Development and Validation of Bioanalytical Assay Methods for Sildenafil in Human Plasma

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List of abbreviations

AOAC	Association of Official Analytical Chemists.
ASTED	Automated Trace Enrichment Dialysates.
CGMP	Cyclic Guanosine MonoPhosphate.
CRM	Certified Reference Material.
CV	Coefficient of Variance.
DAD	Photodiode Array Detector.
DSC	Differential Scanning Calorimetry.
ED	Erectile Dysfunction.
FDA	US Food and Drug Administration.
GC	Gas Chromatography
GTP	Guanosin Triphosphate.
HPLC	High Performance Liquid Chromatography.
ICH	International Conference on Harmonisation.
ISO	International Standard Organisation.
IUPAC	International Union of Pure and Applied Chemistry.
LC-MS/MS	Liquid Chromatography –Mass Spectrometry.
LLE	Liquid – Liquid Extraction.
LLOD	Lower Limit Of Detection.
LLOQ	Lower Limit OF Quantification.
MS	Mass Spectrometry.
NDA	New Drug Application.
PDE	Phosphodiesterase.
QC	Quality Control.
RSD	Relative Standard Deviation.
SFSTP	Societe Francaise Des Sciences et Techniques Pharmaceutiques.

SLD	Sildenafil.
SOP	Standard Operating Procedures.
SPE	Solid Phase Extraction.
SPV	System Performance Verification.
SRM	Standard Reference Material.
STD	Standard.
TRZ	Trazodone
USP	US Pharmacopoeia.
UV	Ultra Violet.

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INTRODUCTION AND OBJECTIVES

Pharmacokinetic and bio-equivalency studies require very precise and accurate assay methods that are well validated to quantify drugs in biological samples. The assay methods have to be sensitive enough to determine the biological sample concentration of the drug and/or its metabolite(s) for a period of about five elimination half-lifes after dosage of the drug. The assay methods also have to be very selective to ensure reliable data, free from interference of endogenous compounds and possible metabolites in the biological samples. In addition, methods have to be as robust and cost effective as possible, making of particular importance to bioequivalent studies. Above all, the assay methods must be able to withstand the scrutiny of national drug registration authorities who judge them on the basis of criteria established by international consensus.

Currently there is a need in the pharmaceutical environment to develop analytical methods for the determination of sildenafil in human plasma. The aim will be to achieve more selectivity, sensitivity and more rapid assay methods than have been previously described. The developed method could then be applied to clinical trials to obtain accurate pharmacokinetic parameters in human plasma.

HPLC-UV, LC-MS / MS, and GC-MS methods have been reported. Some of these methods use complicated extraction instruments, long and tedious extraction procedures, and large amounts of solvents or biological fluids for extraction while other methods have a long turn-around time during analysis.

The main objective of this work is to develop rapid, selective and sensitive HPLC-UV and LC-MS / MS methods that have short and simple extraction procedures, consume small amounts of solvent and biological fluid for extraction and a short turn-around time.

1 LITERATURE REVIEW

1.1 METHOD DEVELOPMENT AND VALIDATION.

INTRODUCTION

Bioanalytical chemistry is the qualitative and quantitative analysis of drug substances in biological fluids (mainly plasma and urine) or tissue. It plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data (Bressolle *et al.*, 1996). The main analytical phases that comprise bioanalytical services are, method development, method validation and sample analysis (method application).

Owing to increased interdependence among countries in recent times it has become necessary for results of many analytical methods to be accepted internationally. Consequently, to assure a common level of quality, the need for and use of validated methods has increased (Hartmann *et al.*, 1998).

Analytical methods are used for product research, product development, process control and chemical quality control purposes. Each of the techniques used, chromatographic or spectroscopic, have their own special features and deficiencies, which must be considered. Whatever way the analysis is done it must be checked to see whether it does what it was intended to do; i.e. it must be validated. Each step in the method must be investigated to determine the extent to which environment, matrix, or procedural variables can affect the estimation of analyte in the matrix from the time of collection up to the time of analysis (Anonymous, May 2001).

A full validation requires a high workload and should therefore only start when promising results are obtained from explorative validation performed during the method development phase. The process of validating a method cannot be separated from the actual development of method conditions, because the developer will not know whether the method conditions

are acceptable until validation studies are performed (Green, 1996). Method development clears the way for the further processes on the validation stage. It must be recognised that proper validation requires a lot of work. However, this effort is repaid by the time saved when running the method routinely during sample analysis.

1.1.1 Method development

A bioanalytical method is a set of all of the procedures involved in the collection, processing, storing, and analysis of a biological matrix for an analyte (Shah *et al.*, 1992). Analytical methods employed for quantitative determination of drugs and their metabolites in biological fluids are the key determinants in generating reproducible and reliable data that in turn are used in the evaluation and interpretation of bioavailability, bioequivalency and pharmacokinetics (Shah *et al.*, 2000).

Method development involves evaluation and optimisation of the various stages of sample preparation, chromatographic separation, detection and quantification. To start these work an extensive literature survey, reading work done on the same or similar analyte and summarising main starting points for future work is of primary importance. Based on the information from this survey, the following can be done.

- The choice of instrument that is suitable for the analysis of your analyte of interest. This includes the choice of the column associated with your instrument of choice, the detector, the mobile phase in the high performance liquid chromatography (HPLC), and the choice of carrier in gas chromatography (GC).
- Choice of internal standard, which is suitable for your study. It must have similar chromatographic properties to your analyte.
- Choice of extraction procedure, which is time economical, gives the highest possible recovery without interference at the elution time of the analyte of interest and has acceptable accuracy and precision.

Method performance is determined primarily by the quality of the procedure itself. The two factors that are most important in determining the quality of the method are selective recovery and standardisation. Analytical recovery of a method refers to whether the analytical method in question provides response for the entire amount of analyte that is contained in a sample. Recovery is usually defined as the percentage of the reference material that is measured, to that which is added to a blank. This should not be confused with the test of matrix effect in which recovery is defined as the response measured from the matrix (e.g.

plasma) as a percentage of that measured from the pure solvent (e.g. water). Results of the experiment that compare matrix to pure solvent is referred to as relative recovery and true test of recovery is referred to as absolute recovery (Karnes *et al.*, 1991).

Another important issue in method development stage is the choice of internal versus external standardisation. Internal standardisation is common in bioanalytical methods especially with chromatographic procedures. The assumption for the use of internal standard is that the partition coefficient of the analyst and the internal standard are very similar (Karnes *et al.*, 1991). For internal standardisation, a structural or isotopic analogue of the analyte is added to the sample prior to sample pre-treatment and the ratio of the response of the analyte to that of the internal standard is plotted against the concentration (Causon, 1997).

Another important point is that the tests performed at the stage of method development should be done with the same equipment that will actually be used for subsequent routine analysis. The differences found between individual instruments representing similar models from the same manufacturer is not surprising and should be accounted for (Bruce *et al.*, 1998).

The following two parameters must be determined at the method development stage as they are the benchmark for further work.

1.1.1.1 Limits of detection and quantification (LLOD and LLOQ)

The US pharmacopoeia (USP) defines the limit of detection (LLOD) as the lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated. They also define the lowest limit of quantification (LLOQ), as the lowest amount of a sample that can be determined (quantitated) with acceptable precision and accuracy under the stated operational condition of the method (Krull & Swartz, 1998).

The limits are commonly associated with the signal to noise ratio (S/N). In the case of LLOD, analysts often use S/N (signal to noise ratio) of 2:1 or 3:1, while a S/N of 10:1 is often considered to be necessary for the LLOQ. Typically the signal is measured from the base line to peak apex and divided by the peak-to-peak noise, which is determined from the blank plasma injection.

The ICH Q2B (international conference on harmonisation) guideline on validation methodology lists two options in addition to the S/N method of determining limits of detection and quantification: visual non-instrumental methods and limit calculations. The calculation is based on the standard deviation of the response (σ) and the slope of the

calibration curve (S) at levels approaching the limits according to equations below (Krull & Swartz, 1998). .

$$LLOD = 3.3 \times \left(\frac{s}{S}\right) \quad (1.1)$$

$$LLOQ = 10 \times \left(\frac{s}{S}\right) \quad (1.2)$$

The standard deviation of the response can be determined based on the standard deviation of the blank, based on the residual standard deviation of the regression line, or the standard deviation of the y-intercept of the regression line. This method can reduce the bias that sometimes occurs when determining the S/N. The bias can result because of difference in opinion about how to determine and measure noise.

1.1.1.2 Calibration line

A calibration line is a curve showing the relation between the concentration of the analyte in the sample and the detected response. It is necessary to use a sufficient number of standards to define adequately the relationship between response and concentration. The relationship between response and concentration must be demonstrated to be continuous and reproducible. The number of standards to be used will be a function of the dynamic range and nature of the concentration-response relationship. In many cases, five to eight concentrations (excluding blank values) may define the standard curve. More standard concentrations may be necessary for non-linear relationships than would be for a linear relationship (Shah *et al.*, 1992).

