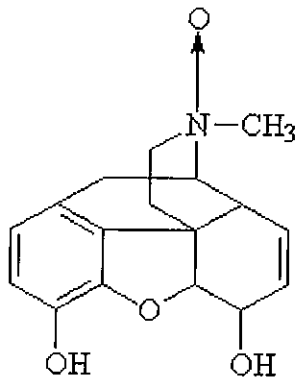

Figure 1.4: Morphine N Oxide

Aqueous morphine solutions exhibit a strong dependence of stability on pH and also on the presence of oxygen. The pH rate profile shows that solutions of morphine are relatively stable at low pH values, but degradation proceeds at a much higher rate in neutral or basic media. Degradation is often accompanied by the appearance of a yellow or brown colour in the solution. Since both pseudomorphine and morphine N-oxide are both colourless, it has been suggested that the colour is due to further decomposition (probably polymerisation) of the original degradation products or possibly the formation of 10-oxomorphine (fig 1.5).

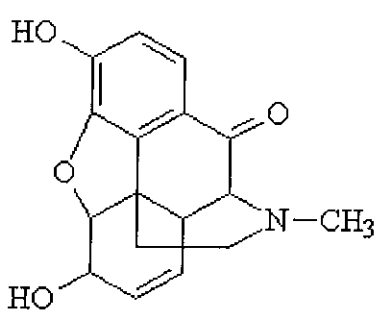

Figure 1.5: 10-oxomorphine

Morphine is an asymmetrical molecule containing several asymmetric centres, and exists naturally as a single enantiomer. When morphine was first synthesised it was made as a racemic mixture of the naturally occurring enantiomer (fig 1.6) plus its mirror image (fig 1.7). These were separated and the unnatural mirror image was tested for analgesic activity. It turned out to have no activity whatsoever.

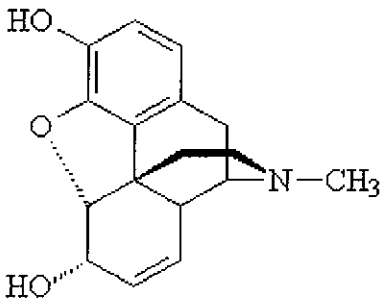

Fig. 1.6: Morphine

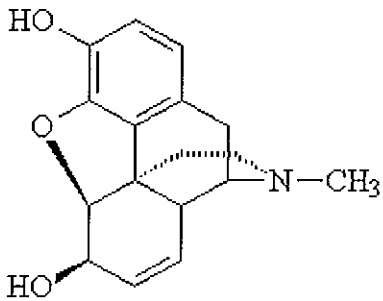

Fig. 1.7: Morphine mirror Image

Presented below is an outline of the different functional groups in the molecule and the effects that these functional groups cause.

Phenolic OH

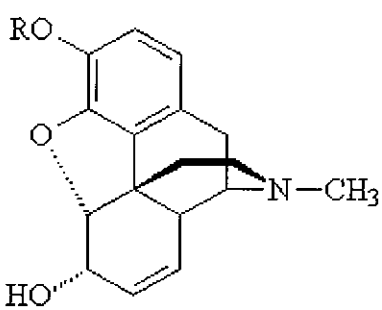

Fig 1.8: morphine molecule showing phenolic OH

The structure above (fig 1.8) is morphine when the R equals hydrogen. By methylating the phenolic OH group the analgesic activity drops remarkably. Incidentally the molecule is now codeine. If the hydrogen is replaced with an ethyl or acetyl group the analgesic activity decreases continually [28].

The alcohol on the six position

By masking or losing the alcohol group on the six position, there are no reported decreases in analgesic activity but depending on the substituted group the analgesic activity can increase [28].

N-Methyl

The literature discusses effects of the nitrogen methyl group and on altering the analgesic properties by altering the CH_3 group but the nitrogen atom is essential to analgesic activity and removal will result in a total loss of all analgesic activity [28].

Aromatic Ring

The aromatic ring is essential. Compounds lacking aromaticity show no analgesic activity [28].

1.4: Structure and Properties of Haloperidol

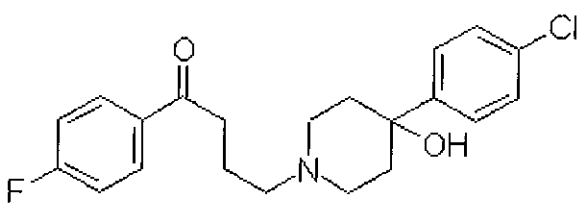

Figure 1.9: Haloperidol structural formula

Compound:	Haloperidol
Chemical Name:	4-[4-(4-chlorophenyl)-4-hydroxypiperidino]-4'-Fluorobutyrophenone
Molecular Formula:	$C_{21}H_{23}ClFNO_2$
Molecular weight:	375.9
Composition:	C=67.1%, H=6.2%, Cl=9.4, F=5.1%, N=3.7%, O=8.5%
Characteristics:	A white or slightly yellowish, amorphous or crystalline powder. It melts at about 150oC. Practically insoluble in water.
Storage:	Haloperidol should be kept in an airtight container and protected from light
Actions and Use :	Antipsychotic [28] [29]

1.5: Stability of Morphine Sulphate in PCAs

Patient controlled analgesia (P.C.A.) is a relatively new technique for managing pain whereby patients have the ability to self administer small controlled doses of opiate analgesic medications when needed [30].

Drug delivery without using a P.C.A. device requires a large dosage of medication to be injected into muscle tissue. This delays the drugs action. The lesser more frequent doses delivered by the P.C.A. infuser control pain more effectively and reduce sedation by releasing medication directly into the bloodstream [30]. The P.C.A device is also advantageous as it reduces the number of times a member of medical staff is required to give a patient an injection.

Figure 1.10 below demostrates the difference between analgesia injection and PCA infusor.

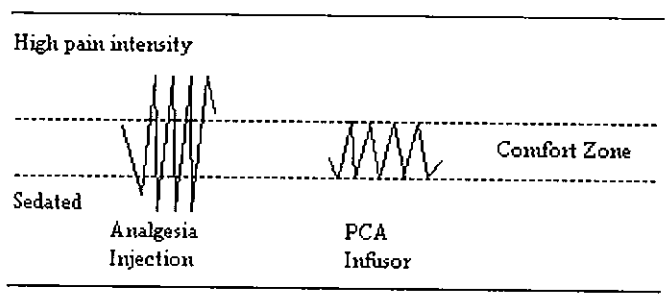

Figure 1.10: Analgesia and PCA injection

The advent of a disposable device allowing the delivery of a controllable amount of analgesic and a "lock-out" offers the opportunity for pharmacy to become more involved in the filling of such devices. Apart from an extension to CIVAs (Centralised IntraVenous Additive) services the lock out mechanism is a safety measure in order to ensure that patients do not overdose themselves.

One of the factors affecting the filling of these devices is the chemical stability of the opiates contained within, and it was the aim of this study to evaluate the chemical stability of morphine sulphate in P.C.A.s. Following evaluation of the chemical

stability it was then possible to establish an appropriate shelf life for morphine P.C.A.s.

High Performance Liquid Chromatography is the gold standard of chemical drug stability testing. The pharmacy department in St. James's Hospital has recently purchased a new Shimadzu HPLC system. Quantitative analysis was performed on this system. Once the HPLC method for the assay of morphine sulphate was developed and validated (see chapters 3 and 4), HPLC analysis was performed under these conditions over a forty-day period (see chapter 5).

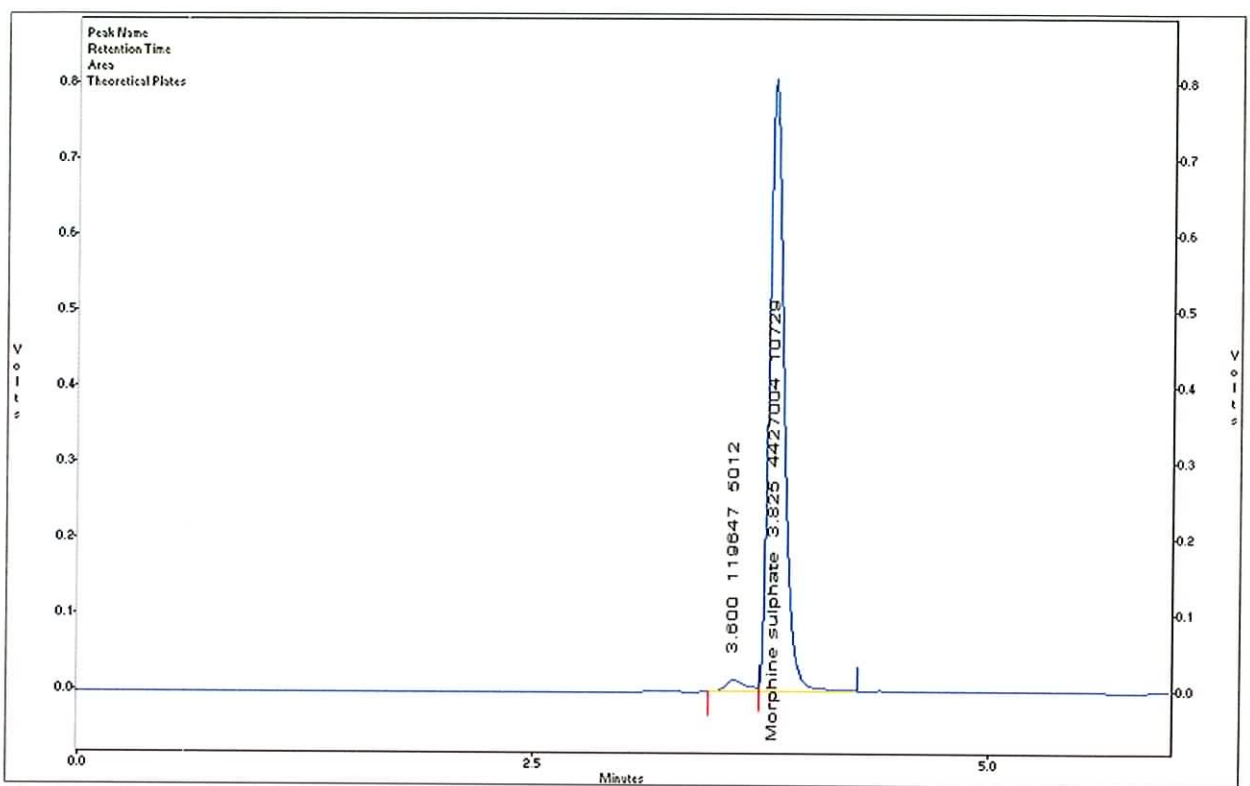

Fig 1.11: Example of a chromatogram of morphine sulphate

1.6: Stability of morphine sulphate in polypropylene syringes

Polypropylene syringes are also used in St James's Hospital for the administration of morphine sulphate. It was discussed and decided that it would be useful to assess the stability of morphine sulphate in the syringes and compare the data to that obtained from the P.C.A. study. The literature has references to stability of morphine in syringes but not with the same preparation used in this project. In this project it was possible to observe the stability patterns in two different environments and compare the data obtained. Again the same HPLC method was used and identical samples were prepared under aseptic conditions.

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CHAPTER 2

METHOD DEVELOPMENT AND VALIDATION OVERVIEW

2.1: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Much of modern chemical technology is based on our ability to separate and analyse complex samples, especially those of interest to the biological and medical sciences. Classical column chromatography invented by Tswett in the early 1900s, experienced a rapid increase in use after its reintroduction in 1930 by Kuhn and Lederer [(1)].

HPLC (High Performance Liquid Chromatography), GC (Gas Chromatography), and TLC (Thin Layer Chromatography) are used today to a much greater extent than are either older or more recent procedures, such as supercritical fluid chromatography (SFC), CE and capillary electro chromatography (CEC). Mass Spectrometry is currently an alternative to chromatographic procedures for analysing some multi-component samples, and we are likely to see an increase in the use of LC/MS (Liquid Chromatography in Conjugate with Mass Spectrometry).

GC gained popularity because of its superior resolution, better automation and more accurate and convenient quantitation. However, GC required volatile samples which ruled out ~75% of all samples. Early versions of HPLC were introduced in the period 1959 – 1964 for the analysis of certain sample types: amino acids [(2)] and synthetic polymers [(3)]. During the mid-1960s, Horwath, Huber and Kirkland developed means for the separation of many samples [(4)] through improvements in equipment and columns. These and other efforts led to the development of what we now call HPLC.

During the 1970s most chemical separations were carried out using a variety of techniques including open column chromatography, paper chromatography, and thin layer chromatography. However these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time pressure liquid chromatography began to be used to decrease flow-through time, thus reducing purification times of compounds being isolated by column chromatography. However flow rates were inconsistent and the question of whether it was better to have a constant flow rate or constant pressure was debated [(5)].

By the 1980s HPLC was commonly used for the separations of chemical compounds. New techniques improved separation, identification, purification and quantification

far above previous techniques. Computers and automation added to the convenience of HPLC [5].

With the commercial introduction of general-purpose equipment a few years later, HPLC began an explosive growth that, by the 1990s, would raise it to first place in total analytical instrument sales and in scientific importance as well. HPLC's present popularity is attributable to its convenient separation and analysis of almost any sample that can be dissolved, and that it can solve various related problems. Applications are generally characterized by exceptional resolving power, speed and detection limits of nanomoles per litre or less. For example, HPLC is a key technology in pharmaceutical R & D for purifying synthetic or natural products; characterizing metabolites; assaying active ingredients, impurities and degradation products generated by accelerated aging.

The practice of HPLC is now 35 years old. Users can choose equipment and columns for almost any application, understand how to best carry out separations, and analyse almost any sample. When the need arises, separations can be completed within a few seconds, or conditions can be optimised to separate every component, in samples containing greater than 40 constituents. With the exception of low-boiling samples that are still better separated by GC and molecules $>10^6$ Da that are better candidates for field-flow fractionation, the typical user today is generally satisfied with the capabilities of HPLC and is unlikely to switch to an alternative chromatographic technique [1].

High performance liquid chromatography is the gold standard of chemical drug stability testing. The basis of all chromatography separations is the differential migration of solutes in a moving phase. In HPLC this differential migration occurs through an equilibrium of the solute between the mobile phase flowing through the column and the column packing or stationary phase. A simple example illustrates this: Compound A and B are injected. Compound A is attracted more strongly to the stationary phase and compound B to the mobile phase. As a result of this B spends more time in the mobile phase than A and travels through the column more quickly. This interaction of solutes with a single particle of stationary phase occurs many times within the HPLC column [6].

2.2: Chromatography Nomenclature

Chromatography nomenclature has evolved over many years. In the late 1950's the first efforts were made to collectively classify the various chromatography terms into a formal nomenclature. These were laid out by the British Chromatography Discussion Group. The various terms are defined as follows [7].

The ***baseline*** is any part of the chromatogram where only the mobile phase is emerging from the column.

The ***peak maximum*** is the highest point on the peak.

The ***injection point*** is that point or time when the sample is placed on the column.

The ***dead point*** is the position of the peak maximum of an unretained solute.

The ***dead time*** (t_o) is the time elapsed between the injection point and the dead point.

The ***dead volume*** (V_o) is the volume of mobile phase passed through the column between the injection point and the dead point.

The ***retention time*** (t_R) is the time elapsed between the injection point and the peak maximum. Each solute will have a characteristic retention time.

The ***retention volume*** (V_R) is the volume of mobile phase passed through the column between the injection point and the peak maximum.

The ***peak height*** (h) is the distance between the peak maximum and the baseline geometrically produced beneath the peak.

The ***peak width*** (w) is the distance between each side of a peak measured at 0.6065 of the peak height. The peak width measured at this height is equivalent to two standard deviations of the Gaussian curve (inflection point) and thus has significance when dealing with chromatography theory. [8]

2.3: Chromatographic Parameters

Four chromatographic parameters, capacity factor, separation factor, column plate number and asymmetry factor are all useful for assessing the quality of the chromatogram.

Capacity Factor

Capacity factor, k' is often a more appropriate measure of retention than retention time, because retention time varies when the column length or flow rate are altered whereas k' does not. The formula for capacity factor is given below in equation (2.1)

$$k' = \frac{t_r - t_0}{t_0} \qquad (2.1)$$

The parameter t_0 represents the column dead time - this is the time it takes for a non-retained compound to pass through the column. This can usually be calculated as the time from sample injection to the first disturbance in the baseline. It may also be necessary to inject a compound, which is known to be non-retained such as uracil for reverse phased HPLC.

Changes in mobile phase composition alter the capacity factor value for a particular chromatogram. A ten percent change in mobile phase composition can give a two to three fold change in k'. Larger k' values give broader and shorter peaks. Altering capacity factor values also alters the retention time of a given eluent. Capacity factors between 2 and 10 are desirable for most separations [6].

Separation Factor

The separation factor, α, is the ratio of capacity factors for two peaks as shown in equation (2.2)

$$\alpha = \frac{k'_1}{k'_2} \qquad (2.2)$$

Where k'_1 and k'_2 are the capacity factors for the first and the second peaks of interest. α is a function of both stationary and mobile phase types which means that changing a column type will also change α. The same applies to variations in the mobile phase. These changes are useful in assisting the separation of two closely eluting bands. Desirable separation factor values are between 1.5 and 3 [9].

Theoretical Plate Number

This is essentially a measure of peak sharpness. It is a useful value calculated often to assess the quality of an existing column or if a new column meets required specifications. Manual calculation is performed using the equation (2.3) shown on the next page.

$$N = 5.54(\frac{t_r}{w_{0.5}})^2 \qquad (2.3)$$

where t_r is the retention time of the eluent and $w_{0.5}$ is the width of the peak at one half the height. Columns with large values of N generally give narrower peaks and overall better separations. Changing the column packing for example from 10-micron particles to one packed with 5 micron particles will double N. Changing the column length, will also vary N proportionally [10].

Asymmetry

A quantitative measure of peak tailing is the asymmetry factor, As. Asymmetry is calculated as shown in figure 2.1.

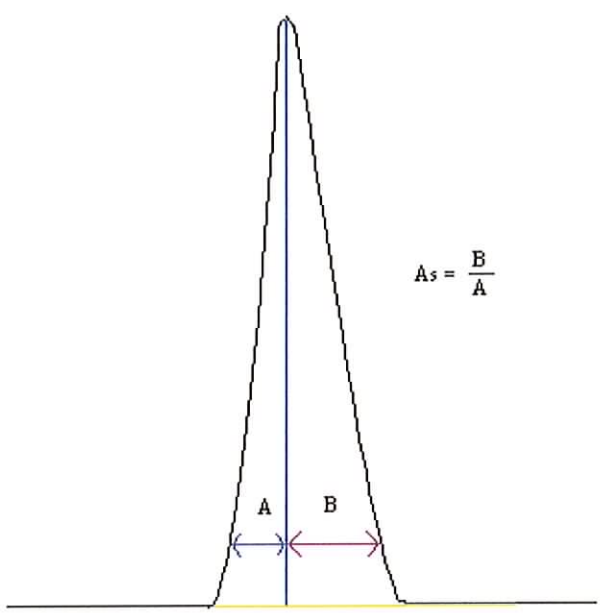

Figure 2.1: Calculating Asymmetry

A vertical line is drawn between the peak maximum and the baseline (Shown in blue). At 10% of the peak height the width leading from this line to the edges of the peak is measured. The ratio of the width of the trailing half to the fronting half defines the asymmetry. Values between 0.9 and 1.2 are desired for optimum column performance. Tailing suppressors such as triethylamine may be added to the mobile phase to reduce the availability of free silanols. pH control through the use of buffers such as ammonium acetate will also help to reduce peak tailing.

Resolution

The requirement for a successful HPLC separation is whether the bands of interest are sufficiently separated from others. This can usually be satisfied by a visual inspection of the chromatograph. Resolution Rs, is a quantitative measure of the separation. Equation (2.4) demonstrates the impact of the various parameters on Rs.

$$Rs = \frac{1}{4}\left(\sqrt{N}\right)\left(\frac{k'}{1+k}\right)(\alpha - 1) \qquad (2.4)$$

A B C

The terms A, B and C have been previously discussed. Term A is the column term which is affected by column length and particle size. Term B is the retention term which relies mostly on the mobile phase composition. Finally term C is the selectivity term, which is dependent on the solvent type and also the stationary phase type.

Analysing the equation shows a square root dependence on the plate number. That means basically to double resolution it is necessary to increase the plate number fourfold. Changing the separation factor is the most powerful way to change resolution but it is not as well understood as changing plate number or capacity factor. Changing the primary solvent in the mobile phase is the most convenient way of changing capacity factor.

The HPLC method used in this project is reverse phased. This means that retention is based on solute polarity. More polar molecules elute first and less polar ones later on. This is because polar molecules interact strongly with the relatively polar mobile phase where less polar solutes are attracted to the non-polar stationary phase. The primary solvent effect in reverse phased HPLC is that the mobile phase polarity controls retention. An important secondary reaction is the solute interaction with silanols groups on the stationary phase. Silanol interaction is responsible for peak tailing when polar compounds are resolved chromatographically.

Most reverse phase HPLC separations are performed under isocratic conditions. This means that the composition of the mobile phase remains constant throughout the chromatographic experiment. Isocratic methods are most commonly used for drug stability studies [10]. The other alternative to isocratic elution is gradient elution. Gradient elution involves changing the composition of the mobile phase throughout a run. For stability studies in which isocratic elution results in long analysis times gradient elution can be used to shorten the retention time.

A resultant chromatogram may look similar to the example below fig (2.2) with time plotted on the x-axis and detector response on the y-axis.

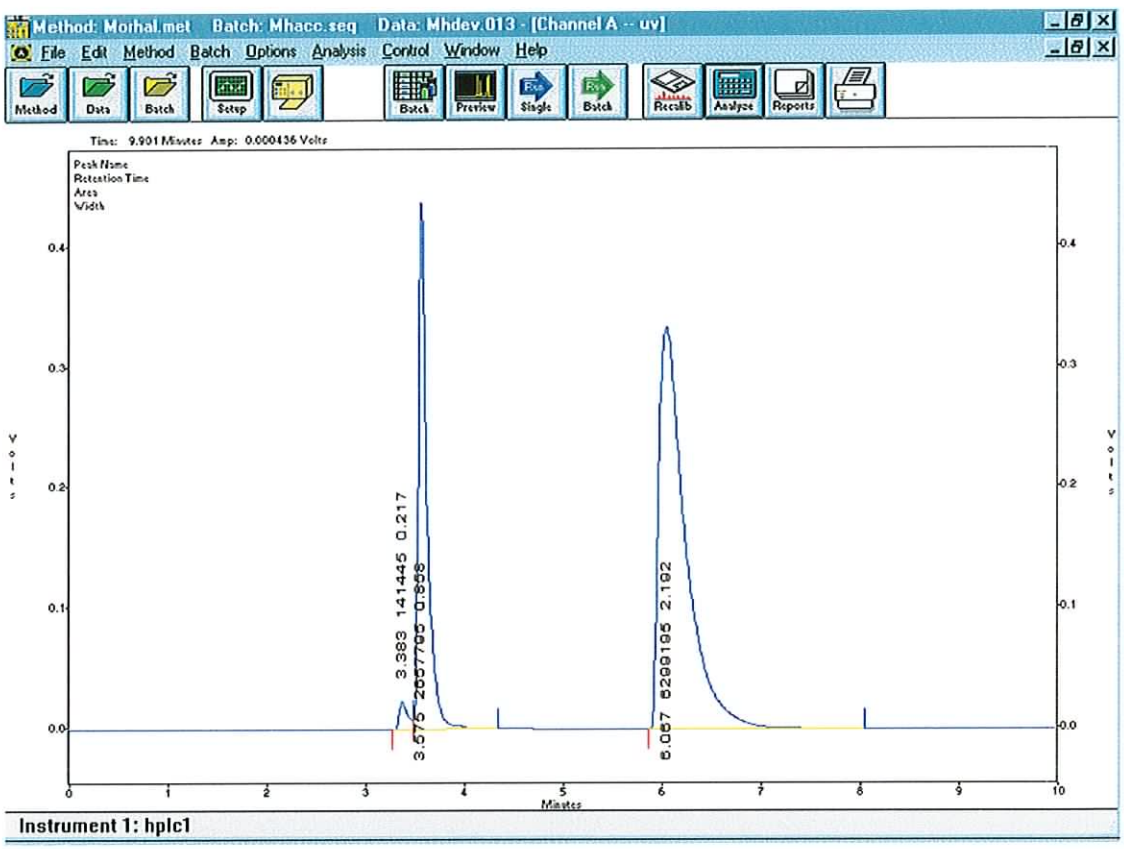

Fig 2.2: Example of a typical HPLC chromatogram

2.4: METHOD DEVELOPMENT INTRODUCTION

The term method development is used to describe how high performance liquid chromatography can be used to provide a reliable way of measuring a solute or solutes. The simplest requirement for a successful method development is an understanding of the nature of the analytical problem the analyst is faced with and knowledge of the parameters controlling the elution and detection of solutes in HPLC [11].

A method development may take on a number of forms. At one extreme, it may involve adapting an existing method, making alterations so that it is suitable for a new application. At the other extreme the analytical chemist may start out with little information and apply expertise and experience to devise a suitable method. It may involve a significant innovation based on novel exploitation of known properties of the analytes. This approach clearly involves a degree of doubt as to the success of the final method. It is not infrequent for method development to involve working on a number of different ideas simultaneously [11].

Good planning is essential in the selection and development of the method for drug analysis. It is necessary to be able to quantify the active components in the samples rapidly with acceptable precision, accuracy and reliability within the cost and other constraints. As the nature of the sample changes (different dosage form or biological fluid), methods can be revised [12].

Thus in HPLC method development it is necessary to separate the solutes under conditions that minimise interference from other materials and result in complete separation of the analytes of interest in the shortest possible time [9].

Before commencing on the practical or experimental work involved it is important to review what is known about the components of interest. Agreement of what is required of the method should be obtained before method development begins.

The goals of the separation should also be defined at this point. For the project concerned the goals of the separation were to develop a suitable method for the separation of morphine sulphate and haloperidol for drug stability studies. The method should be able to discriminate between the two species.

The initial stage in a method development is the characterisation of the samples in question. How many components in the sample, chemical structure, UV spectra and solubility are all important factors that can have a bearing on the final method.

The UV spectrum of the sample is important as it helps to determine if Ultra Violet detection will be used or not. The analyte concentration is also important. The concentration range must be representative of the drug concentration used in clinical practice but considerations must be given to sample preparation [9].

Below are listed some important sample considerations for developing a method.

- Number of compounds present
- Chemical structures of compounds
- Molecular weights of compounds
- PKa values of compounds
- UV spectra of compounds
- Concentration range of samples of interest
- Sample solubility

If a sample has a pK_a or pK_b value, then it will be necessary to use a buffer for optimum results in reversed phase liquid chromatography. [13].

Samples come in different forms such as ready for injection, solutions that require dilution, solids that are soluble in mobile phase, solutions that contain interferences that may damage a column.

With reference to the method development in question the samples concerned, morphine sulphate and haloperidol cannot be injected directly into the HPLC system.

If using reference standard powders, weighing is required. The use of commercial solutions means that diluting of samples may be necessary before injection can occur. It is generally desirable that the final sample solution closely approximates the composition of the mobile phase. This infers dissolving or diluting the sample in mobile phase [14].

2.5: Selecting a HPLC method

Before a stability study is carried out, an HPLC method must be developed that suits the particular needs of the proposed experiment. Time spent refining an existing method can prevent difficulties at a later point of experimentation. A logical and systematic approach is required in method selection and development. This can be outlined in four basis steps:

(1) Generate background data
(2) Review the literature
(3) Develop the method (optimise the experimental conditions) and
(4) Validate the performance of the method before using it routinely.

Methods can be searched for (abstracts, journals, books, pharmacopoeias), recommended by others or developed in-house. The choice of the method depends on facts such as the nature of the drug, the complexity of the sample and the intended use. The choice of the method will also be governed by practical considerations such as the type of equipment, specialist skills and factors such as cost, time, safety and accessibility to reagents will need to be considered. If these are not available or prove too costly then development will be futile.

Stability assays require higher selectivity and sensitivity than routine QC assays [12]. They require non-interference from excipients, process impurities and degradation products. They should be accurate, precise, rapid and capable of automation. Stability assays should also be robust and transferable.

During a literature search several published studies with designs similar to the study under planning may be uncovered. However these studies may not provide a direct answer to the problem in hand.

Similarities between a proposed stability study and a literature report may also be noted in terms of storage temperatures, containers, lighting conditions and compatibility with other drugs. The question then arises as to whether the literature is sufficiently lacking in relevant information to justify carrying out the proposed study.

In some cases, differences between proposed and published studies may be sufficiently important that study is warranted. Examples of such differences are a different storage temperature that is much higher than that previously used, dissimilar solutions and very different drug concentrations.

Some duplication of studies is useful, especially when errors are obvious (or, more important, not obvious), analytical methods are questionable, or experimental design is less than adequate.

If a particular method has been taken from the literature, it may not have been used for the matrix in question, and it is likely that some modification of the method will be required in order to make it suitable for the purpose to which it is to be used. For reliable results it is better to use a technique with which one is familiar and choose the least complex method if this matches the requirements of the intended use. It is best to keep the number of operations to a minimum to reduce contamination possibilities and minimise losses of the analyte.

Although experience is required in method development work, guidance can be obtained from literature sources specialising in method development. These publications may serve as a starting point for the development of methods for drugs and/or metabolites. Alternatively, one can use one of the commercially available software packages (expert systems) to speed up chromatographic method development, particularly HPLC. These systems are developed to assist the chromatographer in the selection and optimisation of chromatographic conditions during method development. With the assistance of these systems, maximum

information can be extracted from a minimum number of experiments. The main advantage of expert systems is that they provide immediate availability of an expert opinion through an inherent knowledge base, and respond to the user's inquiry when presented with a problem [6].

UV-vis spectroscopy is by far the most common detection method used in HPLC, particularly for drugs. Drug molecules normally contain a functional group that absorbs UV-vis radiation; these groups are called chromophores. UV-vis detectors in HPLC are of three types; fixed wavelength, variable wavelength and photodiode array. A fixed-wavelength detector monitors absorbance at a predetermined wavelength, often 254 nm. With a variable-wavelength detector, the wavelength at which the column effluent is monitored can be adjusted to a desired value within a certain range. Photodiode-array detectors continuously monitor the mobile-phase effluent at millisecond intervals over a wide range of wavelengths (e.g. 200 – 600nm). The result is three-dimensional data output; not only is a chromatogram produced, but the UV-vis spectrum of each peak is stored on computer. This is very handy in determining the purity of a peak in question. UV-vis spectra collected throughout the elution time of a peak can be compiled, normalized and overlaid for comparison through the use of computer software. If there are no co-eluting compounds, the spectra should match. The photodiode-array detector does not absolutely rule out co-eluting compounds, since molecules very similar in structure usually have similar UV-vis spectra. Mathematical manipulation of similar spectra can be performed with most accompanying software packages to make small spectral differences more apparent. The three-dimensional photodiode-array output may reveal other clues to the presence of co-eluting compounds, such as a wavelength of maximum absorbance [15].

Fluorescence detectors are of limited use for stability-study purposes because the drug in question must either fluoresce naturally or be tagged (derivatized) with a fluorescent reagent. Fluorescence detection is quite useful for the analysis of drugs and biologicals at therapeutic or trace concentrations in complex matrices because of the enhanced selectivity and sensitivity it can provide. Electrochemical detection is accomplished by applying a potential across two electrodes immersed in the mobile-phase flow stream. If a compound undergoes a reduction-oxidation reaction at the set

potential, a flow of current ensues and a signal is produced. Compounds need not possess a chromophore to be detected by electrochemical methods, just functional groups capable of being oxidized or reduced [15].

When referring to a literature HPLC method, authors should make clear exactly what portions of the method they used. This is particularly true when referring to a method designed to measure a drug in plasma or urine. These methods usually include sample extraction and concentration procedures that are not necessary for determinations of drug stability. Although a method may be validated for the analysis of plasma or urine, it probably does not meet the validation requirements for drug stability studies. The development of HPLC methods can be complex, expensive and time-consuming, especially if one is starting from the very beginning. Software for method development is available from several companies. Until the pharmacist gains experience in HPLC, modification of literature methods is recommended [18].

2.6: Performing the HPLC separation

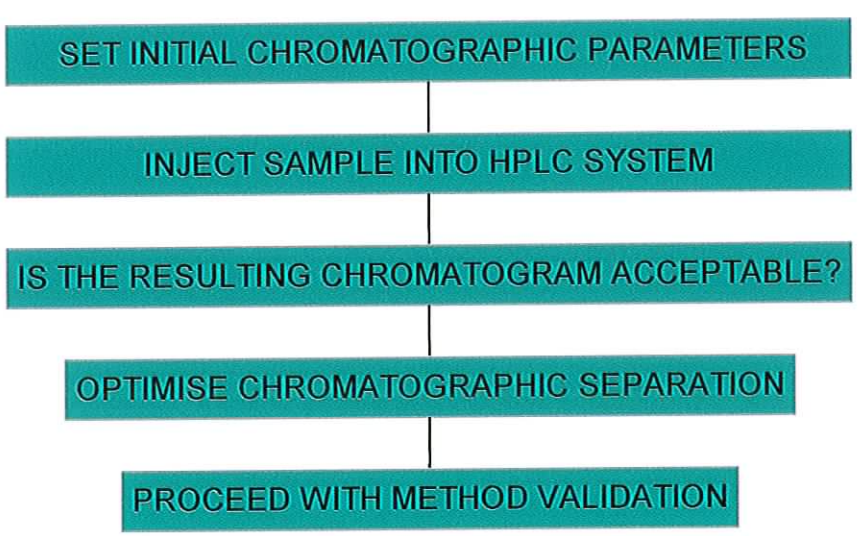

Fig.2.3: Steps in HPLC method development [16]

A suggested flow chart for performing an HPLC separation is shown in Figure 2.3 above. Relevant information should be gathered on the drug of interest and other components of a mixture before any chromatography is performed. This information includes molecular weight, chemical structure, pKa and solubility in the mobile phase. Attention should be paid to the form of drug used, such as free acid, free base, or salt.

On a per-weight basis, a sodium salt, for example, will yield less actual free drug. This information is extremely important when preparing chromatographic standards. The chemical structure of a drug may be of value to some analysts. It provides clues to UV-vis absorbance, possible decomposition products and solubility. Also related to solubility is the pKa, or the pH at which a drug containing a particular functional group is half-ionized. The pKa of a compound is useful if extraction into organic or aqueous solvents is necessary to adjust mobile-phase pH to suppress or induce ionisation of a compound and alter retention behaviour in the column [18]. Before injection, a sample is usually dissolved in or diluted with the mobile phase. If the drug is not soluble in the mobile phase, precipitation will occur, ultimately resulting in an anomalous stability profile. When injectable drugs are diluted, the presence of any preservatives such as benzyl alcohol should be noted. Benzyl alcohol will result in an additional chromatographic peak if standard UV-vis detection is used, possibly interfering with the study compound.

After the initial detection setting and mobile-phase composition are selected, a trial run should be performed. This may be done by injecting a freshly prepared drug solution into the HPLC system. Most reverse-phase HPLC separations are performed under isocratic conditions. Isocratic elution means that the composition of the mobile phase remains constant throughout the chromatographic run. Isocratic methods are most commonly used for drug stability studies. The flow rate of the mobile phase influences the height equivalent of a theoretical plate and therefore the number of theoretical plates, or efficiency (N). Samples can be injected at several flow rates and N calculated for each run to help further optimise a separation.

In contrast to isocratic elution, gradient elution means that the composition of the mobile phase changes throughout a run. Changes in composition may occur stepwise or linearly. Gradient procedures are used to separate mixtures of numerous compounds with a wide range of k' values, resulting in sharper peaks, even for late-eluting compounds. For stability studies in which isocratic elution results in a long analysis time because of, for example, a late-eluting degradation product, gradient elution could be used to shorten the retention time T_r of the degradation product. After gradient elution, time should be allowed for the column to re-equilibrate to the

initial mobile-phase composition. Gradient 'scouting' runs are useful in establishing initial isocratic conditions. The mobile-phase composition at the T_r for the peak of interest can be roughly calculated from the gradient profile to serve as the initial isocratic conditions. Adjustments to these conditions can then be made to optimise the separation.

Once the initial separation is performed, the resulting chromatogram should be examined. How many peaks are present? Does this correspond to the number of expected peaks? The peak representing the drug of interest can be confirmed by spiking a sample with standard drug solution, and observing an increase in the signal after injection. The retention time, t_r should be optimised to allow for adequate separation of the drug and degradation products while minimizing chromatographic analysis time. Again, a compromise must be reached between t_r and resolution. A t_r of 1.5 minutes makes for a very short analysis time, but resolution may be poor, with co-eluting peaks possible. Likewise, a t_r of 30 minutes may ensure that the peaks are separated, but runs are lengthy and peak shape can deteriorate considerably. Once the desired separation is achieved peak symmetry can be assessed.

When using UV-vis detectors in HPLC, it is important to adjust the detection wavelength so that the signal is optimised and noise is minimized. Commonly used solvents, such as methanol, acetonitrile, and isopropanol, absorb shorter-wavelength UV light (190-205 nm). Should the column effluent be monitored with a wavelength in or near this range, a jagged, or noisy, baseline will result. Often, variable-wavelength UV-VIS detectors are set at the λ_{max} for the compound of interest to maximize the signal produced per amount of compound detected. This serves well in most cases, but extraneous peaks and noise may be eliminated or reduced by shifting the detection wavelength away from the λ_{max}. Although the peak for the drug of interest will be smaller, this is normally of little consequence at the concentrations encountered in pharmaceutical dosage forms.

2.7: Mobile Phase

Only highly purified solvents should be used for HPLC work [17]. Most solvents have a description such as HPLC grade. Use of lesser grade solvents may result in alteration of the UV transparency of the solvent.

High performance liquid chromatography method development requires a variety of mobile phase and column parameter optimisation. The pH of the mobile phase is one such consideration. Dramatic changes in the retention and selectivity of acidic and basic compounds can occur when the pH of the mobile phase is altered [20]. When separating acids and bases a buffered mobile phase is usually recommended to maintain consistent retention and selectivity. A buffered mobile phase by definition resists changes in pH so that analytes and silica will be consistently ionised resulting in reproducible chromatography.

Buffers play an additional role in the reproducibility of a separation. Buffer salts help reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion exchange reactions with unprotonated silanols.

In determining an optimum pH for a given mobile phase the role of the column must be considered. At a pH above 7 units the major mechanism is dissolution of the silica in aqueous mobile phases. The rate of dissolution will be affected by the type of silica used for the packing of the column.

$$SIO^- Na^+ + XH \longleftrightarrow SIO^- XH^+ + Na^+ \qquad (2.5)$$

$$X = Basic \quad Compound$$

Equation (2.5): Equation showing dissolution of silica packing of the column

At the other extreme, that of low pH, the mechanism of column degradation differs from that of high pH. Acid hydrolysis of the bonded phase from the silica surface results in changes in solute retention over time. The rate of hydrolysis increases as the pH strength decreases [20].

2.8: HPLC METHODOLOGIES

The following tables summarise some of the important information gathered during the literature survey and lists methodologies for both morphine sulphate and haloperidol. Complete listing of papers and journals are given in the references (see section 2.12).

Ref.	λ (nm)	Flow Rate	Retention Time	Mobile Phase	Column	Conc range
19	280	1mls/min	2.6 mins	25% acetonitrile : 75% 0.05M phosphate buffer (pH 7.5)	Novapak C18 4u 150 x 3.9mm	80-550 µg/ml
20	254	0.5mls/min	4 mins	12% methanol : 88% 0.05M phosphate buffer (pH 7.5)	Novapak C18 5u 150 x 3.9 mm	10-100 µg/ml
21	280	1.5mls/min	7 mins	35% methanol : 65% K_3PO_4 buffer	LC-18DB C18 5u 250 x 4.6mm	70-1510 µg/ml
22	300	1mls/min	3.7 mins	20% acetonitrile : 80% 0.1M sodium phosphate dihydrate (pH 4.2)	Cyano reverse phase 5u 250 x .6mm	N/A
23	284	2.mls/min	0.62 mins	0.01M sodium pentene-sulphonate/acetonitrile/ ortho phosphoric acid	RP; Hypersil ODS 5um 100 x 5mm ID	100-2000 µg/ml
24	280	1 ml/min	5.4 mins	Methanol/ammonium phosphate (40:60, v/v), pH 6.22 with TFA	u-Bondapak C18, 300 x 3.9mm ID	N/A
25	216	1.5mls/min	6.7 mins	50% acetonitrile in 0.02 M potassium phosphate pH 5.4	Spherisorb cyano column 300 x 4.6mm ID, 5um particle size	0.01-0.15 mg/ml
26	280	2.mls/min	2.5 mins	7% acetonitrile and 93% 0.01M sodium phosphate	Hamilton PRP-1 rigid copolymer column	N/A

Table 2:1: Morphine Sulphate methodologies

Ref.	λ (nm)	Flow Rate	Retention Time	Mobile Phase	Column	Conc range
27	284	0.6ml/min	2 mins	65% methanol and 35% water with 0.1% ammonium chloride pH10	Chromospher C8 3.0 x 100mm	10-50µg/ml
28	220	2m/min	N/A	80% ammonium acetate with octane sulphonic acid	Micro-bondapak C18 10um 300 x 3.9mm	N/A
29	287	2ml/min	1.9 mins	80% 0.1M potassium phosphate and 20% acetonitrile, pH 4.6	Novapak C18 4um 100 x 5mm radial pak cartridge	N/A

Table 2.2: Morphine other forms, methodologies

Ref.	λ (nm)	Flow Rate	Retention Time	Mobile Phase	Column	Conc range
30	254	1ml/min	21.5 mins	65%triethylamine, 25% acetonitrile and 10% terahydrofuran, pH7	Supelcosil LC-8, 5um	N/A
31	220	1ml/min	11.8 mins	7% 0.02M Sodium dihydrogen phosphate and 29%tetrahydrofuran	Symmetry C18 3.9 x 150mm	2-20ng/ml
32	196	2mls/min	6.7 mins	55% Acetonitrile and 45% 100mM KH2PO4 pH 3.8-4.0	Reverse Phased octadecyl 10um C18 250 x 4.6mm ID	N/A
33	250	0.5 ml/min	12.5 mins	63% methanol and 37% water with ammonium acetate	Nucleosil C18 5um 250 x 4 mm Internal diameter	N/A
34	EC	N/A	N/A	90% Acetonitrile, 5% methanol, 5% 0.1M ammonium acetate	Utrasphere cyano 5um	> 50pg/mls

Table 2.3: Haloperidol Methodologies

2.9:METHOD VALIDATION INTRODUCTION

The validation of an analytical method is the process used to prove that the method is sufficiently accurate and reliable to provide confidence in the data produced. This process is used to establish that the analytical performance parameters used are adequate for their intended use [36].