The difference between the observed y-value and fitted y-value is called a residual. One of the assumptions involved in linear regression analysis is that the calculated residuals are independent, are normally distributed and have equal variance, which is termed as homoscedasticity. If the variance is not equal, the case is termed as heteroscedasticity, in which case a weighted regression may be performed. The most appropriate weighting factor is the inverse of the variance of the standard, although $1/x$, $1/x^2$,$1/y$ and $1/y^2$ (x = concentration and y = response) are suitable approximations (Lang & Bolton, 1991).

It is important to use a standard curve that will cover the entire range of the concentration of the unknown samples. Estimation of the unknown by extrapolation of standard curve below the lower standard and above the higher standard is not recommended. Instead, it is suggested

that the standard curve be re-determined or sample re-assayed after dilution (Shah *et al.*, 1992).

According to Dadgar *et al*. (1995), the following guidelines can be used for inclusion and exclusion of points from the calibration curve. Provided that the calibration curve consists of at least seven non-zero single standards, up to two non-zero standards may be removed from the calibration if at least one of the following valid reasons exists and a minimum of five non-zero standards remains in the curve.

- Loss of sensitivity.
- Poor chromatography.
- Loss during sample processing.
- If, when included in the calibration curve, it clearly biases the QC result, and the back-calculated standards concentration deviates substantially from its nominal value.

Acceptability of the linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of > 0.999 is generally considered as evidence of an acceptable fit of the data to the regression line (Green, 1996).

1.1.2 Method validation

The search for the reliable range of a method and continuous application of this knowledge is called validation (Bruce *et al*., 1998). It can also be defined as the process of documenting that the method under consideration is suitable for its intended purpose (Hartmann *et al.*, 1998). Method validation involves all the procedures required to demonstrate that a particular method for quantitative determination of the concentration of an analyte (or a series of analytes) in a particular biological matrix is reliable for the intended application (Shah *et al.*, 1992). Validation is also a proof of the repeatability, specificity and suitability of the method.

Bioanalytical methods must be validated if the results are used to support the registration of a new drug or a new formulation of an existing one. Validation is required to demonstrate the performance of the method and reliability of analytical results (Wieling *et al.*, 1996). If a bioanalytical method is claimed to be for quantitative biomedical application, then it is important to ensure that a minimum package of validation experiments has been conducted and yields satisfactory results (Causon, 1997).

Before discussing how to carry out the validation experiment, it is important to stress that validation in bioanalysis should not be considered as an isolated field. A consensus on common terminology for all analytical fields is therefore required. For the moment it is not yet possible to propose a validation terminology that is also in agreement with the recommendations of important international organisations such as the ISO (International Standard Organisation), IUPAC (International Union of Pure and Applied Chemistry) and AOAC (Association of Official Analytical Chemists), since differences exist between their documents (Hartmann *et al.*, 1998).

For the validation of pharmaceutical drug formulations the discussion on a consensus terminology is relatively advanced. It is suggested to follow in general the proposal elaborated for the validation of drug formulation by the joint initiative of the pharmaceutical industry and the regulatory agencies of the three major regulatory authorities (the European Union, the USA and Japan), the International Conference on Harmonisation (ICH). According to them the revised version of terminology to be included are bias (accuracy), precision, specificity, limit of detection, limit of quantification, linearity, range and stability. The term stability is also specifically considered in the validation strate gy for bioanalytical methods, which is prepared by the French group SFSTP (Societe Francaise des Sciences et Techniques Pharmaceutiques) (Hartmann *et al.*, 1998).

On the other hand the guideline for industry by FDA (Anoymous, May 2001) states that the fundamental parameters of validation parameters for a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility and stability. Typical method development and establishment for bioanalytical method includes determination of (1) selectivity, (2) accuracy, (3) precision, (4) recovery, (5) calibration curve, and (6) stability. For a bioanalytical method to be considered valid, specific acceptance criteria should be set in advance and achieved for accuracy and precision for the validation of the QC samples.

Validations are subdivided into the following three categories:

Full validation

This is the validation performed when developing and implementing a bioanalytical method for the first time. Full validation should be performed to support pharmacokinetic, bioavailability, and bioequivalence and drug interaction studies in a new drug application (NDA) (Shah *et al.*, 2000).

Partial validation

Partial validations are performed when modifications of already validated bioanalytical methods are made. Partial validation can range from as little as one intra-assay and precision determination to a nearly full validation. Some of the typical bioanalytical method changes that fall into this category include, bioanalytical method transfer between laboratories or analyst, change in analytical methodology, change of matrix within species, change of species within matrix (Shah *et al*., 2000; Anonymous, May 2001). The decision of which parameters to be revalidated depend on the logical consideration of the specific validation parameters likely to be affected by the change made to the bioanalytical method.

Cross validation

Cross validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross validation would be a situation when the original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator (Shah *et al.*, 2000; Anonymous, May 2001).

1.1.2.1 Selectivity

A method is said to be specific if it produces a response for only a single analyte. Method selectivity is the ability of a method to produce a response for the target analyte distinguishing it from all other interferences. Interferences in biological samples arise from a number of endogenous (analyte metabolite, degradation products, co-administered drugs and chemicals normally accruing in biological fluids) and exogenous sources (impurities in reagents and dirty lab-ware). Zero level interference of the analyte is desired, but it is hardly ever the case. The main thing one must take care of is that, the response of the LLOQ standards should be greater than the response from the blank biological matrix by a defined factor as discussed in section 1.1.1 above. If all the efforts to get rid of interferences in the chromatographic process fail, changing to a more selective detector such as Mass Spectrometry (MS) or MS-MS may give a better result (Dadgar & Burnett, 1995).

According to Dadgar and Burnett (1995), the following practical approach may be used during method development to investigate the selectivity of an analytical method.

- Processing blank samples from different sources will help to demonstrate lack of interference from substances native to the biological sample but not from the analyte metabolite.

- If analyte concentration is sufficiently high, and the chromophores differ sufficiently, the use of photodiode array (DAD) or scanning UV detection under the regular condition can give evidence of peak purity.
- Potential metabolites can be produced *in vitro* by incubation with liver homogenates, and chromatographed to check for potential interference with the analyte of interest.
- Processing of reagent blank in the absence of biological matrix is normally adequate to demonstrate selectivity with regard to exogenous interferences mentioned above.

Although it would be preferable that all tested blanks, if obtained under controlled conditions, be free from interferences, factors like food and beverage intake and cigarette smoking can affect selectivity (Dadgar *et al.*, 1995).

According to Shah *et al.* (1992), the Washington conference on 'Analytical Methods Validation' recommended evaluation of a minimum of six matrix sources to approve the selectivity of the method.

1.1.2.2 Precision

The precision of a bioanalytical method is a measure of random error and is defined as the agreement between replicate measurements of the same sample. It is expressed as the percentage coefficient of variance (% CV) or relative standard deviation (R.S.D.) of the replicate measurements (Causon, 1997).

$$\% \text{ CV} = \left(\frac{s\tan dard\ deviation}{Mean} \right) \text{x } 100 \qquad (1.3)$$

1.1.2.2.1 Intra-Assay Precision

This is also known as repeatability i.e. the ability to repeat the same procedure with the same analyst, using the same reagent and equipment in a short interval of time, e.g. within a day and obtaining similar results.

1.1.2.2.2 Inter-Assay Precision

The ability to repeat the same method under different conditions, e.g. change of analyst, reagent, or equipment; or on subsequent occasions, e.g. over several weeks or months, is covered by the between batch precision or reproducibility, also known as inter-assay-precision. The reproducibility of a method is of prime interest to the analyst since this will give a better representation of the precision during routine use as it includes the variability from a greater number of sources (Causon, 1997).

A minimum of three concentrations in the range of expected concentrations is recommended. The %CV determined at each concentration level, should not exceed 15 % except for the LLOQ, where it should not exceed 20 % (Anonymous, May 2001).

1.1.2.3 Accuracy

The accuracy of a bioanalytical method is a measure of the systematic error or bias and is defined as the agreement between the measured value and the true value. Accuracy is best reported as percentage bias that is calculated from the expression:

$$\% \text{ Bias} = \left(\frac{measured value - true value}{true value} \right) \text{x } 100 \qquad (1.4)$$

Some of the possible error sources causing biased measurement are: error in sampling, sample preparation, preparation of calibration line and sample analysis. The method accuracy can be studied by comparing the results of a method with results obtained, by analysis of certified reference material (CRM) or standard reference material (SRM).

Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15 % of the actual value except at LLOQ, where it should not deviate by more than 20 % (Anonymous, May 2001).