The ability to provide timely, accurate, and reliable data is central to the role of the analytical chemist and is especially true in the discovery, development and manufacture of pharmaceuticals. Analytical data are used to screen potential drug candidates, aid in the development of drug synthesis, support formulation studies, monitor the stability of bulk pharmaceuticals and formulated products and test final products for release. The quality of analytical data is a key factor in the success of a drug development program. The process of method development and validation has a direct impact on the quality of these data [37].

Although a thorough validation cannot rule out all potential problems, the process of method development and validation should address the most common ones.

Method validation is the process of proving that an analytical method is acceptable for its intended purpose. For pharmaceutical methods, guidelines from the United States Pharmacopoeia (USP) (1), International Conference on Harmonisation (ICH) [38-41], and the Food and Drug Administration (FDA) [42-49] provide a framework for performing such validations. The guidance on the interpretation of the EN45000 series of standards and International Standards Organisation ISO/IEC guide 25 [50] includes a chapter on the validation of methods with a list of nine validation parameters. There are variances between regulatory guidelines for analytical method validation. Below in table 2.4 are listed, for example, the specifications for validation in the USP and the ICH.

USP	ICH
Precision	Precision
Accuracy	Accuracy
Linearity and Range	Linearity
Specificity	Specificity
Ruggedness	Range
Robustness	Robustness
Limit of Detection	Limit of Detection
Limit of Quantitation	Limit of Quantitation
	System suitability

Table 2.4: Regulatory differences in method validation parameters

The methods performance characteristics should be based on the intended use of the method. It is usually not necessary to perform all of the various validation parameters. A level of judgement is required to choose those parameters that demonstrate that the method will perform as it should and some parameter validation could be inferred from the validation of others.

The International Standards Organisation definition of validation [50] can be interpreted as being the process of defining an analytical requirement, and confirming that the method under consideration has performance capabilities consistent with that required by application. The judgement of method suitability is important.

Results of validation studies may indicate that a change in the procedure is necessary, which may then require revalidation. During each validation study, key method parameters are determined and then used for all subsequent validation steps. To minimize repetitious studies and ensure that the validation data are generated under conditions equivalent to the final procedure a number of steps must be implemented.

The initial step in the validation of a chromatographic method should be to set minimum requirements, which are essentially acceptable specifications for the method. The validation specifications should be set with acceptable limits and the experimental design should be in accordance with these specifications.

For example, is it critical that method precision (RSD) be $\leq$ 2%? Does the method need to be accurate to within 2% of the target concentration? Is it acceptable to have only one supplier of the HPLC column used in the analysis? During the actual studies and in the final validation, these criteria will allow clear judgement about the acceptability of the analytical method.

If a result of a test cannot be trusted then it has little value. For an analytical result to be fit for its intended purpose it must be sufficiently reliable that any decision based on it can be taken with confidence.

The parameters for method validation have been defined in different working groups of national and international committees and are described in literature[36-49]. There are variances in definitions between the different organisations. An attempt at harmonisation was made for pharmaceutical applications through the International Conference on Harmonisation [38] where representatives from regulatory bodies and from the industrial sector from Europe, the USA, and Japan defined parameters, requirements and to a small extent methodology for analytical method validation.

2.10: Validation Parameters

Specificity

To assess the stability indicating capacity of a HPLC method, samples of the drug solution to be studied must be intentionally degraded at extremes of temperature. This can be done by promoting acid and base hydrolysis, using diluted solutions of hydrochloric acid and sodium hydroxide. Thermal treatment by heating the solutions to high temperature can also be done. Drugs that are known or suspected of undergoing photo degradation should be exposed to intense light for a period of time adequate to cause decomposition through photochemical means. An oxidiser such as hydrogen peroxide can be used to speed up the degradation of drugs known to undergo such reactions.

For chromatographic methods representative chromatograms are inspected for interfering peaks resulting from drug degradation or excipients. Should an interfering peak be present chromatographic conditions are adjusted until a satisfactory

separation is achieved and validation continues on. If the degradation products are well documented and readily available, samples of the solution containing intact drug can be spiked with the degradation products and assayed by HPLC to prove that the assay is stability indicting.

For chromatographic methods, developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response of the analyte in test mixtures containing the analyte and all potential sample components (placebo, formulation, synthesis intermediates, excipients, degradation products, process impurities, etc.) is compared with the response of a solution containing only the analyte. Other potential sample components are generated by exposing the analyte to stress conditions sufficient to degrade it to 80 - 90% purity. For bulk pharmaceuticals, stress conditions such as heat (50°C), light (600 FC), acid (0.1 M HCl), base (0.1 M NaOH), and oxidant (3% H_2O_2) are typical. For formulated products, heat, light and humidity (85%) are often used [37].

The resulting mixtures are then analysed, and the analyte peak is evaluated for peak purity using techniques such as PDA (Photo Diode Array) detection or Mass Spectrometry and resolution from the nearest eluting peak. If an alternate chromatographic column is to be allowed in the final method procedure, it should be identified during these studies. Once acceptable resolution is obtained for the analyte and potential sample components, the chromatographic parameters, such as column type, mobile-phase composition, flow rate and detection mode are considered set.

An example of specificity criteria for an assay method is that the analyte peak will have baseline chromatographic resolution of at least 1.5 from all other sample components. If this cannot be achieved, the unresolved components at their maximum expected levels will not affect the final assay result by more than 0.5%. An example of specificity criteria for an impurity method is, that all impurity peaks that are $\geq$ 0.1% by area will have baseline chromatographic resolution from the main component peak(s) and, where practical, will have resolution from all other impurities.

Linearity

A linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to concentration. For assay methods, this study is generally performed by preparing standard solutions at five concentration levels, from 50% to 150% of the target analyte concentration. Five levels are required to allow detection of curvature in the plotted data. The standards are evaluated using the chromatographic conditions determined during the specificity studies.

Standards should be prepared and analyzed a minimum of three times. The 50% to 150% range for this study is wider than what is required by the FDA guidelines [42-49]. In the final method procedure, a tighter range of three standards is generally used.

A correlation coefficient of >0.99 is generally considered as an acceptable fit of the data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level [36].

Another approach to linearity is to divide signal data by their respective concentrations yielding the relative responses. A graph is plotted with the relative responses on the y-axis and the corresponding concentrations on the x-axis, on a log scale. The obtained line should be horizontal over the full linear range. At higher concentrations, there will typically be a negative deviation from linearity. Parallel horizontal lines are drawn in the graph corresponding to, for example, 95 percent and 105 percent of the horizontal line. The method is linear up to the point where the plotted relative response line intersects the 95 percent line [51].

Accuracy

The accuracy of a method is the closeness of the measured value to the true value for the sample. Accuracy can also be described as the closeness of agreement between the value that is adopted, either as a conventional, true or accepted reference value, and the value determined [52].

Accuracy expresses the closeness of a result to a true value. Method validation seeks to quantify the likely accuracy results by assessing systematic and random effects on results. Accuracy is, therefore normally studied as two components trueness and

precision. The trueness of a method is expressed as how close the mean of a set of results are to the true value. Trueness is normally described in terms of bias where precision is interpreted as how close results are to each other and is usually expressed by measures such as standard deviation.

Practical assessment of accuracy relies on comparison of mean results from a method with known values, that is accuracy assessed against a reference value. Two basic techniques are available: checking against reference values from characterised sample or from another characterised method. Reference values are ideally traceable to international standards. Certified reference materials are generally accepted as providing traceable values. To check accuracy using a reference material the mean and standard deviation of a set of replicate samples are determined and compared with the characterised value for the reference material. The ideal reference material is a certified, natural mix reference material, closely similar to the samples of interest. Clearly the availability of such samples is limited. Reference materials for validation may accordingly be:

- Prepared by spiking materials with pure certified reference materials or other materials of suitable purity and stability.
- Commercially available secondary standards, with certified traceability, whose preparation is ILAB accredited.
- Typical, well-characterised materials checked in-house for stability and retained for in-house quality control.

The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range.

Accuracy is usually determined in one of four ways. Analysing a sample of known concentration, and comparing the measured value to the true value can assess accuracy. National Institute of Standards and Technology (NIST) reference standards are often used [59]. However, such a well-characterized sample is usually not available for new drug-related analytes. The second approach is to compare test

results from the new method with results from an existing alternative method that is known to be accurate. Again, for pharmaceutical studies, such an alternate method is usually not available.

There are other approaches used to assess accuracy and the third and fourth of these are based on the recovery of known amounts of analyte spiked into sample matrix. The third approach involves spiking the analyte in blank matrices. For assay methods, spiked samples are prepared in triplicate at three levels over a range of 50 - 150% of the target concentration. If potential impurities have been isolated, they should be added to the matrix to mimic impure samples. For impurity methods, spiked samples are prepared in triplicate at three levels over a range that covers the expected impurity content of the sample, such as 0.1 - 2.5 wt%. The analyte levels in the spiked samples should be determined using the same quantitation procedure as will be used in the final method procedure (i.e. same number and levels of standards, same number of sample and standard injections, etc.). The percent recovery should then be calculated.

An example of an accuracy criteria for an assay method is that the mean recovery will be 100 $\pm$ 2% at each concentration over the range of 80 - 120% of the target concentration.

Detection Limit

The terms limit of detection and "detection limit" are not generally accepted although they are used in some regulatory documents. IUPAC uses the term minimum detectable true value.

The detection limit of a method is the lowest analyte concentration that produces a response detectable above the noise level of the system, typically, three times the noise level. The detection limit needs to be determined only for impurity methods in which chromatographic peaks near the detection limit will be observed. An example of the detection limit criteria is that, at the 0.05% level, an impurity will have a signal to noise ratio greater than or equal to three [37].

The ICH guidelines on detection limit state that visual evaluation may be used. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. If detection limit is determined on visual evaluation or based on signal to noise ratio the presentation of the relevant chromatogram is considered acceptable for justification [38-41].

The other approaches concerning detection limit are based on

- Signal to noise ratio: a signal to noise ratio between 3 or 2:1 is considered suitable for estimating the detection limit.
- Standard deviation of the response of the slope: detection limit (DL) is expressed as

$$DL = \frac{3.3\sigma}{S}$$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

Quantitation Limit

The quantitation limit is the lowest level of analyte that can be accurately and precisely measured. This limit is required only for impurity methods and is determined by reducing the analyte concentration until a level is reached where the precision of the method is unacceptable. If not determined experimentally, the quantitation limit is often calculated as the analyte concentration that gives S/N = 10. An example of quantitation limit criteria is that the limit will be defined as the lowest concentration level for which a percentage relative standard deviation (%RSD) less than or equal to twenty percent is obtained when an intra-assay precision study is performed.

Stability

Stability data are required to show that the concentration of the analyte in the sample at the time of analysis corresponds to the concentration of analyte at the time of sampling The stability of the analyte in stock solutions should be established.

An example of stability criteria for assay methods is that sample and standard solutions and the mobile phase will be stable for 48 hrs under defined storage conditions. Acceptable stability is $\leq$ 2% change in standard or sample response relative to freshly prepared standards.

For impurity methods, the sample and standard solutions and the mobile phase will be stable for 48 hours under defined storage conditions. Acceptable stability is $\leq$ 2% change in standard or sample response, relative to freshly prepared standards. The mobile phase is considered to have acceptable stability if aged mobile phase produces equivalent chromatography (capacity factors, resolution, or tailing factor) and assay results are within 2% of the value obtained with fresh mobile phase [37].

Precision

There are numerous ways of assessing precision. These include repeatability, intra-assay precision and reproducibility.

The precision study used for this project was reproducibility [37] which is determined by testing homogeneous samples in multiple laboratories, often as part of inter-laboratory crossover studies. The evaluation of reproducibility results often focuses more on measuring bias in results than on determining differences in precision alone. Statistical equivalence is often used as a measure of acceptable inter-laboratory results. An alternative, more practical approach is the use of "analytical equivalence" in which a range of acceptable results is chosen prior to the study and used to judge the acceptability of the results obtained from the different laboratories.

An example of reproducibility criteria for an assay method could be that the assay results obtained in multiple laboratories will be statistically equivalent or the mean results will be within 2% of the value obtained by the primary testing laboratory. For an impurity method, results obtained in multiple laboratories will be statistically equivalent or the mean results will be within 10% (relative) of the value obtained by

the primary testing lab for impurities > 1wt%, within 25% for impurities from 0.1 - 1.0 wt%, and within 50% for impurities < 0.1 wt% [37].

System suitability

According to the United States Pharmacopoeia [36], system suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole.

System suitability is the checking of the system to ensure system performance before and during the analysis of unknowns. All chromatographic analytical procedures should include system suitability testing and criteria. Parameters such as plate count, tailing factor, capacity factor, retention time, injection reproducibility, resolution, etc., are determined and compared against preset specifications for the method. Some system suitability parameters as defined and discussed by the CDER reviewer guidance on Validation of Chromatographic Methods (Nov 1994) [51]. These include theoretical plate number, retention time and peak area.

Range [53]

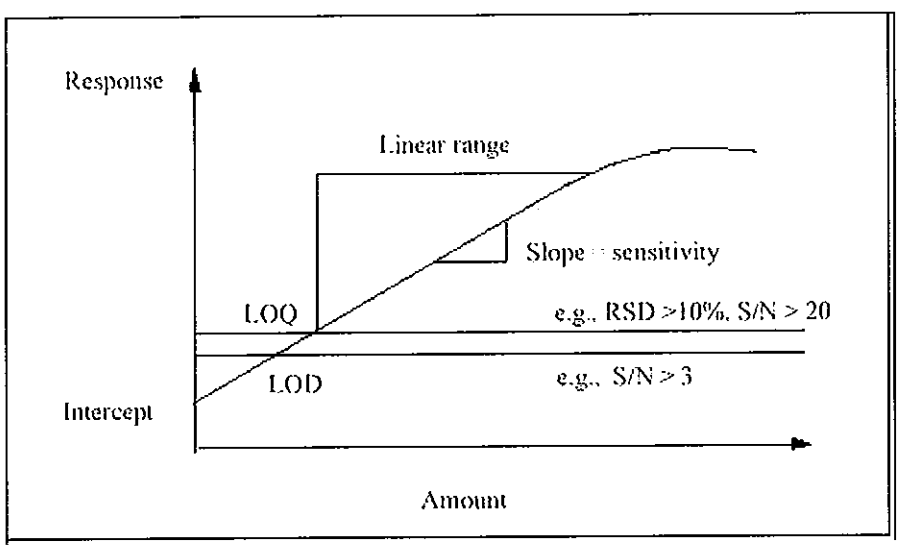

Figure 6.1: graphical representation of range of an analyte response

The range of an analytical procedure is the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The range closely related to linearity is the range of results from the analytical procedure.

Various guidelines suggest a number of minimum specified ranges[36,41].

- 80-120 percent of the test concentration for the assay of an active substance or a finished product.
- 70-130 percent of the test concentration for the content uniformity for the majority of formulations.
- From their reported level to 120 percent of the specification for impurities.
- From their reported level to 120 percent of the assay specification if a 100 percent standard is used and the assay and purity test are combined.

Robustness

The robustness of a method is its ability to remain unaffected by small changes in parameters such as percent organic content and pH of the mobile phase, buffer concentration, temperature and injection volume. These method parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment [54]. Obtaining data on the effects of these parameters may allow a range of acceptable values to be included in the final method procedure. For example, if column performance changes over time, adjusting the mobile-phase strength to compensate for changes in the column may be allowed if such data are included in the validation.

An example of robustness criteria is that the effects of the following changes in chromatographic conditions will be determined: methanol content in mobile phase adjusted by $\pm$ 2%, mobile-phase pH adjusted by $\pm$ 0.1 pH units, and column temperature adjusted by $\pm$ 5^0C. If these changes are within the limits that produce acceptable chromatography, they will be incorporated in the method procedure [37].

Ruggedness

Ruggedness is not addressed in the ICH documents [38-41]. Its definition has been replaced by reproducibility which has the same meaning as ruggedness, as defined by the USP [36] to be: the degree of reproducibility of results obtained under a variety of conditions, including different laboratories, different analysts, different instruments, environmental conditions, operators and materials.

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts different instruments, different lots of reagents , different elapsed assay times, different assay temperatures, different days, etc. Ruggedness is normally expressed as the lack of influence on test results of operational and environmental variables of the analytical method. Ruggedness is a measure of reproducibility of test results under the variation of conditions normally expected from laboratory to laboratory and from analyst to analyst [56].

Reference Standards

A reference standard is a highly purified compound that is well characterised. Chromatographic methods rely heavily on reference standards to provide accurate data. Therefore the quality and purity of the reference standard is important. Chromatographic test methods use either external or internal standards for quantitation [59].

An external standard method is used when the standard is analysed on a separate chromatogram from the sample. Quantitation is based on a comparison of peak height or peak area of the sample to that of a reference standard of the analyte of interest. The external standard method is more appropriate for the following samples:

- Sample with a single target concentration and narrow concentration range
- Simple Sample preparation procedure
- Increased baseline time for detection of potential extraneous peaks [51]

An internal standard is a compound that is added to a sample before pre-treatment and analysis to increase the precision of the assay. Detector response is expressed as the ratio of the peak area for the drug to the area of the peak for the internal standard. The internal standard method is more appropriate for samples as follows:

- Complex sample preparation procedures
- Low concentration sample
- Wide range of concentrations expected in the sample for analysis

There is not a lot of benefit in using the internal standard method if an automatic injector is used in HPLC [55]. As most drug samples require little pre-treatment and have good reproducibility of repeat injections the external standard method seems an appropriate choice. Although it is not specified whether a method must use an external or internal standard for quantitation, it is commonly observed that with HPLC methods for release and stability the use of external standards is employed [51].

A standard curve is used to determine the concentration of a drug in the samples and should be prepared on each day of sampling. Drug standards used in preparing these graphs should be purchased from a chemical supplier or a certified sample can be obtained from the manufacturer of the parent dosage form. Substituting pharmaceutical dosage forms for certified drug standards is regarded somewhat bad practice unless some justification is presented [57,58].

2.11: Summary

Performing a thorough method validation can be a tedious process, but the quality of data generated with the method is directly linked to the quality of this process. Time constraints often do not allow for sufficient method validations. Many researchers have experienced the consequences of invalid methods and realized that the amount of time and resources required to solve problems discovered later exceeds what would have been expended initially, if the validation studies had been performed properly.

Validation is a constant, evolving process starting before an instrument is placed on-line and is continuous after method development and transfer. Different regulatory guidelines give varied suggestions and insights into method validation. There is a great deal of information left open to interpretation by the analyst. By approaching method development, optimisation and validation in a logical and stepwise manner, laboratory resources can be used productively with great efficiency.

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44) Http **Summary**: Parameters For Validation of HPL Chromatographic methods for drug substance and for drug product, *02-16-98, http://www.fda.gov/cder/guidance/cmc3.pdf*

45) **Summary**: wpd 12/ 14/ 98 Guidance for Industry Bioanalytical Methods Validation for Human Studies DRAFT GUIDANCE This guidance document is being distributed for comment purposes only. wpd 12/ 14/ 98 Guidance for Industry Bioanalytical Methods Validation for Human Studies, *01-05-99, http://www.fda.gov/cder/guidance/2578dft.pdf*.

46) **Summary**: Guidance for Industry Q2B Validation of Analytical Procedures: Methodology Additional copies are available from: the Drug Information Branch (HFD- 210), Center for Drug Evaluation and Research (CDER), 5600 Fishers Lane, Rockville, MD 20857 (Tel) 301- 827, *06-19-98, http://www.fda.gov/cder/guidance/1320fnl.pdf*.

47) **Summary**: wpd 08/ 29/ 00 Guidance for Industry Analytical Procedures and Methods Validation Chemistry, Manufacturing, and Controls Documentation Draft Guidance , *08-30-00, http://www.fda.gov/cder/guidance/2396dft.pdf*

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CHAPTER 3

METHOD DEVELOPMENT

3.1: Materials used in Method Development

HPLC Equipment

SCL-10A VP Shimadzu system controller,SPD-10A VP Shimadzu UV-Vis detector,SIL-10A VP Shimadzu auto injector, LC-10AT VP Shimadzu liquid chromatograph, FCV-10AL VP Shimadzu low pressure gradient control valve, Shimadzu on line degassing device GA STORR 154, Class-VP Chromatography data analysis system

Column

Hichrom NC100-5C18-3109
250mm X 4.6mm ID 5µ particle size

pH meter

Jenway pH Meter 3310

Balances

Taurus Mettler balance model BP110

Taurus Mettler balance model Bl120S

Reagents

Acetonitrile, 99.93%, HPLC grade, Sigma-Aldrich., Ammonium Acetate 97.3%, Sigma-Aldrich Lot 58H0060, Phosphoric Acid, ACS reagent 85% Lot 90609017, Deionised Water

Morphine Sulphate

Commercially prepared solutions (supplied by Antigen pharmaceuticals, Roscrea, Tipperary) were used as both standards and in the filling of the PCAs required for testing. Ampoules of variable concentration were required for dilution (10mg/ml, 15mg/ml, 30mg/ml, 60mg/ml).

Morphine Sulphate reference standard, Sigma-Aldrich Lot MPT-394A

Haloperidol

Commercially prepared solutions (Supplied by Norton Healthcare, Waterford) were used as both standards and for sampling (20mg in 2mls)

Haloperidol reference standard, Sigma-Aldrich Lot 18H0408

3.2: Method Development Results and Discussion

There are many variables to consider in developing or optimising a HPLC method. Therefore the initial approach decided upon was to limit as many variables as possible. It was necessary to examine the starting materials and see what variables could be controlled or fixed that would allow a suitable chromatogram to be produced. There were two components in the sample morphine sulphate and haloperidol so the expected chromatogram should yield two peaks. The method of detection was Ultra-Violet (UV) spectroscopy.

The starting point was to determine a suitable UV absorbance to work with. Morphine sulphate has UV absorbance maxima of 220, 254 and 280-285nm. Haloperidol has a UV absorbance maximum of 247nm. Ultra Violet absorbance spectra were taken of Morphine sulphate, haloperidol and the combination of both. The spectrum for morphine sulphate and haloperidol in mobile phase is presented in appendix 7 (page 175). From the ultra-violet spectroscopy data it can be seen that the most suitable wavelength for detection is around 247-248nm.

A literature search was conducted to examine if there was an existing method for the separation of morphine sulphate and haloperidol (as seranace®) which could be adapted. A number of methods were discovered for the HPLC analysis of morphine sulphate and there were but a few methods for the HPLC analysis of haloperidol. Some methods indicated separation of derivative drugs of both haloperidol and morphine but none could be found that were deemed appropriate at the time for adaptation. Finally a method was found for the separation of haloperidol [1], which could possibly be adapted for the purposes of this project. The separation was based on a reverse phased HPLC method and the method incorporated the use of a Hypersil C18 (5u) 150 X 5mm column, a mobile phase consisting of 55% of 1% ammonium acetate buffer and 45% acetonitrile. The flow rate was 2mls/min and detection wavelength was 247nm. This wavelength had already been chosen for the separation under consideration.

Obviously different resources were available which limit the ability to adapt the method described above. The column used for this project was a Hichrom Nucleosil C18 stainless steel column of length 25 cm, with internal diameter of 4.6mm and external diameter 5mm and 5 micron packing size. This column was chosen because a C18 column was desirable for the reverse phased HPLC method and from a previous stability assay this type of column gave sharp well resolved peaks.

The next step was to determine a suitable pH for the mobile phase. Taking into account the impact of pH on the column a suitable pH must lie between 3.0 units and 7 pH units [2,3]. Examination of the literature showed a wide variance in the pH values chosen for various mobile phase compositions. It was decided to adjust the pH of the mobile phase to 3.6 pH units. An ammonium acetate buffer solution was prepared (1%) and was combined with acetonitrile (55%:45%).

Injection of a standard solution of morphine sulphate (0.75mg/ml) and haloperidol (0.4mg/ml) yielded the chromatogram in fig 3.1 below.

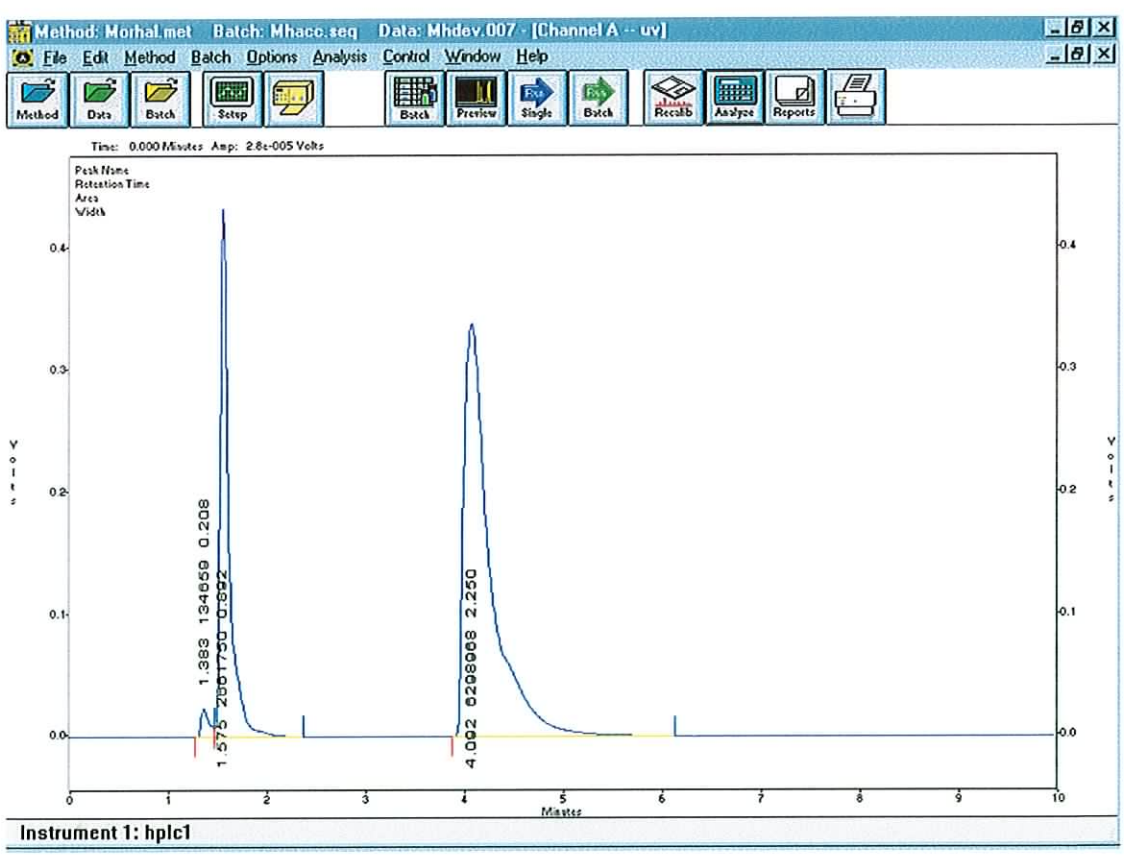

Fig 3.1: Chromatogram 1

Morphine sulphate eluted very close to the solvent front so modifications to the acetonitrile component of the mobile phase were made. This was ineffective in delaying the elution of morphine sulphate. A time program was employed to adjust the flow rate of the mobile phase throughout the run. For the first three minutes a flow rate of 0.5mls/min was selected in order to delay the elution of morphine sulphate, this flow rate was then increased to 1ml/min from three to ten minutes. This subsequently delayed the retention time for haloperidol also but kept the retention time for both drugs under ten minutes and provided better resolution between the two. The column was replaced with a new Hichrom C18 (5µ) 250 x 4.6mm column, and the outcome was the separation of morphine sulphate and haloperidol as sharp well resolved peaks. The chromatogram in fig 3.2 below shows this.

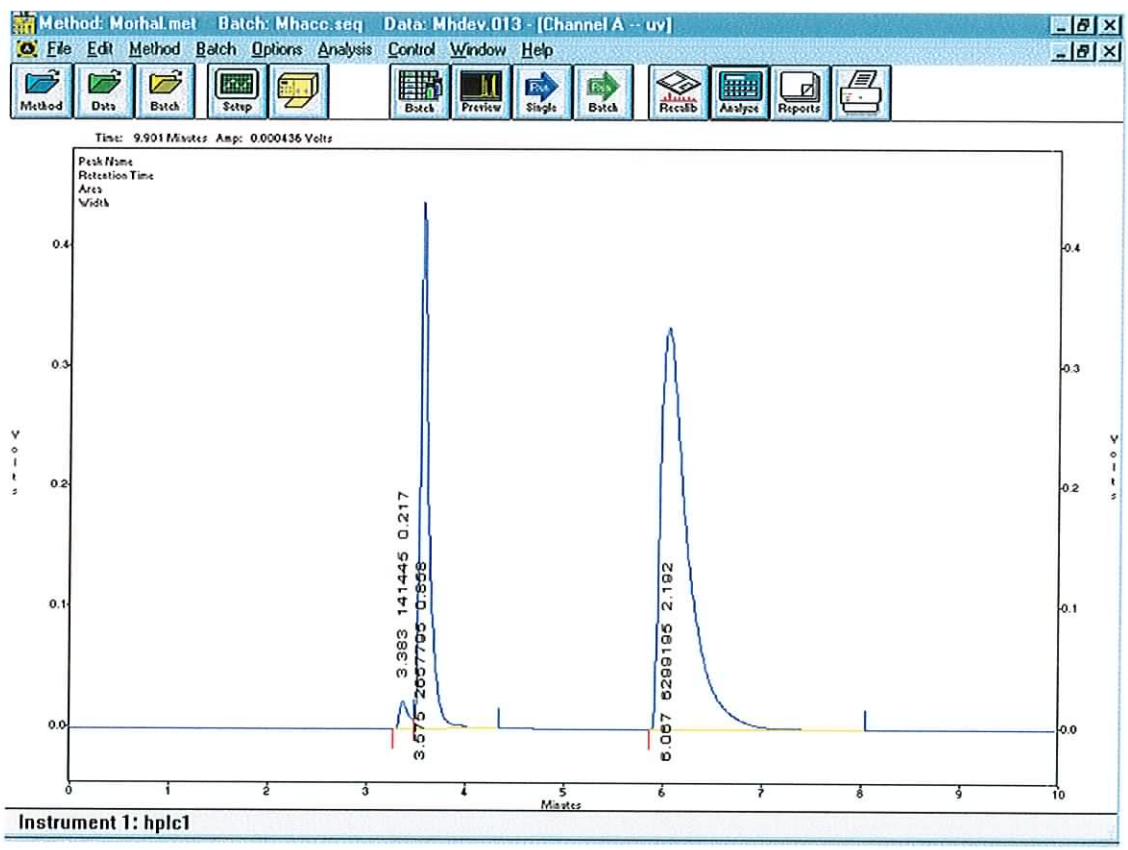

Fig 3.2: Chromatogram 2

The mobile phase was acetonitrile and ammonium acetate buffer with a flow rate of 1.5 mls/min. This chromatogram looks suitable for the analysis in question as there are two peaks and they are well resolved from each other. However both peaks show

tailing, particularly the haloperidol peak. At this point it was necessary to optimise the separation and also to show that the mobile phase was suitable.

To begin with alterations to the mobile phase were made and also to the detection wavelength. A mobile phase was prepared without the ammonium acetate buffer and a 50:50 acetonitrile/water mixture was prepared. The pH was adjusted to 3.7 and an injection of the standard solution performed at 210nm. The resulting chromatogram is shown below in fig 3.3:

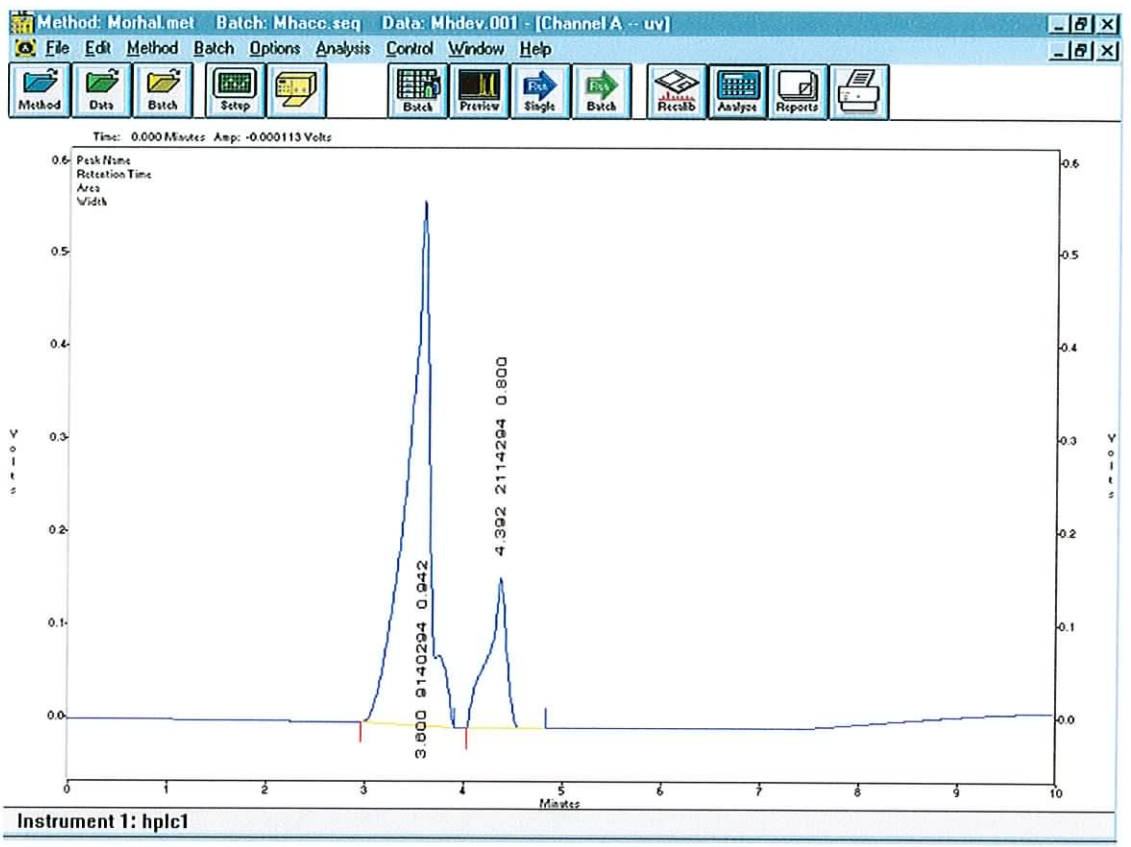

Fig 3.3: Chromatogram 3

Inspection of this chromatogram clearly shows that this is a poor separation with poor peak shape and resolution for compounds concerned. From this chromatogram it can be concluded that the wavelength is unsuitable. The chromatogram overleaf, fig 3.4, shows data from injection of the standard solution but at 270nm. This chromatogram looks similar to the previous one and is of poor quality.

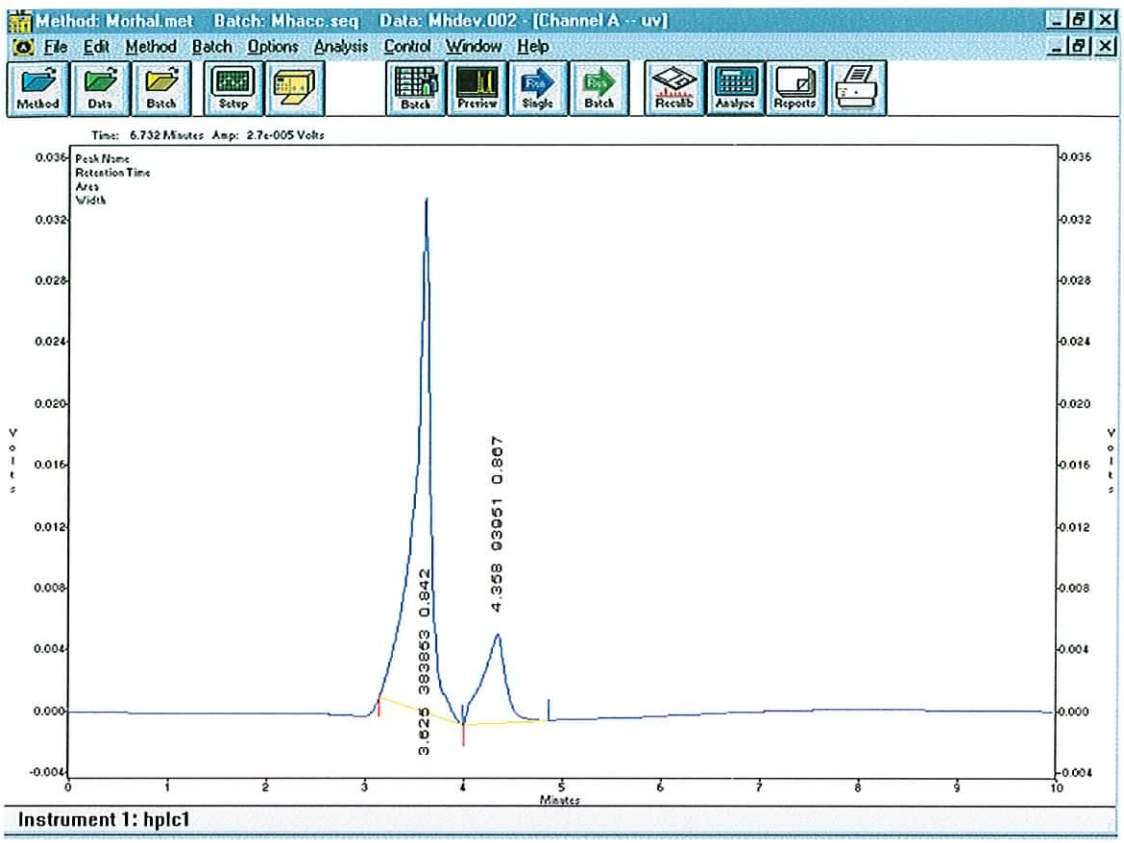

Fig 3.4: Chromatogram 4

The chromatograms represented by figures 3.5 and 3.6 below were generated using the 50:50 acetonitrile/water mobile phase mixture. This time however the standard solution was injected at 247nm for both. The sample represented by chromatogram 3 had an injection volume of 20 micro litres, and that of chromatogram 4 had an injection volume of 30 micro litres. Inspection of chromatograms 3 and 4 again shows very poor separation of both components. Increasing the injection volume (chromatogram 4, fig 3.4) has little significance here. It is clear that by altering the parameters discussed above there will be little effect on the separation. The reason for this is that the mobile phase is entirely unsuitable in this instance. The addition of the ammonium acetate buffer is significant in obtaining a better separation.

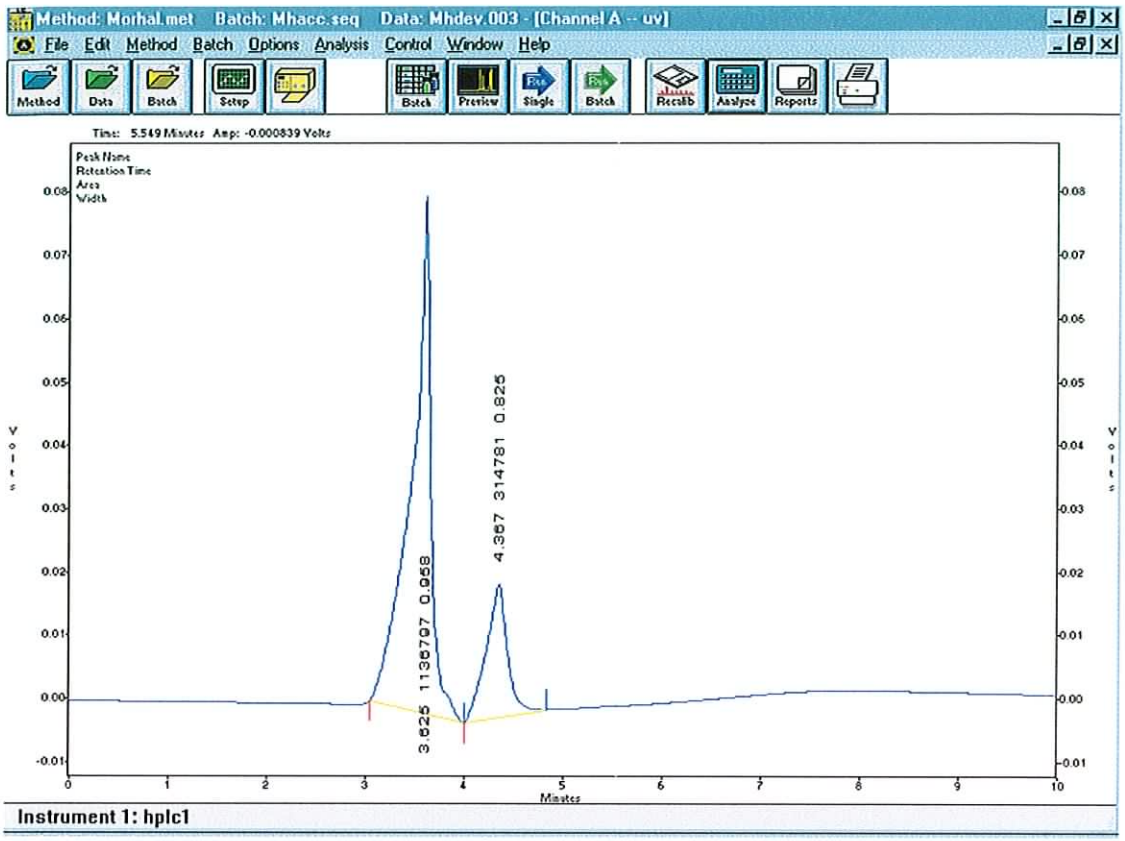

Fig 3.5: Chromatogram 5

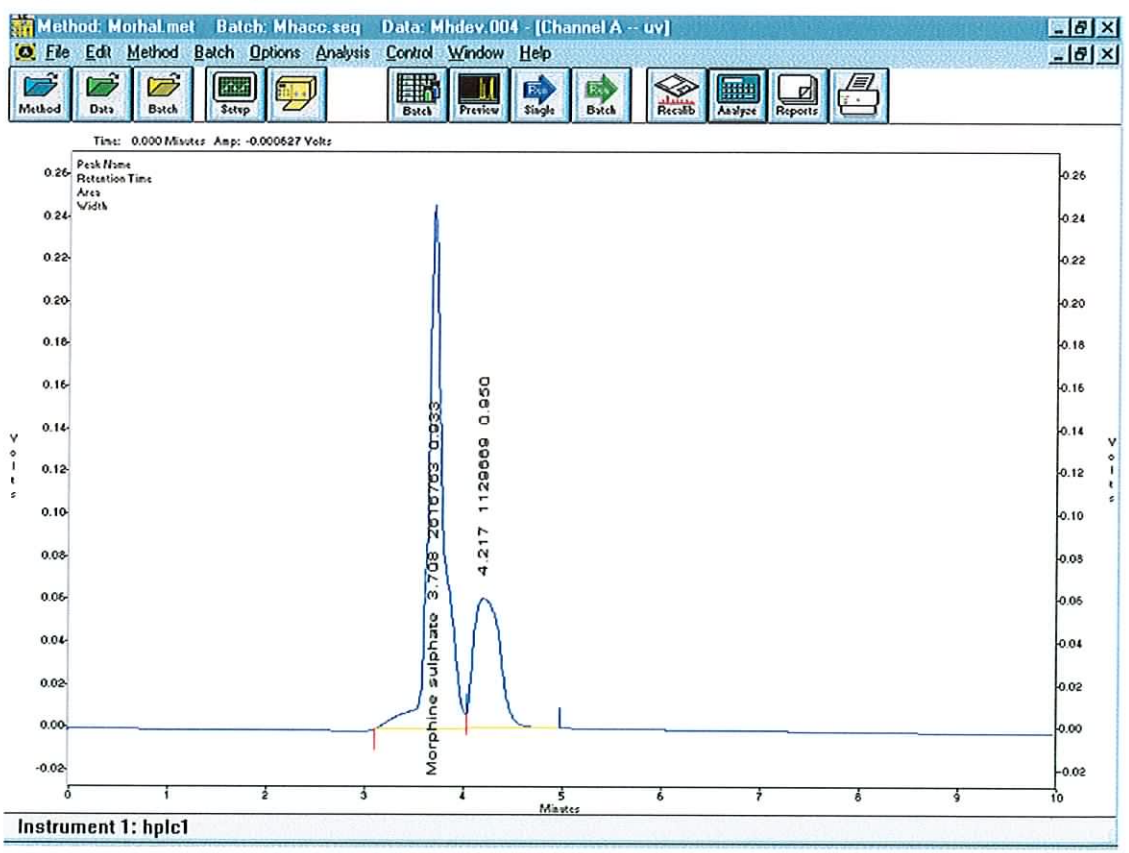

Fig 3.6: Chromatogram 6

Having established that a suitable mobile phase is ammonium acetate (1%w/v) and acetonitrile some other parameters were investigated. The flow rate was altered next. Chromatograms 7-9 (figures 3.7,3.8,and 3.9) were generated using constant flow rates of 0.5mls/min, 1.0mls/min, 1.5mls/min respectively. Samples were injected using 55% ammonium acetate (1%w/v) buffer and 45% acetonitrile and the pH of the mobile phase adjusted to 3.6 with phosphoric acid. Chromatogram 7 results from injection at a constant flow rate of 0.5mls/min. Morphine sulphate eluted at approximately 3.84 minutes and haloperidol eluted later at approximately 7.57 minutes. At a flow rate of 1ml/min the retention times for both components decreased but the peak shape for haloperidol was suspect. This was even more apparent on increasing the flow rate to 1.5mls/min where morphine sulphate was very poorly retained the haloperidol peak showed some severe tailing.

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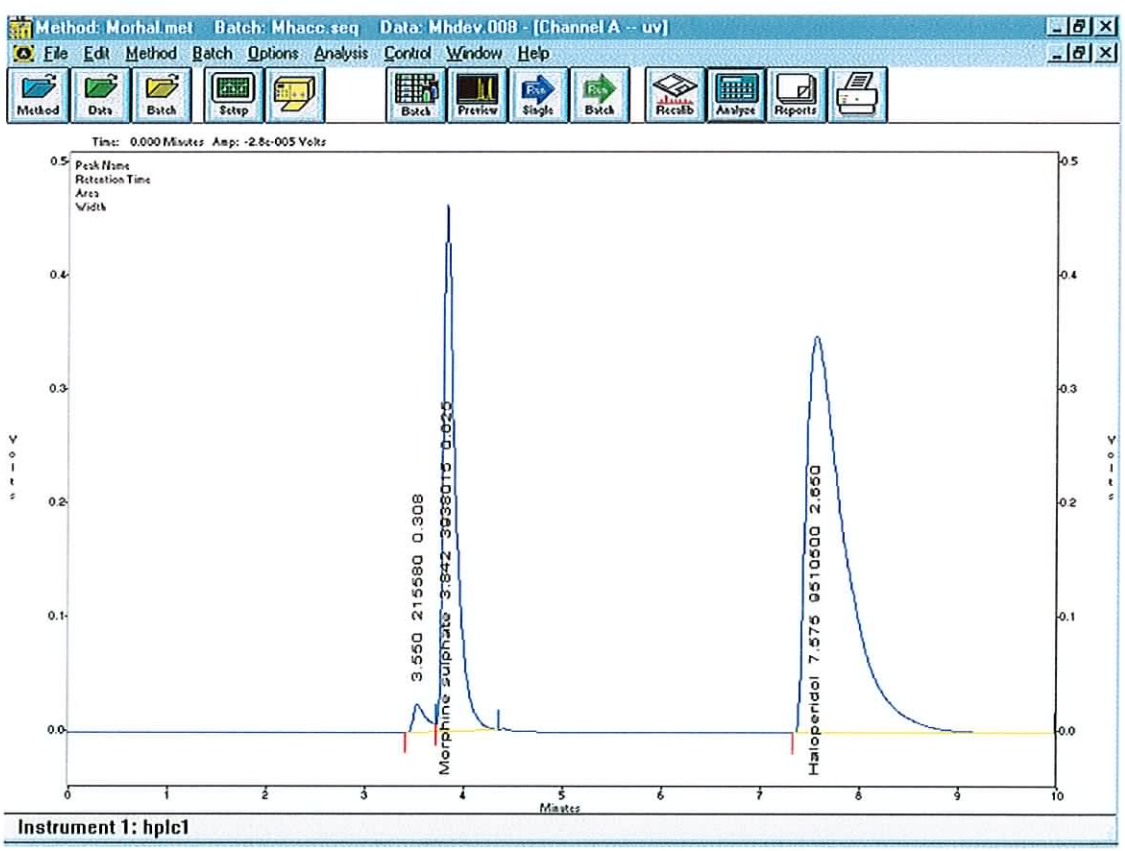


Fig 3.7: Chromatogram 7

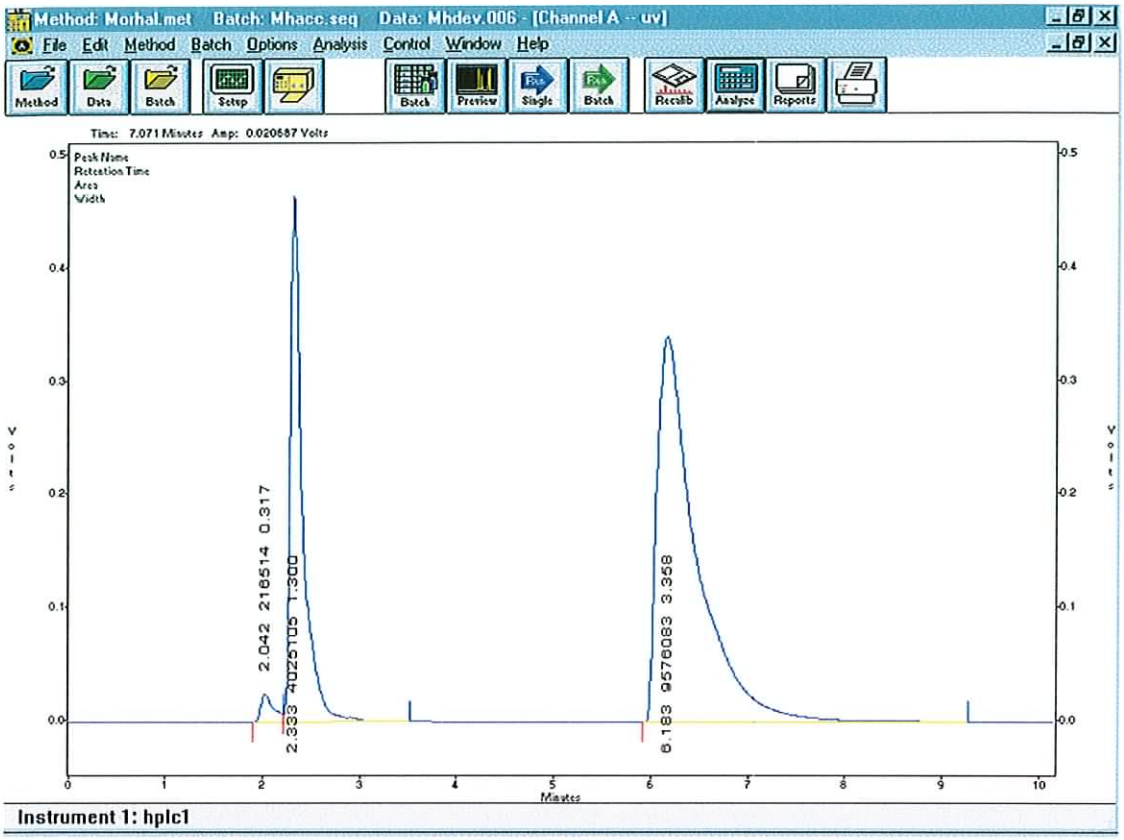


Fig 3.8: Chromatogram 8

It was decided that it would be advantageous to decrease the haloperidol retention time but steps were required to delay the elution of morphine sulphate whilst maintaining acceptable resolution. Later elution of haloperidol also showed broadening of the peak, so the problem was to obtain a reduced retention time without compromising the chromatography. To do this a time program was employed (table 3.1). The flow rate was set initially to 0.5mls/min for three minutes and increased to 1ml/min until ten minutes. The resulting chromatogram can be seen below in figure 3.9. The retention time for haloperidol was reduced to approximately 7.5 minutes.

Time	Function	Flow Rate mls/min
0.01	TOTAL FLOW	0.5
3.00	TOTAL FLOW	0.5
3.01	TOTAL FLOW	1.0
10.00	TOTAL FLOW	1.0
10.01	STOP	0.0

Table 3.1: Time program used for HPLC method

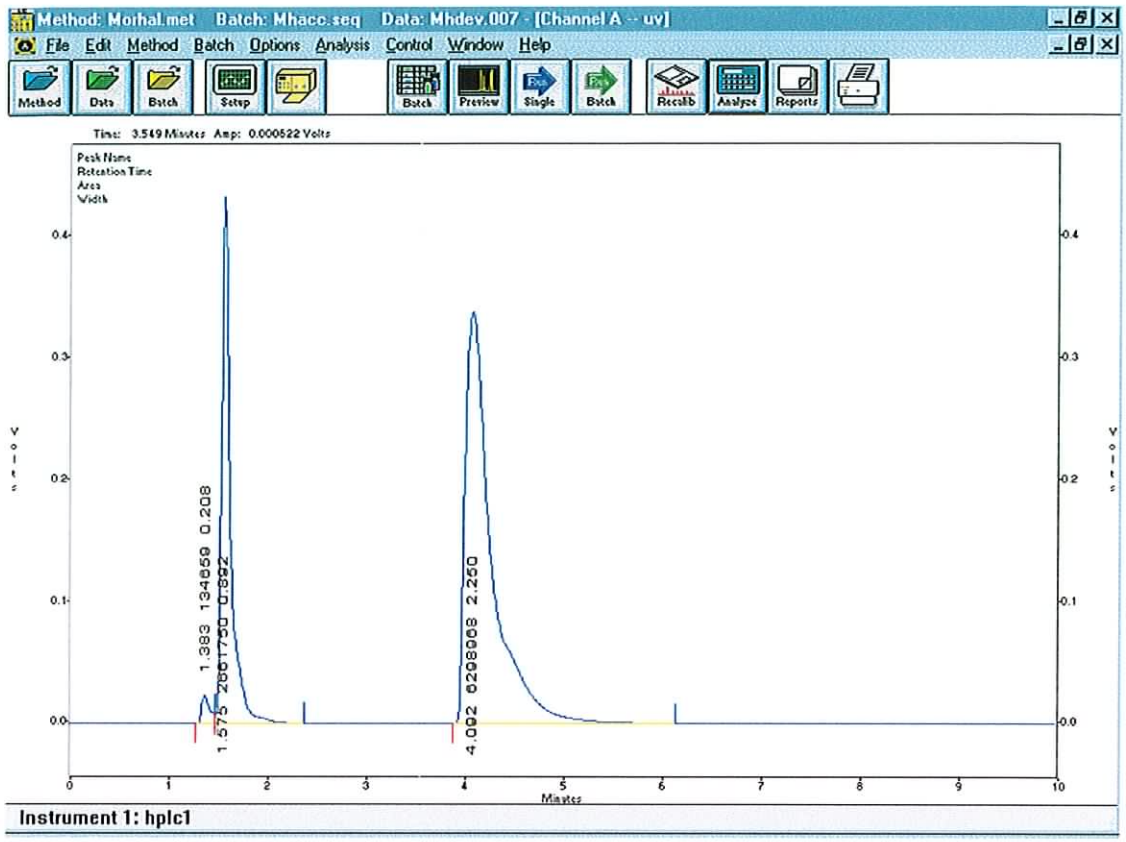


Fig 3.9: Chromatogram 9

Using the time program (table 3.1) and the ammonium acetate acetonitrile mobile phase the injection volume was altered. A sample of the standard solution of morphine sulphate and haloperidol was injected. The injection volume previously was 20 micro litres so 10 microlitres and 30 micro litre samples were injected to see the effect on the chromatogram produced.

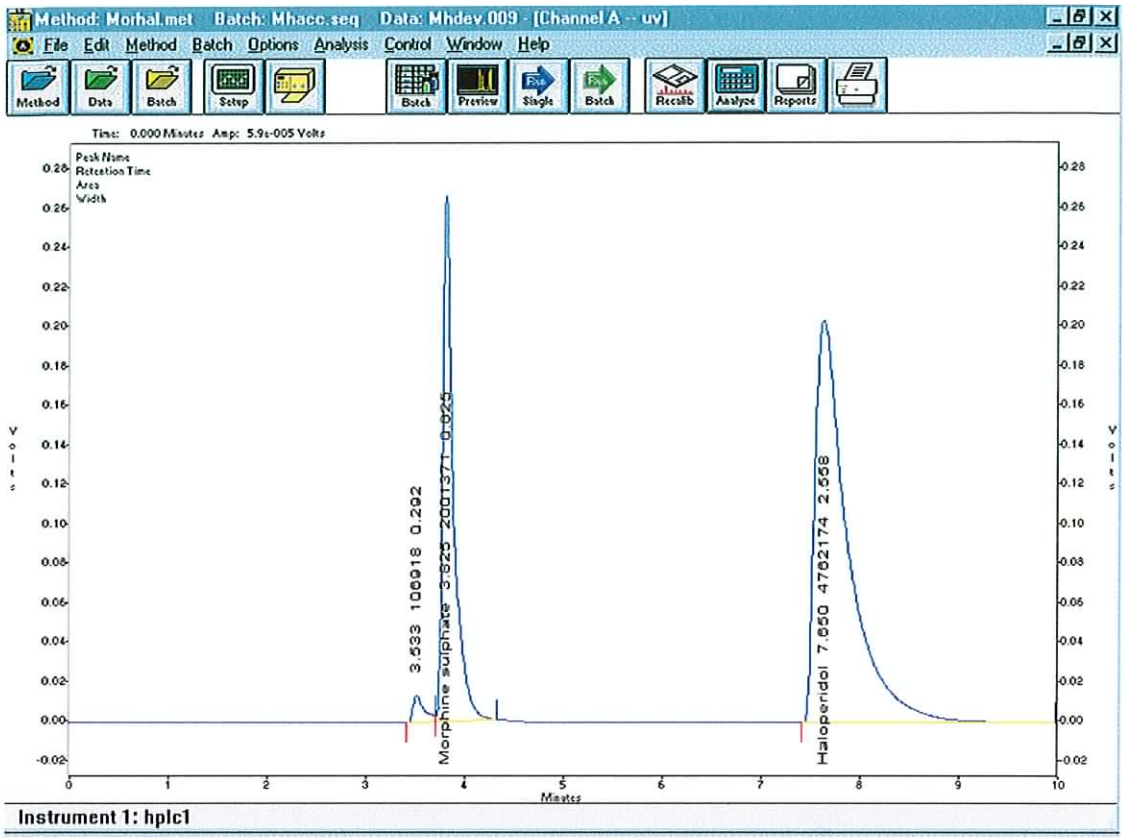


Fig 3.10: Chromatogram 10

Chromatogram 10 (fig 3.10) above represents a 10 micro litre injection of the standard solution. A 30 micro litre injection was also performed (fig 3.11). Varying the injection volume has the effect of increasing or decreasing the peak response for larger and smaller injection volumes respectively. Injections were performed altering the volume to check for an overload effect. There was no change in any other of the chromatograms property. An injection volume of 20 micro litres was determined to be an acceptable volume for the purposes of this project.

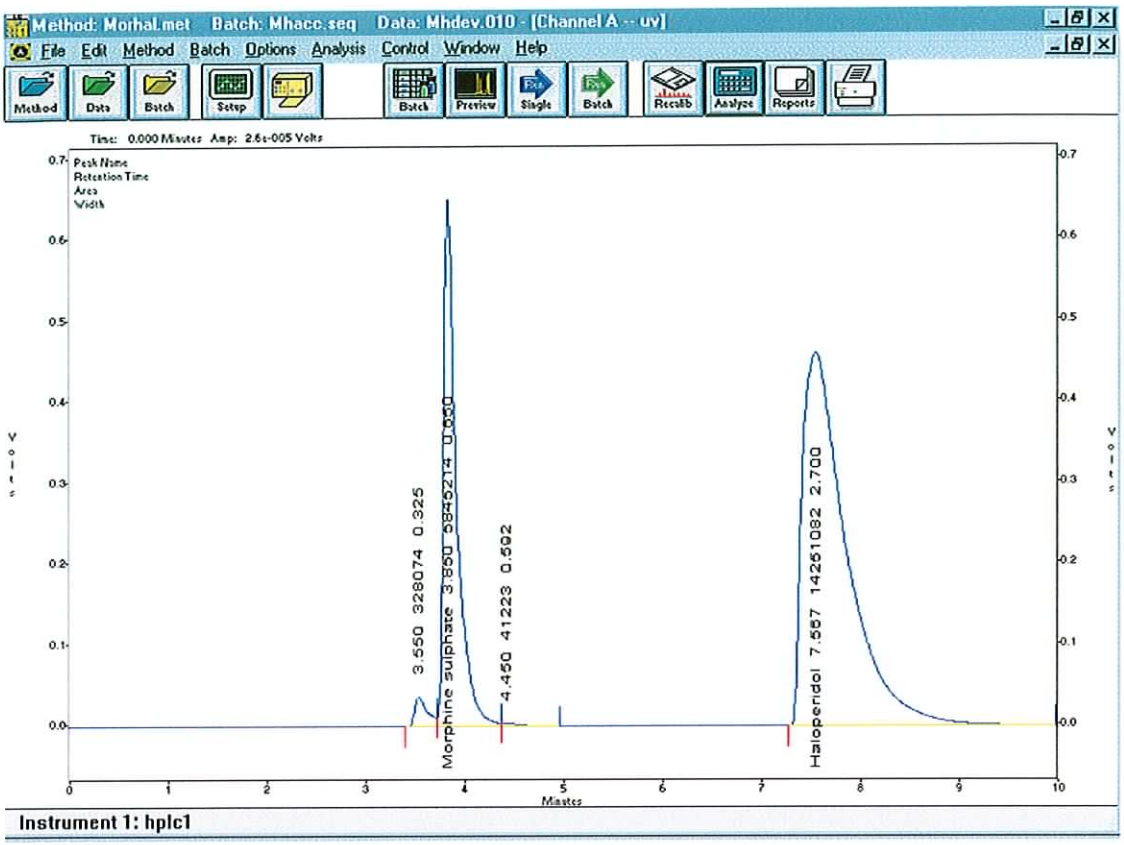


Fig 3.11: Chromatogram 11

Two injections were now performed using the mobile phase mentioned previously. The wavelength was set at 210nm for the first injection and to 270nm for the second one. These wavelengths were chosen to see the effect of varying the wavelength around 247nm at bother a higher and lower wavelength. The resultant chromatograms are shown in figures 3.12 and 3.13. Chromatogram 12 represents injection of the standard at 210nm. From this chromatogram it is clear that the wavelength is unsuitable. The morphine sulphate peak is very unsymmetrical and it appears that the solvent front, which is minimal at 247nm, shows a much larger response on the chromatogram at 210nm. The haloperidol peak appears to have no interfering peaks at this wavelength. Chromatogram 13 results from the injection of the standard at 270nm. There are no apparent interfering peaks similar to the injection at 210nm. The peak response is lower at this wavelength than that at 247nm. Thus 247nm is the most suitable to use for detection of morphine sulphate and haloperidol.

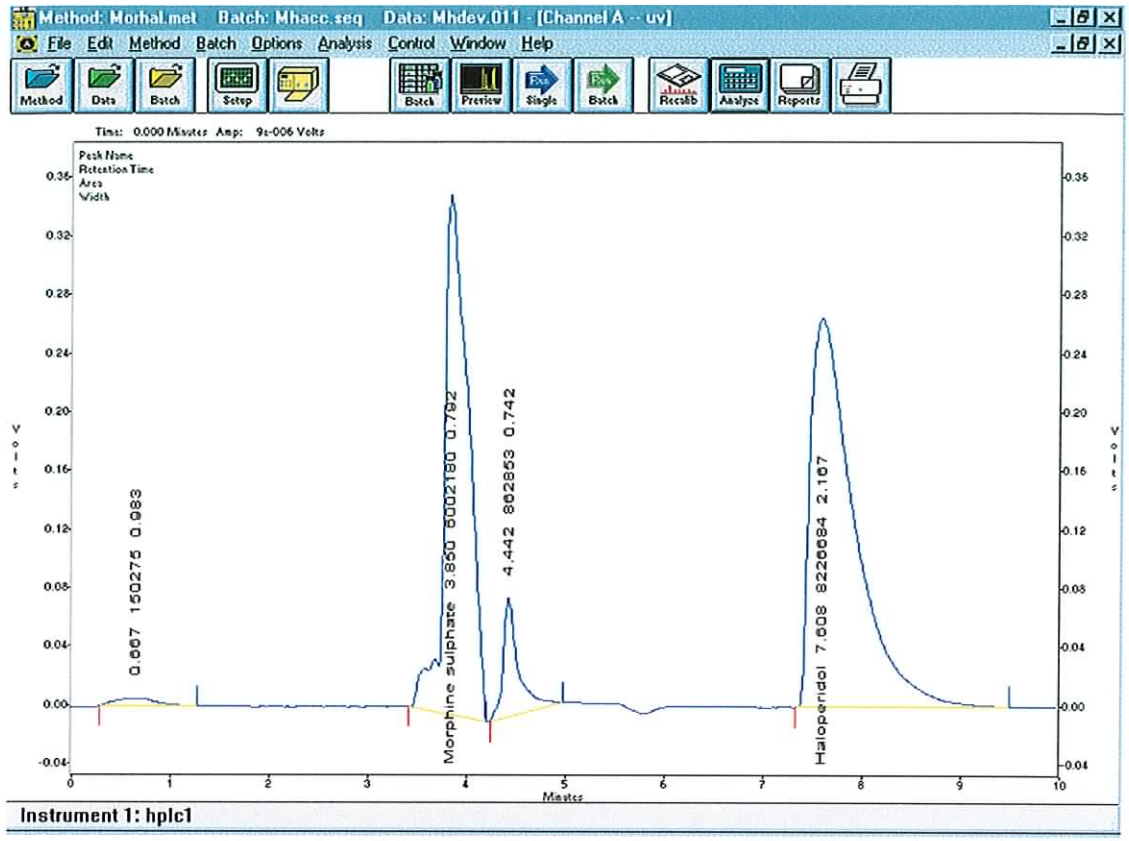


Fig 3.12: Chromatogram 12

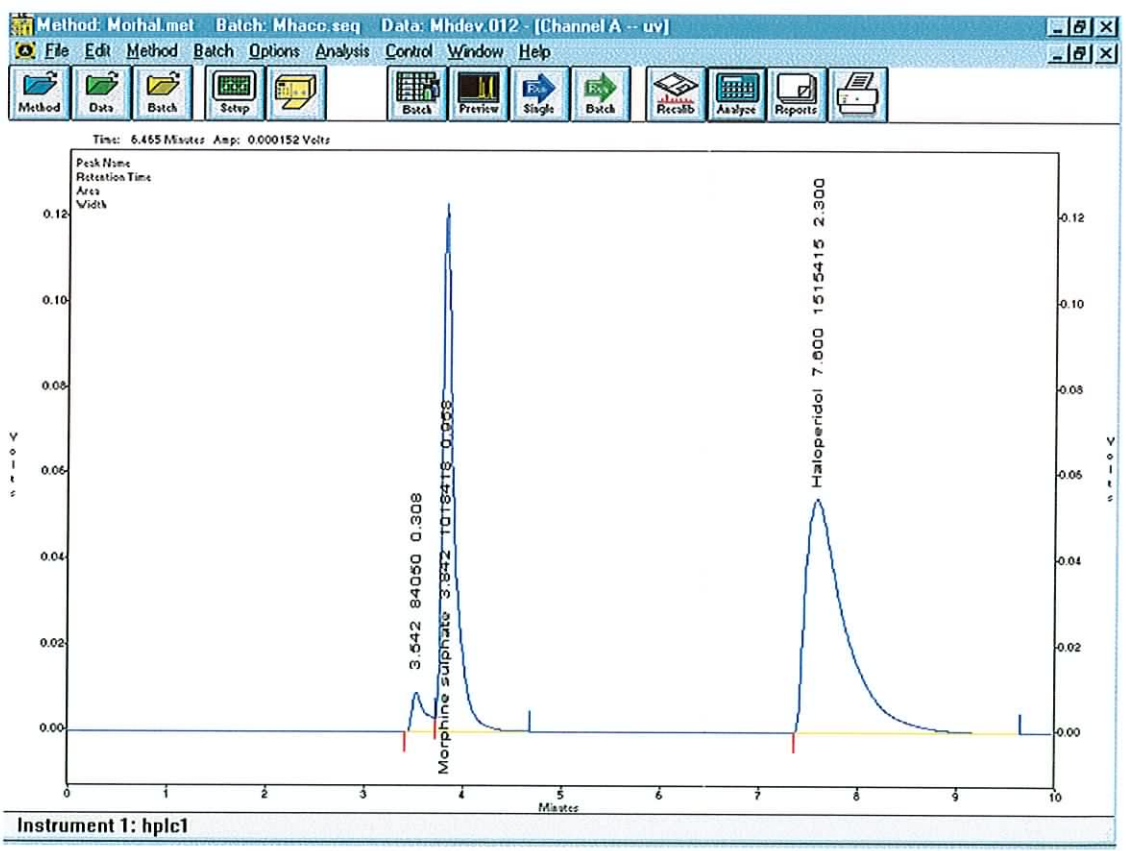


Fig 3.13: Chromatogram 13

Chromatogram 14 results from an injection of the standard solution (see fig 3.14). The mobile phase sample injection volume and detection wavelength were all set as described above. This time the time program was altered. The flow rate was changed after three minutes to 1.5mls/min. From the chromatogram it can be seen that there is no significant change in the peak shapes and the retention time is reduced slightly for both components. However considering that the HPLC pump is more robust operating at lower flow rates it was decided that the previously mentioned time program was sufficient to provide adequate separation of the components of interest.

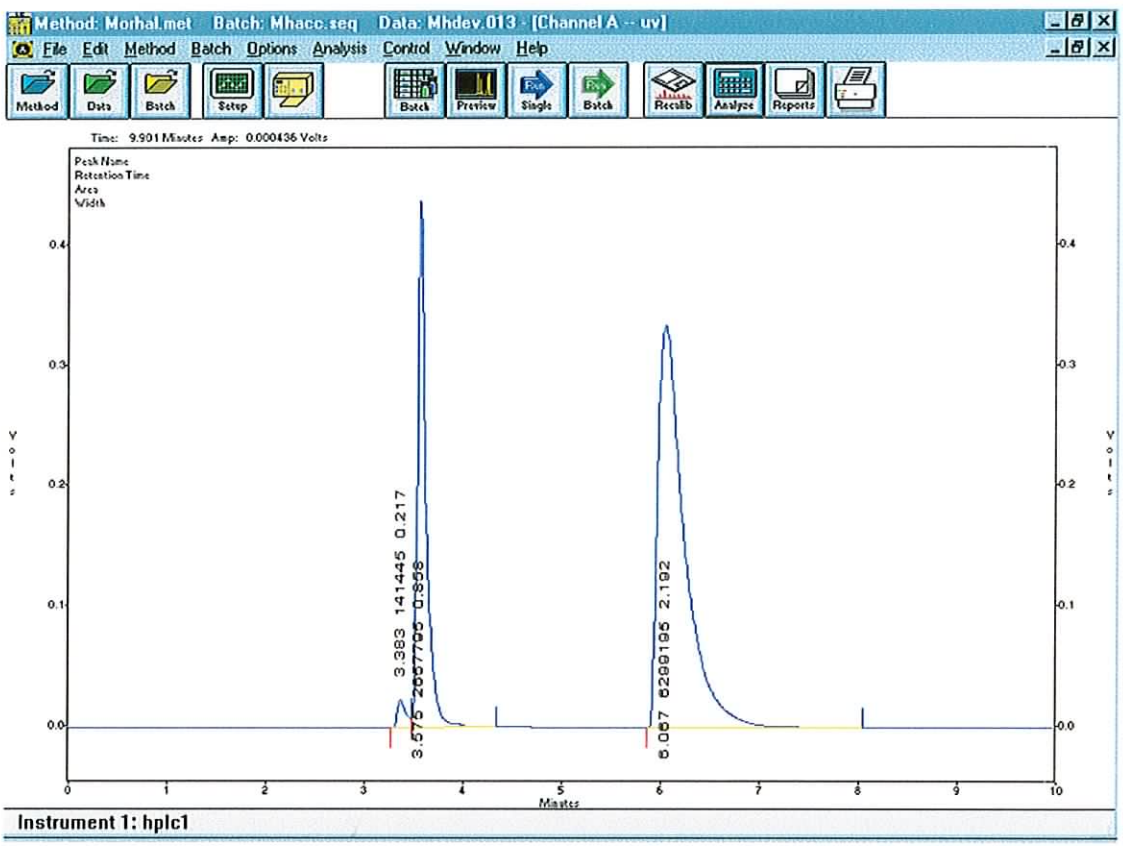


Fig 3.14: Chromatogram 14

The final developed method is summarised below as follows:

Equipment used:	Shimadzu SCL 10 A vp
Column used:	Hichrom NC100-5-C18, 250 X 4.6mm ID
Column Packing:	C18 5µ particle size
Mobile phase:	55% ammonium acetate (1%) buffer
	45% acetonitrile, pH adjusted to 3.6
Injection volume:	20 micro litres
Detection wavelength:	247nm
Sensitivity	2.0 AUFS, Attenuation 516
Flow rate:	time program, 0-3 mins at 0.5mls/min and
	From 3-10mins a flow rate of 1mls/min
Typical Retention times	Morphine Sulphate 3.8 minutes
	Haloperidol 7.5 minutes

A chromatogram resulting from the injection of morphine sulphate (0.75 mg/ml) and haloperidol (0.4 mg/ml) using this developed method is presented below in figure 3.15 (chromatogram 15).

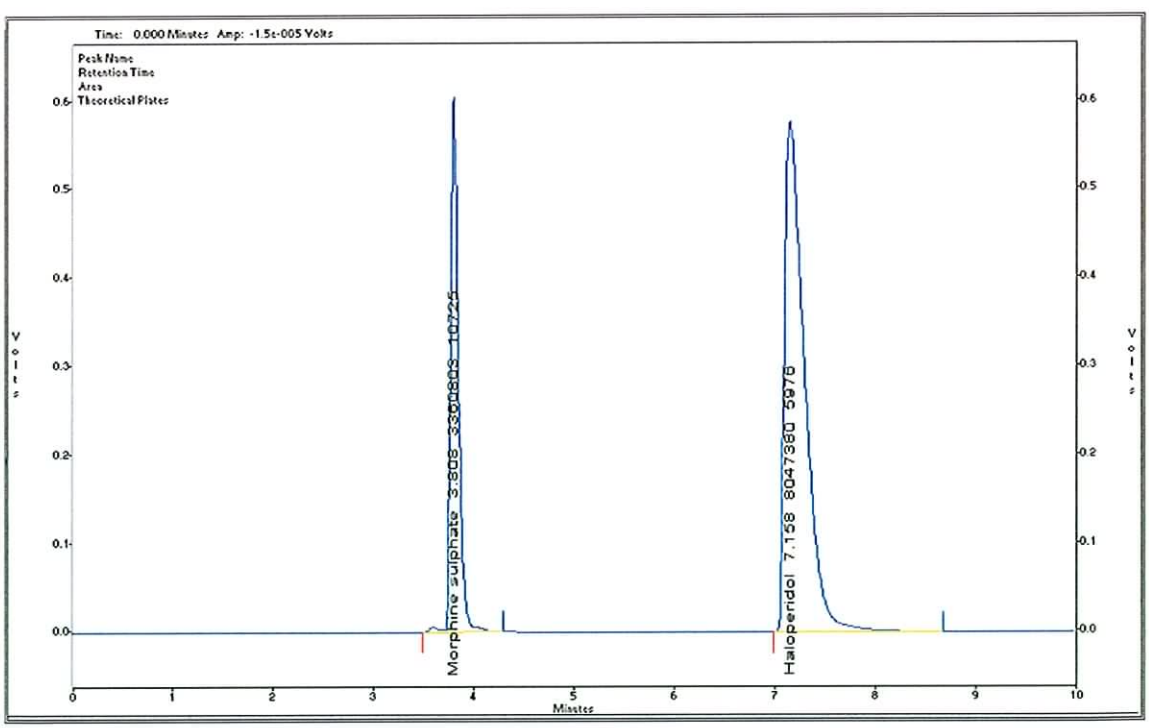


Fig 3.15: Chromatogram 15

3.3: Troubleshooting

Method development is a long and tedious process. It is very important to outline the goals of the development undertaking. Experience is vital in developing a successful approach as is a thorough literature search very important. Limited resources and machine breakdown require knowledge of troubleshooting. The following discussion lists a variety of problems and solutions during the process of the method development. Whilst a number of these symptoms and solutions can relate to everyday HPLC they are discussed with reference to the automated Shimadzu system used in this project. Troubleshooting is a part of any process in HPLC and is well documented in the literature [9,10,14,16] but in a method development situation when no peaks have yet been processed much experience is gained in troubleshooting. If there is a simple blockage or a poor column and these symptoms cannot be identified, then it may not be possible to generate appropriate peaks with correct conditions. Even if peaks have been generated at this stage there are usually a lot of possible causes for a particular problem. Experience helps to discriminate and identify particular problems which economises time and effort. In an HPLC system problems can arise from many sources. The first objective is to define the problem, then isolate its source. A process of elimination will usually provide the ability to specify the cause of the problem and implement corrective action.

Mobile phase problems can occur although this is more usually associated with gradient elution. Water is the most common source of contamination in reverse phase analysers. Only highly purified or deionised water should be used when preparing a mobile phase. However several common deionisers introduce organic contaminants into the water. Removal of these types of contaminants is done by passing the deionised water through activated charcoal or a C18 cartridge. Only HPLC grade solvents should be used. Cleaning lower quality solvents is time consuming and trace levels of contaminants can often remain. These trace contaminants can cause problems when a high sensitivity ultraviolet or fluorescence detector is used. The mobile phase should be degassed prior to use to help prevent bubbles in the system. The equipment used in this project had a degassing device attached. Filtering the mobile phase through a 0.2 or 0.45-micron filter eliminates gas and also removes particulate matter that can produce a noisy baseline or plug the column. One final

point should be made in relating to preparing mobile phases. Solvents that are immiscible or incompatible should be avoided also.

The pumping mechanism must deliver a constant flow of solvent to the column over a wide range of conditions. Pumping system problems were found to be easy to identify and correct. Some of the more common symptoms observed were erratic retention times and a noisy baseline. It was possible to identify leaks, and loose connectors, by observing the buffer precipitating out at some of the fittings.

The injector rapidly introduces the sample into the system with a minimal disruption to the solvent flow. Mechanical problems involving the injector were also quite easily identified during the project. Problems such as motor slips and worn seals were some of the problems encountered.

Presented below is a small discussion on some of the problems that occurred during the duration of the project and the logic applied that allowed for identification of the problem and the application of corrective action.

Carryover peaks

During the development carryover peaks occurred from a previous sample. This was a cause for concern. There was possibly a contamination in the injector or the column. To combat this problem the injector was programmed for a flush between analyses. The mobile phase was changed and the column washed with isopropyl alcohol to purge the flow lines of contamination.

Broad Peaks

When peaks show broadening throughout a given run a number of parameters could cause this. The mobile phase composition may have changed. In the case of the method in question good mixing of the mobile phase is essential to ensure the content is uniform. The mobile phase flow rate could be too low. The slower the mobile phase velocity the broader the peak shape as a rule. A possible alternative could also be that the peak is not an individual but two or more poorly resolved compounds. There could also have been column contamination or the column worn out. By making up a new mobile phase the problem was not resolved. However the problem

was resolved quite simply. On careful inspection of the column it was seen that there was a small leak at a nut at the top of the column between the column and the detector. By adjusting the fitting the problem was overcome.

Baseline Noise

An initial problem with the chromatography system was the baseline. There was a problem with baseline drift. On removal and inspection of the detector cell a small air bubble and a contaminant was observed in the window of the cell. By detaching the detector cell from the system and flushing the cell out with methanol the problem was resolved.

At one stage during development the baseline noise became irregular. This may have been caused by the deterioration or contamination of the mobile phase. Replacing the mobile phase reduced the erratic noise but did not satisfactorily resolve the problem. An inspection of the pump for leaks yielded little success. The system was flushed thoroughly with methanol and this brought the baseline back to a normal level. This indicated that an air bubble was trapped in the system.

Fronting peaks

On investigation of the injection volume fronting peaks and rounded peaks were observed on the chromatogram. This may have been caused by an incompatibility between the sample injected and the mobile phase or interference in the sample. Making up fresh samples did not have any impact on the peak shape. The peak fronting ceased when the injection volume was reduced. Column overload was the problem.

Variable Retention Times

During an experiment the peak retention times for morphine sulphate and haloperidol began to fluctuate. It was thought that the column may have been the problem but this is not a common cause of erratic retention. As a column ages retention times usually gradually decrease. Another explanation for the variance in retention time was an air bubble on the pump head. The pump and check valves were purged and this provided a solution.

No Peaks/very small peaks

This was one of the very first difficulties to overcome. There were two incidents that required troubleshooting where no peaks were visible. The first of these was where there was no peak signal at all for any conditions set out. The lamp was on and the mobile phase was pumping through the system. Changing on the attenuation on the detector eliminated this problem. The second problem with no peaks occurred at a later interval. Changing attenuation on the detector had no effect. The problem was identified. There was a loose wire between the detector and the computer recorder. This required an electrical repair.