1.1.2.4 Recovery

Absolute recovery of a bioanalytical method is the measured response of a processed spiked matrix standard expressed as a percentage of the response of a pure standard, which has not been subjected to sample pre-treatment and indicates whether the method provides a response for the entire amount of the analyte that is present in the sample (Bressolle *et al.*, 1996).

$$\text{Absolute recovery} = \left(\frac{response\ of\ spiked\ plasma\ (processed)}{response\ of\ standard\ solution\ (unprocessed)} \right) \text{x } 100 \qquad (1.5)$$

The matrix effect can also be studied by comparing the response of extracted samples spiked before extraction with the response of the extracted blank matrix sample to which analyte has been added at the same nominal concentration just before injection (Causon, 1997). Good precision and accuracy can be obtained from methods with moderate recoveries, provided they have adequate sensitivity. Indeed it may be desirable to intentionally sacrifice high recovery in order to achieve better selectivity with some sample extraction procedure. Solvents such as ethyl acetate normally give rise to high recovery of analyte, however these

solvents simultaneously extract many interfering compounds. Therefore, provided that an adequate sensitive detection limit is attained with good precision and accuracy, the extent of recovery should not be considered an issue in bioanalytical method development and validation (Dadgar *et al.*, 1995).

1.1.2.5 Stability

The stability of the analyte is often critical in biological samples even over a short period of time. Degradation is not unusual even when all precautions are taken to avoid specifically known stability problems of the analyte (e.g. light sensitivity). It is therefore important to verify that there is not sample degradation between the time of collection of the sample and their analysis that would compromise the result of the study. Stability evaluation is done to show that the concentration of analyte at the time of analysis corresponds to the concentration of the analyte at the time of sampling (Hartmann *et al.*, 1998).

An essential aspect of method validation is to demonstrate that analyte(s) is (are) stable in the biological matrix and in all solvents encountered during the sample work-up process, under the conditions to which study samples will be subjected (Dadgar & Burnett, 1995).

According to the recommendations on the Washington conference report by Shah *et al.* (1992), the stability of the analyte in matrix at ambient temperature should be evaluated over a time that encompasses the duration of typical sample preparation, sample handling and analytical run time. Similarly Dagar & Brunett (1995) gave the following details to be investigated.

1.1.2.5.1 Long term stability

This is done to assess whether the analyte is stable in the plasma matrix under the sample storage conditions for the time period required for the samples generated in a clinical study to be analysed.

1.1.2.5.2 Standard stock solution stability

The stability test for the standard stock solution must be done at the same temperature, container and solvent as that to be used for the study. The time period should be at least 6 hours.

1.1.2.5.3 Short term matrix stability

This must be evaluated following the storage under laboratory conditions used for sample work-up for a period of e.g. 6 h to 24 h, and compared with data from the same samples prepared and analysed without delay.

1.1.2.5.4 On-instrument sample stability

This should be evaluated over the maximum time from completion of sample work-up to completion of data collection, with allowance for potential delay in analysis due to equipment failure. This stability study is conducted at the temperature at which processed study samples will be held prior to data collection.

1.1.2.5.5 Freeze -thaw stability

This stability test is done to ensure that the sample remains stable after it is subjected to multiple freeze-thaw cycles in the process of the study. This can be done by thawing samples at high, medium and low concentrations unassisted and allowing them to froze again for at least 12-24 hrs. The cycle is repeated twice and the sample is processed at the end of the third cycle and its result is compared with freshly prepared sample. If the analyte is not stable after three cycles, measures must be taken to store adequate amounts of aliquots to permit repeats, without having to freeze and thaw the sample more than once.

Acceptable stability is 2 % change in standard solution or sample solution response relative to freshly prepared standard. Acceptable stability at the LLOQ for standard solution and sample solution is 20 % change in response relative to a freshly prepared sample (Green, 1996).

1.1.2.6 Sensitivity

According to IUPAC as cited in Roger Causon, (Causon, 1997), a method is said to be sensitive if small changes in concentration cause large changes in the response function. Sensitivity can be expressed as the slope of the linear regression calibration curve, and it is measured at the same time as the linearity tests. The sensitivity attainable with an analytical method depends on the nature of the analyte and the detection technique employed (Bruce *et al.,* 1998). The sensitivity required for a specific response depends on the concentrations to be measured in the biological specimens generated in the specific study.

1.2 BACKGROUND INFORMATION ON SILDENAFIL

1.2.1 Introduction

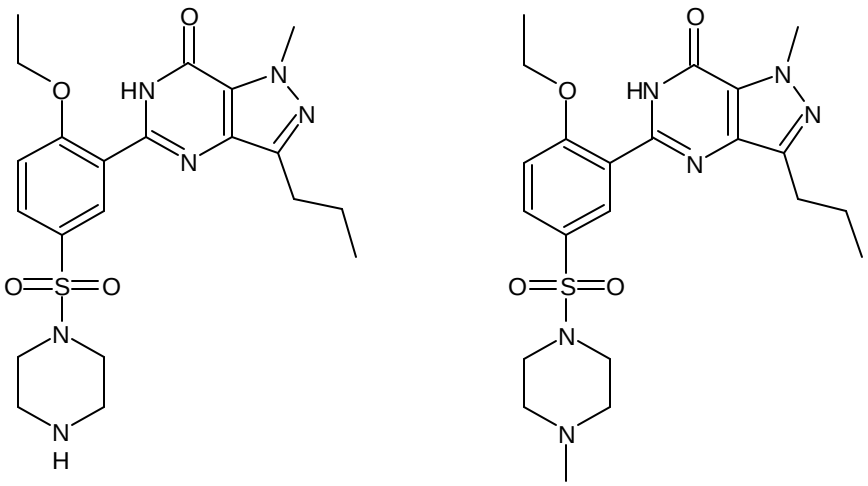
Sildenafil citrate marketed as Viagra™ (Pfizer) was approved as a drug for treating male erectile dysfunction (ED) by the US Food and Drug Administration on 27 March 1998. The release of this drug on the world market had a very large impact as could be judged by the attention the media gave it. This was due to the fact that the drug was a breakthrough for men suffering from ED. They represent a significant part of the male population: it is estimated that 10% of men suffer from erectile dysfunction, and that this figure is as much as 52 % for men between 40 and 70 years old. Sildenafil has been the fastest-selling drug in pharmaceutical history since its release in 1998. Viagra™ was discovered by a research team in the Pfizer Sandwich site in Kent (WWW.ch,ic.as.uk,Department).

Sildenafil is a potent and selective inhibitor of (type V)-specific phosphodiesterase (PDE 5) that is responsible for degradation of cyclic guanosine monophosphate (cGMP). Sildenafil has no direct relaxant effect on isolated human corpus cavernosum, but enhances the effect of nitric oxide by inhibiting PDE-5. Increase in the level of cGMP in the corpus cavernosum results in smooth muscle relaxation and inflow of blood to the corpus cavernosum, improving the penile erectile function (Anonymous, 1998).

Accidental deaths have been reported when sildenafil was used in combination with organic nitrates. In the USA, 123 deaths after sildenafil use were reported to the FDA for the 7 month following the launch of Viagra™ (sildenafil citrate) in 1998. Also in Japan two deaths due to sildenafil use were reported a few months after marketing commenced in 2000 (Anonymous, March 2001). Investigation of sildenafil in biological fluids and other parts of the human body is therefore important.

1.2.2 Structure and chemical properties of sildenafil

The chemical name of sildenafil (SLD) is 5-[2-ethoxy-5- (4-methylpiperazin-1-ylsulfonyl) phenyl]-1-methyl-3-propyl-1, 6-dihydro-7H-pyrazolo [4,3-d] pyrimidin-7-one and its empirical chemical formula is $C_{22}H_{30}N_6O_4S$. The melting point of sildenafil is 186-190 ^oC. Its solubility is 3.5 mg/ml in water. Sildenafil citrate is twice as soluble in methanol than in water. Its solubility decreases with pH up to 9 when it starts to increase again. The N-desmethylated metabolite of sildenafil is called UK-103, 320 (Badwan *et al.*, 2001).



UK-103, 320 **Sildenafil**

FIG. 1.1: Chemical structures of sildenafil (SLD) and UK-103, 320.

According to Differential Scanning Calorimetry (DSC) measurements done, the melting process of sildenafil appear to take place with decomposition. Thermogravimetric analysis of sildenafil citrate shows no weight loss below 200 ^{o}C illustrating the anhydrous nature of the analyte (Badwan *et al.*, 2001).

Both sildenafil and UK-103-320 have a basic functional group with a pK_a 8.7 (NH-piperazine). Difficulties may arise while analyzing compounds with basic properties. So to increase the transfer to the extracting solvent, the addition of dilute sodium hydroxide is recommended (Cooper *et al.*, 1997; Lee & Min, 2001). A weak acidic moiety is, however, also present on the parent compound with pK_a 9.6-10.1 (HN-amide) which will be completely ionized above pH values of 11 resulting in a decreased partition coefficient of sildenafil.