Pressure Problems

Pressure problems were one of the largest difficulties associated with this method development. Fluctuations can arise from a great number of sources. Blockages in the needle cause pressure fluctuations. One problem to rapidly identify is large pulsing in the pressure of the system. This symptom is quite serious and is not as simple as an air bubble. This symptom is usually a sign that one or both of the pump pistons are cracked or broken. When a piston breaks the pressure will break down completely and go from zero to a high pressure at each piston stroke. If the pressure was lower than usual it could indicate a leak present or air trapped in the pump head.

From the various experiments conducted and through a process of troubleshooting and maintenance it was possible to obtain a chromatogram that had two identifiable peaks, one of morphine sulphate with a retention time of approximately 3.8 minutes and the other of haloperidol with a retention time of approximately 7.5 minutes. It was now possible to continue with the project and validate this method.

3.4: References

1) Baker Norton Data Sheet, 2000

2) http://matamaticas.udea.edu.co/~carlopez/chromatography/ph/95121tb.html/ "The Impact of pH on HPLC Method Development, 2000.

3) Stiles ML, Tu Y-H, Allen LV Jr. "Stability of morphine sulphate in portable pump reservoirs during storage and simulated administration. Am J Hosp Pharm. 1989; 46, 1404-07.

CHAPTER 4

METHOD VALIDATION

4.1 Materials used in Method Validation

HPLC Equipment

SCL-10A VP Shimadzu system controller

SPD-10A VP Shimadzu UV-Vis detector

SIL-10A VP Shimadzu auto injector

LC-10AT VP Shimadzu liquid chromatograph

FCV-10AL VP Shimadzu low pressure gradient control valve

Shimadzu on line degassing device GA STORR 154

Class-VP Chromatography data analysis system

Column

Hichrom NC100-5C18-3109

pH meter

Jenway pH Meter 3310

Balances

Taurus Mettler balance model BP110

Taurus Mettler balance model Bl120S

Heating Bath

Grant heating bath, model jB1, Vestar ltd.

Reagents

Acetonitrile, 99.93%, HPLC grade, Sigma-Aldrich.

Ammonium Acetate 97.3%, Sigma-Aldrich Lot 58H0060

Phosphoric Acid, ACS reagent 85% Lot 90609017

Hydrochloric Acid, Sigma Aldrich

Hydrogen Peroxide, Sigma Aldrich

Sodium hydroxide, Sigma Aldrich

Deionised Water

Morphine Sulphate

Commercially prepared solutions (supplied by Antigen pharmaceuticals, Roscrea, Tipperary) were used as both standards and in the filling of the PCAs required for testing. Ampoules of variable concentration were required for dilution (10mg/ml, 15mg/ml, 30mg/ml, 60mg/ml).

Morphine Sulphate reference standard
Sigma-Aldrich Lot MPT-394A

Haloperidol

Commercially prepared solutions (Supplied by Norton Healthcare, Waterford) were used as both standards and for sampling (20mg in 2mls)

Haloperidol reference standard
Sigma-Aldrich Lot 18H0408

4.2: Comparison study of reference standards against commercial solutions

At the outset a major challenge was faced before evaluation of the validation parameters was possible. This was the need to use a more economical and viable material (morphine sulphate and haloperidol) to use as a reference standard due to the time and cost to obtain pure morphine sulphate. There was a three month ordering process to obtain the morphine sulphate standard powder as well as the need for a licence to purchase it. Added to this was a considerable cost factor (€650 per g). Due to the nature of the work it was not easy to know exactly how much powder would be required and it was not viable to order a large quantity of the powder that may not be used. Following discussion and assessment of the situation and the obstacle faced with, it was decided to use the commercial solutions used for sampling as reference standards due as the commercial solutions were cost effective and there was a supply in stock in the pharmacy department.

It was necessary to perform a comparison study of the commercial solutions of morphine sulphate and haloperidol against their respective reference standard

powders. This was performed by making up the appropriate solutions listed in tables 4.1 and 4.2 below. Two sets of linear plots were generated for each drug to cover the experimental range and the peak areas were compared for reference standard against commercial solution for each drug.

Concentration $\mu g/cm^3$	Commercial solution used	Volume of cs* diluted to $10cm^3$	Wt of Reference std powder diluted to $100cm^3$
100	$10mg/cm^3$	100µl	10mg
200	$10mg/cm^3$	200µl	20mg
270	$30mg/cm^3$	90µl	27mg
510	$30mg/cm^3$	170µl	51mg
750	$60mg/cm^3$	125µl	75mg
1000	$10mg/cm^3$	$1cm^3$	100mg
1100	$10mg/cm^3$	$1.1cm^3$	110mg

Table 4.1: Morphine Sulphate sample preparation. *Commercial solution

Concentration $\mu g/cm^3$	Volume of cs* ($10mg/cm^3$) diluted to $10cm^3$	Wt of Reference std powder diluted to $100cm^3$
100	100µl	10
200	200µl	20
400	400µl	40
600	600µl	60
750	750µl	75
1000	$1cm^3$	100

Table 4.2: Haloperidol sample preparation. *Commercial Solution

4.3: Preparation of the Standard Solution

The standard solution consisted of morphine sulphate ($0.75mg/cm^3$) and haloperidol 0.4mg/ml. This was prepared by pipetting 250ul of a $30mg/cm^3$ morphine sulphate solution (Antigen) and 400ul of a 20mg/ml haloperidol solution (Serenace) and diluting to $10cm^3$ with mobile phase.

4.4: System Suitability

System suitability [1] was carried out on each day of testing. At the start of any given HPLC run six replicate injections of the standard solution of morphine sulphate and

haloperidol were performed. It was defined that the peak areas should have a coefficient of variation (CV) or relative standard deviation (RSD) of ≤ 2% and that the retention times for the elution of morphine sulphate and haloperidol should be not vary appreciatively from 3.8 and 7.5 minutes respectively. Test samples and standards were injected in duplicate and a single injection of the standard was performed after every six injections.

4.5: Linearity Study

Linearity standards [2-10] were prepared at six concentration levels in the range of 0.27-1.5mg/ml for morphine sulphate and 0.1-1.0mg/ml for haloperidol. The table 4.3 below summarises the preparation of solutions for the linearity study. All samples were analysed using the developed HPLC method parameters.

Standard	Volume of Morphine Sulphate	Volume of 10mg/cm^3 haloperidol	Final Volume*	Morphine Sulphate Final Concentration µg/ml	Haloperidol final concentration µg/ml
1	90ul of 30mg/cm^3	100	10cm^3	270	100
2	170ul of 30mg/cm^3	200	10cm^3	510	200
3	250ul of 30mg/cm^3	400	10cm^3	750	400
4	1cm^3 of 10mg/cm^3	600	10cm^3	1000	600
5	1.2cm^3 of 10mg/cm^3	750	10cm^3	1200	750
6	1cm^{3}of 15mg/cm^3	1cm^3	10cm^3	1500	1000

Table 4.3: Preparation of linearity solutions.

4.6: Robustness

For assessment of the robustness the standard solution was prepared and all injections were performed from this solution. Two parameters were chosen for alteration [11].

- Adjustment of pH of mobile phase by ±0.1 pH unit using phosphoric acid
- Modification of the acetonitrile component in the mobile phase by ±1%

Parameter	Proposed Conditions	Upper	Lower
PH	3.6	3.7	3.5
Mobile phase ratio*	Standard Volume Ratio	Upper Volume Ratio	Lower Volume Ratio
	$200cm^3$:$165cm^3$	200cm3:$168cm^3$	200cm3:163cm3

Table 4.4: Robustness study sample preparation.

*The mobile phase ratio is expressed as a volume ratio. The proposed conditions for the developed method would entail a ratio of buffer to acetonitrile. This is expressed in the table 4.4 as volume of ammonium acetate buffer (1%) to volume of acetonitrile. Only the organic volume was altered for the upper and lower alterations.

Analyses:

The standard was injected in duplicate onto the HPLC column at first using the proposed standard conditions for the developed method. Then two further injections of the standard were performed using the modified mobile phases listed in the table above. Finally the mobile phase was replaced using the upper and lower pH solutions. Peak areas were then compared between standard conditions and robustness conditions. The same standard solution was injected each time to keep uniformity to the comparison. Each mobile phase was only used after the previous one was purged from the HPLC system.

4.7: Preparation of Accuracy Standards

Samples of morphine sulphate and haloperidol were prepared using sample concentrations in the linear range using commercial solutions (tables 4.5 and 4.6) . These samples were injected onto the column and peak area determined. Samples were injected in duplicate. The accuracy samples were prepared by spiking a replicate set of solutions to those above with morphine sulphate reference standard powder. The percentage recovery was calculated using the equation:

$$\%\,\mathrm{Recovery} = \left(\frac{MeasuredValue}{TrueValue}\right)\times 100$$

morphine sulphate concentration µg/ml	Commercial Solution Volume diluted to 10mls	Commercial solution spiked with reference standard powder vol/wt diluted to 10 mls
270	90ul of 30mg/ml	90ul of 30mg/ml
510	90ul of 30mg/ml	90ul of 30mg/ml + 2.4mg ref std powder
750	90ul of 30mg/ml	90ul of 30mg/ml + 4.8mg ref std powder
1000	90ul of 30mg/ml	90ul of 30mg/ml + 7.3mg ref std powder
1200	90ul of 30mg/ml	90ul of 30mg/ml + 9.3mg ref std powder

Table 4.5: Morphine sulphate sample preparations for accuracy study

Haloperidol concentration µg/ml	Commercial Solution Volume diluted to 10mls	Commercial solution spiked with reference standard powder vol/wt diluted to 10 mls
270	100ul of 10mg/ml	100ul of 10mg/ml
510	100ul of 10mg/ml	100ul of 10mg/ml + 1mg std ref powder
750	100ul of 10mg/ml	100ul of 10mg/ml + 3mg std ref powder
1000	100ul of 10mg/ml	100ul of 10mg/ml + 5mg std ref powder
1200	100ul of 10mg/ml	100ul of 10mg/ml + 6.5mg std ref powder

Table 4.6: Haloperidol sample preparations for accuracy study

4.8: Degradation Studies

Solutions of morphine sulphate 6mg/ml and haloperidol 1mg/ml were prepared and diluted in 0.1M sodium hydroxide, 0.1M hydrochloric acid, and 3% hydrogen peroxide (Representative chromatograms presented in appendix 2, page 133). These samples were left at room temperature and 90°C for over three hours. Samples were then analysed twenty four hours later by HPLC using the proposed developed method. The high concentrations were used to magnify the baseline and to show any traces of byproducts.

A second HPLC run was performed using the standard solution of morphine sulphate and haloperidol. Again samples were prepared in 0.1 M sodium hydroxide, 0.1 M hydrochloric acid and 3% hydrogen peroxide solution. They were also subjected to thermal stress and stored at room temperature.

4.9: Preparation of Precision Standards

Six solutions were individually prepared of the standard solution and duplicate injections of each sample were performed. Peak areas of each of the six solutions were analysed and compared.

4.10: Stability of Mobile Phase

A standard solution was prepared and the mobile phase as described in the method development chapter. Six injections of the standard solution were performed using the proposed HPLC conditions. The test was repeated for three consecutive days with a fresh standard solution prepared on each day as the compatibility of morphine and haloperidol had not been assessed yet. Peaks were compared over the duration of the three days for changes in retention time or peak area or shape.

4.11: Results and Discussion - Justification for replacing reference standard with commercial solutions

A problem arose during the development of the method with regard to the use of the morphine sulphate reference standard powder. It is a difficult powder to obtain, needing a licence for permission to obtain it and it is also extremely expensive (€650 per gram). Also the powder comes in very small quantities and takes quite some time to obtain. Thus use of morphine sulphate reference standard powder is not practical for the analysis required and the resources that that were available. It was necessary to replace the existing powder with a practical alternative. This issue has been discussed in literature and it is strongly advised against. Thus it was decided that following the method development, that commercial solutions of morphine sulphate and haloperidol would be used in place of their corresponding reference standards. The reasoning behind this was that if a fresh standard was prepared on each test day during a stability run then we would be comparing the aseptically prepared solutions with fresh samples of an identical nature to the ones already under analysis. To keep the experimental design uniform the haloperidol reference standard powder was also replaced with commercial solutions.

To satisfy the concerns and advice from literature it was necessary to justify this replacement of the morphine sulphate and haloperidol reference standard powder. It was decided to conduct an experimental study i.e. to construct a linear calibration plot covering the concentration ranges to be used later for both morphine sulphate and haloperidol and to compare the plots and peak areas for both drugs (Representative chromatograms are presented in Appendix 1, pages 125-128).

Before experimental considerations are discussed it is important to note that the commercial solutions used are those used for reconstitution in clinical applications. Many drugs can be dangerous to a patient if they receive an overdose so an accurate drug concentration is required without question. Therefore this would seem to be justification for the use of commercial solutions as reference standards. Also when performing drug stability studies, the ideal situation for the analyst would be to use commercial solutions as standards as the exact same solutions are used in the clinical application. This means the same materials are used so the stability study is at an optimum.

Analysis of the two potential standards (reference standard powder / commercial solutions) was the first stage in this justification. It was decided to compare the mean peak area for the commercial solution against that of the reference standard powder at each concentration. This was done using the equation below

$$\left(\frac{reference.std.peak.area - commercial.solution.peak.area}{reference.std.peak.area}\right)\times 100$$

It was assumed that the reference standard mean peak area was at one hundred percent concentration or a representation of "true concentration". This is because certified reference standards are traceable to another source and they are free from additives or interfering components. The percentage variance between this "true value" and the value represented by the commercial solution was then established. An acceptance value such that the percentage variance should not exceed five percent between the solutions at all concentrations tested was decided upon. Furthermore it was considered inappropriate to replace reference standard powders with commercial

solutions if this value was exceeded as the commercial solutions could only be deemed unsuitable in this case. Likewise the variance in the slopes of the linear plots for reference standard and commercial solutions should not exceed two percent variation. By comparing the slopes we are investigating the linear properties of each curve. If the concentrations of the standards are near identical then the slopes of the standard curves should also demonstrate similar behaviour.

Concentration µg/cm^3	Commercial Solution mean peak area	Reference Standard mean peak area	% Variation of results
100	2040451	2080920	1.94
200	4019738	3963903	-1.40
400	8124964	8019032	-1.32
600	12479702	12129338	-2.88
750	15405984	15533858	0.82
1000	20565103	20617683	0.25

Table 4.7: Haloperidol reference standard and commercial solution data.

Concentration µg/cm^3	Commercial Solution mean peak area	Reference Standard mean peak area	% Variation of results
100	468597	468529	-0.01
200	930002	937058	0.75
270	1265127	1273982	0.69
510	2354073	2350495	-0.15
750	3354488	3390169	1.05
1000	4360191	4520225	3.54
1100	4796210	4799936	0.08

Table 4.8: Morphine Sulphate reference standard and commercial solution data.

Firstly plots of mean peak area against concentration were created using the data in the tables above. This was done to prove that the plots for the commercial solutions were linear just like those of the reference standard powder. Also the benefit of creating these linear comparison plots can demonstrate visually that the commercial solutions show an almost identical slope to that of the reference standard powder. This can be clearly demonstrated by superimposing the linear graph for commercial solution onto that of the reference standard.

Inspection of the slopes of the graphs (fig 4.2) for haloperidol shows variance in the slope of the graph of less than 0.3%. For morphine sulphate the variance between the slopes of the graphs for reference standard powder and commercial solutions is 1.8% (fig 4.1). It is clear from the reference standard plot that the 1mg/cm3 mean peak area value has altered the slope of the graph and contributed to this high value

On analysis of the mean peak areas presented in tables 4.7 and 4.8, it can be clearly seen that the percentage difference between the concentrations of the reference standard powder to that of the commercial solution is below five percent. In fact on analysis of haloperidol all peak area values are below two percent variance except for the 600µg/ml solution. This value is nearer three percent suggesting that there was a dilution or weighing error in the sample preparation. Similar analysis of morphine sulphate results shows peak area values well below two percent variation. The exception was the 1mg/ml solutions, which had a variation of over three percent. This again suggests a weighing or diluting error.

It has been shown that the commercial solutions for morphine sulphate and haloperidol yielded results within the limits agreed upon that would justify using them in place of reference standard powders for the remainder of this project.

Fig 4.1: Morphine Sulphate (Ref. Std. versus Commercial Soln.)

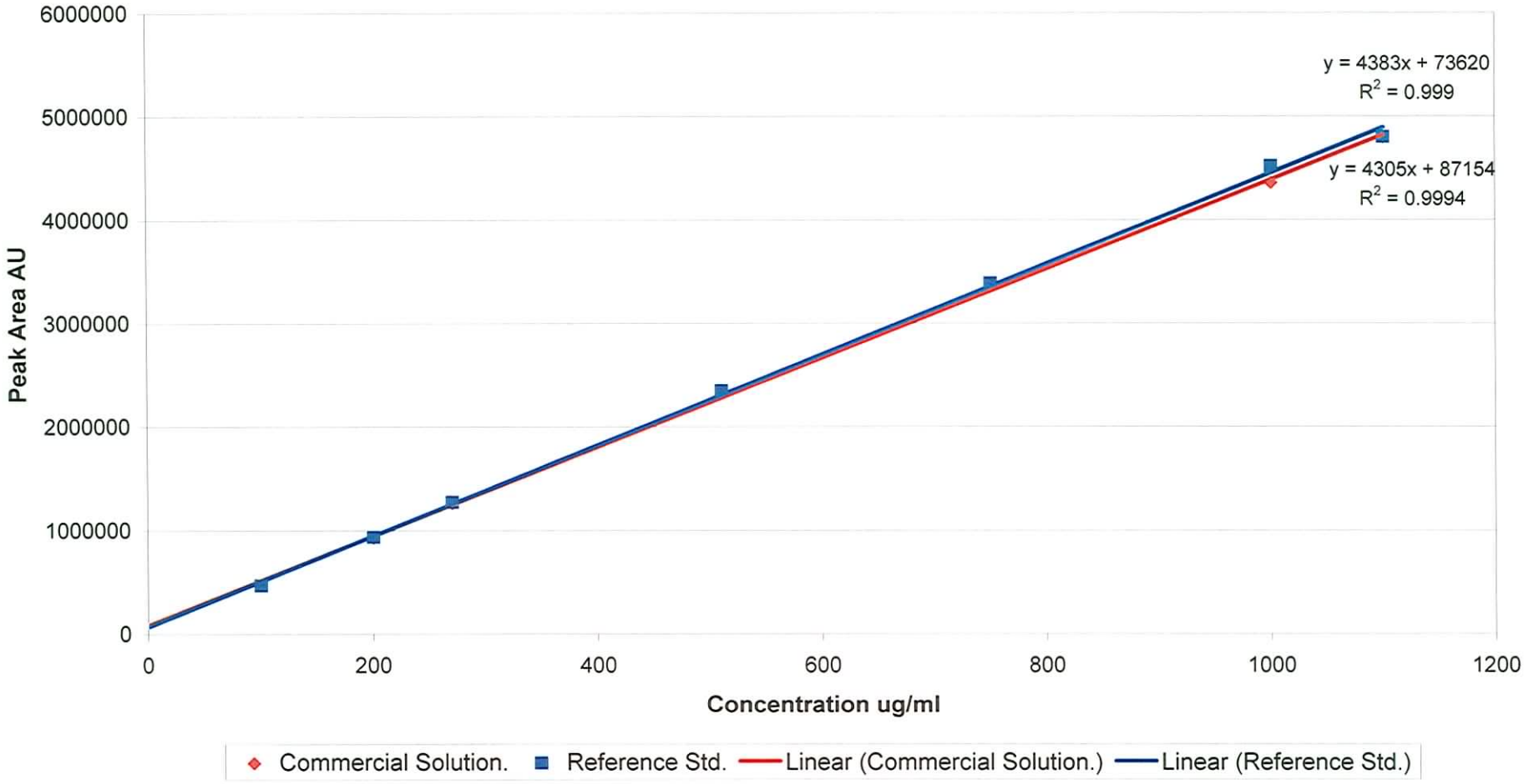
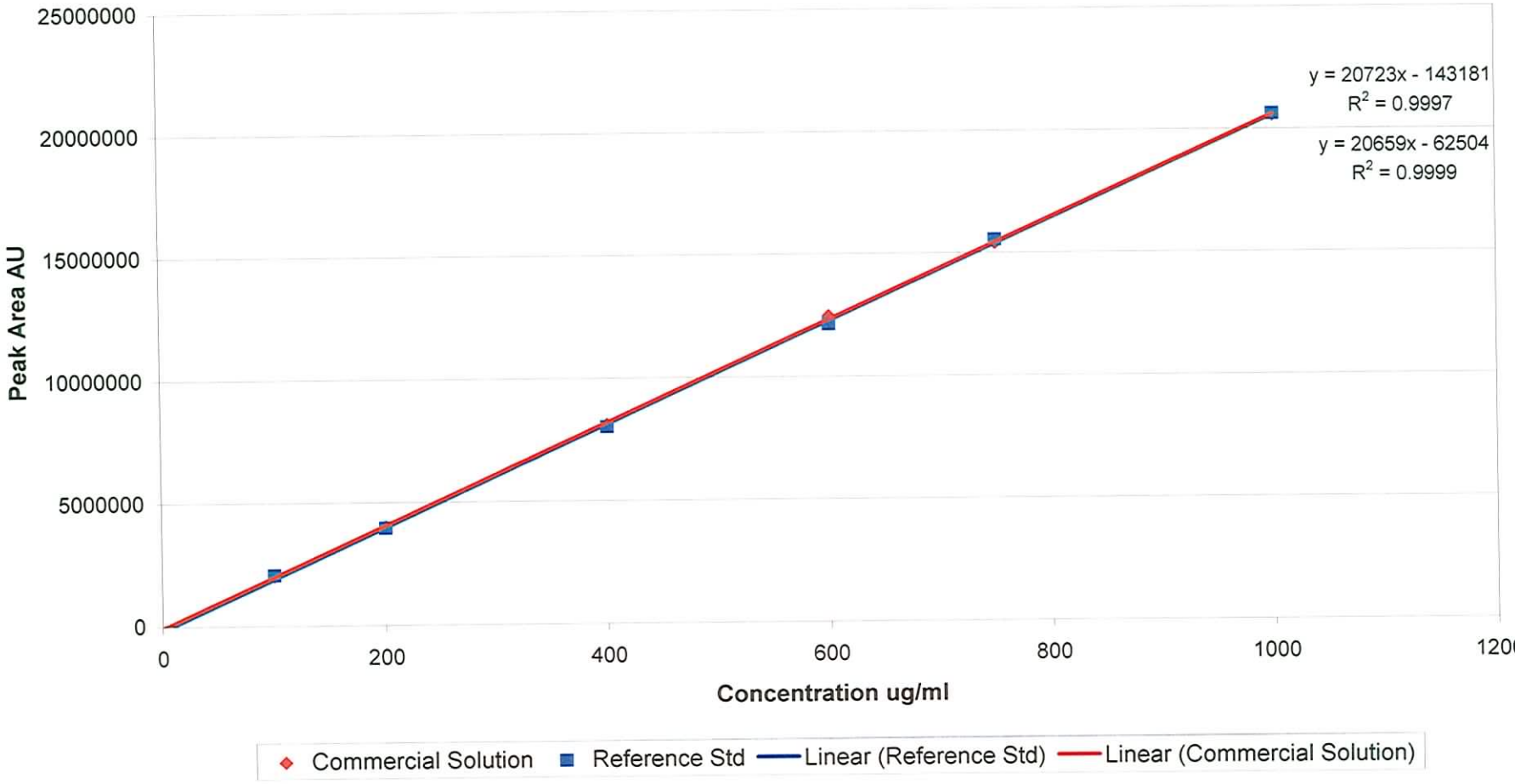


Fig 4.2: Haloperidol (Ref. Std. versus Commercial soln.)



4.12: METHOD VALIDATION PARAMETERS DISCUSSION

Linearity

Table 8.3 and the graphs (figures 4.3 and 4.4) summarise the results for the linearity study (Representative chromatogram is presented in Appendix 1, page 131).

Sample No.	Morphine sulphate concentration µg/cm3	Morphine sulphate mean peak area	Haloperidol concentration µg/cm3	Haloperidol mean peak area
1	270	1248720	100	2059511
2	510	2429914	200	4111558
3	750	3454802	400	8267536
4	1000	4327435	600	12043970
5	1200	5156609	750	15207005
6	1500	6232389	1000	20320064

Table 4.9: Linearity study mean peak areas for morphine sulphate and haloperidol

From the table above the concentration ranges for morphine sulphate (270-1500µg/ml) and haloperidol (100-1000µg/ml) were investigated for linearity. These linearity plots are represented by figures 4.3 and 4.4. The correlation coefficient for both the morphine sulphate and haloperidol were calculated. In both cases the correlation coefficient exceeded 0.99 which was the preset value that would indicate if the concentration values taken were within the linear range. The correlation coefficient value for the morphine sulphate plot was 0.9987, and for the haloperidol plot this value was 0.999. From interpretation of these results it is assumed that the developed HPLC method demonstrates linearity.

It is not practical to rely on the numerical values to assess the linearity because what statistically is correct may not be the case. A visual examination of the linear plots is also important as you can tell whether all data points lie close to the plotted line or if outliers are giving a misleading representation. Visual examination of the linear plots for this project shows that all points lie close to the plotted line so the method islinear.

Fig 4.3: Linearity: Morphine Sulphate

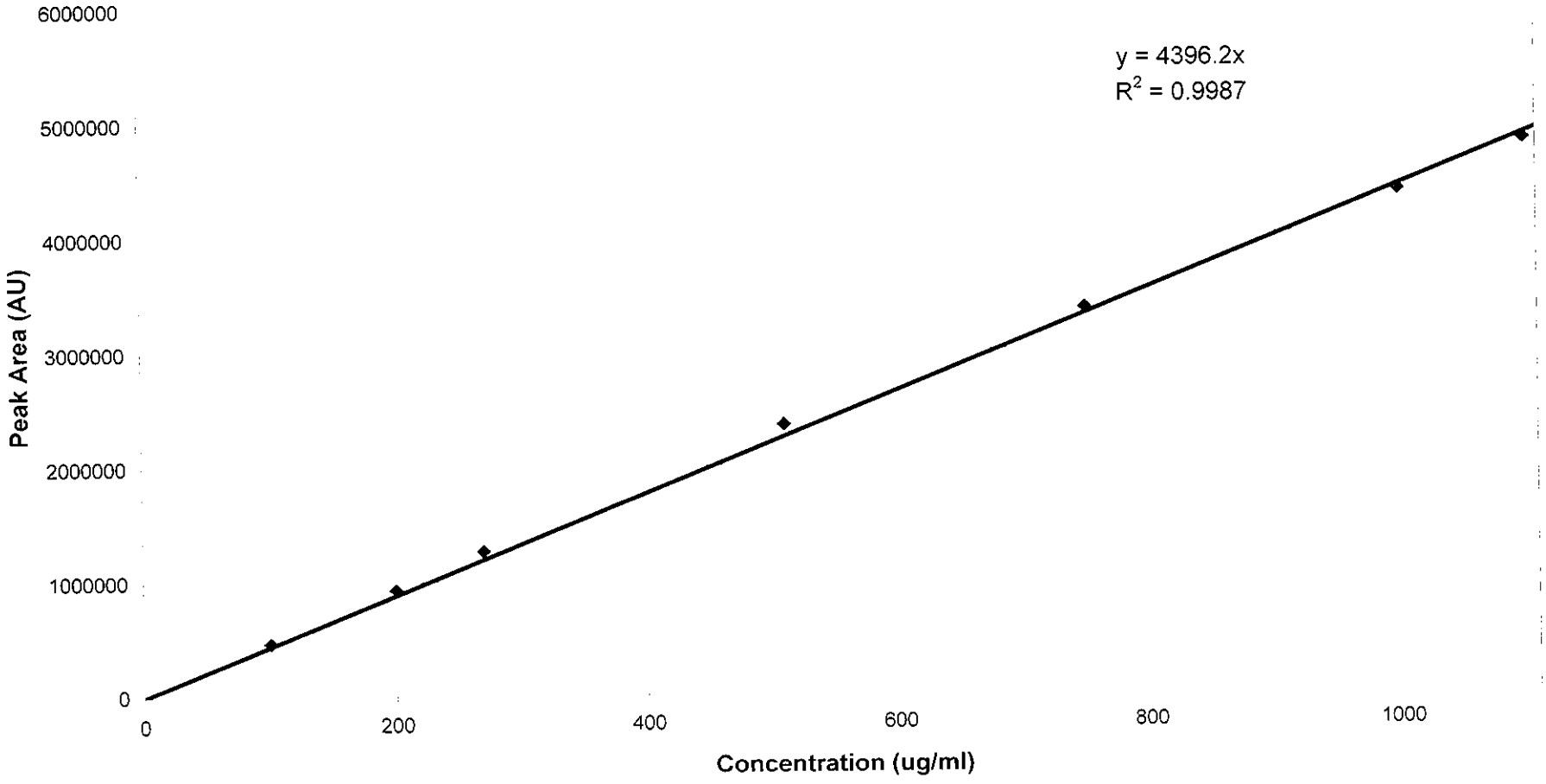
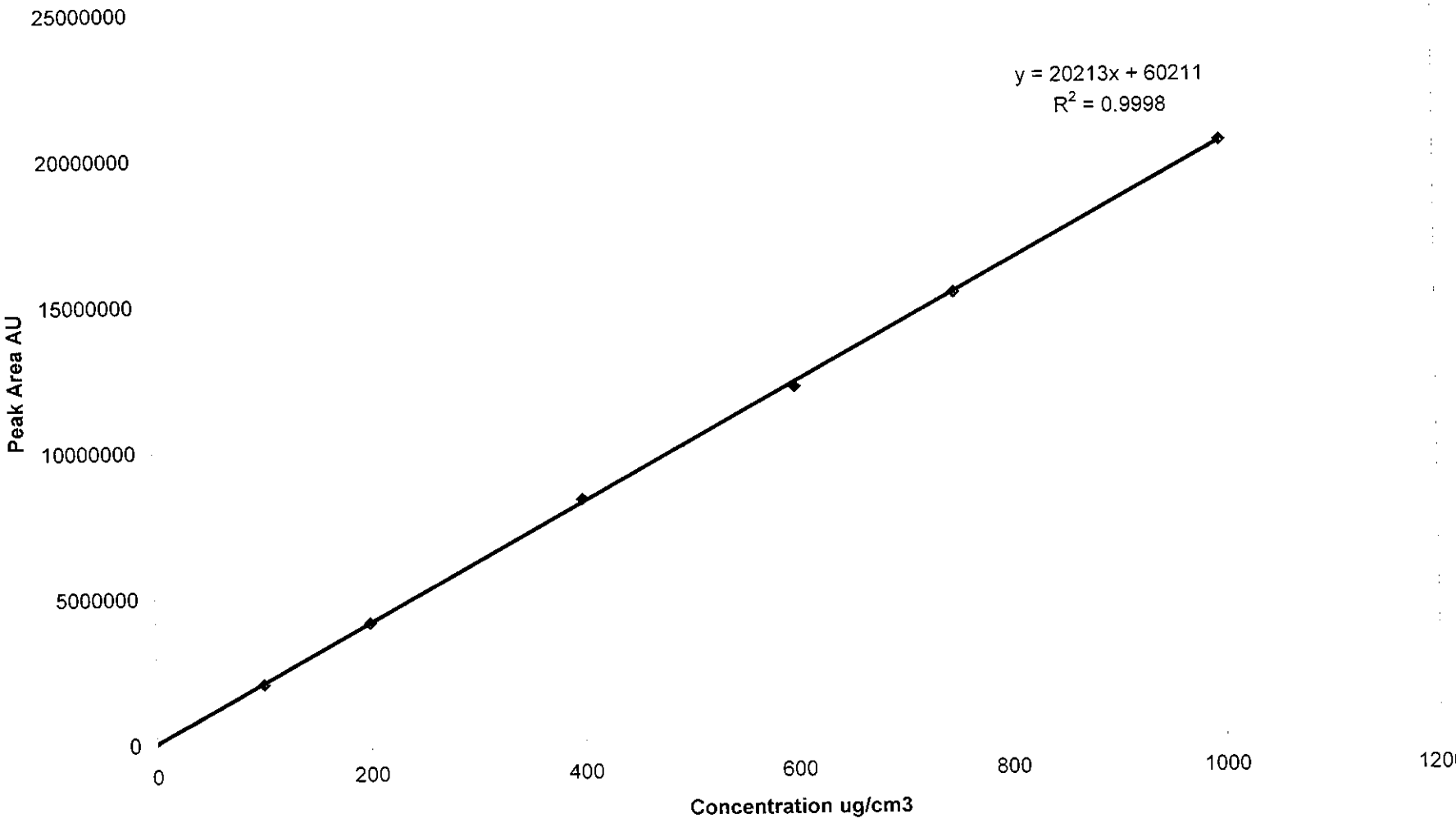


Fig 4.4: Linearity: Haloperidol



Precision

The type of precision study used in this project is termed repeatability. Whilst satisfying a mathematical requirement this type of precision puts a value on the sort of variability that can be expected when a method is performed by a single analyst on one piece of equipment over a short time, i.e. the sort of variability to be expected between results when a sample is analysed in duplicate. Results are shown for the precision study in tables 4.10 and 4.11 below (Representative chromatogram is presented in Appendix 1 page 130).

Sample	Morphine sulphate Mean Peak Area	Std Deviation	Coefficient of variation %
1			
2	3330535	19886	0.60
3	3310566	23610	0.71
4	3318178	9234	0.28
5	3363877	15057	0.45
6	3376196	1246	0.04
Mean (n=6)	Average mean peak area	Average std	Average CV
	3340232	25754	0.77

Table 4.10: Morphine Sulphate precision results

Sample	Haloperidol Mean Peak Area	Std Deviation	Coefficient of variation %
1	8098037	12467	0.15
2	8156995	35691	0.44
3	8057879	63176	0.78
4	8143222	3490	0.04
5	8240639	9983	0.12
6	8145025	40369	0.50
Mean (n=6)	Average mean peak area	Average Std Dev	Average CV
	8140299	61574	0.76%

Table 4.11: Haloperidol precision results

Analysis of the results shows that the method is precise. For the six homogeneous samples of morphine sulphate prepared the coefficient of variation is 0.77% which falls well below the predefined limit of 2%. Similarly for haloperidol the coefficient of variation for the six identical samples is 0.76%, again well below the limit of 2%. This type of precision is known as "between day" or "inter-day" precision. Another type of precision is termed "between day" or "intra-day" precision. As part of system suitability six replicate injections of the standard solution were made during each batch run. After every six sample injections this standard was injected again. As described above a relative standard deviation of 2% was chosen as limitation for precision. All results maintained a value below the set limit [11].

Accuracy

The tables 4.12 and 4.13 below summarises the results from the accuracy study (Representative chromatogram is presented in Appendix 1 page 132). Samples prepared using commercial solutions at three concentrations were used and compared against three accurately prepared reference powered samples. The accuracy of morphine sulphate is within 3.30% (see table 4.12, fig 4.5) and the accuracy of haloperidol is within 2.93% (see table 4.13, fig 4.6). Further conclusions can be derived from this accuracy study in support of replacing reference standard powders with commercial solutions. These conclusions are that there was acceptable level of recovery when spiking reference standard solutions with their commercial counterparts. This data supports the justification study described previously.

Morphine sulphate Concentration µg/ml	Spiked Solution mean peak area	Commercial Solution mean peak area	% Difference
270	1248170	1265127	1.34
510	2293115	2354073	2.59
750	3243767	3354488	3.30
1000	4287033	4360191	1.68
1200	5245596	5232229	-0.26

Table 4.12: Morphine Sulphate accuracy study results

Haloperidol Concentration µg/ml	Spiked Solution mean peak area	Commercial Solution mean peak area	% Difference
100	2043701	2040451	-0.16
200	4131838	4019738	-2.79
400	8089078	8124964	0.44
600	12114134	12479702	2.93
750	15342198	15405984	0.40

Table 4.13: Haloperidol accuracy study results

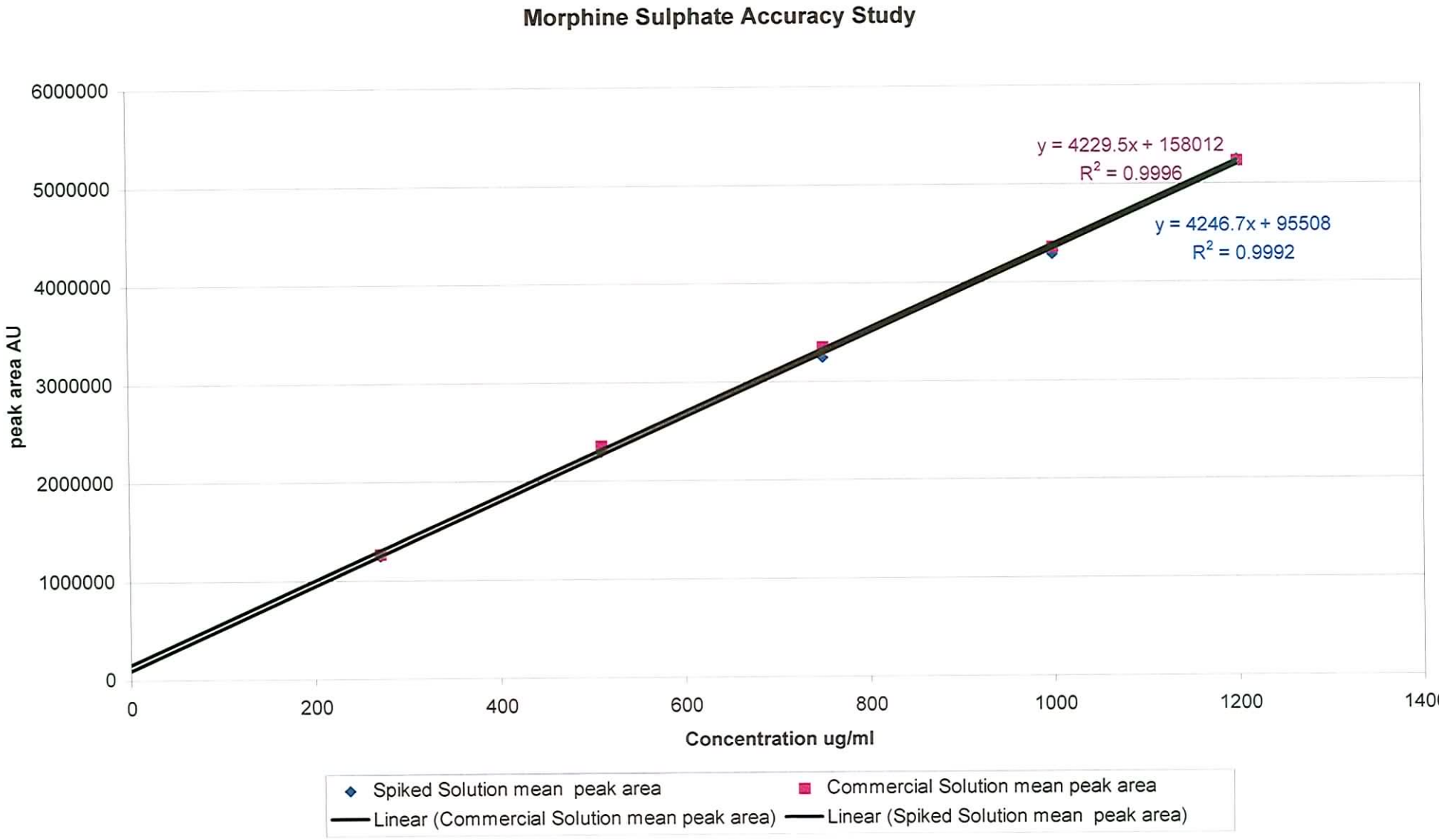


Figure 4.5: Morphine Sulphate Accuracy Study

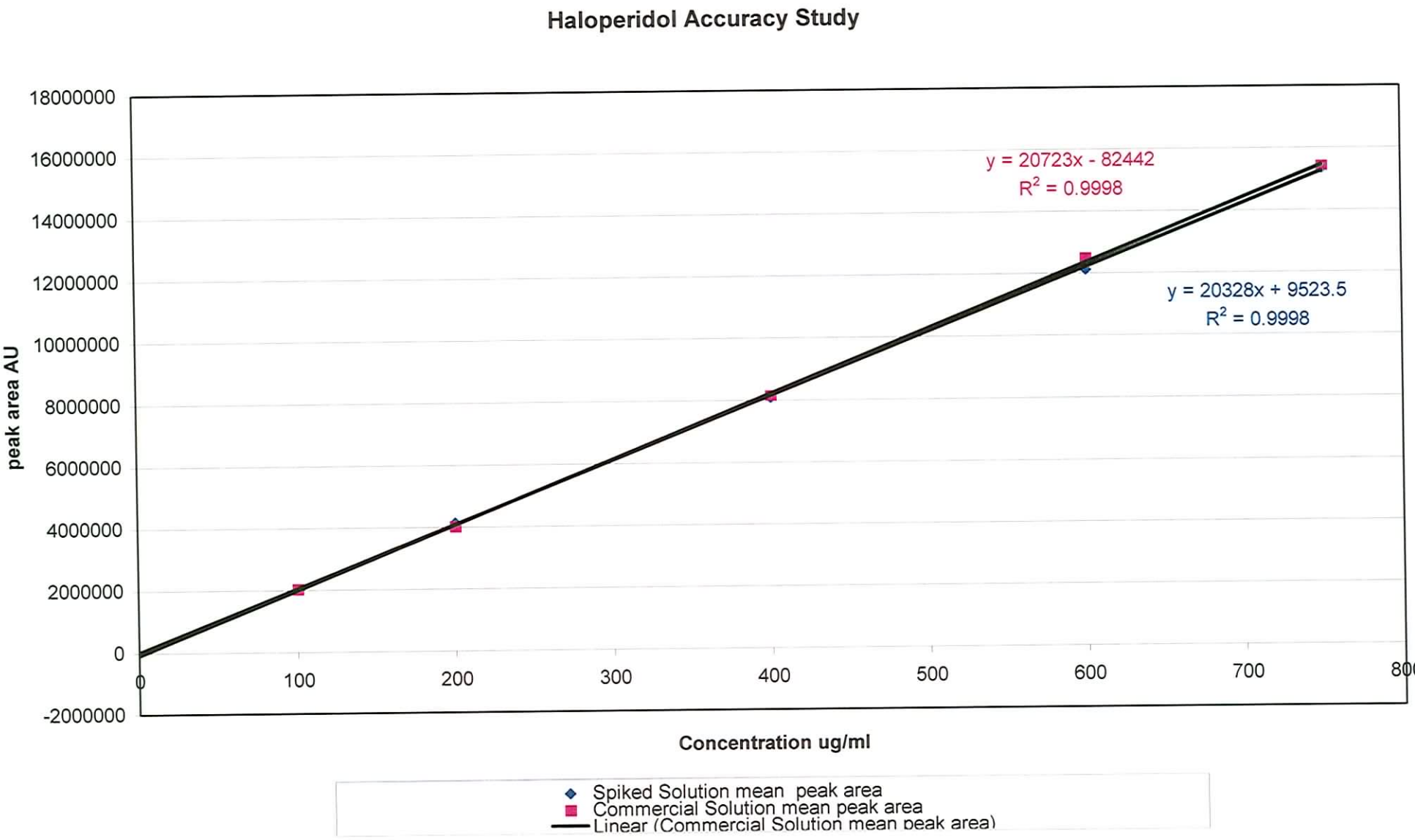


Fig 4.6: Haloperidol Accuracy Study

System Suitability

As discussed previously a system suitability check was performed on each day of testing. This was done to ensure that the method was performing appropriately. Six injections of the standard solution were performed and after six subsequent injections of sample or standard one injection of the standard was performed. System suitability was ensured if the coefficient of variation of the six initial injections was less than two percent. This also implied that the percentage coefficient of variation of any subsequent injections should not deviate by more than two percent of the mean of the first six injections. The system suitability parameters chosen were peak area and retention time. Table 4.14 below demonstrates an example of a system suitability check during the experiment.

	Morphine Sulphate Retention Time	Haloperiol Retention Time	Morphine Sulphate Peak Area	Haloperidol Peak Area
1	3.84	7.69	330180	8075110
2	3.84	7.70	3345819	8100906
3	3.83	7.70	3339227	8112126
4	3.84	7.73	3350339	8096141
5	3.84	7.79	3342882	8104232
6	3.83	7.73	3343782	8099707
Mean	3.84	7.72	3342038	8098037
Std Dev	0.005	0.037	6863	12467
%CV	0.12	0.48	0.2	0.15

Table 4.14: example of system suitability results

From the table 4.14 above it is clear that the results above prove the system to be suitable for the parameters chosen. The coefficient of variation for both parameters is less than two percent.

Robustness

Sample	Morphine Sulphate		Haloperidol	
	Peak Area	Retention Time	Peak Area	Retention Time
	AU	Mins	AU	Mins
Standard				
1	3382866	3.833	8078195	7.383
2	3394507	3.833	8106126	7.383
Mean	3388687	3.833	8092161	7.383
Std Dev	8231	0.000	19750	0.000
CV	0.24	0.00	0.24	0.00
A				
1	3254346	3.850	7921109	7.900
2	3331479	3.842	8130173	7.825
3	3328577	3.842	8091060	7.783
Mean	3304801	3.845	8047447	7.836
Std Dev	43719	0.005	111146	0.059
CV	1.32	0.12	1.38	0.76
B				
1	3333879	3.842	8003639	7.433
2	3385901	3.825	8121627	7.517
3	3384636	3.817	8126205	7.533
Mean	3368139	3.828	8083824	7.494
Std Dev	29676	0.013	69480	0.054
CV	0.88	0.33	0.86	0.72
C				
1	3357982	3.833	8098381	7.467
2	3336260	3.833	8062532	7.458
3	3368199	3.833	8092509	7.458
Mean	3354147	3.833	8084474	7.461
Std Dev	16311	0.000	19228	0.005
CV	0.49	0.00	0.24	0.07
D				
1	3379214	3.817	8130739	7.425
2	3389560	3.817	8177121	7.433
3	3380523	3.808	8155905	7.442
Mean	3383099	3.814	8154588	7.433
Std Dev	5634	0.005	23219	0.009
CV	0.17	0.14	0.28	0.11

Table 4.15: Robustness results

The robustness of the HPLC method was assessed and the results presented in table 4.15 above. The standard solution of morphine sulphate and haloperidol was again used for all injections in the robustness study. The initial standard injection was performed under normal conditions. The samples A-D represent the injections of the standard solutions performed under varying conditions from the same vial.

- A represents injection where pH of the mobile phase was 3.5
- B represents injection where pH of the mobile phase was 3.7
- C represents injection where acetonitrile composition was 47% of the mobile phase
- D represents injection where acetonitrile composition was 43% of the mobile phase

Again the robustness was assessed by the coefficient of variation or percentage residual standard deviation. This value was obtained by comparing the mean peak areas and mean retention times for both morphine sulphate and haloperidol against the same parameters for the standard solution under normal conditions. From the table it can be seen that all coefficients of variation fell below the two percent limit set for robustness. It is clear that the method is robust under these conditions.

Obtaining data on the effects mentioned above, helps to assess whether a method needs to be revalidated when one or more parameters are changed, for example to compensate for column performance over time. In the ICH document [12] it is recommended to consider the evaluation of a method's robustness during the development phase, and any results that are critical for the method should be documented. This is not, however, required to be included as part of a registration application.

Specificity

Because of the intended use of the HPLC method, i.e. stability indicating assay it was decided to degrade samples of the standard solution under various condition. Samples were thermally degraded (90°C) using hydrogen peroxide, hydrochloric acid and sodium hydroxide solutions. Samples were also prepared and left at room temperature. The samples were injected after twenty-four hours and analysed by

HPLC. The resultant chromatograms were inspected and compared to a standard chromatogram. It was not possible to determine interfering compounds within the experiment or to inject known decomposition products. A photodiode array detector is a useful tool for testing the homogeneity of a chromatographic peak and it would be useful to look at the developed method using a photodiode array detector.

Inspection of the resultant chromatograms for the specificity study yielded several interesting results. Chromatograms for this study are listed in the appendix 2 (pages 133-135). Haloperidol precipitated out of solution as soon as it was diluted in 0.1 M sodium hydroxide. It was known that the precipitate was haloperidol because the resultant cloudy solution was filtered and the chromatogram produced had no haloperidol peak.

In acidic solution both morphine sulphate and haloperidol remained in solution and no interfering peaks were interpreted from the chromatogram produced. Samples degraded in hydrogen peroxide at room temperature and at 90°C yielded an unidentified peak at about 6.2 minutes between morphine sulphate and haloperidol.

The terms *selectivity* and *specificity* are often used interchangeably. Although it is not consistent with ICH, the term *specific* generally refers to a method that produces a response for a single analyte only, while the term *selective* refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term *selectivity* is usually more appropriate. The USP monograph [1] defines selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be expected to be present in the sample matrix. Selectivity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength. Besides chromatographic separation, the sample preparation step can also be optimized for best selectivity. It is a difficult task, in chromatography, to ascertain whether the

peaks within a sample chromatogram are pure, or consist of more than one compound. Therefore the analyst should know how many compounds are in the sample, or whether procedures for detecting impure peaks should be used. While in the past chromatographic parameters, such as mobile phase composition or the column were modified, now the application of spectroscopic detectors coupled on-line to the chromatograph are being used. UV/Visible diode-array detectors and mass-spectrometers acquire spectra on-line throughout the entire chromatogram. The spectra acquired during the elution of a peak are normalized and overlaid for graphical presentation. If the normalized spectra are different, the peak consists of at least two compounds. The principles of diode-array detection in HPLC and their application and limitations with regard to peak purity are described in literature. While a chromatographic signal indicates no impurities in either peak, spectral evaluation can identify a peak as impure. The level of impurities that can be detected with this method depends on the spectral difference, on the detector's performance and on the software algorithm. Under ideal conditions, peak impurities of 0.05 to 0.1% can be detected.

Stability of the mobile phase

Stability of the mobile phase is essential to the validation process and it was necessary to determine for how long should a prepared mobile phase be used. To assess the stability of the mobile phase a standard solution was prepared. This was injected three times on consecutive days. The results were then compared over each day. The percentage variation between standard injections each day should not exceed two percent.

It was found that there was no appreciable change in peak area of the solutions injected over a three-day period and the integrity of the mobile phase was intact. It was decided that three days was adequate time for use of mobile phase as the volume of liquid used did not exceed two days usage. In conclusion a three day validity period on the mobile phase has been established.

4.13: References

1) United States Pharmacopoeia 23 1776-7,1982-1984.
2) **Summary**: 97D 0448 International Conference on Harmonisation; Draft Guidance on Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances AGENCY: FDA, 1998, *http://www.fda.gov/cder/guidance/1862dft.pdf*.
3) **Summary**: Types of Analytical Procedures To Be Validated, *02-16-98, http://www.fda.gov/cder/guidance/ichq2a.pdf*.
4) **Summary**: Parameters For Validation of HPL Chromatographic Methods for Drug Substance and for drug Product, *02-16-98, http://www.fda.gov/cder/guidance/cmc3.pdf*
5) **Summary**: wpd 12/ 14/ 98 Guidance for Industry Bioanalytical Methods Validation for Human Studies DRAFT GUIDANCE This guidance document is being distributed for comment purposes only. wpd 12/ 14/ 98 Guidance for Industry Bioanalytical Methods Validation for Human Studies, *01-05-99, http://www.fda.gov/cder/guidance/2578dft.pdf*.
6) **Summary**: Guidance for Industry Q2B Validation of Analytical Procedures: Methodology Additional copies are available from: the Drug Information Branch (HFD- 210), Center for Drug Evaluation and Research (CDER), 5600 Fishers Lane, Rockville, MD 20857 (Tel) 301- 827, *06-19-98, http://www.fda.gov/cder/guidance/1320fnl.pdf*.
7) **Summary**: wpd 08/ 29/ 00 Guidance for Industry Analytical Procedures and Methods Validation Chemistry, Manufacturing, and Controls Documentation DRAFTGUIDANCE,*08-30-00, http://www.fda.gov/cder/guidance/2396dft.pdf*
8) **Summary**: Product performance qualification - Establishing confidence through appropriate testing that the finished product produced by a specified process meets all release requirements for functionality and safety. Validation protocol, *05-06-98, http://www.fda.gov/cder/guidance/pv.htm*.

9) **Summary**: A regulatory analytical procedure is the analytical procedure used to evaluate a defined characteristic of the drug substance or drug product, *08-30-00, http://www.fda.gov/cder/guidance/2396dft.htm*.

10) Reviewer Guidance "Validation of Chromatographic Methods", Center for Drug Evaluation and Research (CDER), November 1994. http://www.fda.gov/cder/guidance/index.htm .

11) Green, M., J., "A Practical Guide to Analytical Method Validation", Analytical Chemistry, 1996, 68, 305A-309A.

12) Johnson CE, Christen C, Perez MM, et al. Compatibility of bupivicaine hydrochloride and morphine sulphate. Am J Health Syst Pharm. 1997; 54: 61-64.

CHAPTER 5

STABILITY STUDIES

5.1: Materials Used in Stability Studies

HPLC Equipment
SCL-10A VP Shimadzu system controller, SPD-10A VP Shimadzu UV-Vis detector,SIL-10A VP Shimadzu auto injector, LC-10AT VP Shimadzu liquid chromatograph, FCV-10AL VP Shimadzu low pressure gradient control valve, Shimadzu on line degassing device GA STORR 154, Class-VP Chromatography data analysis system.

Column
Hichrom NC100-5C18-3109

pH meter
Jenway pH Meter 3310

Balances
Taurus Mettler balance model BP110

Taurus Mettler balance model Bl120S

Reagents
Acetonitrile, 99.93%, HPLC grade, Sigma-Aldrich, Ammonium Acetate 97.3%, Sigma-Aldrich Lot 58H0060, Phosphoric Acid, ACS reagent 85% Lot 90609017, Deionised Water

Morphine Sulphate
Commercially prepared solutions (supplied by Antigen pharmaceuticals, Roscrea, Tipperary) were used as both standards and in the filling of the P.C.As required for testing. Ampoules of variable concentration were required for dilution (10mg/ml, 15mg/ml, 30mg/ml, 60mg/ml).

Morphine Sulphate reference standard, Sigma-Aldrich Lot MPT-394A

Haloperidol
Commercially prepare solutions (Supplied by Norton Healthcare, Waterford) were used as both standards and for sampling (20mg in 2mls)

Haloperidol reference standard, Sigma-Aldrich Lot 18H0408

5.2: Methods for Stability of morphine Sulphate in P.C.A.s and in polypropylene syringes

Chromatographic Conditions

The solutions were analysed using the developed and validated HPLC method. Analysis was performed using the Shimadzu HPLC system. The mobile phase consisted of 55% ammonium acetate (1%) and 45% acetonitrile. The pH was adjusted to 3.6 using phosphoric acid. The flow rate was a step program from 0.5mls/min (1-3mins) and 1.0 ml/min (3-10 mins). Stationary phase was a Hichrom NC100-5C18-3109 column (250×4.6mm, 5μm particle size).

UV detection was performed at 247nm and the injection volume was 20μl. The retention time for morphine sulphate was about 3.8 minutes.

Physical Stability

The physical stability was visually controlled by inspection of morphine sulphate solutions contained in the silicone balloon infusers on each test day. Solutions were visually inspected for indications of discoloration or precipitation [1].

Variations in pH during the study were considered as a significant indicator for instability. The pH was measured every test day using a Jenway 3310 pH meter that was calibrated using pH 4 and pH 7 buffers.

Calibration Curve

Before the analysis was performed a calibration curve was constructed to show a linear plot of peak area versus concentration. The concentration range for this curve was from 100μg/ml to 1100μg/ml and the correlation coefficient was greater than 0.99.

Storage Conditions and Sampling

The P.C.A.s and syringes were filled in aseptic conditions in the compounding unit. The concentrations tested were 2mg/ml and 10mg/ml morphine sulphate, which were prepared in duplicate. The P.C.A.s and syringes were stored at room temperature (22±3°C) and refrigerated at 4°C [2].

On each test day samples were diluted from 2mg/ml and 10mg/ml to 200µg/ml and 1mg/ml respectively. Two standards at each of these concentrations were prepared also and used as a comparative reference of 100% stability.

The prepared solutions were analysed using the validated high performance liquid chromatography method described above. Each injection was performed in duplicate and each run had a duration of ten minutes.

5.3: Preparation of solutions for Stability of morphine sulphate and haloperidol in polypropylene syringes

Test solutions were prepared by mixing, appropriately and accurately, the volumes of morphine sulphate and haloperidol in 10ml polypropylene syringes, and making the volume to 10mls with water for injection. At time zero one of the duplicate syringes was analysed by HPLC immediately. One set of syringes was stored in the dark and the others left in the light for 24 hours. These samples were then analysed. Concentrated samples were diluted in mobile phase to bring them within the analytical range.

The table 5.1 overleaf shows the preparation of test samples. The contents of the polypropylene syringes were inspected at time zero and after 24 hours. The presence of cloudiness particulate matter or colour change was defined as incompatibility.

The mean peak area of the test samples was divided by the mean peak area of the standard solution and then multiplied by the standard concentration. Samples at time zero were normalised to 100% and samples at 24 hours were calculated against this.

Calculations used

$C_{initial}$ = (peak area of sample / peak area of standard) x standard concentration

C_{24hrs} = (peak area of sample/ peak area of standard) x standard concentration

% concentration remaining = $(C_{24hrs}/C_{initial})$ x 100

Sample	Volume of morphine sulphate	Volume of halo-peridol 10mg/ml ampoule	Final conc morphine sulphate mg/ml	Final conc haloperidol mg/ml
1	500ul of 10mg/ml	250ul	0.5	0.25
2	1ml of 10mg/ml	250ul	1.0	0.25
3	1ml of 15 mg/ml	250ul	1.5	0.25
4	1ml of 30 mg/ml	250ul	3.0	0.25
5	1ml of 60 mg/ml	250ul	6.0	0.25
6	2mls of 60 mg/ml	250ul	2.0	0.25
7	500ul of 10 mg/ml	500ul	0.5	0.50
8	1ml of 10mg/ml	500ul	1.0	0.50
9	1ml of 15 mg/ml	500ul	1.5	0.50
10	1ml of 30 mg/ml	500ul	3.0	0.50
11	1ml of 60 mg/ml	500ul	6.0	0.50
12	2mls of 60 mg/ml	500ul	12.0	0.50
13	500ul of 10 mg/ml	750ul	0.5	0.75
14	1ml of 10mg/ml	750ul	1.0	0.75
15	1ml of 15 mg/ml	750ul	1.5	0.75
16	1ml of 30 mg/ml	750ul	3.0	0.75
17	1ml of 60 mg/ml	750ul	6.0	0.75
18	2mls of 60 mg/ml	750ul	12.0	0.75
19	500ul of 10 mg/ml	1ml	0.5	1.0
20	1ml of 10 mg/ml	1ml	1.0	1.0
21	1ml of 15 mg/ml	1ml	1.5	1.0
22	1ml of 30 mg/ml	1ml	3.0	1.0
23	1ml of 60 mg/ml	1ml	6.0	1.0
24	2ml of 60 mg/ml	1ml	12.0	1.0
25	1ml of 30 mg/ml	1ml	3.0	2.0
26	1ml of 60 mg/ml	2ml	6.0	2.0
27	2ml of 60 mg/ml	2ml	12	2.0

Table 5.1: Preparation of test samples for drug combination study

5.4: Stability of morphine sulphate in P.C.A.s: Results and Discussion

No visual change was noted in any of the samples tested over the forty days. There was no visual evidence of discoloration, precipitation or cloudiness in any of the solutions. There was no significant change in the pH of any of the solutions of morphine sulphate tested.

The tested concentrations of morphine sulphate in P.C.A.s were shown to be chemically stable, that is the concentrations tested retained greater than 90% of initial concentration after forty days under refrigerated conditions (4°C) and room temperature (20-24°C) [3][4].

The results of the stability test are presented in table 5.2 and figure 5.1 (Representative chromatograms are presented in appendix 3, pages 137-142). The values represent the mean of each sample assayed in duplicate. Peak areas are presented in appendix 5, pages 148-161.

Day	Percentage Concentration of Morphine Sulphate			
	2mg/ml Room Temperature	2mg/ml 4°C	10mg/ml Room Temperature	10mg/ml 4°C
0	97.8	100.2	99.3	97.4
5	103.2	97.7	99.1	98.1
7	105.4	96.3	99.0	98.2
10	104.8	97.7	102.6	100.1
12	103.2	97.7	99.1	98.1
14	109.2	97.5	107.0	99.1
17	102.8	99.2	102.7	99.6
21	104.3	105.2	100.0	98.3
28	102.3	101.5	100.5	100.0
35	104.8	101.2	98.8	99.9
40	108.0	102.8	101.5	96.9

Table 5.2: Stability of morphine sulphate in P.C.A.s results

The concentration of morphine sulphate in each sample was calculated as a percentage of the concentration of the standard solutions assayed on each test day. From the results it is clear that morphine sulphate retained greater than 90% of initial concentration over the test period.

This indicates that the silicone material in the infuser is an acceptable material to use and does not affect the chemical stability of morphine sulphate.

A point to note on examination of the results is that on some of the test days there was a high value for the percentage concentration of morphine sulphate. These values were almost as high as 110%. No explanation can be given for this beyond the deduction of experimental error but further investigation may be necessary.

Thus morphine sulphate (2mg/ml and 10mg/ml) is chemically stable in P.C.A. silicone balloon infusers at room temperature (22±2°C) and in refrigerated conditions (4°C) and a 28 day shelf life may be placed on these syringes.

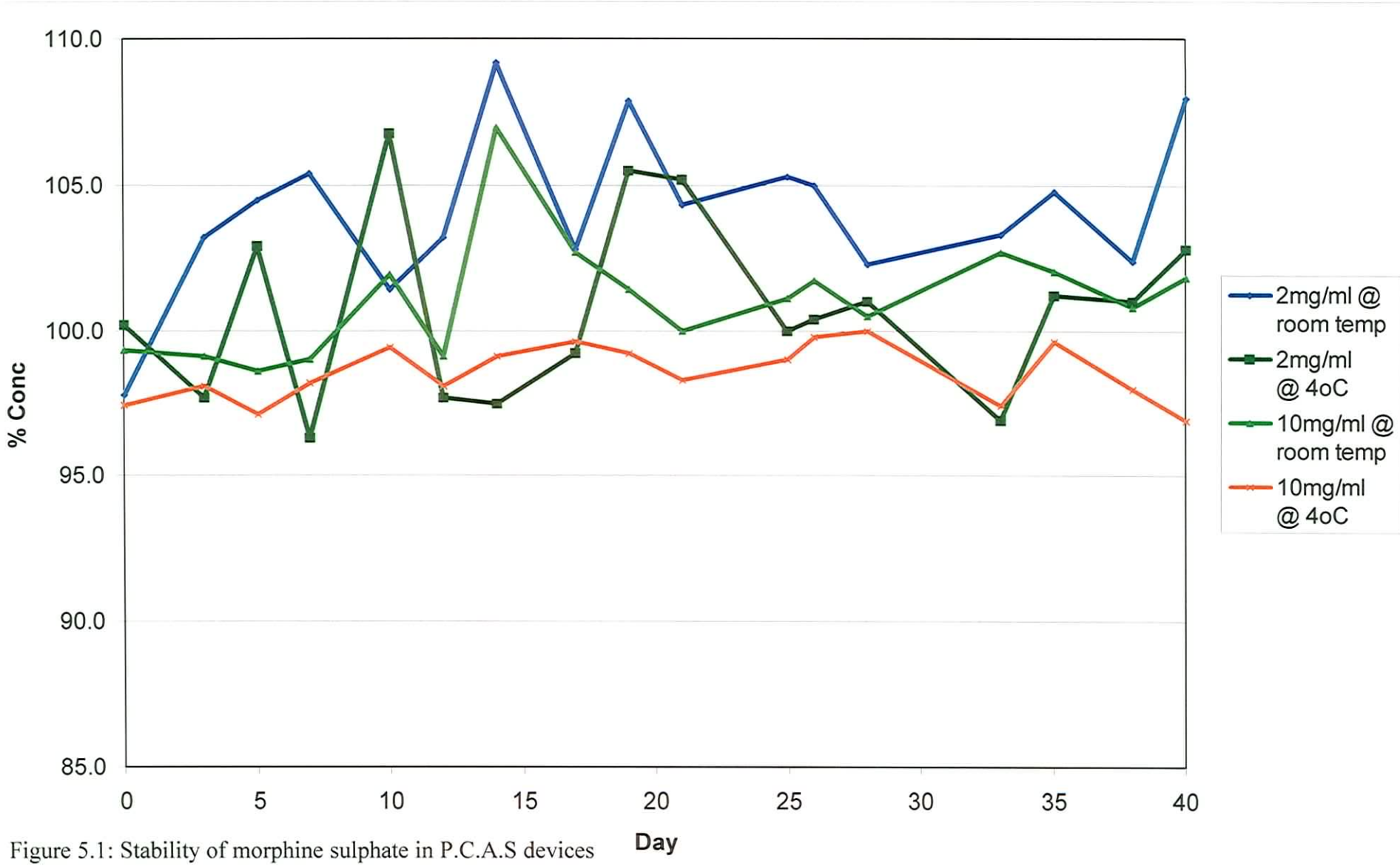


Figure 5.1: Stability of morphine sulphate in P.C.A.S devices

5.5: Stability of morphine sulphate in polypropylene syringes: Results and Discussion

No visual change was noted in any of the samples tested over the forty days. There was no visual evidence of discoloration, precipitation or cloudiness in any of the solutions. There was no significant change in the pH of any of the solutions of morphine sulphate tested

The results for this study are presented in table 5.3 and graphed in figure 5.2 (Representative chromatograms are presented in appendix 4, pages 143-147). Analysis of these results show that the concentration of morphine sulphate remained greater than 90% over the 30 days of testing. The reason that this stability study is shorter than the P.C.A study is that microbiological considerations of the aseptic compounding unit must be taken into account. The P.C.A.s and syringes are prepared under aseptic conditions in this unit and microbiological validation of the unit allows a maximum shelf life of 28 days to be placed on any given product. It was only after the P.C.A. study that this information was learned. Peak areas are presented in appendix 6, pages 162-174.

Day	**Percentage Concentration of Morphine Sulphate**			
	2mg/ml Room Temperature	2mg/ml 4°C	10mg/ml Room Temperature	10mg/ml 4°C
0	102.6	100	98.3	99.3
3	103.0	101.0	97.5	98.7
6	102.8	99.7	98.4	98.5
8	102.3	100.6	96.7	98.8
10	103.0	99.9	98.3	99.9
13	102.2	99.7	98.9	100.6
15	103.3	100.6	99.7	101.3
17	103.1	100.4	99.1	101.0
21	102.0	99.0	99.0	100.5
22	102.2	100.9	98.5	102.0
24	103.7	99.6	98.0	99.3
28	104.0	100.2	97.5	99.0
30	103.1	98.8	98.2	100.1

Table 5.3: Stability of morphine sulphate in polypropylene syringes, results

Thus morphine sulphate (2mg/ml and 10mg/ml) is chemically stable in polypropylene syringes at room temperature (22±2°C) and in refrigerated conditions (4°C) and a 28-day shelf life may be placed on these syringes.

The data given in this study will allow for the comparison of the stability of morphine sulphate in both P.C.A.s and polyethylene syringes which are used in some centres as patient controlled analgesia with the aid of an in-vitro pump. There are some points of interest to note. Firstly one would expect drugs stored at lower temperatures to be more stable than those at higher temperatures. Looking at both studies whether from the graphical representation or the tables of values no distinct pattern is evident at all here. The results show no pattern between refrigerated samples and those stored at room temperature.

Comparing the two graphs (figures 5.1 and 5.2) and the stability pattern allows some interesting observations. Firstly if the P.C.A stability graph is looked at, there is a distinct variation in the concentrations for samples on consecutive test days. The percentage concentration fluctuates by several percent on some days to the time zero concentration. No explanation readily presents itself. Initially it was thought that this concentration fluctuation was due to variance in sample preparation and experimental error. But the variance is consistent with all samples tested under all conditions which rules out random error.

If a consistent error were occurring then one would expect a similar pattern whilst conducting the stability testing for morphine sulphate in polypropylene syringes. This pattern did not occur in this second stability test. The results over all concentrations tested did not show as high a variance between concentrations on consecutive test days. Therefore it is assumed that this observed fluctuation in concentration is dependent on the delivery device. It is beyond the scope of this experiment to offer explanation as to this phenomenon but it is noteworthy and may warrant investigation. Perhaps a second P.C.A. study would show if this variance in concentration is consistent. It should be stated that morphine sulphate did retain chemical stability in both devices and experimental data generated has demonstrated this.

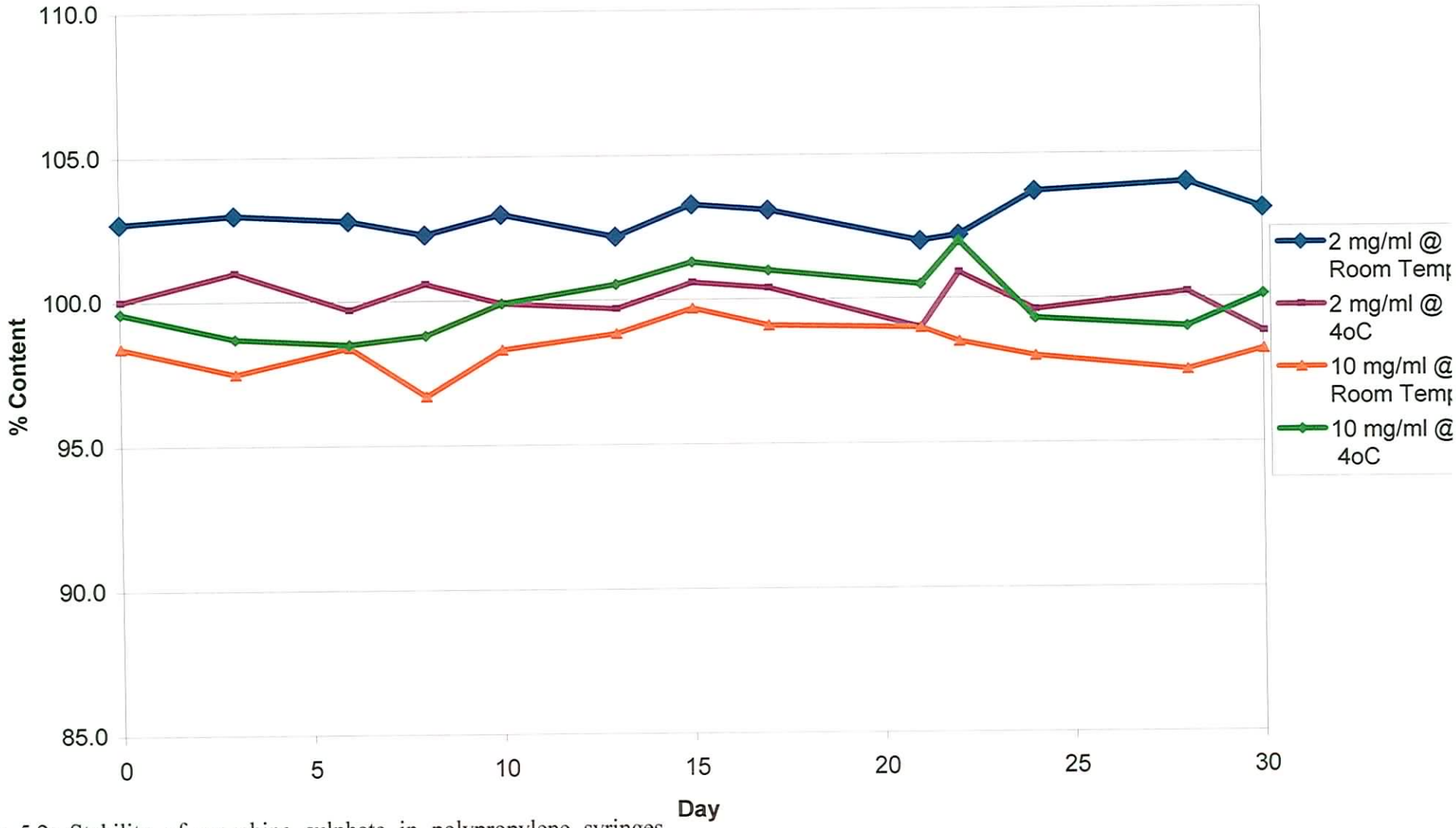


Fig 5.2: Stability of morphine sulphate in polypropylene syringes.

5.6: Stability of morphine Sulphate and Haloperidol in Combination: Results and Discussion

A chemical stability of morphine sulphate and haloperidol in combination in polypropylene syringes was performed over a range of concentrations [4][5][6]. A time zero concentration of each sample tested was taken and after twenty four hours samples were then analysed again. The results are expressed this time as a percentage of the initial concentration remaining. Each syringe was visually assessed for the indications of precipitation or colour change.

Haloperidol concentration	Morphine Sulphate (Light)	Haloperidol (Light)	Morphine Sulphate (Dark)	Haloperidol (Dark)
0.25mg/ml	103.7	103.3	101.8	100.0
0.50mg/ml	101.6	100.0	101.3	100.0
0.75mg/ml	100.0	102.7	98.2	101.4
1.0mg/ml	98.2	104.2	96.4	101.1

Table 5.4: Results of 24 hour drug combination stability

Morphine sulphate concentration was fixed at 0.5mg/ml and the haloperidol concentration varied (table 5.4). No precipitation was observed.

Haloperidol concentration	Morphine Sulphate (Light)	Haloperidol (Light)	Morphine Sulphate (Dark)	Haloperidol (Dark)
0.25mg/ml	101.4	100.0	100.7	96.3
0.50mg/ml	101.2	101.0	99.5	98.0
0.75mg/ml	100.0	102.7	101.0	102.7
1.0mg/ml	100.0	103.1	99.0	102.0

Table 5.5: Results of 24 hour drug combination stability

Morphine sulphate concentration was fixed at 1mg/ml with varying concentrations of haloperidol after 24 hours (table 5.5). No precipitation or colour change observed.

Haloperidol concentration	Morphine Sulphate (Light)	Haloperidol (Light)	Morphine Sulphate (Dark)	Haloperidol (Dark)
0.25mg/ml	100.5	100.0	103.3	103.3
0.50mg/ml	102.5	101.6	99.6	98.8
0.75mg/ml	100.7	102.7	102.8	104.1
1.0mg/ml	100.7	104.1	95.8	101.0

Table 5.6: Results of 24 hour drug combination stability

Morphine sulphate concentration was constant at 1.5mg/ml and haloperidol concentration was varied (table 5.6). No precipitation or colour change observed.

Haloperidol concentration	Morphine Sulphate (Light)	Haloperidol (Light)	Morphine Sulphate (Dark)	Haloperidol (Dark)
0.25mg/ml	99.0	98.4	100.0	96.0
0.50mg/ml	97.4	97.1	98.0	96.0
0.75mg/ml	103.1	107.9	104.1	105.5
1.0mg/ml	100.3	103.9	99.0	103.0
2.0mg/ml	105.1	104.0	103.7	102.6

Table 5.7: Results of 24 hour drug combination stability

Morphine sulphate fixed concentration of 3mg/ml with varying haloperidol concentration (table 5.7). No precipitation or colour change evident

Haloperidol concentration	Morphine Sulphate (Light)	Haloperidol (Light)	Morphine Sulphate (Dark)	Haloperidol (Dark)
0.25mg/ml	96.3	104.0	92.4	96.0
0.50mg/ml	94.1	96.0	104.3	108.0
0.75mg/ml	96.9	96.0	97.4	98.7
1.0mg/ml	103.3	99.0	98.6	99.0
2.0mg/ml	99.2	109.5	105.2	105.3

Table 5.8: Results of 24 hour drug combination stability

Morphine sulphate fixed concentration of 6mg/ml with varying haloperidol concentration (table 5.8). No precipitation or colour change evident.

Haloperidol concentration	Morphine Sulphate (Light)	Haloperidol (Light)	Morphine Sulphate (Dark)	Haloperidol (Dark)
0.25mg/ml	109.5	116.7	99.8	108.3
0.50mg/ml	100.9	106.0	98.2	102.0
0.75mg/ml	99.1	105.4	97.3	102.7
1.0mg/ml	102.9	103.1	104.9	109.3
2.0mg/ml	97.3	97.1	94.3	94.1

Table 5.9: Results of 24 hour drug combination stability

Morphine sulphate concentration was fixed at 12mg/ml with varying concentrations of haloperidol (table 5.9). No evidence of precipitation or colour change was observed.

The concentration ranges chosen reflect the common doses used over twenty four hours in clinical practice. From the results of the HPLC analysis and physical inspection it can be concluded that morphine sulphate (0.5-12mg/ml) and haloperidol (as Seranace©) (0.25-2mg/ml) in combination are chemically stable at the concentration range tested. All samples have greater than 90% of initial concentration after 24 hours in light and dark at room temperature (22±2°C). From table 5.9 there are some high percentage concentrations that are almost 10% greater than initial rates. No explanation can be given for these high values at this point, save the suggestion of experimental error, and may merit further investigation.

An important point to note on drug stability studies is that a time zero determination of drug concentration is critical. If an initial concentration cannot be assessed there is no reference point. It then becomes clear that definitive conclusions regarding changes in drug concentration and hence drug stability are difficult to make.

The simple assumption that the initial concentration is the intended target concentration is not a valid one. There are a numerous opportunities for errors to occur such as human error and variations in drug fill volume to solution volume overfilling and syringe variability. These factors amount to an uncertain starting point. Therefore an importance must be placed in assessing the time zero concentration at the beginning of a stability study is very important.

5.7: References

1) Trissel, L., A., "Handbook on Injectable Drugs", Am soc of Health-Syst Pharm Inc 1997.

2) Guidance for Industry, "Bioanalytical methods Validation for Human Studies", US Department of Health and Human Services, FDA, CDER, December 1998.

3) Stella, V., J., "Chemical and Physical bases determining the instability and incompatibility of formulated injectable drugs", J. of Paranteral Sci. Technology, 1986.

4) Zachrisson, U., Furst, C., J., "Drug infusors in palliative medicine: A Swedish inquiry", J. Pain Symptom Manage, 1998, 15 5.

5) Murray, M., "The delivery of subcutaneous infusions by syringe pump in a palliative care setting in St. James's Hospital", MSc. Hospital Pharmacy Thesis, 1997.

6) McQuillan, R., "The use of subcutaneous infusions", Palliative care today, Irish supplement, 1998, Issue 2.

CHAPTER 6

CONCLUSIONS

6.1: Conclusions: Method Development

The initial objective of the project was to develop a method for the separation of morphine sulphate and haloperidol. A literature search was conducted and the samples characterised. Using High performance liquid chromatography a suitable method was developed for the separation of morphine sulphate and haloperidol. This optimised method is presented below with a typical chromatogram (see fig 6.1).

Equipment used:	Shimadzu SCL 10 A vp
Column used:	Hichrom NC100-5-C18
Column Packing:	C18 5µ
Mobile phase:	55% ammonium acetate (1%) buffer
	45% acetonitrile, pH adjusted to 3.6
Injection volume:	20 micro litres
Detection wavelength:	247nm
Sensitivity	2.0 AUFS, Attenuation 516
Flow rate:	time program, 0-3 mins at 0.5mls/min and
	From 3-10mins a flow rate of 1mls/min
Typical Retention times	Morphine Sulphate 3.8 minutes, Haloperidol 7.5 minutes

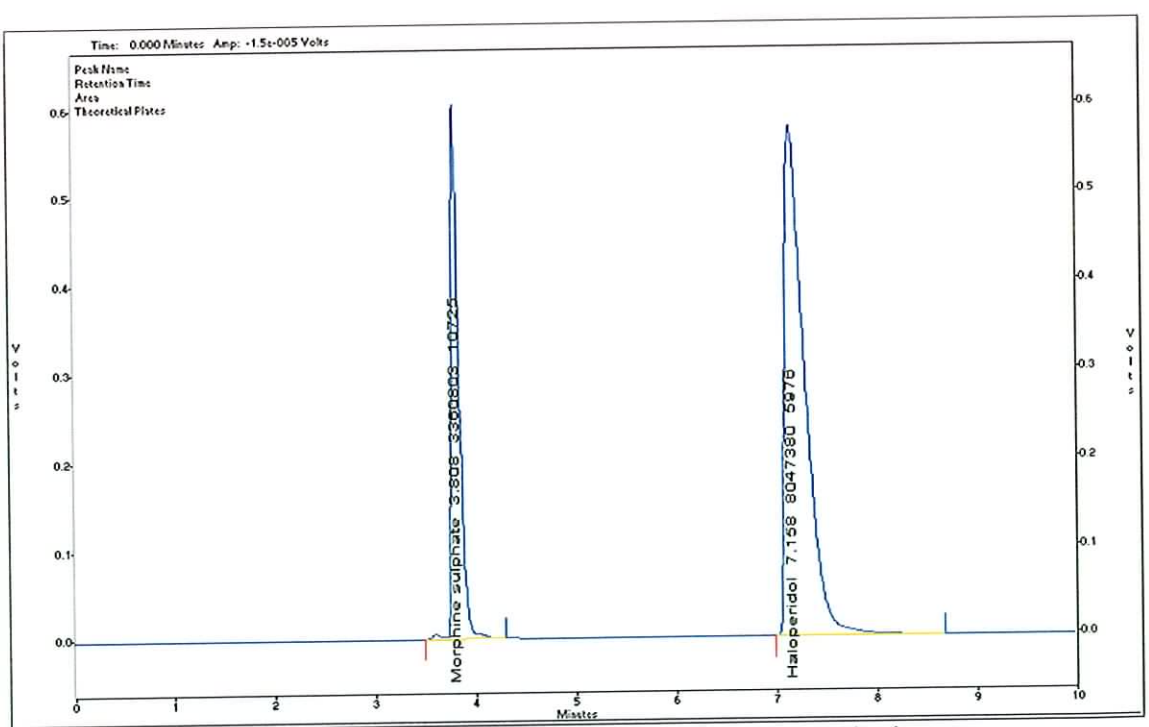


Fig 6.1: Typical Chromatogram of morphine sulphate and haloperidol in combination

6.2: Conclusions: Method Validation

High performance liquid chromatography methods must be validated. This allows confidence to be placed in the chromatograms produced, and ultimately, in the resulting profile of the stability of drugs of interest. To do this a number of parameters were investigated. Initially it was necessary to justify the use of commercial solutions of morphine sulphate and haloperidol in place of reference standard powders. The experimental results obtained (see chapter 4) have justified the use of commercial solutions in place of reference standard powders. This justification is only relevant to this project and was necessary as it was not feasible to work with the materials suggested by literature. It does not suggest that commercial solutions should be used above reference standard powders. Careful thought and discussion was needed to suggest an alternative to the problems faced with the difficulty in obtaining morphine sulphate reference standard powder. Any other project faced with a similar problem must in that particular case likewise justify using commercial solutions in place of reference standards.