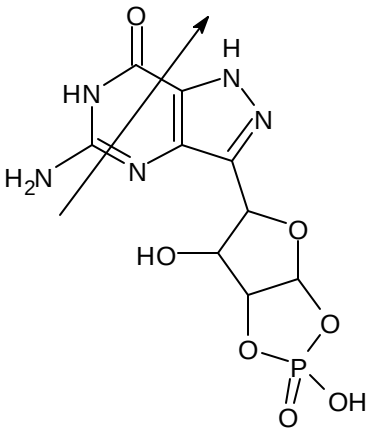
According to Jeong *et al*. (2001), the more acidic the mobile phase in liquid chromatography is, the shorter the analysis time. However, mobile phase (pH 4) gave more interference in column switching and therefore they set the pH of mobile phase at pH 4.5 instead of pH 4.

The chromatographic peak shape of sildenafil depends on the pH of the mobile phase. According to Liaw & Chang (2001), the best peak shape and resolution were obtained when the mobile phase (32 % acetonitrile with 0.2 % H_3PO_4 in water) pH was between 5.0 and 6.0. A mobile phase pH greater than 6.0 gave rise to irreproducible peak height and poor peak shape.

1.2.2.1 Pharmacophoric group of sildenafil

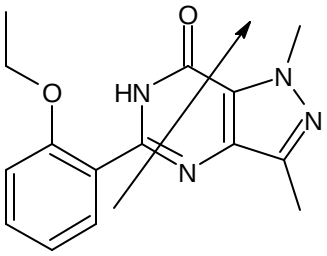
Sildenafil aims at inhibiting the enzyme phosphodiesterase, PDE5. It must therefore have a structure that is similar in some places to the substrate. However, there are many other constraints as there are several different types of PDE enzymes that are found in different parts of the body. Of the 7 types of PDE, three selectively hydrolyse cGMP relative to cAMP. PDE5 itself can be found in several parts of the body: the lungs, platelets and various forms of smooth muscle. Selectivity was a very important factor in the research for an inhibitor of PDE5. Sildenafil gave an excellent combination of enzyme inhibitory potency, selectivity, solubility and in *vivo* characteristics (WWW.ch,ic.as.uk,Department).

The following 2 dimensional models are the results of modelling studies on cGMP and the pyrazolo [4,3-d] pyrimidin-7-one. It shows their similarities. The second molecule was one of the steps in the discovery of sildenafil.



5-amino-3-(2,6-dihydroxy-2-oxidotetrahydrofuro[2,3-*d*][1,3,2]dioxaphosphol-5-yl)-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one

cyclic GMP



5-(2-ethoxyphenyl)-1,3-dimethyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one

FIG. 1.2: Relationship between pharmacophoric sub-structures of cyclic GMP and sildenafil.

1.2.2.2 Mode of action of sildenafil

Nitric Oxide (NO) is released with sexual stimulation from nerve endings and endothelial cells in the spongy erectile tissue, the corpus cavernosum of the penis. This release of NO activates the enzyme guanylate cyclase. The enzyme guanylate cyclase then converts guanosine triphosphate (GTP) into cGMP causing the smooth muscle to relax, which causes

an inflow of blood, which then leads to an erection. Cyclic guanosine monophosphate (cGMP) is then hydrolysed back to the inactive GMP by phosphodiesteras type 5 (PDE5) (Anonymous, 1998).

The level of cGMP is therefore controlled by the activation of cyclic nucleotide cyclase and the breakdown by PDE5. It is the latter that sildenafil acts upon. Men who suffer from erectile dysfunction often produce too little amounts of NO. This means that the small amount of cGMP they produce is broken down at the same rate and therefore does not have time to accumulate and cause a prolonged vasodilatation effect. Sildenafil works by inhibiting the enzyme PDE5 by occupying its active site. This means that cGMP is not hydrolysed as fast and this allows the smooth muscle to relax leading to increased blood flow into the organ and therefore penile erection (WWW.ch,ic.as.uk,Department).

1.2.3 Metabolism of sildenafil

Metabolism is the mechanism of elimination of foreign and undesirable compounds from the body and the control of desirable compounds such as vitamins in the body. The metabolism reactions are catalysed by a group of enzymes known as the cytochrome P450 (Gunaratna, 2000). Sildenafil is metabolised predominantly by cytochrome P450 / CYP3A4 in the liver and is converted to an active metabolite, N-desmethyl-sildenafil, that has approximately 50 % of the efficacy of the parent compound. Plasma concentration of this metabolite is approximately 40 % of the parent molecule, so that the metabolite accounts for about 20 % of the pharmacological effect of sildenafil. Metabolism of sildenafil in liver by CYP3A4 is significant because this is responsible for metabolism of many therapeutic agents (Walker *et al*., 1999; Badwan *et al.*, 2001). Five metabolism pathways were identified in rat, rabbit, dog and man, i.e. piperazine N- demethylation, pyrazole N- demethylation, N.N'-deethylation, oxidation of the piperazine ring and aliphatic hydroxylation. The piperazine N-desmethyl metabolite, UK –103,320 was identified as a major metabolite having a similar potency to sildenafil in dog, mouse, rat and man (Walker *et al.*, 1999).

1.2.4 Pharmacokinetics of sildenafil

Pharmacokinetics is the study of the way in which the body handles the administered drug. The major pharmacokinetic variables are absorption, clearance, volume of distribution, half life ($t_{1/2}$), C_{max} (maximum concentration) and t_{max} (time required to attain the maximum concentration) (Katzung, 1987)].

Sildenafil is rapidly and incompletely absorbed after oral administration, with absolute bioavailability of approximately 40 % (Berzas *et al.*, 2000). Similarly, Nichols *et al.* (2002) found the mean absolute oral bioavailability of sildenafil to be 41% in the fasted state. They also found that when sildenafil was taken with a high fat meal, the t_{max} of sildenafil was delayed by approximately 1h, but the total amount of sildenafil absorbed was not significantly changed. Dose proportional increase in plasma concentration of sildenafil was seen with increase in dose over the range of 25-200 mg. Sildenafil demonstrates near ideal pharmacokinetics for oral pharmacotherapy. It is rapidly absorbed after oral administration and has a rapid onset of action, usually within 1hr of dosing. Sildenafil has a plasma half-life of approximately 4hours (Berzas *et al.*, 2000; Montorsi *et al.*, 1999).

According to the new drug application (NDA) submitted by Pfizer research laboratories, peak concentration of sildenafil (C_{max}) in the elderly population was reported to be 303 ng/ml with a 25 mg sildenafil capsule and that sildenafil has dose proportional pharmacokinetics. Maximum plasma concentrations are reached within 30 to 60 minutes of oral dosing in the fasted state (Anonymouse, 1998).

The mean steady state volume of distribution (V_{ss}) for sildenafil is 105 litres, indicating distribution into the tissue. Sildenafil and its major circulating N-desmethyl metabolite are both approximately 96 % bound to plasma proteins. Protein binding is independent of total drug concentrations (Anonymous, 1998).

The major elimination routes of sildenafil are faeces (80 %), followed by the kidney (13 %) and semen (0.001 %). According to an excretion study done by Walker *et al.* (1999) using [^{14}C] labelled sildenafil in man following oral doses, it has been demonstrated that 79 % of radioactivity was excreted in the faeces, while 12 % was excreted in urine over 5days. Lewis and Johnson (2000) supported these data in their study done on post mortem specimens. They found that the sildenafil and its metabolite concentrations were found high in bile rather than kidney, liver, heart, and muscle specimens of two victims. Hair analysis is also a useful method for analysis in toxicology cases involving sildenafil, because the drug remains in hair after its disappearance from the urine and blood (Saisho *et al.*, 2001). Many studies have been reported on pharmacokinetics of sildenafil, a summary is given in Table 1.1.

1.2.4.1 Pharmacokinetic interactions of sildenafil

Co-administration of sildenafil with drugs that inhibit the cytochrome P450 CYP3A4 enzyme can interfere with the metabolism of sildenafil. As a consequence of concurrent treatment with multiple doses of erythromycin, the mean AUC (area under curve) and the maximum

plasma concentration (C_{max}) of sildenafil increased by 2.8-fold and 2.6-fold, respectively. No significant pharmacokinetic interaction occurred when sildenafil was co-administered with azithromycin, a less potent cytochrome P450 CYP3A4 inhibitor. So when sildenafil is administered with P450 CYP34A inhibitors, a lower starting dose (25mg) is recommended (Badwan *et al.*, 2001; Muirhead *et al.*, 2002).

Similarly sildenafil shows interaction with some diets. According to Lee and Min (2001), grapefruit juice appears to increase the C_{max} of sildenafil by 42 % without significantly increasing AUC. The explanation for the lesser effect on AUC could be because sildenafil has a relatively high bioavailability (40 %) and that grapefruit juice has a less prominent effect on such drugs. Grapefruit juice is a good example of dietary constituents that inhibit CYP 3A4, which appears to be the main reason for the high C_{max} of sildenafil in plasma when it is taken with grapefruit juice (Gunaratna, 2000). Summaries of pharmacokinetic data from literature are given on Table 1.1.

1.2.4.2 Stability of sildenafil

Using automated sequential trace enrichment dialysis (ASTED); Cooper *et al.* (1997) found no obvious degradation of the sildenafil and its metabolite plasma over 24hrs. at room temperature. Fifty percent losses of both compounds in aqueous media were observed within a 30 min. period after storing in plastic compared 2 % loss in glass. Addition of 20 % (v/v) methanol to the aqueous solutions negated the losses in plastic. These difficulties are generally over-come when the analytes are retained in plasma, especially when high protein binding occurs as is the case for sildenafil and UK-103, 320.

The stock solution made up in methanol was stable for minimum of 9 days at 4 °C with 0.6 % loss measured at the concentration of 250 ng/ml. Stock solutions at room temperature lost 7.5 % of its volume being left on the bench for 6 hrs. Based on previous results, the stock solution was stored at 4 °C and the stock solution was prepared every 9 days during the study period. Sildenafil in plasma was found to be stable (within 95 %) for as long as 6 hrs at room temperature (Lee & Min, 2001).

QC (quality control) samples stored in a freezer at −20 °C remained stable for at least 3 months. Extracted calibration standards and QCs were allowed to stand at ambient temperature for 48 hrs. prior to injection. No effect on quantitation of the calibration standards or QCs was observed. When stock solution methanol: water (1:1) was stored at 2-8 °C, the analytes were stable for 3 months (Eerkes, *et al.*, 2002)

TABLE 1.1: Summary of pharmacokinetic studies from literature survey

Author of article	Dose of Sildenafil	Pharmacokinetic data reported in study		
		PK parameter	**SLD with Water**	**SLD with Grape fruit juice**
Lee & Min	100mg p.o.	T_{max} (hr)	1	0.666
		C_{max} (ng /ml)	1067.7	1517
		K_e (h^{-1})	0.142	0.1306
		$T_{1/2}$ (hr)	4.9	5.3
		AUC (mg.hr /ml	4082.9	4171.9
		MRT (hr)	5.1	6.2
		Cl/F (l /h)	24.5	24
		PK parameter	**SLD**	**UK-103**
Jeong *et al.*	50 mg p.o.	C_{max} (ng/ml)	419±150	86±27
		T_{max} (h)	1	1.13±.25
		$t_{1/2}$ (h)	1.07±.21	1.26±.35
		AUC (ng h/ml)	870±160	217±74
		PK parameter	**SLD**	
Jung *et al.*	50 mg p.o.	MRT (hr)	16.5±0.5	
		K_e (hr^{-1})	.01±.03	
		C_{max} (ng /ml)	835±107	
		T_{max} (hr)	.8±0.0	
		$T_{1/2}$ (hr)	7.4±2.1	
		AUC (mg.hr /ml	12.9±2.6	

T_{max}, time to reach maximum concentration: $C_{max,}$ Maximum concentration: K_e, elimination rate constant: $T_{1/2}$, elimination half-life: AUC, area under time-concentration curve: MRT, mean residence time: Cl/F, apparent total clearance.

1.2.5 Side effects of sildenafil

Blood pressure is transiently reduced by oral administration of 100mg of sildenafil followed by the possible adverse effect of colour (blue /green) discrimination, headaches, flushing, and nasal congestion, all events were mild and short-lived in nature. No significant adverse cardiac or cerebrovascular events were observed (MoreiraJr *et al*., 2000). Typical delivery through a local tissue area could be considered for alternative administration, instead of orally, to avoid these adverse effects, to shorten on-set time, and to sustain effect for a longer period (Liaw & Chang, 2001).

1.2.6 Bioanalytical assay methods overview of sildenafil

Various methods have been published for the analysis of sildenafil in biological fluids. These include HPLC, LC-MS/MS and GC methods using variety extraction procedures. A summary of these methods in chronological order is given below.

[1] Cooper et al., ASTED – HPLC

An automated sequential trace enrichment dialysis (ASTED) that is an accelerated dialysis by continual movement of the recipient solvent was used to extract 650 µl spiked plasma to which 150 µl monochloroacetic acid solution of UK/108,302 (IS) was added. Potassium phosphate buffer (10 mmol/l, pH 7) or water was used as donor solvent while 10 % methanol in potassium phosphate buffer (10 mmol/ml, pH 7) was used as solvent on the receptor compartment. Mobile phase composed of acetonitrile: potassium phosphate buffer (500 mmol/ml, pH 4.5) : water (28 : 4 : 68) was utilized. Ethylamine hydrochloride 10 mmol/ml was added to the water or buffer prior to the addition of acetonitrile. Unlike the problem associated with non-specific binding of bases, the ion-exchange properties of these compounds can be used to advantage. The use of non-end capped short alkyl chain silica ensures that enrichment is probably due to ion–exchange mechanisms between the positively charged amine moiety of the base and the exposed charged silanols. The separation was done on HPLC column (100 x 4.6 mm I.D.) that was packed with 5 µm Kromasil C_4.

[2] Bhoir et al., HPLC- UV detector.

1 ml. Plasma was extracted using 4 ml. of dichloromethane after alkalinising the plasma with 100 µl of NaOH (1 M). 3 ml. Of the supernatant was evaporated and reconstituted in 200 µl of mobile phase. Mobile phase composed of methanol : H_3PO_4 (70 : 30) adjusted to pH 5.2 with tri-ethyl amine and column Inertsil-ODS-3, (250 x 4.6 mm) 5 µm was used for separation of the analytes.

[3] Lewis and Johnson, HPLC- MS/MS and HPLC -MS/MS/M S

The authors develop a method for identification and quantification of sildenafil and UK-103-320 in post-mortem fluids (urine, blood and bile) and tissue samples (liver, kidney, heart and muscle). The highest level of sildenafil and UK-103, 320 were found in bile. They used a combination process of precipitation and solid phase extraction (SPE) methods for analyte extraction. The protein in 3 ml. biological fluid was precipitated with 9 ml. of acetonitrile and the volume of the supernatant was decreased to 1 ml. by evaporating under nitrogen. The residue was loaded onto an SPE cartridge using 4 ml. of (0.1 M) phosphate buffer, washed

with 1 ml. of acetic acid (1 M) followed by 6 ml. of methanol and eluted with 4 ml. of 2 % ammonium hydroxide in ethyl acetate. The eluate was evaporated and the residue reconstituted in 50 µl of acetonitrile before injection onto the HPLC-MS-MS system where separation was done on Supelcosil LC_{18} (150 x 4.6 mm I.D., 3 µM particles) column.

[4] Jeong et al., HPLC- Column switching.

A fully automated narrow bore column HPLC method with a three column-switching system for determination of sildenafil and its metabolite in human plasma was developed. This method is an on-line trace enrichment technique that can directly analyse small volumes (50 µl plasma) biological samples. The two primary columns were used for deproteinisation and concentration while separation was performed on the third phenyl-hexyl (100 x 2 mm I.D.) analytical column using mobile phase composition 36 % acetonitrile in 10 mM phosphate solution (pH 4.5). The whole process took 17 min. per sample.

[5] Lee and Min, HPLC- UV.

The authors develop an HPLC - UV method for the determination of sildenafil in plasma. In addition a case study for the effect of grapefruit juice on the pharmacokinetics of sildenafil was presented. 200 µl of plasma sample and 50 µl of IS (1 µg/ml) was alkalinised with 300 µl NaOH (0.01M) and extracted with 400 µl of diethyl ether. The organic layer was evaporated under nitrogen, the residue reconstituted in 200 µl 80 % methanol, and 20 µl was injected onto the HPLC with mobile phase composition of acetonitrile : 500 mmol/ml potassium phosphate buffer containing 10 mmol/ml diethyl amine hydrochloride (32 : 68) and containing a Kromasil C_4 (150 x 4.6 mm I.D., 5 µm) analytical column.

[6] Lalla et al., Automated sequential trace enrichment dialysis-HPLC.

They modified the method developed by Cooper *et al.* (1997) by improving some of the parameters used. Conditions such as receptor compartment solvent, mobile phase and column were changed to 10 % dimethyl formide (DMF) in acetate buffer (10 mM/l) pH 7.2, acetonitrile : buffer (500 mm/l, pH 4.5) : water : diethyl amine (38 : 4 : 58 : 0.05) and Waters µ-Bond pak, C_{18} (300x 3.9 mm) respectively. The retention time and LLOQ results are higher than that of Cooper *et al.*'s method.