The next step was to validate the developed method. Analytical method validation in the pharmaceutical industry is influenced by regulation from the United States Food and Drug Administration (FDA), the International Conference of Harmonisation (ICH) and similar bodies form Canada, Japan and other countries. Many of the principles, procedures and requirements of validation are common to the majority of analytical methods. The validation parameters investigated were

- Accuracy: Samples throughout the linear range were prepared using reference standard solution and the percentage accuracy determined. All data generated showed an acceptable percentage difference between data.
- Precision: Coefficient of variation of six homogeneous samples of the standard solution less than or equal to two percent.
- Linearity : Correlation coefficient of linear range was not less than 0.99 for calibration curves produced for morphine sulphate and haloperidol.
- Specificity: Samples of the standard solution were degraded in acid, base and hydrogen peroxide. These solutions were subjected also to thermal degradation under these conditions. Results for acid hydrolysis indicate no interference from by-products.

- System suitability: Correlation coefficient of six repeated injections of standard and subsequent injections does not exceed two percent. Parameters analysed are peak area and retention time.
- Robustness: Changes made to pH and percentage acetonitrile in the mobile phase were assessed. The correlation coefficient did not exceed two percent.
- Range: the concentration range for the experiment for morphine sulphate was 100µg/ml to 1100µg/ml and for haloperidol the range was100µg/ml to 1000µg/ml.

From the results of the specificity study some elements of the study may need further investigation at a later point. The use of a photodiode detector may be advisable to assess the purity of some of the peaks particularly samples degraded in hydrogen peroxide.

Although system suitability is listed as a parameter in validation it has uses outside the scope of validation. Method validation occurs at the method development stage and afterwards whilst system suitability checks the performance of the system. System suitability checks can detect normal changes (wear and tear) in the equipment or the column during usage. If a method is robust it should not fail such tests. System suitability tests are useful in routine quality control work and also in stability studies.

Validation does not imply that the developed method is free from errors. It merely confirms that the method used is suitable for the purpose to which it is intended. It is therefore necessary to revalidate the method if any changes to it are implemented that alter the methods suitability. Increased modification of the method requires a greater need for thorough revalidation. As the method was to be used for drug stability studies of morphine sulphate alone and in combination with haloperidol it has been satisfactorily validated. It was now possible to continue to the next phase of the project and perform drug stability studies.

6.3: Conclusions: Drug Stability

Having successfully developed and validated a HPLC method for the separation of morphine sulphate and haloperidol it was now possible to assess the chemical stability of the following:

- Morphine sulphate in P.C.A silicone balloon infuser. Concentrations tested were 2mg/ml and 10mg/ml at room temperature and refrigerated conditions.
- Morphine sulphate in polypropylene syringes. Concentrations tested were 2mg/ml and 10mg/ml at room temperature (22±2°C) and refrigerated (4°C) conditions.
- Morphine sulphate (0.5-12mg/ml) and haloperidol (0.25-2mg/ml) in combination in polypropylene syringes. Samples were tested in light and dark conditions.

Morphine sulphate (2mg/ml and 10mg/ml) in P.C.A infusers retained stability over the forty days of testing (room temperature (22±2°C) and 4°C). Morphine sulphate P.C.A.s will be prepared in the St. James's central compounding unit. A 28-day shelf life has been allocated to morphine P.C.A.s as a result of this work, and haven taken into account microbiological validation of the compounding unit in St James's Hospital. Ready to use P.C.A.s are now available in batches for in-house use, outside hospital use, and home healthcare use.

Morphine sulphate (2mg/ml and 10mg/ml) retained chemical stability in polypropylene syringes over 30 days of testing (room temperature (22±2°C) and 4°C). Comparison of results between both stability studies shows that a more erratic drug concentration variation in PCAs is evident than in polypropylene syringes but well within acceptable limits. It is suggested that this variation is a function of the PCA device

.

This project shows that it is feasible to conduct small-scale stability studies using HPLC within a hospital pharmacy department, with significant impact on clinical practice.

APPENDIX 1

REPRESENTATIVE CHROMATOGRAMS FOR CHAPTER 4

METHOD VALIDATION

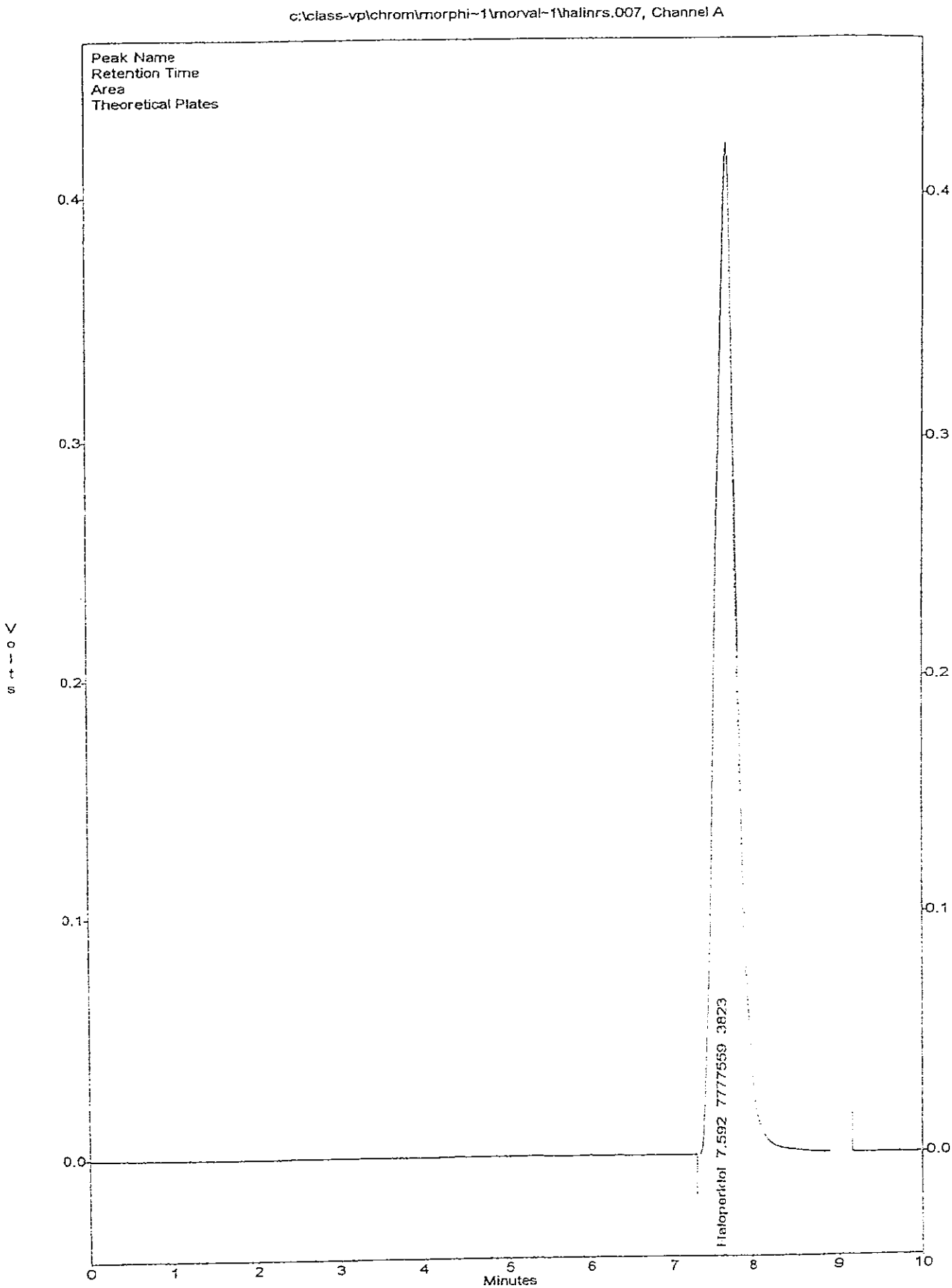


Fig A1 1: chromatogram of haloperidol reference standard

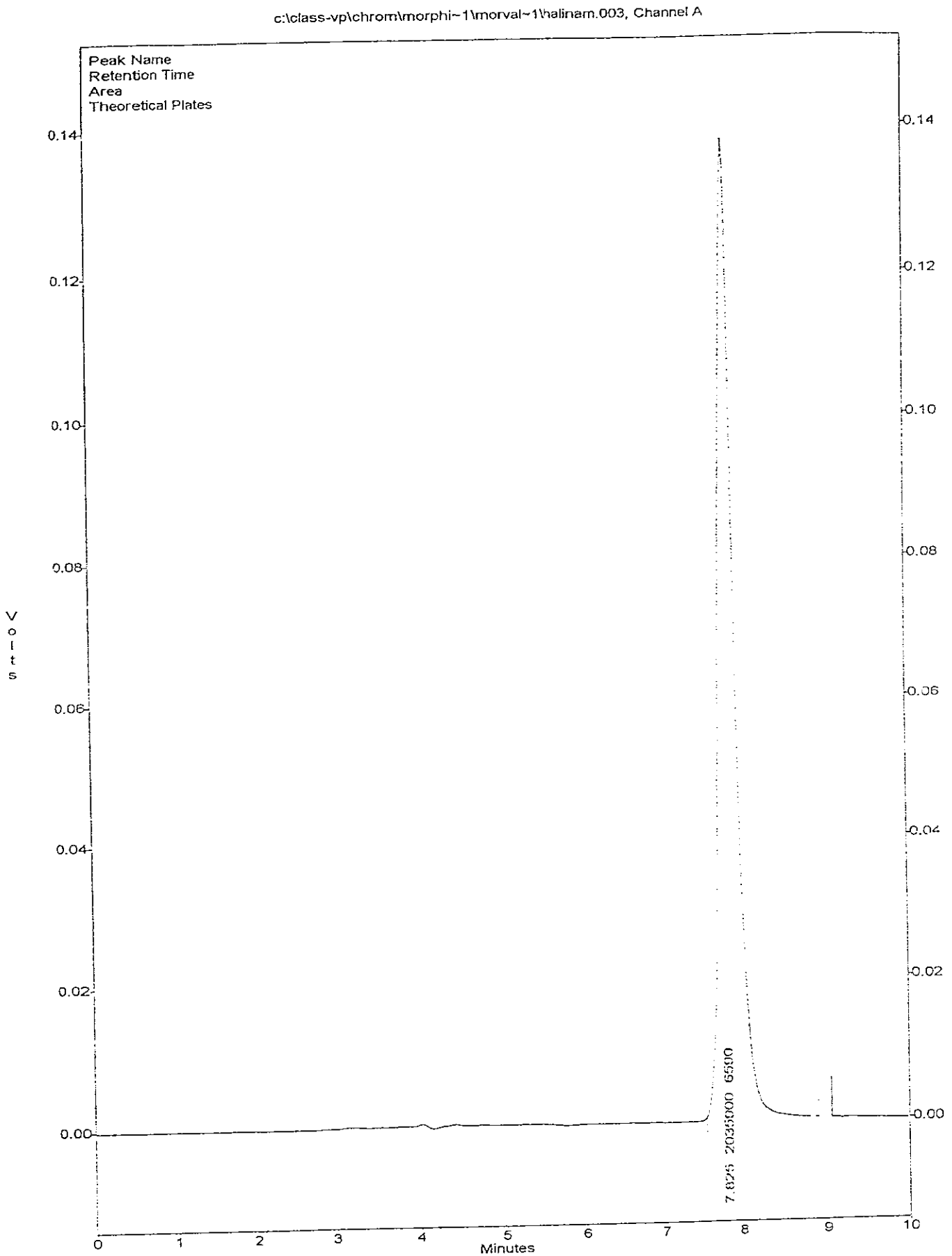


Fig A1.2: chromatogram haloperidol commercial solution

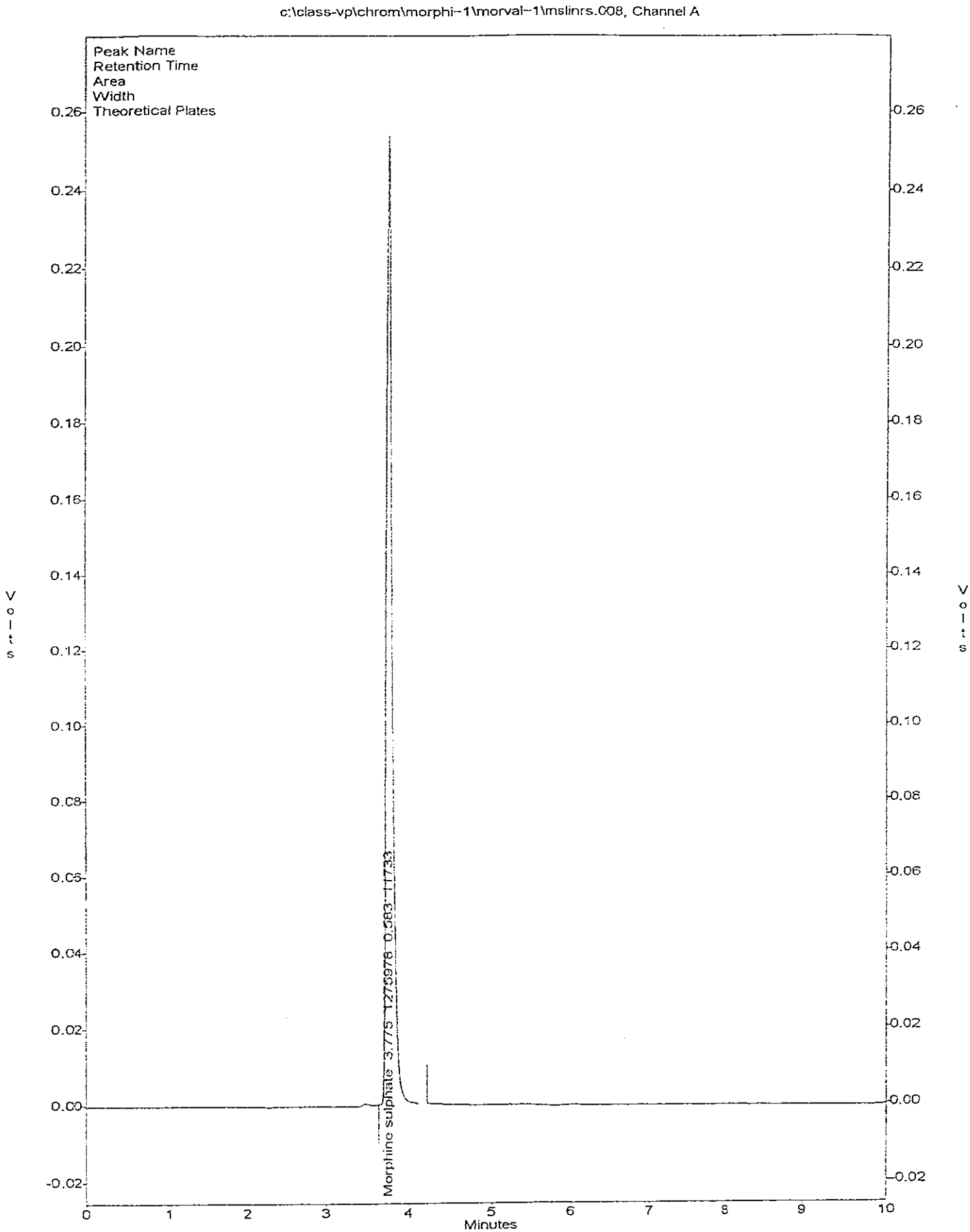


Fig A1.3: chromatogram morphine sulphate reference standard

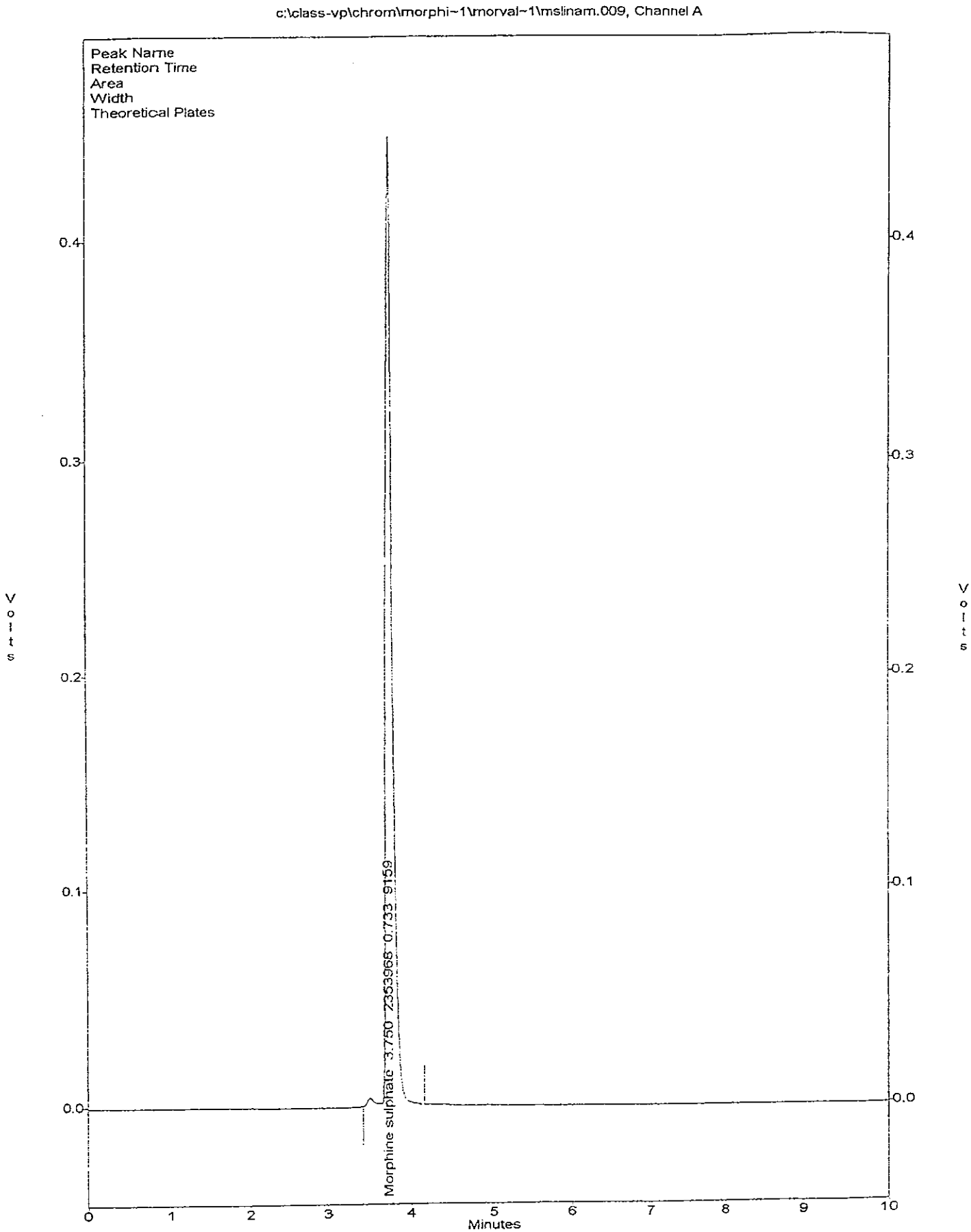
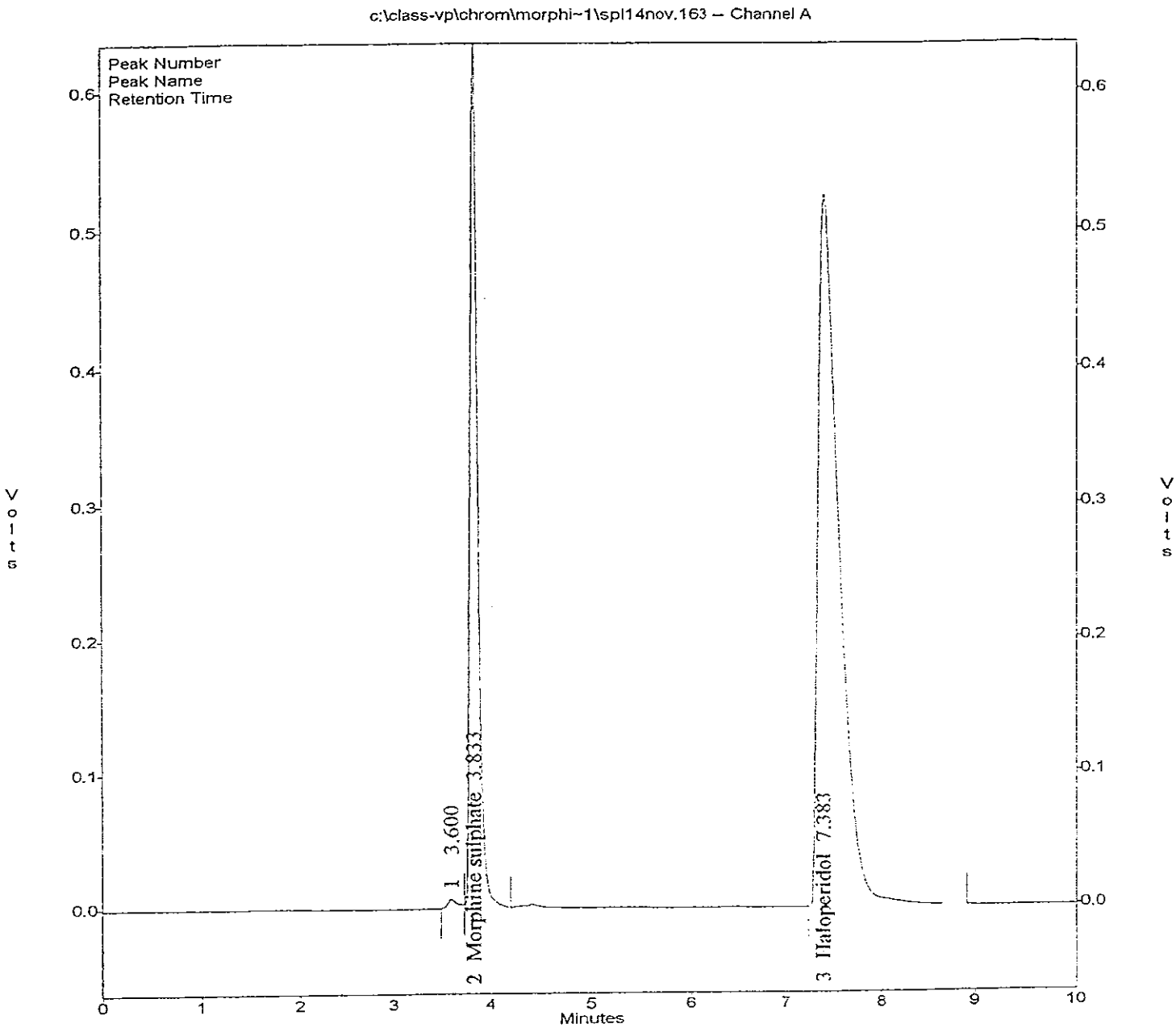


Fig A1.4: chromatogram morphine sulphate commercial solution

Vial : 0
Volume : 20
Acquired : Nov 16, 1999 02:54:44
Printed : Jan 16, 2001 15:31:37
User : System

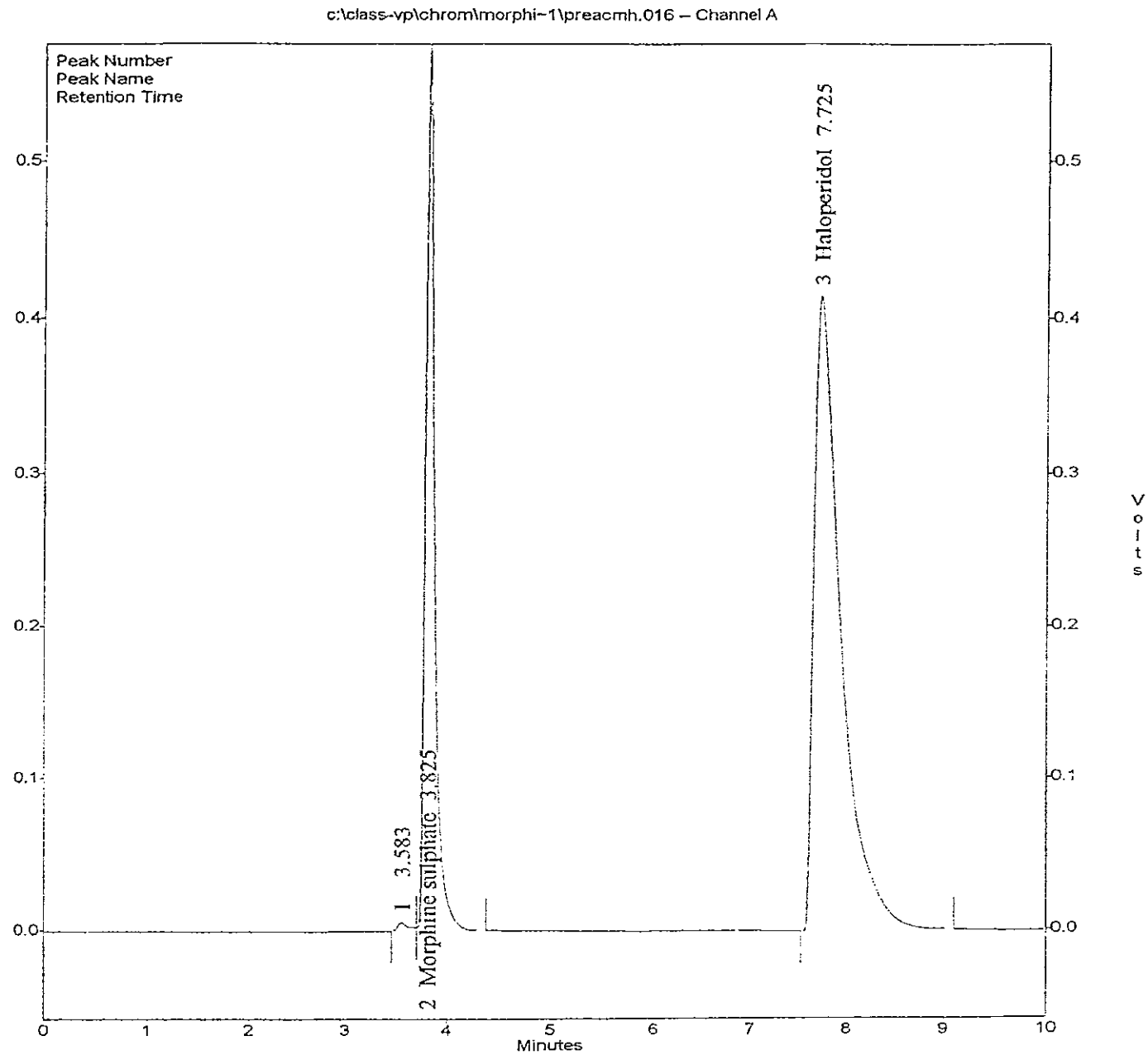


Channel A Results

Peak name	Peak Number	Time	Area
Morphine sulphate	2	3.833	3394507
Haloperidol	3	7.383	8106126

Fig A1.5: chromatogram morphine sulphate and haloperidol

Vial : 5
Volume : 20
Acquired : Dec 21, 2000 16:06:44
Printed : Jan 08, 2001 16:13:46
User : System

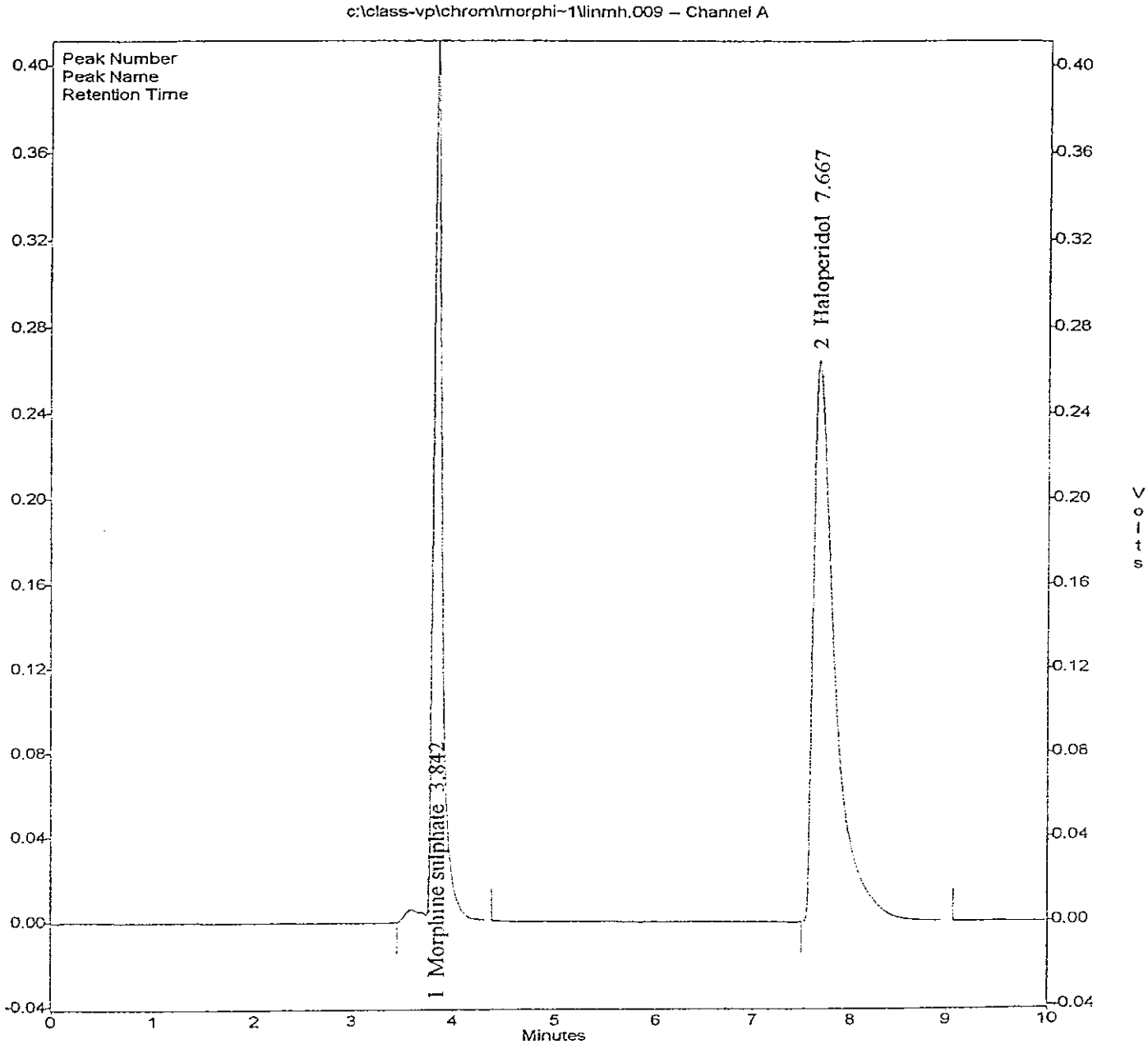


Channel A Results

Peak name	Peak Number	Time	Area
Morphine sulphate	2	3.825	3377077
Haloperidol	3	7.725	8116479

Fig A1.6: Chromatogram of morphine sulphate and haloperidol precision standard

Vial : 2
Volume : 20
Acquired : Dec 20, 2000 16:13:37
Printed : Jan 08, 2001 15:47:48
User : System

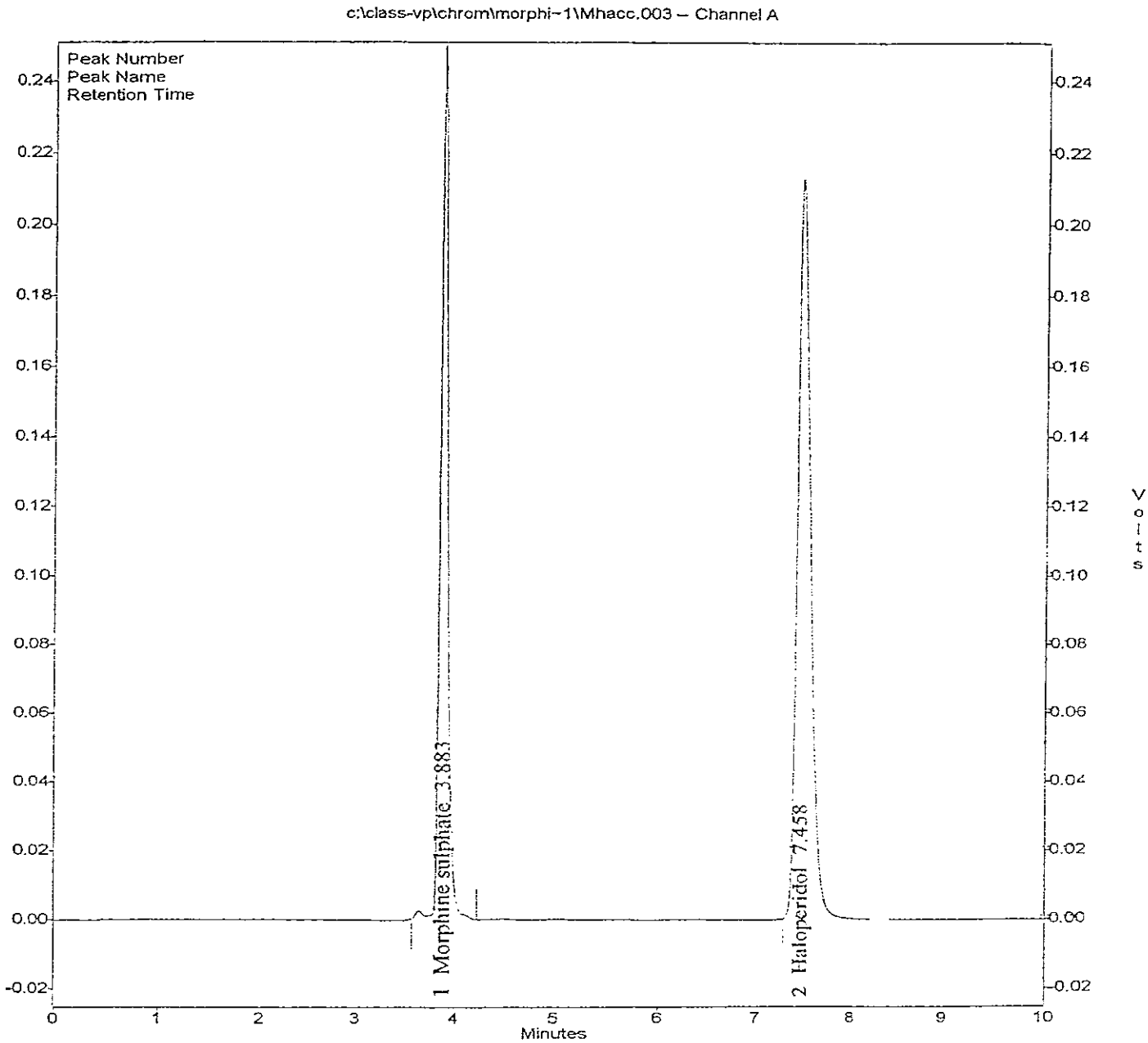


Channel A Results

Peak name	Peak Number	Time	Area
Morphine sulphate	1	3.842	2433839
Haloperidol	2	7.667	4115716

Fig A1.7: Chromatogram of morphine sulphate and haloperidol from linearity study

Vial : 6
Volume : 20
Acquired : Jun 08, 2001 10:09:12
Printed : Jun 08, 2001 10:19:14
User : System



Channel A Results

Peak name	Peak Number	Time	Area
Morphine sulphate	1	3.883	1249327
Haloperidol	2	7.458	2036749

Fig A1.8: Chromatogram of morphine sulphate and haloperidol from accuracy study

APPENDIX 2

REPRESENTATIVE CHROMATOGRAMS FOR CHAPTER 2

METHOD VALIDATION
SPECIFICITY

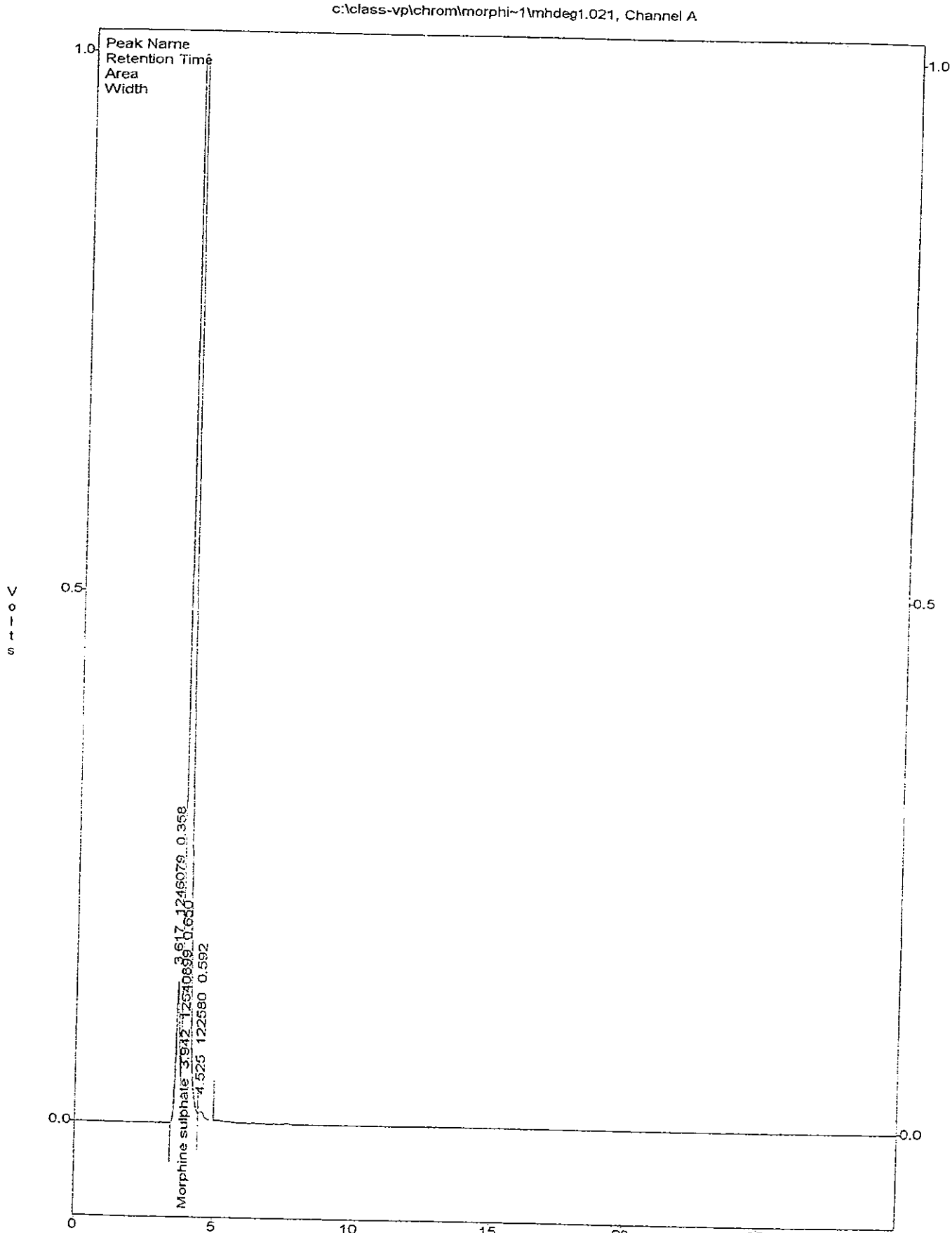


Fig A2.1: Specificity morphine sulphate and haloperidol degraded in sodium hydroxide