[7] Saisho et al., GC-MS

A GC-MS method for determination of sildenafil and its metabolite in human and rat hair was developed. 25 mg Hair was extracted with 1.5 ml. methanol : 5 M HCl (20 : 1). The 0.1 M phosphate buffer (pH 6) reconstituted solution of the extract was purified on a Varian

Bond elute certify column and the residue of the fluent derivatised with silylating reagent consisting of trimethylsilylimidazole : N, O-bis (trimethylsilyltrifluoroacetamide): trimethylchlorosilane (3 : 3 :2) at 90 ^{0}C for 30 min. The GC-MS analysis was carried out with a 15 m x 0.25 mm I.D., 0.25 µm cross-linked methylsilicone fused silica column (DB-5MS). . The rat hair concentration of sildenafil and its metabolite were 5.8 ng/mg and 17.6 ng/mg respectively with 25 mg/kg sildenafil intraperitoneal (I.P) administration and 3.6 ng/ml and 9.1 ng/ml respectively with 25 mg/kg sildenafil oral administration.

[8] Jung et al., HPLC - UV

A solid phase extraction was developed for analysis of sildenafil and demethylated metabolite in cat plasma. 1 ml. Plasma and 20 µl methanolic solution of internal standard (10 µg/ml) was deproteinised with 2 ml. of acetonitrile. 2.5 ml. of the supernatant was loaded onto a C_{18} sep Pak cartridge that was conditioned with 5 ml. of methanol followed by 5 ml. of water. The analyte was washed with 5 ml. of water and eluted with 4 ml. of methanol. After evaporating the solvent, the residue was reconstituted with 90 µl of acetonitrile and 60 µl was injected onto the HPLC system with mobile phase composition, acetonitrile : 0.5M KH_2PO_4 containing 10 mM diethylamine (pH 4.5) : Water (30 : 64 : 4) and a Capcellpak UK 120 (150 x 5 mm I.D., 5 µm) analytical column.

[9] Liaw and Chang, HPLC - UV

An HPLC method was developed to assess the transdermal permeability of sildenafil of fresh nude mouse skin to develop a proposal for a method of local tissue administration of sildenafil. Extraction was performed by precipitating 0.4 ml. of nude mouse skin constituent in phosphate buffer saline collected from the Frenz cell receiver compartment with 0.8 ml. of acetonitrile. The supernatant was transferred to another container and evaporated under a nitrogen stream. The residue was reconstituted with 100 µl mobile phase (32 % acetonitrile in water with 0.2 % phosphoric acid, pH 5.3) and 20 µl was injected onto Luna C_{18} (150x4.6 mm I.D., 5 µm) analytical column.

[10] Eerkes et al., LC-MS/MS

An LC - MS/MS-solid phase extraction on 96-channel programmable liquid handling work-station (Quadra 96) using C_8 and cation exchange mixed mode sorbent was developed. 350 µl of sample and 20 µl of internal standard was loaded onto each column of a 1 ml. deep column 96-well plate and automated SPE was carried out on a Tomtec Quadra 96-320 robotic

analyser. An aqueous – organic mobile phase and a Betasil silica (50 x 3 mm I.D., 5 µm) analytical column were used for the separation.

[11] Kim et al., LC-MS/MS

100 µl Plasma was alkalinised using 100 µl of NaOH (50 mM) and extracted with 700 µl of dichloromethane. After evaporating the organic solvent, the residue was reconstituted in 50 µl of acetonitrile and 10 µl was injected onto the HPLC. Separation was performed on Luna phenylhexyl (100 x 2 mm I.D., 3 µm) column with the mixture acetonitrile : ammonium formate (10 mM, pH 6)(60 : 40) as mobile phase.

[12] Tracqui and Ludes, HPLC –MS

3 ml. Biological fluid and 3 µl of internal standard was alkalinised by 2 ml. of NH_4Cl buffer (pH 9.5) and extracted with 3 ml. of chloroform/2-propanol/n-heptane (25 : 10 :65). The organic solvent was evaporated, the residue was reconstituted in 25 µl methanol, and 2 µl was injected onto the HPLC. Separation was carried on NovaPak C_{18} (150 x 2 mm I.D., 4 µm) column with a gradient mobile phase composed of (acetonitrile and 10 µg/ml trimethylamine) in 2 mM NH_4COOH pH 3 buffer (35 % : 70 % in 9 min.).

A summary of the analytical results of the above methods reported in the literature is presented in Table 1. 2.

TABLE 1.2: Assay Methods Overview and Performance Summary

Reference & Assay mode	Analytes	Conc. range (ng/ml)	Correlation coefficient	LLOQ (ng/ml)	LLOD (ng/ml	t_R (min)	Recovery (%)	Precision (CV) %	
								Intra-day	Inter-day
[1] ASTED-HLPC -UV (plasma)	Sildenafil	1-250	0.998	1	NR	5	NR	11.2-1.3	13.5-3.69
	UK-103 -320	1-250	0.9977	1	NR	4	NR	8.2-1.1	13.6-3.49
	UK-108-302*					7			
[2] HPLC-UV (plasma)	Sildenafil	25-1000	0.9999	25	10	8.92	88	14.25-0.65	1.1-17.87
	NI.								
[3] LC-MS/MS & MS/MS/MS (bio-fluids)	Sildenafil	2-800	0.999	2	1	2	83	1.0-2.0	1.0-4.0
	UK-103 -320	4.8	0.999	4	1	2.5	78	4.0-6.0	5.0-14
	Medazepam*								
[4] Narrow-bore HPLC -UV (plasma)	Sildenafil	10-1000	0.999	10	NR	14	93.5	2.1-1.5	2.3-1.8
	UK-103 -320	10-100	0.999	10		10	93.2	2.2-1.8	2.1-1.8
	NI.							(RSD %)	(RSD %)
[5] HPLC-UV (plasma)	Sildenafil	10-2500	0.999	10	7.12	10.2	87-90.8	12.6-1.8	9.1-3.9
	UK-114542-27*		& 0.9982			11.4	88.6		
			2 calibrations						
[6] ASTED-HPLC -UV (plasma)	Sildenafil	12-500	0.999	12	NR	12	91.7	0.33-0.13	0.34-0.15
	NI.								
[7] GC - MS (hair)	Sildenafil	0.1-50ng/mg	0.999	0.1	0.05	10.4		4.8	
	UK-103 -320	0.1-50ng/mg	0.998	0.1	0.2	12	NR	3.7	
	Trazodone*			(ng/mg)	(ng/mg)	2			
[8] HPLC-UV (cat plasma)	Sildenafil	50-1000	0.999	50	NR	9	88	13-3	15-1.9
	UK-103 -320	50-1000	0.999	50		7.5	93	9-1.2	14-0.86
	NN					13			
[9] HPLC-UV (Mouse skin)	Sildenafil	5-500	0.999	5	NR	8.8	NR	10-4.6	14-6.4
	Ethylparaben*	(50µl plasma)				10.5			
[10] LC/MS-MS (plasma)	Sildenafil	1-500	0.9988	1	NR	1.68	70	7.4-.3	4.7-0.7
	UK-103 -320	1-500	0.9976	1		1.58	67	9.1-0.3	5.7-2.0
	NN	1-500				1.6	69		
[11] LC/MS-MS (plasma)	Sildenafil	2-1000	0.997	2	NR	2.8	59-63	5.5-7.6	8.1-6.9
	UK-103 -320	2-1000	0.995	2	NR	2.2	74-79	3.4-6.9	6.7-4.1
	DA-8159*					2.5	55		
[12] LC-MS (biological fluids)	Sildenafil	0.2-1000	0.992	0.5	0.2	4.2	92-92	9.6-4.8	
	Buprenor-					5.07	(blood)		
	phine-d_4*						87(urine &		
							oral fluid)		

* = Internal standard (I.S).

NI = No internal standard is given.

NN = No nam e is given for the internal standard used.

2 EXPERIMENTAL

2.1 HPLC-UV ASSAY METHOD FOR SILDENAFIL

2.1.1 Method development

Method development involves evaluation and optimisation of the various stages of sample preparation, chromatographic separation, detection and quantification. Optimisation of various parameters was performed in order to develop a selective and sensitive method for analysis of sildenafil on HPLC using UV detection. A 0.05 M Phosphate buffer based mobile phase was first investigated to develop a fully validated assay method using UV detection. Since an assay method using MS/MS detection was also to be developed, the preliminary development of an HPLC-UV method utilising a 0.05 M ammonium acetate buffer based mobile phase was also undertaken. The full validation of this latter method was later performed on an LC-MS/MS system.

2.1.1.1. Materials

Sildenafil citrate was purchased from SynFine Research (Richmond, Ontario, Canada). While awaiting the arrival of the sildenafil citrate from SynFine Research, a 100mg tablet Viagra™ was purchased from Pfizer (Sandton, South Africa) and was used during the initial method development stage. t-C18 Sep-Pak® cartridges for solid phase extraction (SPE) were purchased from Waters Corporation (Milford, Massachusetts, USA). All reagents used were of analytical grade and used without further purification.

2.1.1.2 Instruments

HPLC analysis was performed using an Agilent 1100 Series quaternary pump combined with a Hewlett Packard (HP) 1100 series photo diode array detector (Germany), an HP 1100 series autosampler, and an Agilent 1100 series vacuum degasser (Germany). The column used was

Discovery® C_{18} bonded 5 µm silica, (15 cm x 2.1 mm) (Supelco, USA) with a mobile phase flow rate of 0.3 ml/min. An HP 1100 series thermostat column compartment (Germany) was used to control the temperature. The solid phase extractions were done on a SPEEDISK® 48 pressure processor ((Mallinckrodt Baker, Holland). All solvent evaporations were carried out in a Savant, Speed Vac® Plus SC 210A vacuum evaporator connected to a refrigerated vapour trap RvT4104 (Thermo Savant, USA). The freezing process during liquid/liquid extraction (LLE) was done on a FRYKA Kältetechnik Esslingen cooling bath at about –20 °C. UV spectra were obtained on an Agilent 8453 UV-Visible Spectroscopy system. Analyte weighing for preparation of calibration standards and quality controls was done on a microbalance, Mettler-Toledo AG 1993, Type PB 602 from Mettler Toledo (Switzerland). Other weighings were also done on a Mettler-Toledo, Type PB 303 balance.

2.1.1.3 Preparation of the stock solutions

Two types of stock solutions were used in the method development stage. Stock solution A one was prepared from the Viagra™ tablet and stock solution B was from the standard sildenafil-citrate (R392) material. The method development work was started using stock solution A. This stock solution was prepared by weighing the tablet and grinding it in a porcelain mortar. Powder (321.5 mg) equivalent to 50mg sildenafil was then shaken thoroughly with 100 ml. of ethanol on a mechanical shaker to obtain a solution of 500 µg/ml sildenafil. The insoluble excipients of the tablet were removed by centrifugation.

Stock solution B was prepared by dissolving 1.980 mg sildenafil citrate (equivalent to 1.41 mg sildenafil base) in 11.16 g (14.1 ml.) methanol.

Based on the work by Saisho *et al.* (2001), Trazodone was chosen to be the internal standard. 3.61 mg Trazodone.HCl was dissolved in 14.24 gm (18 ml.) methanol to give a solution of 200 µg/ml. All stock solutions were stored in a refrigerator at 4 °C freezer.

2.1.1.4 Spectrophotometric Measurement

In order to determine the wavelength at which the HPLC detector system must operate, UV spectra of 10 µg/ml sildenafil and 10 µg/ml trazodone in methanol solution were measured using a spectrophotometer (Agilent, Germany) over a wavelength range of 196 – 380 nm. Absorbance maximum was found to lie between 200 nm to 260nm. Fig. 2.1. The $^{1\%}E_{1cm}$ of methanol solutions of sildenafil and trazodone at 230nm wavelength were calculated to be 593AU and 261AU respectively.

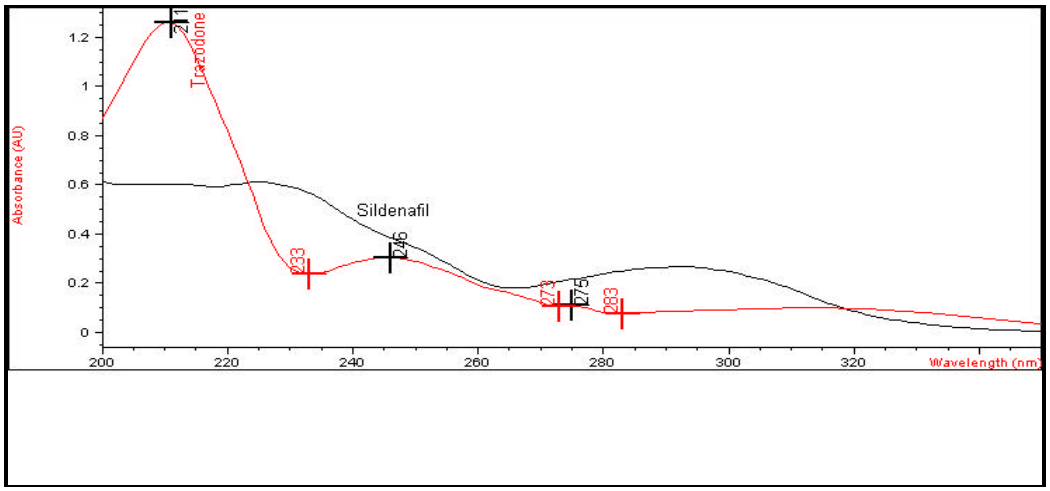


FIG. 2.1: UV-Spectra of 10 mg/ml sildenafil and 10 mg/ml trazodone in methanol solution.

The UV spectrum of sildenafil is pH dependent, it shifts slightly when neutral solution is basified or acidified using 10 M NaOH and concentrated HCl respectively. This experiment was done to confirm the change of spectra shown when the pH of the mobile phase is changed. To 19.69 µg/ml sildenafil in water solution, 200 µl of 10 M NaOH or 200 µl of concentrated HCl was added and the following spectra were recorded. Fig. 2.2.

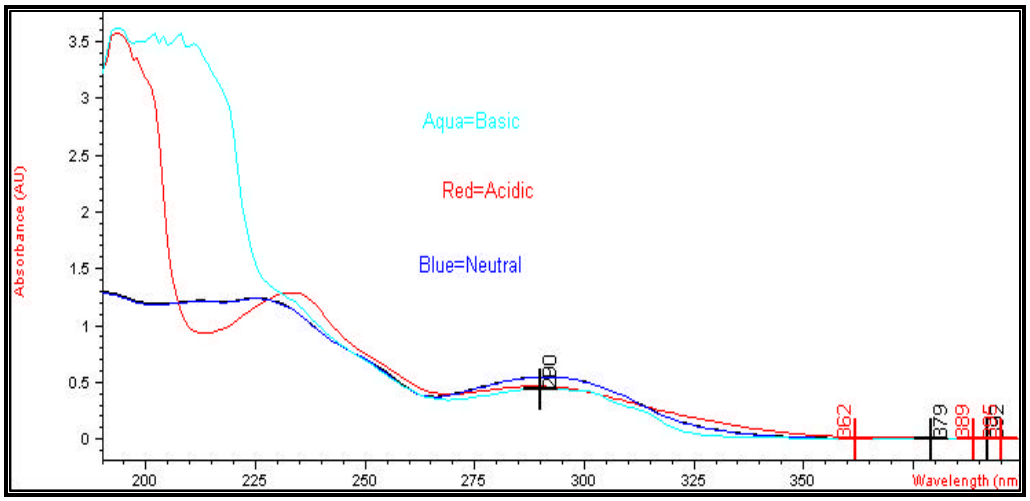


FIG. 2.2: UV Spectra of 20 mg/ml sildenafil in neutral, acidic and basic solutions in methanol.

A preliminary chromatographic separation of sildenafil and trazodone was performed to determine the best wavelength at which to monitor the eluent.

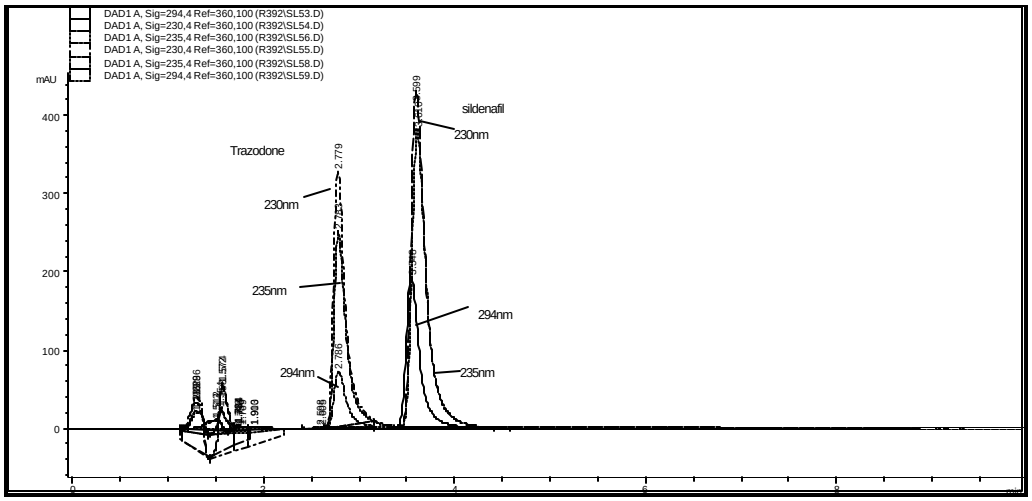


FIG. 2.3: Overlaid chromatograms of sildenafil and trazodone obtained with various detection wavelengths (230, 235, and 294 nm). Mobile phase: 30% acetonitrile in 0.05 M ammonium acetate buffer pH 5.8.