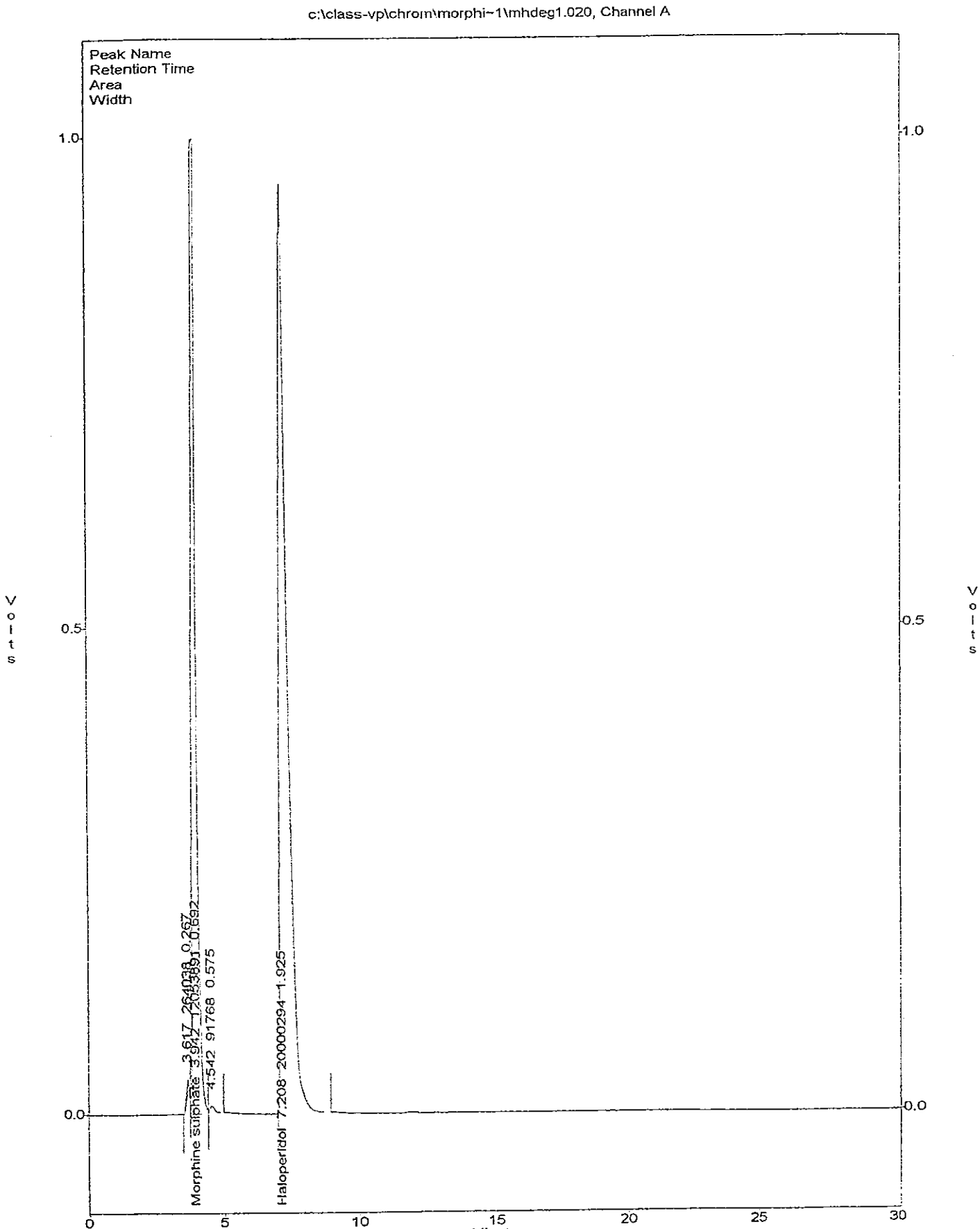


Fig A2.2: Specificity morphine sulphate and haloperidol degraded in hydrochloric acid

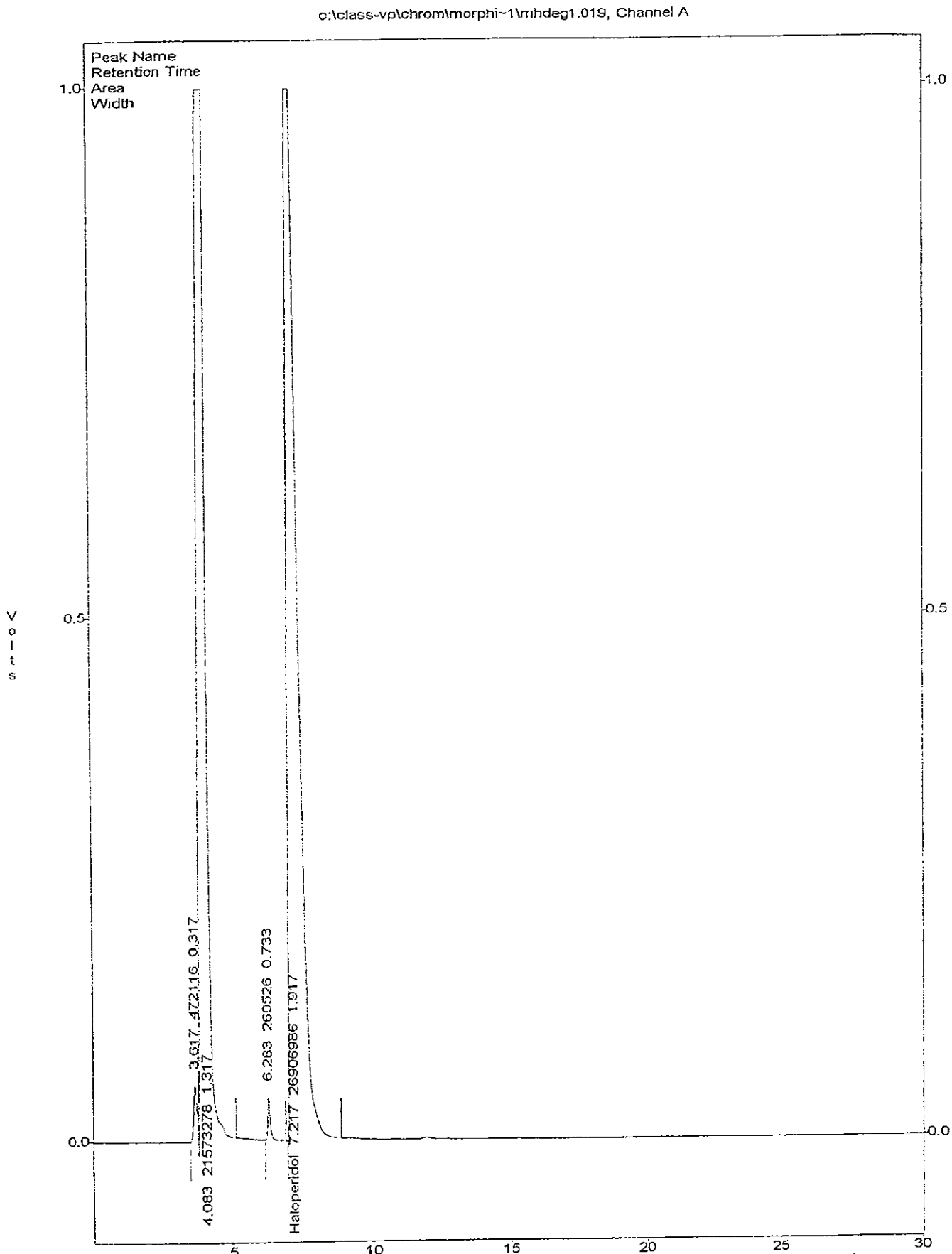


Fig A2.3: Specificity morphine sulphate and haloperidol degraded in hydrogen peroxide

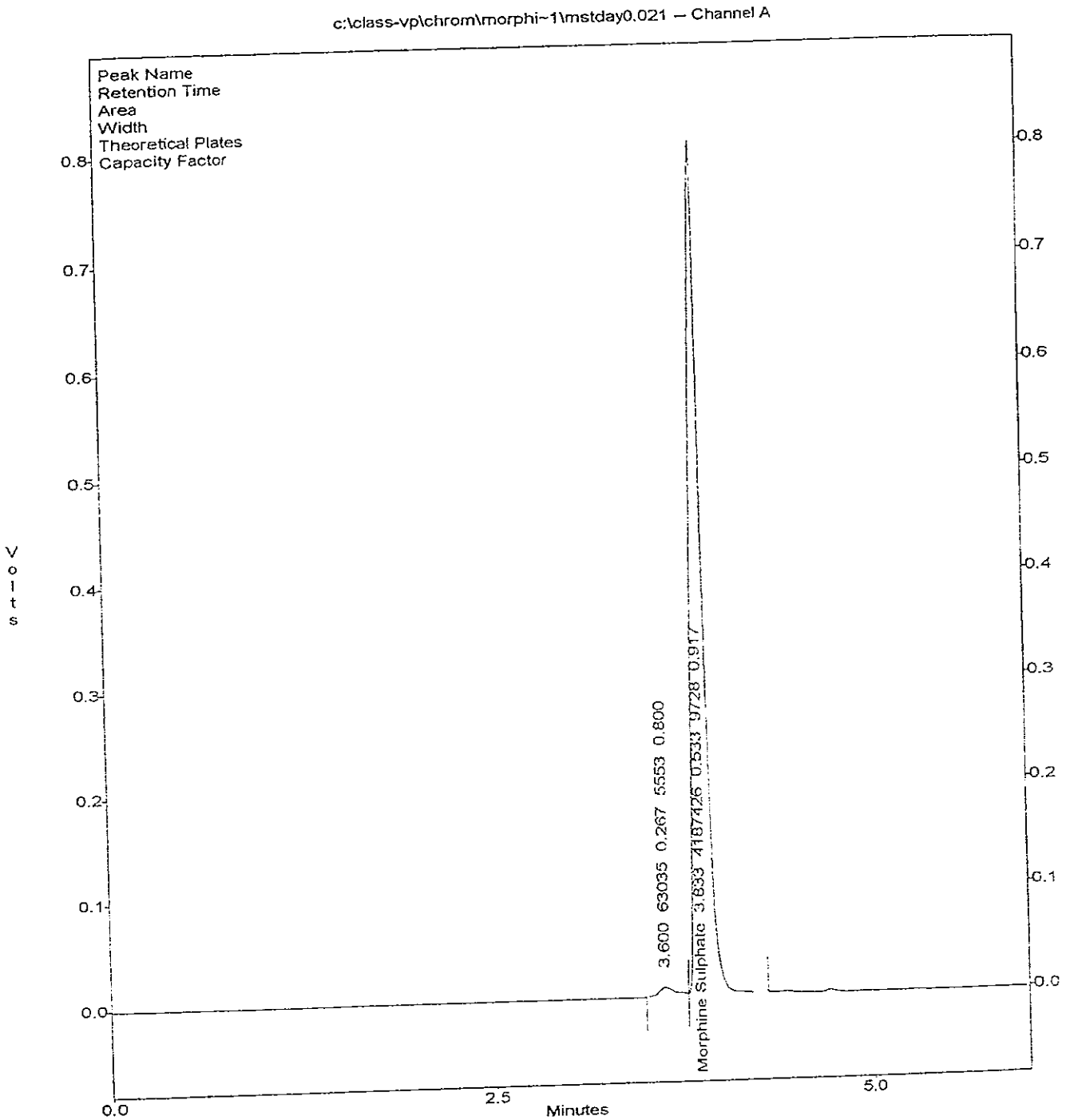
APPENDIX 3

REPRESENTATIVE CHROMATOGRAMS FOR SECTION 3

DRUG STABILITY STUDIES

STABILITY OF MORPHINE SULPHATE IN PCAs

Vial : 5
Volume : 20
Acquired : Sep 24, 1999 17:55:33
Printed : Nov 16, 1999 09:45:46
User : System



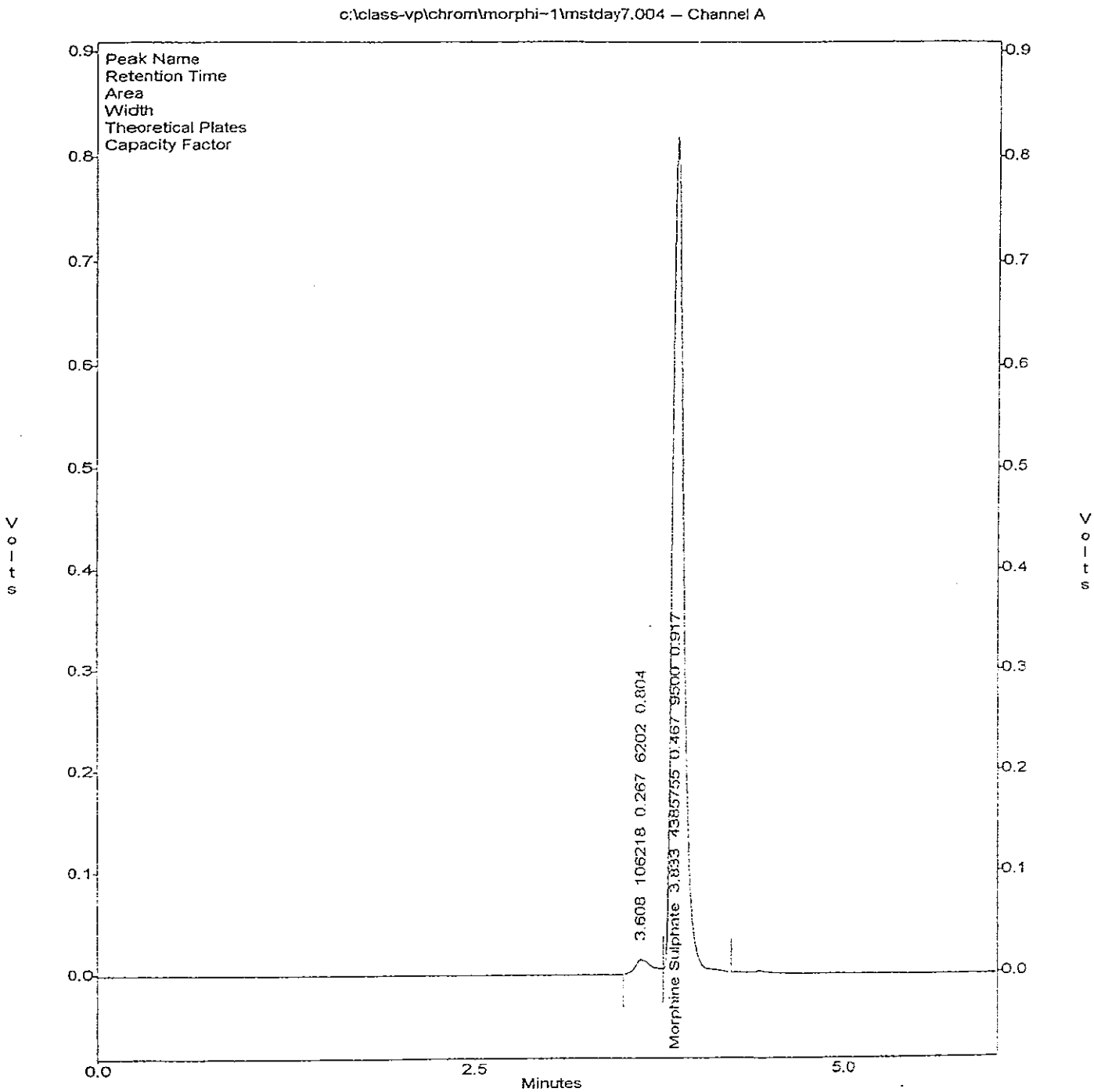
Channel A Results

Peak	Time	Area	Width	Theor Plates	Capac Factor
2	3.833	4187426	0.533	9728	0.917

Totals : 4187426

Fig A3.1: Chromatogram of morphine sulphate test day 0

Vial : 0
Volume : 20
Acquired : Oct 01, 1999 17:27:11
Printed : Nov 16, 1999 15:10:25
User : System



Channel A Results

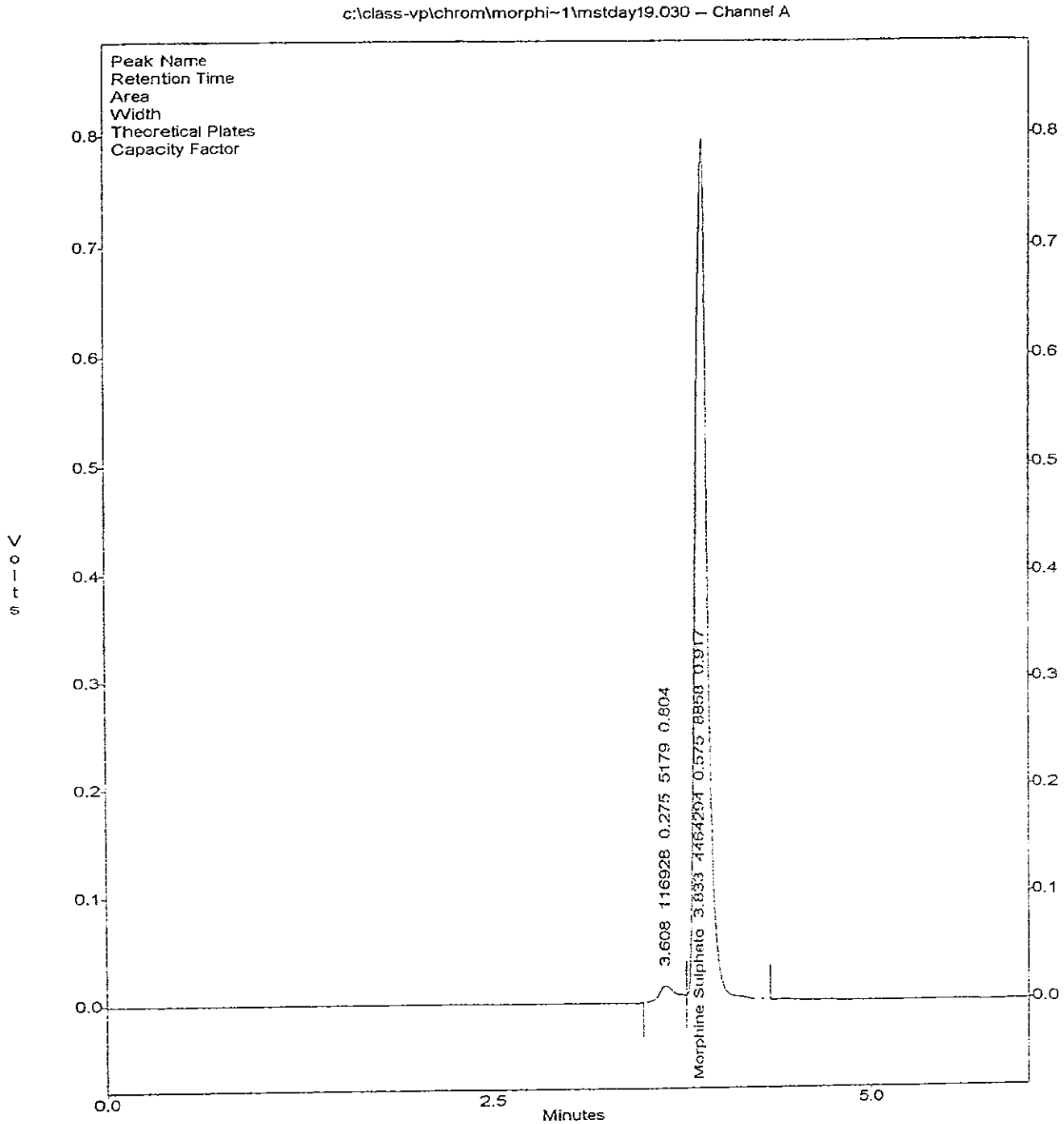
Peak	Time	Area	Width	Theor Plates	Capac Factor
2	3.833	4385755	0.467	9501	0.917

Totals :

4385755

Fig A3.2: Chromatogram of morphine sulphate test day 7

Volume : 20
Acquired : Oct 13, 1999 20:24:46
Printed : Nov 24, 1999 10:31:54
User : System



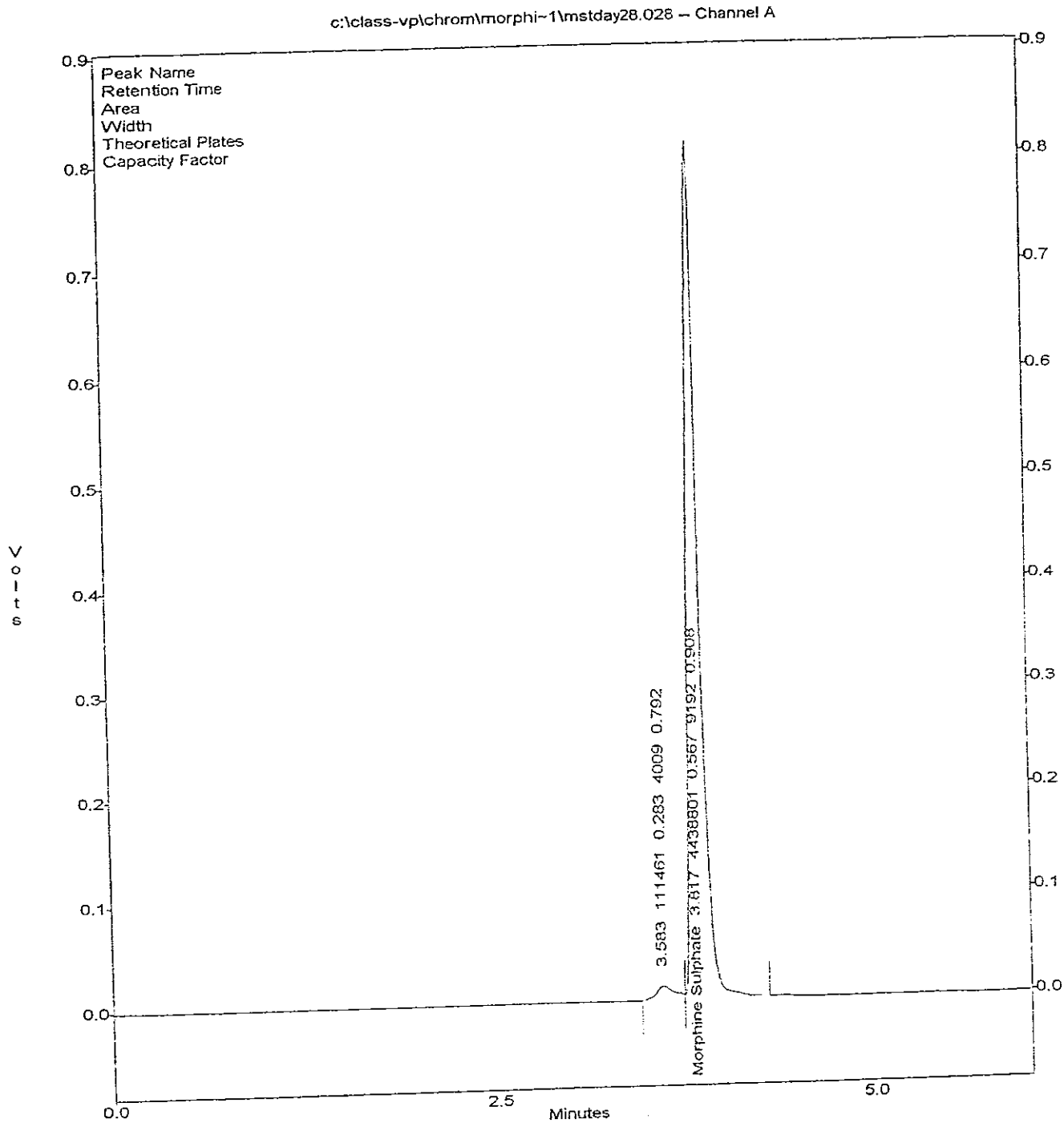
Channel A Results

Peak	Time	Area	Width	Theor Plates	Capac Factor
2	3.833	4464294	0.575	8859	0.917

Totals : 4464294

Fig A3.3: Chromatogram of morphine sulphate test day 19

Vial : 7
Volume : 20
Acquired : Oct 22, 1999 20:43:33
Printed : Nov 17, 1999 16:06:45
User : System



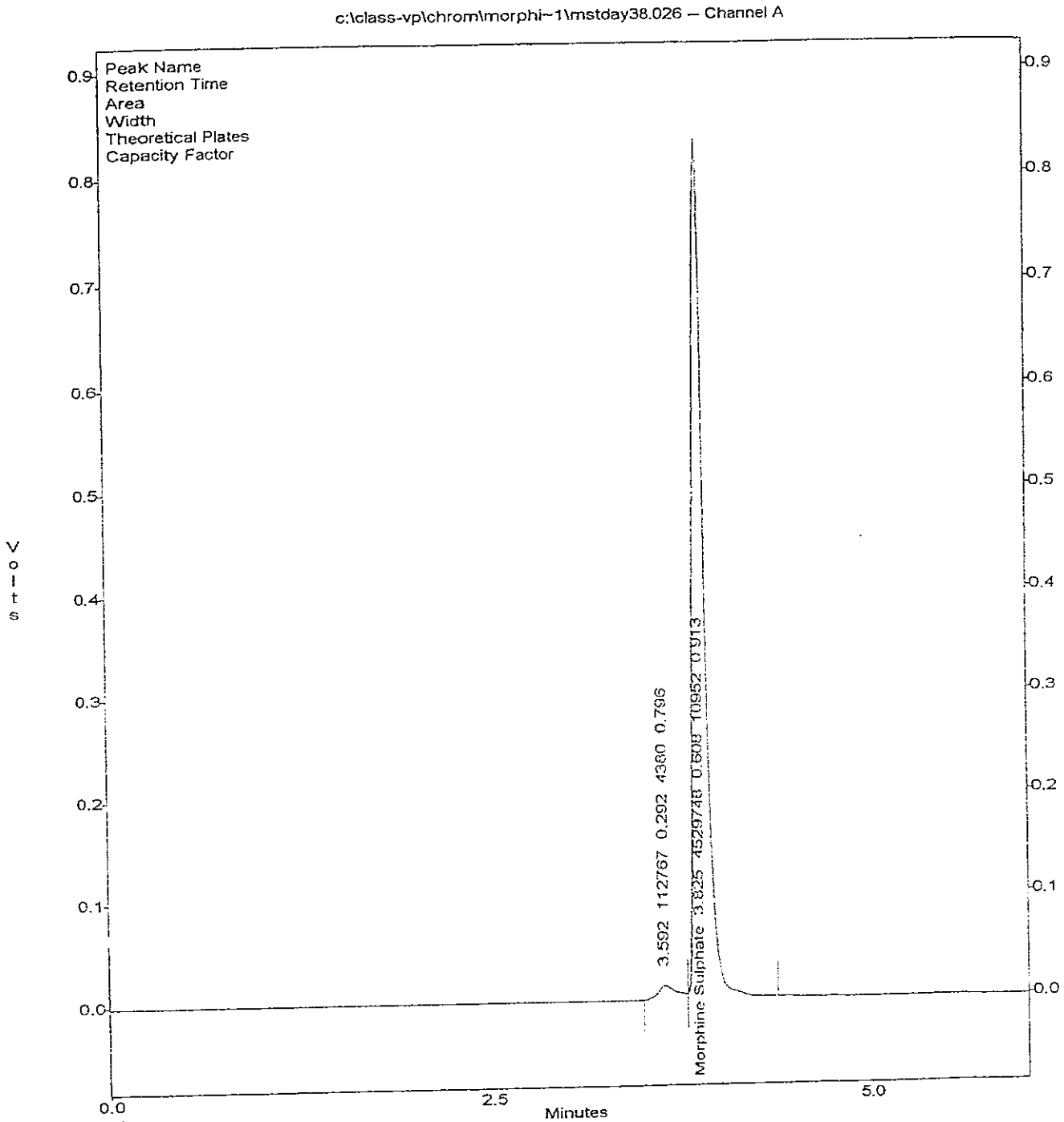
Channel A Results

Peak	Time	Area	Width	Theor Plates	Capac Factor
2	3.817	4438801	0.567	9192	0.908

Totals : 4438801

Fig A3.4: Chromatogram of morphine sulphate test day 28

Vial : 6
Volume : 20
Acquired : Nov 01, 1999 20:27:13
Printed : Nov 23, 1999 15:46:51
User : System



Channel A Results

Peak	Time	Area	Width	Theor Plates	Capac Factor
2	3.825	4529748	0.608	10952	0.913

Totals : 4529748

Fig A3.5. Chromatogram of morphine sulphate test day 38

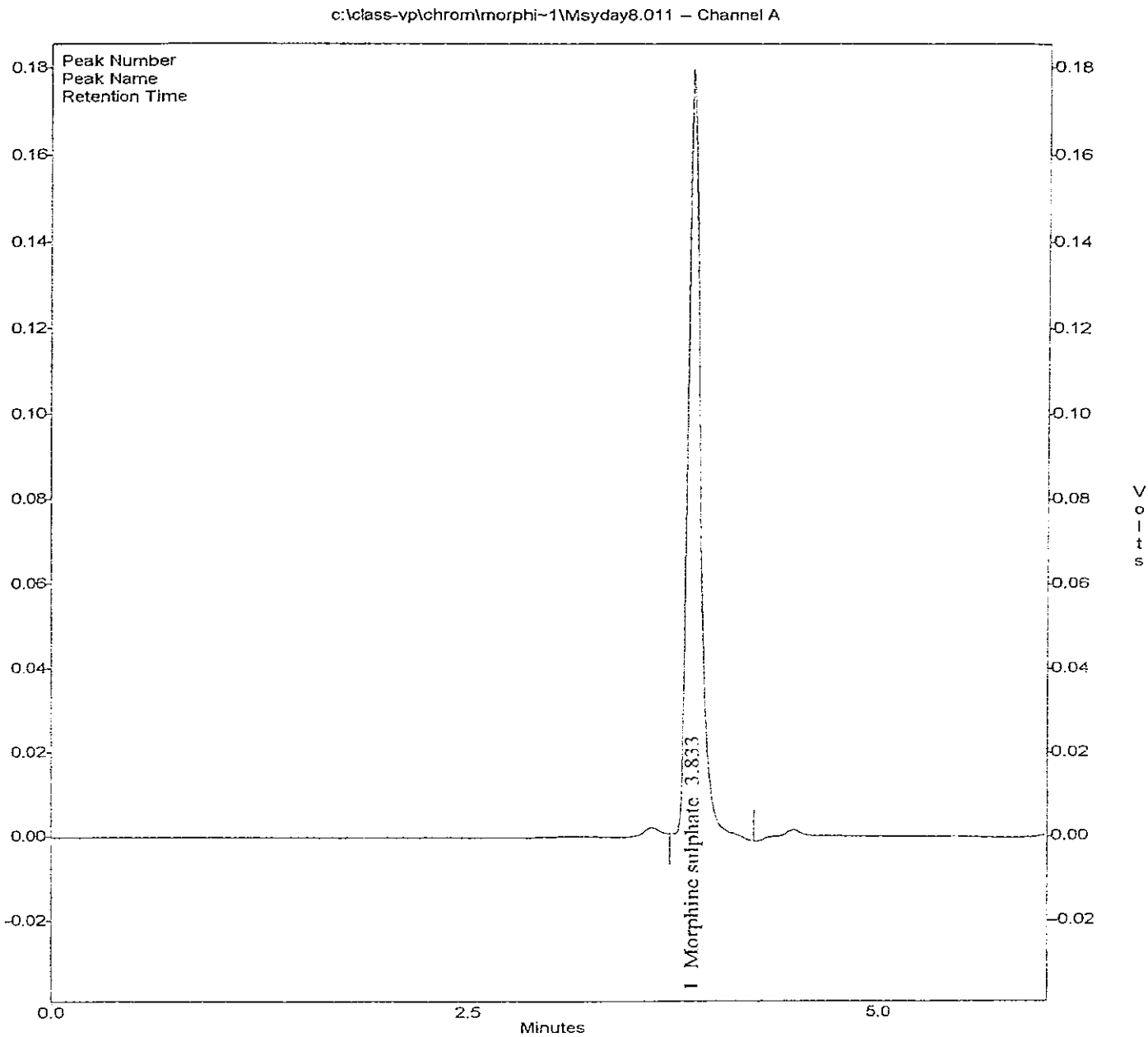
APPENDIX 4

REPRESENTATIVE CHROMATOGRAMS FOR SECTION 3

DRUG STABILITY STUDIES

STABILITY OF MORPHINE SULPHATE IN POLYPROPYLENE SYRINGES

Vial : 3
Volume : 20
Acquired : Sep 20, 2000 13:19:26
Printed : Sep 20, 2000 13:25:30
User : System

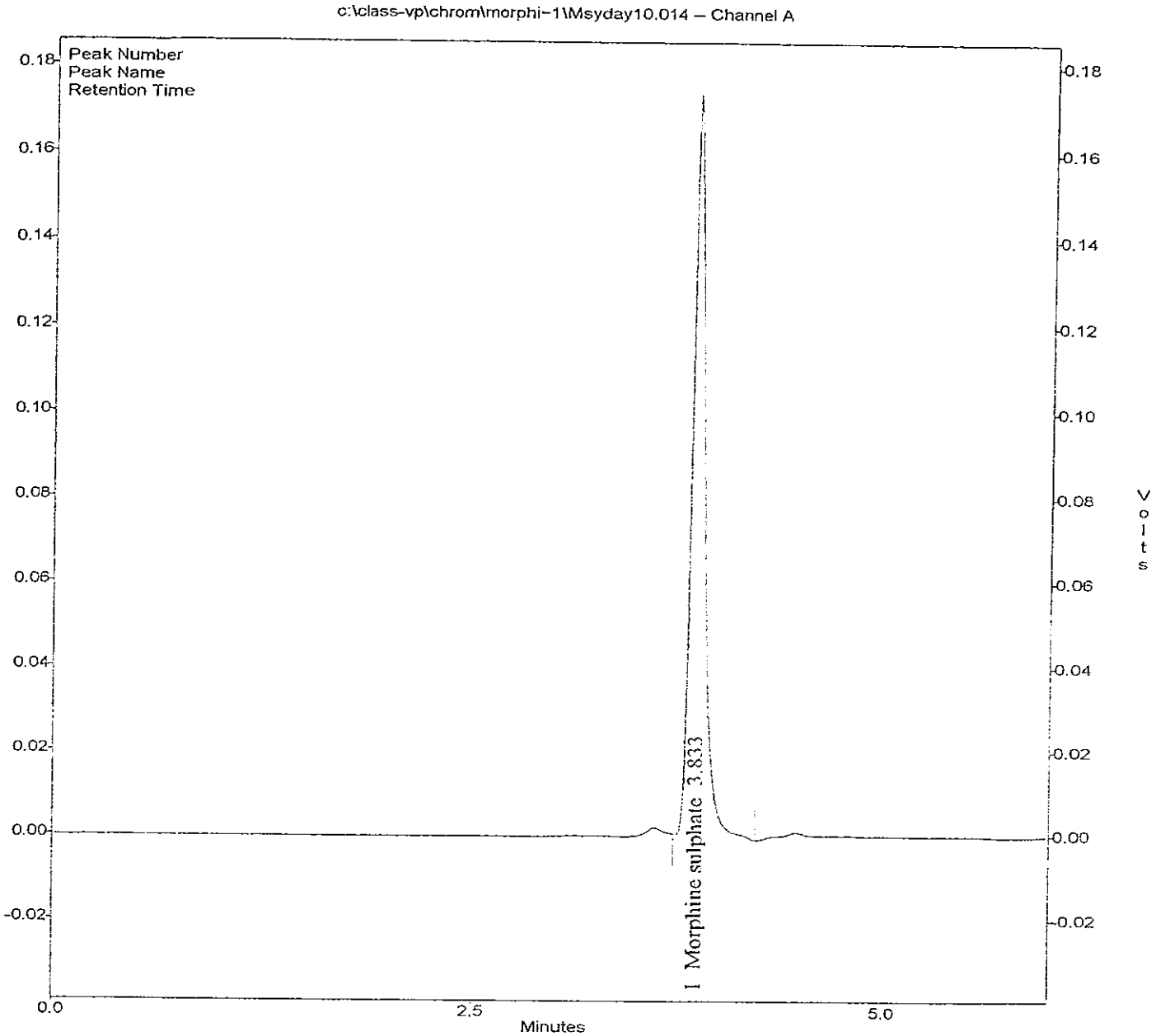


Channel A Results

Peak name	Peak Number	Time	Area
Morphine sulphate	1	3.833	1005017
Haloperidol	--	7.300	0

Fig A4.1: Chromatogram of morphine sulphate test day 8

Vial : 4
Volume : 20
Acquired : Sep 22, 2000 12:57:59
Printed : Sep 22, 2000 13:04:02
User : System

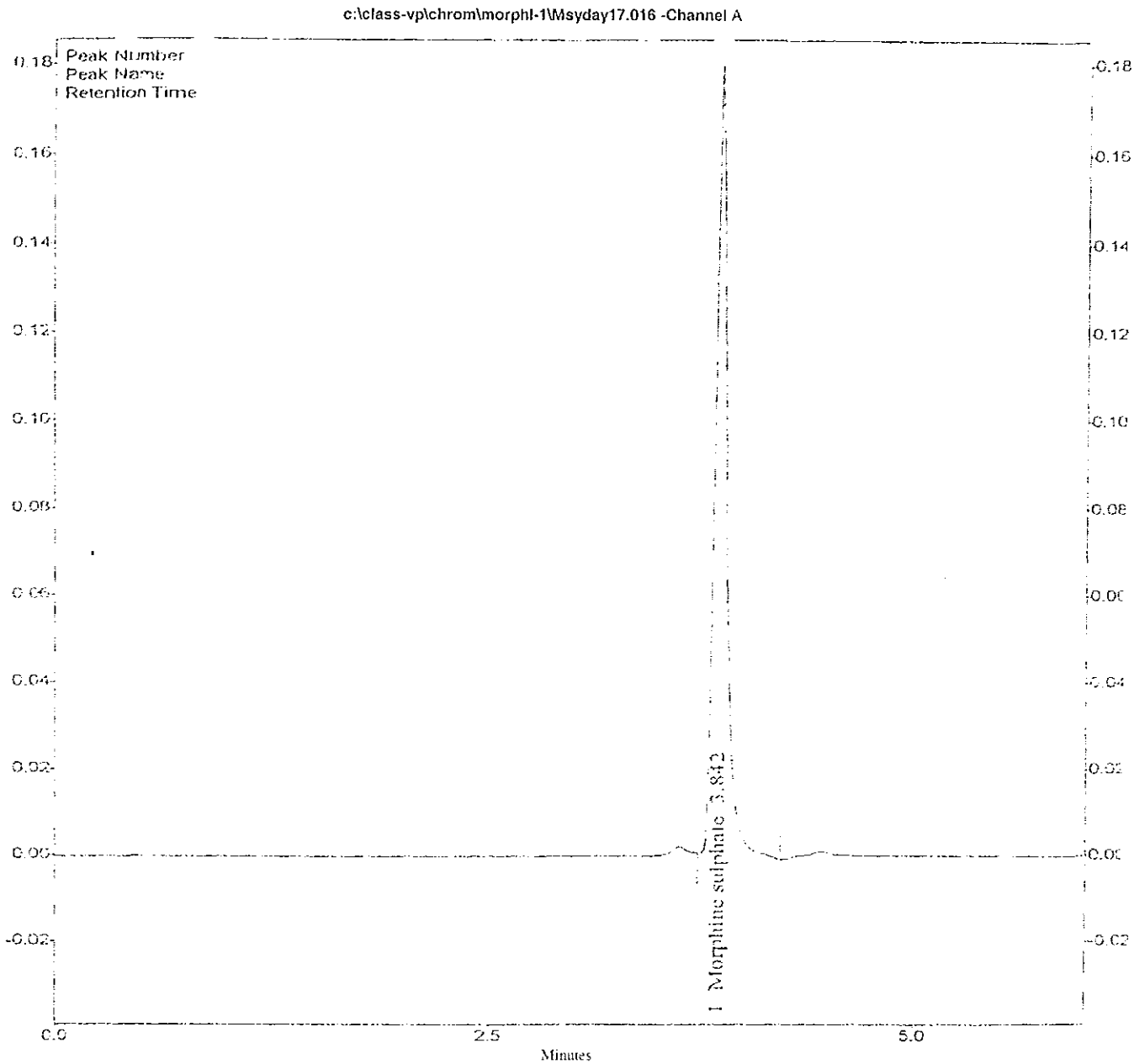


Channel A Results

Peak name	Peak Number	Time	Area
Morphine sulphate	1	3.833	983952
Haloperidol	--	7.300	0

Fig A4.2: Chromatogram of morphine sulphate test day 10

nple ID : Morphine Sulphate
3.1 : 5
lume : 20
quired : Sep 29, 2000 12:19:16 : Sep
inted 29, 2000 12:25:18 : System
er

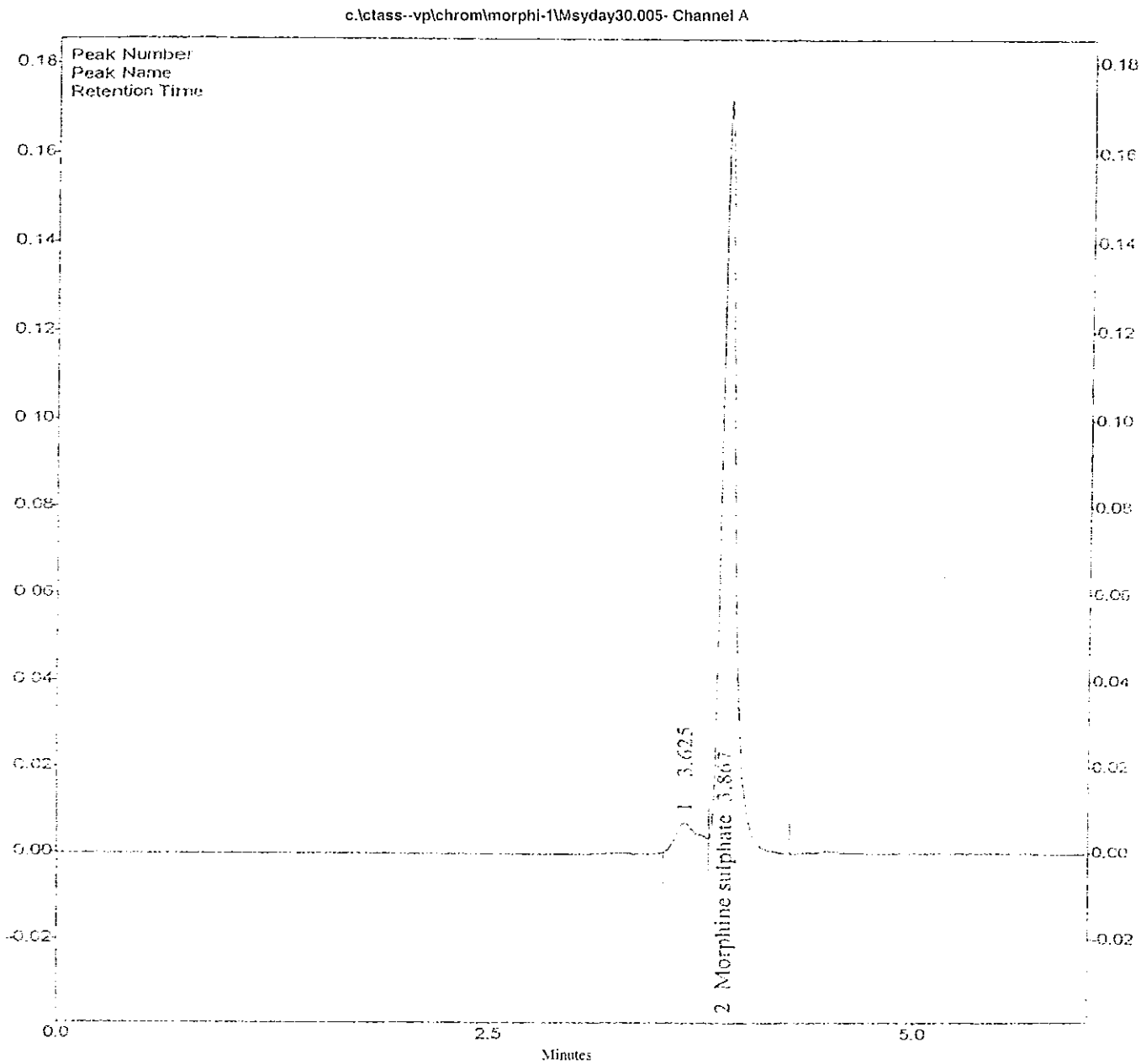


Channel A Results

Peak name	Peak Number	Time	Area
Morphine sulphate	1	3.842	992509
Haloperidol		7.300	0

Fig A4.3: Chromatogram of morphine sulphate test day 17

.mple ID .al : morpnJ.ne SU.I.plidl..t:
llume :quired : 0
:inted ;er : 20
: Oct 12, 2000 15:21:10 : Oct
12, 2000 15:27:11 : System



Channel A Results

Peak name	Time	Area
Morphine sulphate	3.867	990761
Haloperidol	7.300	C

Peak Number 2

Fig A4.4: Chromatogram of morphine sulphate test day 30

APPENDIX 5

TABLES OF DATA FOR STABILITY OF MORPHINE SULPHATE IN PCAs

		TEST DAY 0		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		4311820	
	2		4347832	
	3		4306707	
	4		4314843	
	5		4330297	
	6		4326537	
		MEAN	**4323006**	
		STD DEV	**15096**	
Std 0.2mg/ml	1			
	2		952514	
	3		969410	
		MEAN	**960962**	
		STD DEV	**11947**	
A1	1		939888	97.8
	2		939781	97.8
		MEAN	**939835**	**97.8**
		STD DEV	**76**	**0.0**
A2	1		939908	97.8
	2		939041	97.7
		MEAN	**939475**	**97.8**
		STD DEV	**613**	**0.1**
B1	1		964594	100.4
	2		963766	100.3
		MEAN	**964180**	**100.3**
		STD DEV	**585**	**0.06**
B2	1		959688	99.9
	2		961662	100.1
		MEAN	**960675**	**100.0**
		STD DEV	**1396**	**0.15**
C1	1		4298343	99.4
	2		4291305	99.3
		MEAN	**4294824**	**99.3**
		STD DEV	**4977**	**0.12**
C2	1		4284282	99.1
	2		4291917	99.3
		MEAN	**4288100**	**99.2**
		STD DEV	**5399**	**0.12**
D1	1		4287617	99.2
	2		4295292	99.4
		MEAN	**4291455**	**99.3**
		STD DEV	**5427**	**0.13**
D2	1		4141949	95.8
	2		4125690	95.4
		MEAN	**4133820**	**95.6**
		STD DEV	**11497**	**0.3**

Table A5.1: Stability of morphine sulphate in PCAs, test day 0

		TEST DAY 3		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		4466969	
	2		4436196	
	3		4453020	
	4		4427172	
	5		4455611	
	6		4453745	
		MEAN	**4448786**	
		STD DEV	**14460**	
Std 0.2mg/ml	1			
	2		988391	
	3		989861	
		MEAN	**989126**	
		STD DEV	**1039**	
A1	1		1021624	103.3
	2		1022080	103.3
		MEAN	**1021852**	**103.3**
		STD DEV	**322**	**0.03**
A2	1		1019839	103.1
	2		1020803	103.2
		MEAN	**1020321**	**103.2**
		STD DEV	**682**	**0.1**
B1	1		963689	97.4
	2		961765	97.2
		MEAN	**962727**	**97.3**
		STD DEV	**1360**	**0.14**
B2	1		968653	97.9
	2		973260	98.4
		MEAN	**970957**	**98.2**
		STD DEV	**3258**	**0.33**
C1	1		4408386	99.1
	2		4426931	99.5
		MEAN	**4417659**	**99.3**
		STD DEV	**13113**	**0.29**
C2	1		4394227	98.8
	2		4398664	98.9
		MEAN	**4396446**	**98.8**
		STD DEV	**3137**	**0.07**
D1	1		4447640	100.0
	2		4445722	99.9
		MEAN	**4446681**	**100.0**
		STD DEV	**1356**	**0.03**
D2	1		4273849	96.1
	2		4285918	96.3
		MEAN	**4279884**	**96.2**
		STD DEV	**8534**	**0.2**

Table A5.2: Stability of morphine sulphate in PCAs, test day 3

		TEST DAY 5		
SAMPLE	**INJ**		**PEAK AREA**	**% CONTENT**
Std 1mg/ml	1			
	2		5810524	
	3		5755832	
	4		5797130	
	5		5771575	
	6		5832707	
		MEAN	**5793554**	
		STD DEV	**30577**	
Std 0.2mg/ml	1			
	2		1320578	
	3		1327046	
		MEAN	**1323812**	
		STD DEV	**4574**	
A1	1		1377024	104.0
	2		1376847	104.0
		MEAN	**1376936**	**104.0**
		STD DEV	**125**	**0.01**
A2	1		1404643	106.1
	2		1372489	103.7
		MEAN	**1388566**	**104.9**
		STD DEV	**22736**	**1.7**
B1	1		1331859	100.6
	2		1331094	100.6
		MEAN	**1331477**	**100.6**
		STD DEV	**541**	**0.04**
B2	1		1394332	105.3
	2		1393276	105.2
		MEAN	**1393804**	**105.3**
		STD DEV	**747**	**0.06**
C1	1		5665281	97.8
	2		5650835	97.5
		MEAN	**5658058**	**97.7**
		STD DEV	**10215**	**0.18**
C2	1		5774274	99.7
	2		5750894	99.3
		MEAN	**5762584**	**99.5**
		STD DEV	**16532**	**0.29**
D1	1		5636062	97.3
	2		5684191	97.28
		MEAN	**5636062**	**97.3**
		STD DEV	**34032**	**0.00**
D2	1		5611026	96.8
	2		5609518	96.8
		MEAN	**5610272**	**96.8**
		STD DEV	**1066**	**0.0**

Table A5.3: Stability of morphine sulphate in PCAs, test day 5

		TEST DAY 7		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1			
	2		4350247	
	3		4389114	
	4		4385755	
	5		4410845	
	6		4420360	
		MEAN	**4391264**	
		STD DEV	**27145**	
Std 0.2mg/ml	1			
	2		974707	
	3		976607	
		MEAN	**975657**	
		STD DEV	**1344**	
A1	1		996302	102.1
	2		998038	102.3
		MEAN	**997170**	**102.2**
		STD DEV	**1228**	**0.1**
A2	1		1058824	108.5
	2		1061260	108.8
		MEAN	**1060042**	**108.6**
		STD DEV	**1723**	**0.2**
B1	1		939280	96.3
	2		938053	96.1
		MEAN	**938667**	**96.2**
		STD DEV	**868**	**0.09**
B2	1		940758	96.4
	2		939360	96.3
		MEAN	**940059**	**96.4**
		STD DEV	**989**	**0.10**
C1	1		4355089	99.2
	2		4353310	99.1
		MEAN	**4354200**	**99.2**
		STD DEV	**1258**	**0.03**
C2	1		4338076	98.8
	2		4340275	98.8
		MEAN	**4339176**	**98.8**
		STD DEV	**1555**	**0.04**
D1	1		4303556	98.0
	2		4317756	98.3
		MEAN	**4310656**	**98.2**
		STD DEV	**10041**	**0.23**
D2	1		4314521	98.3
	2		4315023	98.3
		MEAN	**4314772**	**98.3**
		STD DEV	**355**	**0.0**

Table A5.4: Stability of morphine sulphate in PCAs, test day 7

		TEST DAY 10		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1			
	2		4432754	
	3		4432092	
	4		4444170	
	5		4389315	
	6		4364071	
		MEAN	**4412480**	
		STD DEV	**34205**	
Std 0.2mg/ml	1			
	2		988391	
	3		989861	
		MEAN	**989126**	
		STD DEV	**1039**	
A1	1		1003559	101.5
	2		1002721	101.4
		MEAN	**1003140**	**101.4**
		STD DEV	**593**	**0.1**
A2	1		1001322	101.2
	2		1003245	101.4
		MEAN	**1002284**	**101.3**
		STD DEV	**1360**	**0.1**
B1	1		1056956	106.9
	2		1055466	106.7
		MEAN	**1056211**	**106.8**
		STD DEV	**1054**	**0.11**
B2	1		1138396	115.1
	2		1140324	115.3
		MEAN	1139360	115.2
		STD DEV	1363	0.14
C1	1		4550994	103.1
	2		4551983	103.2
		MEAN	**4551489**	**103.2**
		STD DEV	**699**	**0.02**
C2	1		4436011	100.5
	2		4438711	100.6
		MEAN	**4437361**	**100.6**
		STD DEV	**1909**	**0.04**
D1	1		4389392	99.5
	2		4391769	99.5
		MEAN	**4390581**	**99.5**
		STD DEV	**1681**	**0.04**
D2	1		4379410	99.3
	2		4377417	99.2
		MEAN	**4378414**	**99.2**
		STD DEV	**1409**	**0.03**

Table A5.5: Stability of morphine sulphate in PCAs, test day 10

		TEST DAY 12		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		4466969	
	2		4436196	
	3		4453020	
	4		4427172	
	5		4455611	
	6		4453745	
		MEAN	**4448786**	
		STD DEV	**14460**	
Std 0.2mg/ml	1			
	2		988391	
	3		989861	
		MEAN	**989126**	
		STD DEV	**1039**	
A1	1		1021624	103.3
	2		1022080	103.3
		MEAN	**1021852**	**103.3**
		STD DEV	**322**	**0.0**
A2	1		1019839	103.1
	2		1020803	103.2
		MEAN	**1020321**	**103.2**
		STD DEV	**682**	**0.1**
B1	1		963689	97.4
	2		961765	97.2
		MEAN	**962727**	**97.3**
		STD DEV	**1360**	**0.14**
B2	1		968653	97.9
	2		973260	98.4
		MEAN	**970957**	**98.2**
		STD DEV	**3258**	**0.33**
C1	1		4408386	99.1
	2		4426931	99.5
		MEAN	**4417659**	**99.3**
		STD DEV	**13113**	**0.295**
C2	1		4394227	98.8
	2		4398664	98.9
		MEAN	**4396446**	**98.8**
		STD DEV	**3137**	**0.07**
D1	1		4447640	100.0
	2		4445722	99.9
		MEAN	**4446681**	**100.0**
		STD DEV	**1356**	**0.03**
D2	1		4273849	96.1
	2		4285918	96.3
		MEAN	**4279884**	**96.2**
		STD DEV	**8534**	**0.2**

Table A5.6: Stability of morphine sulphate in PCAs, test day 12

		TEST DAY 14		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		4286788	
	2		4335645	
	3		4353698	
	4		4383846	
	5		4384366	
	6		4393233	
		MEAN	**4356263**	
		STD DEV	**40437**	
Std 0.2mg/ml	1		962828	
	2		977694	
	3		977871	
		MEAN	**972798**	
		STD DEV	**8634**	
A1	1		1068405	109.8
	2		1065464	109.8
		MEAN	**1068405**	**109.8**
		STD DEV	**2080**	**0**
A2	1		1053513	108.3
	2		1060429	109.0
		MEAN	**1056971**	**108.7**
		STD DEV	**4890**	**0.5**
B1	1		931491	95.8
	2		930646	95.7
		MEAN	**931068.5**	**95.7**
		STD DEV	**598**	**0.06**
B2	1		965070	99.2
	2		966509	99.4
		MEAN	**965790**	**99.3**
		STD DEV	**1018**	**0.10**
C1	1		4683270	107.5
	2		4679962	107.4
		MEAN	**4681616**	**107.5**
		STD DEV	**2339**	**0.05**
C2	1		4640454	106.5
	2		4640029	106.5
		MEAN	**4640242**	**106.5**
		STD DEV	**301**	**0.0**
D1	1		4288612	98.4
	2		4304751	98.8
		MEAN	**4296682**	**98.6**
		STD DEV	**11412**	**0.26**
D2	1		4335969	99.5
	2		4336219	99.5
		MEAN	**4336094**	**99.5**
		STD DEV	**177**	**0.0**

Table A5.7: Stability of morphine sulphate in PCAs, test day 14

		TEST DAY 17		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1			
	2		4447929	
	3		4366090	
	4		4451669	
	5		4418387	
	6		4455918	
		MEAN	**4427999**	
		STD DEV	**37623**	
Std 0.2mg/ml	1			
	2		972310	
	3		972922	
		MEAN	**972616**	
		STD DEV	**433**	
A1	1		995935	102.4
	2		996840	102.5
		MEAN	**996388**	**102.4**
		STD DEV	**640**	**0.1**
A2	1		1002320	103.1
	2		1005302	103.4
		MEAN	**1003811**	**103.2**
		STD DEV	**2109**	**0.2**
B1	1		963330	99.0
	2		964123	99.1
		MEAN	**963727**	**99.1**
		STD DEV	**561**	**0.06**
B2	1		956566	98.3
	2		975181	100.3
		MEAN	**965874**	**99.3**
		STD DEV	**13163**	**1.35**
C1	1		4555229	102.9
	2		4556723	102.9
		MEAN	**4555976**	**102.9**
		STD DEV	**1056**	**0.02**
C2	1		4534790	102.4
	2		4544134	102.6
		MEAN	**4539462**	**102.5**
		STD DEV	**6607**	**0.15**
D1	1		4412929	99.7
	2		4407100	99.5
		MEAN	**4410015**	**99.6**
		STD DEV	**4122**	**0.09**
D2	1		4420887	99.8
	2		4396759	99.3
		MEAN	**4408823**	**99.6**
		STD DEV	**17061**	**0.4**

Table A5.8: Stability of morphine suphate in PCAs, test day 17

		TEST DAY 19		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		4452758	
	2		4443345	
	3		4428557	
	4		4404567	
	5		4428980	
	6		4414363	
		MEAN	**4428762**	
		STD DEV	**17784**	
Std 0.2mg/ml	1			
	2		969719	
	3		969049	
		MEAN	**969384**	
		STD DEV	**474**	
A1	1		1050698	108.4
	2		1052856	108.6
		MEAN	**1051777**	**108.5**
		STD DEV	**1526**	**0.2**
A2	1		1040655	107.4
	2		1040075	107.3
		MEAN	**1040365**	**107.3**
		STD DEV	**410**	**0.0**
B1	1		1023942	105.6
	2		1024098	105.6
		MEAN	**1024020**	**105.6**
		STD DEV	**110**	**0.01**
B2	1		1020666	105.3
	2		1021720	105.4
		MEAN	**1021193**	**105.3**
		STD DEV	**745**	**0.08**
C1	1		4507925	101.8
	2		4503565	101.7
		MEAN	**4505745**	**101.7**
		STD DEV	**3083**	**0.07**
C2	1		4464111	100.8
	2		4479065	101.1
		MEAN	**4471588**	**101.0**
		STD DEV	**10574**	**0.24**
D1	1		4379521	98.9
	2		4385395	99.0
		MEAN	**4382458**	**99.0**
		STD DEV	**4154**	**0.09**
D2	1		4408545	99.5
	2		4408255	99.5
		MEAN	**4408400**	**99.5**
		STD DEV	**205**	**0.005**

Table A5.9: Stability of morphine sulphate in PCAs, test day 19

		TEST DAY 21		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		4438210	
	2		4420755	
	3		4427004	
	4		4390487	
	5		4431986	
	6		4440064	
		MEAN	**4424751**	
		STD DEV	**18238**	
Std 0.2mg/ml	1			
	2		968949	
	3		968168	
		MEAN	**968559**	
		STD DEV	**552**	
A1	1		1002481	103.5
	2		1001870	103.4
		MEAN	**1002176**	**103.5**
		STD DEV	**432**	**0.0**
A2	1		1020438	105.4
	2		1017632	105.1
		MEAN	**1019035**	**105.2**
		STD DEV	**1984**	**0.2**
B1	1		1020465	105.4
	2		1018250	105.1
		MEAN	**1019357.5**	**105.2**
		STD DEV	**1566**	**0.16**
B2	1		1018489	105.2
	2		1017822	105.1
		MEAN	**1018156**	**105.1**
		STD DEV	**472**	**0.05**
C1	1		4398835	99.4
	2		4398682	99.4
		MEAN	**4398759**	**99.4**
		STD DEV	**108**	**0.002**
C2	1		4450618	100.6
	2		4452069	100.6
		MEAN	**4451344**	**100.6**
		STD DEV	**1026**	**0.02**
D1	1		4357958	98.5
	2		4353402	98.4
		MEAN	**4355680**	**98.4**
		STD DEV	**3222**	**0.07**
D2	1		4339358	98.1
	2		4347606	98.3
		MEAN	**4343482**	**98.2**
		STD DEV	**5832**	**0.1**

Table A5.10: Stability of morphine sulphate in PCAs, test day 21

		TEST DAY 25		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		4416416	
	2		4447640	
	3		4447751	
	4		4452664	
	5		4453955	
	6		4454731	
		MEAN	**4445526**	
		STD DEV	**14585**	
Std 0.2mg/ml	1		971436	
	2		973750	
	3		986971	
		MEAN	**977386**	
		STD DEV	**8381**	
A1	1		1025962	105.0
	2		1013752	103.7
		MEAN	**1019857**	**104.3**
		STD DEV	**8634**	**0.9**
A2	1		1037955	106.2
	2		1039987	106.4
		MEAN	**1038971**	**106.3**
		STD DEV	**1437**	**0.1**
B1	1		977532	100.0
	2		977563	100.0
		MEAN	**977547.5**	**100.0**
		STD DEV	**22**	**0.00**
B2	1		975921	99.9
	2		978894	100.2
		MEAN	**977408**	**100.0**
		STD DEV	**2102**	**0.22**
C1	1		4500345	101.2
	2		4497948	101.2
		MEAN	**4499147**	**101.2**
		STD DEV	**1695**	**0.038**
C2	1		4492353	101.1
	2		4491134	101.0
		MEAN	**4491744**	**101.0**
		STD DEV	**862**	**0.02**
D1	1		4405077	99.1
	2		4400752	99.0
		MEAN	**4402915**	**99.0**
		STD DEV	**3058**	**0.07**
D2	1		4395310	98.9
	2		4395261	98.9
		MEAN	**4395261**	**98.9**
		STD DEV	**35**	**0.0**

Table A5.11: Stability of morphine sulphate in PCAs, test day 25

		TEST DAY 26		
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		4391455	
	2		4397163	
	3		4404369	
	4		4388609	
	5		4384857	
	6		4399272	
		MEAN	**4394288**	
		STD DEV	**7266**	
Std 0.2mg/ml	1		975318	
	2		962963	
	3		963794	
		MEAN	**967358**	
		STD DEV	**6906**	
A1	1		1019086	105.3
	2		1020214	105.5
		MEAN	**1019650**	**105.4**
		STD DEV	**798**	**0.1**
A2	1		1012872	104.7
	2		1011890	104.6
		MEAN	**1012381**	**104.7**
		STD DEV	**694**	**0.1**
B1	1		973035	100.6
	2		973250	100.6
		MEAN	**973142.5**	**100.6**
		STD DEV	**152**	**0.02**
B2	1		971007	100.4
	2		969372	100.2
		MEAN	**970190**	**100.3**
		STD DEV	**1156**	**0.12**
C1	1		4532304	103.1
	2		4523199	102.9
		MEAN	**4527752**	**103.0**
		STD DEV	**6438**	**0.147**
C2	1		4403689	100.2
	2		4410742	100.4
		MEAN	**4407216**	**100.3**
		STD DEV	**4987**	**0.11**
D1	1		4343573	98.8
	2		4336422	98.7
		MEAN	**4339998**	**98.8**
		STD DEV	**5057**	**0.12**
D2	1		4428352	100.8
	2		4433678	100.9
		MEAN	**4431015**	**100.8**
		STD DEV	**3766**	**0.1**

Table A5.12: Stability of morphine sulphate in PCAs, test day 26

		TEST DAY 28		
SAMPLE	**INJ**		**PEAK AREA**	**% CONTENT**
Std 1mg/ml	1		4398687	
	2		4386938	
	3		4380895	
	4		4398390	
	5		4385939	
	6		4406009	
		MEAN	**4392810**	
		STD DEV	**9629**	
Std 0.2mg/ml	1		964928	
	2		972640	
	3		968213	
		MEAN	**968594**	
		STD DEV	**3870**	
A1	1		989477	102.2
	2		989315	102.1
		MEAN	**989396**	**102.1**
		STD DEV	**115**	**0.01**
A2	1		990842	102.3
	2		993724	102.6
		MEAN	**992283**	**102.4**
		STD DEV	**2038**	**0.2**
B1	1		973834	100.5
	2		975049	100.7
		MEAN	**974441.5**	**100.6**
		STD DEV	**859**	**0.09**
B2	1		982156	101.4
	2		982353	101.4
		MEAN	**982255**	**101.4**
		STD DEV	**139**	**0.01**
C1	1		4393690	100.0
	2		4391661	100.0
		MEAN	**4392676**	**100.0**
		STD DEV	**1435**	**0.03**
C2	1		4438801	101.0
	2		4439950	101.1
		MEAN	**4439376**	**101.1**
		STD DEV	**812**	**0.0**
D1	1		4370680	99.5
	2		4366452	99.4
		MEAN	**4368566**	**99.4**
		STD DEV	**2990**	**0.07**
D2	1		4413955	100.5
	2		4416336	100.5
		MEAN	**4415146**	**100.5**
		STD DEV	**1684**	**0.0**

Table A5.13: Stability of morphine sulphate in P.C.As, test day 28

APPENDIX 6
TABLES OF DATA FOR STABILITY OF MORPHINE SULPHATE IN POLYPROPYLENE SYRINGES

TEST DAY 0				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		992561	
	2		991581	
	3		991175	
	4		988258	
	5		990024	
	6		992459	
		MEAN	**991010**	
		STD DEV	**1637**	
Std 0.2mg/ml	1			
	2		4401195	
	3		4417438	
		MEAN	**4409317**	
		STD DEV	**11486**	
A1	1		1018540	23.1
	2		1022971	23.2
		MEAN	**1020756**	**103.0**
		STD DEV	**3133**	**0.1**
A2	1		1011529	22.9
	2		1015591	23.0
		MEAN	**1013560**	**102.3**
		STD DEV	**2872**	**0.1**
B1	1		990144	22.5
	2		991987	22.5
		MEAN	**991066**	**100.0**
		STD DEV	**1303**	**0.03**
B2	1		988806	22.4
	2		994572	22.6
		MEAN	**991689**	**100.1**
		STD DEV	**4077**	**0.09**
C1	1		4320025	435.9
	2		4345540	438.5
		MEAN	**4332783**	**98.3**
		STD DEV	**18042**	**1.82**
C2	1		4338452	437.8
	2		4331987	437.1
		MEAN	**4335220**	**98.3**
		STD DEV	**4571**	**0.46**
D1	1		4352462	439.2
	2		4387595	442.7
		MEAN	**4370029**	**99.1**
		STD DEV	**24843**	**2.51**
D2	1		4405200	444.5
	2		4374473	441.4
		MEAN	**4389837**	**99.6**
		STD DEV	**21727**	**2.2**

Table A6.1: Stability of morphine sulphate in polypropylene syringes, test day 0

TEST DAY 3				
SAMPLE	**INJ**		**PEAK AREA**	**% CONTENT**
Std 1mg/ml	1		975823	
	2		972262	
	3		971054	
	4		973010	
	5		972077	
	6		973021	
		MEAN	**972875**	
		STD DEV	**1616**	
Std 0.2mg/ml	1		4463613	
	2		4469821	
		MEAN	**4466717**	
		STD DEV	**4390**	
A1	1		1003834	22.5
	2		1006443	22.5
		MEAN	**1005139**	**103.3**
		STD DEV	**1845**	**0.04**
A2	1		1004981	22.5
	2		1006330	22.5
		MEAN	**1005656**	**103.4**
		STD DEV	**954**	**0.0**
B1	1		985096	22.1
	2		983361	22.0
		MEAN	**984229**	**101.2**
		STD DEV	**1227**	**0.03**
B2	1		985184	22.1
	2		986744	22.1
		MEAN	**985964**	**101.3**
		STD DEV	**1103**	**0.02**
C1	1		4310010	443.0
	2		4322461	444.3
		MEAN	**4316236**	**96.6**
		STD DEV	**8804**	**0.90**
C2	1		4359734	448.1
	2		4376364	449.8
		MEAN	**4368049**	**97.8**
		STD DEV	**11759**	**1.21**
D1	1		4408955	453.2
	2		4428234	455.2
		MEAN	**4418595**	**98.9**
		STD DEV	**13632**	**1.40**
D2	1		4386834	450.9
	2		4407151	453.0
		MEAN	**4396993**	**98.4**
		STD DEV	**14366**	**1.5**

Table A6.2: Stability of morphine sulphate in polypropylene syringes, test day 3

TEST DAY 6				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		984513	
	2		982504	
	3		980532	
	4		983039	
	5		984068	
	6		986571	
		MEAN	**983538**	
		STD DEV	**2039**	
Std 0.2mg/ml	1			
	2		4477561	
	3		4485958	
		MEAN	**4481760**	
		STD DEV	**5938**	
A1	1		1017067	22.7
	2		1017741	22.7
		MEAN	**1017404**	**103.4**
		STD DEV	**477**	**0.01**
A2	1		1004446	22.4
	2		1006401	22.5
		MEAN	**1005424**	**102.2**
		STD DEV	**1382**	**0.0**
B1	1		981266	21.9
	2		982720	21.9
		MEAN	**981993**	**99.8**
		STD DEV	**1028**	**0.02**
B2	1		976585	21.8
	2		981142	21.9
		MEAN	**978864**	**99.5**
		STD DEV	**3222**	**0.07**
C1	1		4401098	447.5
	2		4417922	449.2
		MEAN	**4409510**	**98.4**
		STD DEV	**11896**	**1.21**
C2	1		4403378	447.7
	2		4420299	449.4
		MEAN	**4411839**	**98.4**
		STD DEV	**11965**	**1.22**
D1	1		4399944	447.4
	2		4413189	447.36
		MEAN	**4399944**	**98.2**
		STD DEV	**9366**	**0.00**
D2	1		4410851	448.5
	2		4433434	450.8
		MEAN	**4422143**	**98.7**
		STD DEV	**15969**	**1.6**

Table A6.3: Stability of morphine sulphate in polypropylene syringes, test day 6

TEST DAY 8				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		986396	
	2		991880	
	3		992940	
	4		991320	
	5		991945	
	6		991835	
		MEAN	**991053**	
		STD DEV	**2341**	
Std 0.2mg/ml	1			
	2		4434078	
	3		4459291	
		MEAN	**4446685**	
		STD DEV	**17828**	
A1	1		1016970	22.9
	2		1024583	23.0
		MEAN	**1020777**	**103.0**
		STD DEV	**5383**	**0.1**
A2	1		1005017	22.6
	2		1008258	22.7
		MEAN	**1006638**	**101.6**
		STD DEV	**2292**	**0.1**
B1	1		996320	22.4
	2		997905	22.4
		MEAN	**997113**	**100.6**
		STD DEV	**1121**	**0.03**
B2	1		998272	22.4
	2		998773	22.5
		MEAN	**998523**	**100.8**
		STD DEV	**354**	**0.01**
C1	1		4286332	432.5
	2		4309907	434.9
		MEAN	**4298120**	**96.7**
		STD DEV	**16670**	**1.68**
C2	1		4283835	432.3
	2		4319186	435.8
		MEAN	**4301511**	**96.7**
		STD DEV	**24997**	**2.52**
D1	1		4372476	441.2
	2		4403120	444.3
		MEAN	**4387798**	**98.7**
		STD DEV	**21669**	**2.19**
D2	1		4383054	442.3
	2		4409086	444.9
		MEAN	**4396070**	**98.9**
		STD DEV	**18407**	**1.9**

Table A6.4: Stability of morphine sulphate in polypropylene syringes, test day 8

TEST DAY 10				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		981053	
	2		986393	
	3		985979	
	4		984816	
	5		986432	
	6		986168	
		MEAN	**985140**	
		STD DEV	**2088**	
Std 0.2mg/ml	1		4391669	
	2		4404829	
		MEAN	**4398249**	
		STD DEV	**9306**	
A1	1		1025075	23.3
	2		1027302	23.4
		MEAN	**1026189**	**104.2**
		STD DEV	**1575**	**0.0**
A2	1		1003170	22.8
	2		1004601	22.8
		MEAN	**1003886**	**101.9**
		STD DEV	**1012**	**0.0**
B1	1		983952	22.4
	2		983953	22.4
		MEAN	**983953**	**99.9**
		STD DEV	**1**	**0.00**
B2	1		982879	22.3
	2		984159	22.4
		MEAN	**983519**	**99.8**
		STD DEV	**905**	**0.02**
C1	1		4281063	434.6
	2		4298446	436.3
		MEAN	**4289755**	**97.5**
		STD DEV	**12292**	**1.25**
C2	1		4357725	442.3
	2		4359213	442.5
		MEAN	**4358469**	**99.1**
		STD DEV	**1052**	**0.11**
D1	1		4404564	447.1
	2		4411570	447.8
		MEAN	**4408067**	**100.2**
		STD DEV	**4954**	**0.50**
D2	1		4390219	445.6
	2		4396977	446.3
		MEAN	**4393598**	**99.9**
		STD DEV	**4779**	**0.49**

Table A6.5: Stability of morphine sulphate in polypropylene syringes, test day 10

TEST DAY 13				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		969297	
	2		975508	
	3		973693	
	4		976438	
	5		972454	
	6		973349	
		MEAN	**973457**	
		STD DEV	**2509**	
Std 0.2mg/ml	1			
	2		4329571	
	3		4337673	
		MEAN	**4333622**	
		STD DEV	**5729**	
A1	1		1008834	23.3
	2		1011031	23.3
		MEAN	**1009933**	**103.7**
		STD DEV	**1554**	**0.0**
A2	1		980441	22.6
	2		983304	22.7
		MEAN	**981873**	**100.9**
		STD DEV	**2024**	**0.0**
B1	1		969549	22.4
	2		971851	22.4
		MEAN	**970700**	**99.7**
		STD DEV	**1628**	**0.04**
B2	1		968519	22.3
	2		973289	22.5
		MEAN	**970904**	**99.7**
		STD DEV	**3373**	**0.08**
C1	1		4256994	437.3
	2		4287743	440.5
		MEAN	**4272369**	**98.6**
		STD DEV	**21743**	**2.234**
C2	1		4280608	439.7
	2		4313339	443.1
		MEAN	**4296974**	**99.2**
		STD DEV	**23144**	**2.38**
D1	1		4332232	445.0
	2		4369056	448.8
		MEAN	**4350644**	**100.4**
		STD DEV	**26039**	**2.67**
D2	1		4358140	447.7
	2		4367486	448.7
		MEAN	**4362813**	**100.7**
		STD DEV	**6609**	**0.7**

Table A6.6: Stability of morphine sulphate in polypropylene syringes, test day 13

TEST DAY 15				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		974962	
	2		973003	
	3		974523	
	4		972582	
	5		975056	
	6		976773	
		MEAN	**974483**	
		STD DEV	**1524**	
Std 0.2mg/ml	1		4328866	
	2		4345761	
		MEAN	**4337314**	
		STD DEV	**11947**	
A1	1		1010037	23.3
	2		1015191	23.3
		MEAN	**1010037**	**103.6**
		STD DEV	**3644**	**0**
A2	1		999320	23.0
	2		1001503	23.1
		MEAN	**1000412**	**102.7**
		STD DEV	**1544**	**0.0**
B1	1		982071	22.6
	2		986070	22.7
		MEAN	**984070.5**	**101.0**
		STD DEV	**2828**	**0.07**
B2	1		976305	22.5
	2		976521	22.5
		MEAN	**976413**	**100.2**
		STD DEV	**153**	**0.00**
C1	1		4335205	444.9
	2		4342264	445.6
		MEAN	**4338735**	**100.0**
		STD DEV	**4991**	**0.51**
C2	1		4303833	441.7
	2		4322546	443.6
		MEAN	**4313190**	**99.4**
		STD DEV	**13232**	**1.4**
D1	1		4359914	447.4
	2		4383038	449.8
		MEAN	**4371476**	**100.8**
		STD DEV	**16351**	**1.68**
D2	1		4404240	452.0
	2		4428627	454.5
		MEAN	**4416434**	**101.8**
		STD DEV	**17244**	**1.8**

Table A6.7: Stability of morphine sulphate in polypropylene syringes, test day 15

TEST DAY 17				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		986823	
	2		984395	
	3		988058	
	4		985817	
	5		988546	
	6		989554	
		MEAN	**987199**	
		STD DEV	**1898**	
Std 0.2mg/ml	1			
	2		4411688	
	3		4361712	
		MEAN	**4386700**	
		STD DEV	**35338**	
A1	1		1020791	23.3
	2		1023904	23.3
		MEAN	**1022348**	**103.6**
		STD DEV	**2201**	**0.1**
A2	1		1012724	23.1
	2		1015562	23.2
		MEAN	**1014143**	**102.7**
		STD DEV	**2007**	**0.0**
B1	1		985163	22.5
	2		991326	22.6
		MEAN	**988245**	**100.1**
		STD DEV	**4358**	**0.10**
B2	1		992509	22.6
	2		994808	22.7
		MEAN	**993659**	**100.7**
		STD DEV	**1626**	**0.04**
C1	1		4325153	438.1
	2		4353397	441.0
		MEAN	**4339275**	**98.9**
		STD DEV	**19972**	**2.02**
C2	1		4335805	439.2
	2		4379022	443.6
		MEAN	**4357414**	**99.3**
		STD DEV	**30559**	**3.10**
D1	1		4405410	446.3
	2		4418236	447.6
		MEAN	**4411823**	**100.6**
		STD DEV	**9069**	**0.92**
D2	1		4444275	450.2
	2		4460758	451.9
		MEAN	**4452517**	**101.5**
		STD DEV	**11655**	**1.2**

Table A6.8: Stability of morphine sulphate in polypropylene syringes, test day 17

TEST DAY 21				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		1032678	
	2		1027748	
	3		1028795	
	4		1026571	
	5		1026938	
	6		1026319	
		MEAN	**1028175**	
		STD DEV	**2383**	
Std 0.2mg/ml	1			
	2		4558065	
	3		4492168	
		MEAN	**4525117**	
		STD DEV	**46596**	
A1	1		1047484	23.1
	2		1050459	23.2
		MEAN	**1048972**	**102.0**
		STD DEV	**2104**	**0.0**
A2	1		1048410	23.2
	2		1051720	23.2
		MEAN	**1050065**	**102.1**
		STD DEV	**2341**	**0.1**
B1	1		1017720	22.5
	2		1019571	22.5
		MEAN	**1018646**	**99.1**
		STD DEV	**1309**	**0.03**
B2	1		1015894	22.5
	2		1018620	22.5
		MEAN	**1017257**	**98.9**
		STD DEV	**1928**	**0.04**
C1	1		4456753	433.5
	2		4466850	434.4
		MEAN	**4461802**	**98.6**
		STD DEV	**7140**	**0.69**
C2	1		4485648	436.3
	2		4499184	437.6
		MEAN	**4492416**	**99.3**
		STD DEV	**9571**	**0.93**
D1	1		4521189	439.7
	2		4533260	440.9
		MEAN	**4527225**	**100.0**
		STD DEV	**8535**	**0.83**
D2	1		4557323	443.2
	2		4570958	444.6
		MEAN	**4564141**	**100.9**
		STD DEV	**9641**	**0.938**

Table A6.9: Stability of morphine sulphate in polypropylene syringes, test day 21

TEST DAY 22				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		936086	
	2		938132	
	3		939789	
	4		937563	
	5		938825	
	6		940494	
		MEAN	**938482**	
		STD DEV	**1586**	
Std 0.2mg/ml	1			
	2		4369298	
	3		4361149	
		MEAN	**4365224**	
		STD DEV	**5762**	
A1	1		962427	22.0
	2		962394	22.0
		MEAN	**962411**	**102.5**
		STD DEV	**23**	**0.0**
A2	1		953842	21.9
	2		954512	21.9
		MEAN	**954177**	**101.7**
		STD DEV	**474**	**0.0**
B1	1		948860	21.7
	2		953021	21.8
		MEAN	**950940.5**	**101.3**
		STD DEV	**2942**	**0.07**
B2	1		965062	22.1
	2		969918	22.2
		MEAN	**967490**	**103.1**
		STD DEV	**3434**	**0.08**
C1	1		4262521	454.2
	2		4295636	457.7
		MEAN	**4279079**	**98.0**
		STD DEV	**23416**	**2.495**
C2	1		4301715	458.4
	2		4334017	461.8
		MEAN	**4317866**	**98.9**
		STD DEV	**22841**	**2.43**
D1	1		4505310	480.1
	2		4542286	484.0
		MEAN	**4523798**	**103.6**
		STD DEV	**26146**	**2.79**
D2	1		4370443	465.7
	2		4396137	468.4
		MEAN	**4383290**	**100.4**
		STD DEV	**18168**	**1.9**

Table A6.10: Stability of morphine sulphate in polypropylene syringes, test day 22

TEST DAY 24				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		985753	
	2		988836	
	3		986857	
	4		987913	
	5		986726	
	6		985391	
		MEAN	**986913**	
		STD DEV	**1296**	
Std 0.2mg/ml	1		4412428	
	2		4429292	
		MEAN	**4420860**	
		STD DEV	**11925**	
A1	1		1034458	23.4
	2		1034512	23.4
		MEAN	**1034485**	**104.8**
		STD DEV	**38**	**0.0**
A2	1		1021254	23.1
	2		1001905	22.7
		MEAN	**1011580**	**102.5**
		STD DEV	**13682**	**0.3**
B1	1		982658	22.2
	2		985371	22.3
		MEAN	**984014.5**	**99.7**
		STD DEV	**1918**	**0.04**
B2	1		978211	22.1
	2		983212	22.2
		MEAN	**980712**	**99.4**
		STD DEV	**3536**	**0.08**
C1	1		4302791	436.0
	2		4317768	437.5
		MEAN	**4310280**	**97.5**
		STD DEV	**10590**	**1.073**
C2	1		4346845	440.4
	2		4357631	441.5
		MEAN	**4352238**	**98.4**
		STD DEV	**7627**	**0.77**
D1	1		4398939	445.7
	2		4419480	447.8
		MEAN	**4409210**	**99.7**
		STD DEV	**14525**	**1.47**
D2	1		4370398	443.6
	2		4377975	443.6
		MEAN	**4377975**	**99.0**
		STD DEV	**5358**	**0.0**

Table A6.11: Stability of morphine sulphate in polypropylene syringes, test day 24

TEST DAY 28				
SAMPLE	INJ		PEAK AREA	% CONTENT
Std 1mg/ml	1		957741	
	2		965107	
	3		964273	
	4		964143	
	5		963480	
	6		962245	
		MEAN	**962832**	
		STD DEV	**2670**	
Std 0.2mg/ml	1			
	2		4388065	
	3		4401086	
		MEAN	**4394576**	
		STD DEV	**9207**	
A1	1		998254	22.7
	2		997804	22.7
		MEAN	**998029**	**103.7**
		STD DEV	**318**	**0.0**
A2	1		1004761	22.9
	2		1005259	22.9
		MEAN	**1005010**	**104.4**
		STD DEV	**352**	**0.0**
B1	1		964207	21.9
	2		967578	22.0
		MEAN	**965892.5**	**100.3**
		STD DEV	**2384**	**0.05**
B2	1		961839	21.9
	2		966518	22.0
		MEAN	**964179**	**100.1**
		STD DEV	**3309**	**0.08**
C1	1		4242734	440.7
	2		4260110	442.5
		MEAN	**4251422**	**96.7**
		STD DEV	**12287**	**1.276**
C2	1		4314859	448.1
	2		4322199	448.9
		MEAN	**4318529**	**98.3**
		STD DEV	**5190**	**0.54**
D1	1		4343696	451.1
	2		4366051	453.5
		MEAN	**4354874**	**99.1**
		STD DEV	**15807**	**1.64**
D2	1		4335301	450.3
	2		4354081	452.2
		MEAN	**4344691**	**98.9**
		STD DEV	**13279**	**1.4**

Table A6.12: Stability of morphine sulphate in polypropylene syringes, test day

APPENDIX 7
UV SPECTRUM OF MORPHINE SULPHATE AND HALOPERIDOL IN MOBILE PHASE