The chromatograms above (Fig. 2.3) indicate that detection at 230 nm would be the most sensitive wavelength to work at but this is not necessarily the optimum wavelength if an endogenous interfering peak were to absorb at the same wavelength.

2.1.1.5 Optimisation of the mobile phase

The following four solutions/solvents were prepared, degassed and connected to the four inlets of the quaternary HPLC pump:

- A: Methanol.
- B: Acetonitrile.
- C: 0.05 M H_3PO_4.
- D: 0.05 M phosphate buffer prepared by titrating 0.05 M H_3PO_4 with 5 M NaOH solution to pH 7.

To obtain some information about the required composition of the buffer, and organic modifier, gradient elutions were first run at 0.3 ml/min from 0 to 90 % in 20 min. using organic modifier (acetonitrile or methanol) and pH 7 phosphate buffer D injecting 2 µl of the stock solution A. Sildenafil eluted at 76 % methanol (t_R = 19.56 min.) and at 65 % acetonitrile (t_R = 13.79 min.). With an isocratic run using 76 % methanol in pH 7 buffer sildenafil eluted at 2.22 min. and this retention time increased to 5.08 minutes when the methanol content of the mobile phase was reduced to 60%. An isocratic run with 65% acetonitrile in pH 7 buffer, eluted sildenafil with a retention time of 1.98 minutes.

The above preliminary information was used to investigate the influence of the buffer pH on the elution of sildenafil. The quaternary pump system is ideal for the acquisition of automated chromatographic runs as acquisition methods can be set up with practically any combinations of the connected mobile phase solutions. Thus, different combinations of solvents C and D would yield buffers with different pHs and combinations of solutions A, C and D would deliver mobile phases at different pHs and organic modifier A. To first determine the pHs of specific combinations of solutions C and D, they were mixed in various proportions and the pH of the resultant buffer solution measured on a pH meter. The results of this experiment are presented in Table 2.1.

TABLE 2.1: pH Values of buffer solutions obtained when mixing different %ages of 0.05 M H_3PO_4 and 0.05M phosphate buffer of pH 7.

Volume of solution C (.05M H_3PO_4) (ml.)	Volume of solution D. (.05MPO4 buffer) (ml.)	% of C in final Buffer	pH of Buffer
10	0	100	1.66
9	1	90	1.77
8	2	80	1.92
7	3	70	2.1
6	4	60	2.3
5	5	50	2.63
4	6	40	3.42
3	7	30	6.02
2	8	20	6.45
1	9	10	6.73
0	10	0	7

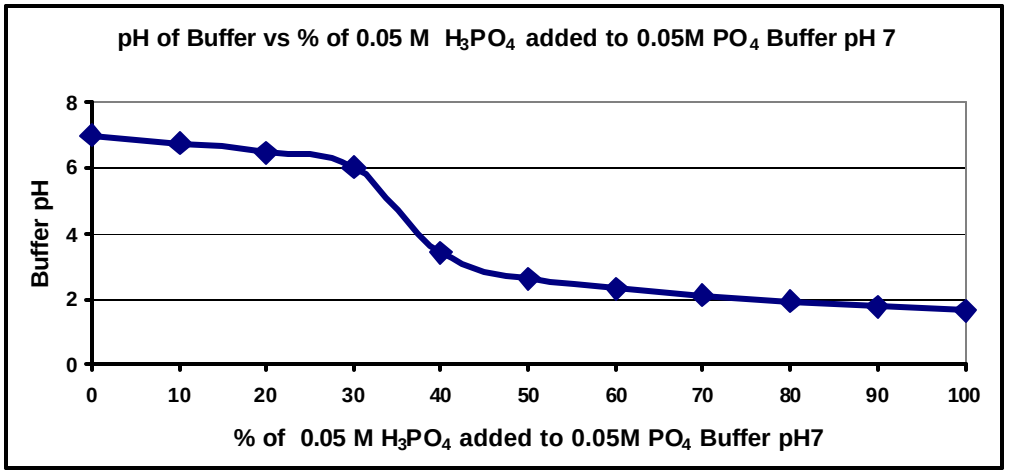


FIG. 2.4: Graphical representation of the data in Table 2.1.

Using Fig. 2.4 as guide for determining the amount of solution C required for buffers of specific pH values, several chromatographic runs were programmed using 60 % methanol in the mobile phase with various pHs and the detector wavelength set at 230 nm. Table 2.2 below shows the results obtained.

TABLE 2.2: Effect of mobile phase buffer pH on retention time of sildenafil with methanol content of mobile phase kept at 60%.

% of C in buffer	pH of buffer	Retention time (min.).
20	6.4	5.76
30	6	5.087
33	5	4.91
37	4	4.275
40	3.4	3.043
60	2.3	3.04

Graphically it can be seen that the retention time of sildenafil increases with increasing pH of the buffer (Fig. 2.5). This is in agreement with the literature.

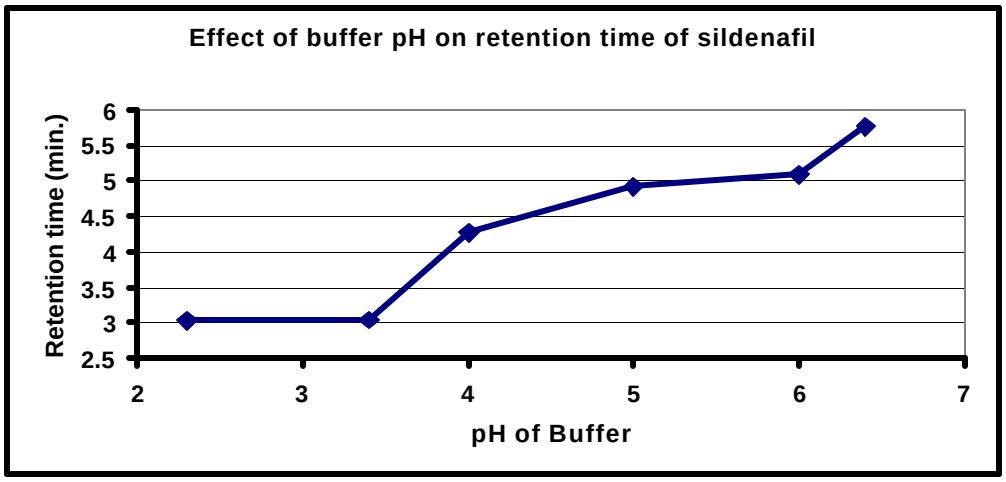


FIG. 2.5: Graphical presentation of data in Table 2.2.

The same experiment was performed with an isocratic mobile phase containing 35% acetonitrile but injecting both sildenafil and trazodone. The results are presented in Table 2.3 and Fig. 2.6

TABLE 2.3: Retention times at different buffer pH of mobile phase containing 35% acetonitrile.

Buffer pH	Retention time (min.)	
	SLD	TRZ
2.2	2.34	2.14
3	2.47	2.25
4	2.59	2.37
5	2.9	2.49
6	5.13	3.84
7	9.49	8.24

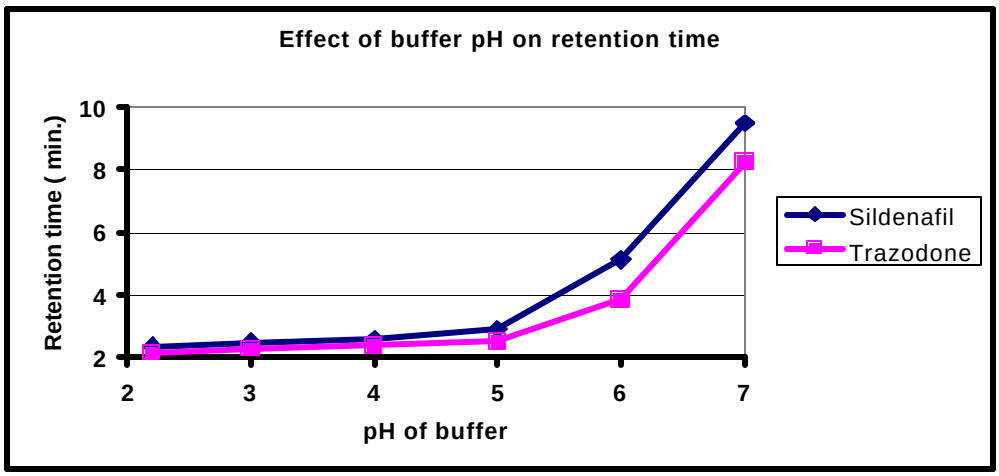


FIG. 2.6: Graphical presentation of data in Table 2.3.

The strong dependence of retention times of both analytes is clearly evident. In the mobile phase containing methanol as organic modifier, trazodone also elutes before sildenafil at all mobile phase pHs.

A typical chromatogram of sildenafil (16.67 µg/ml) and trazodone (45.8 µg/ml) using this mobile phase is depicted in Fig. 2.7.

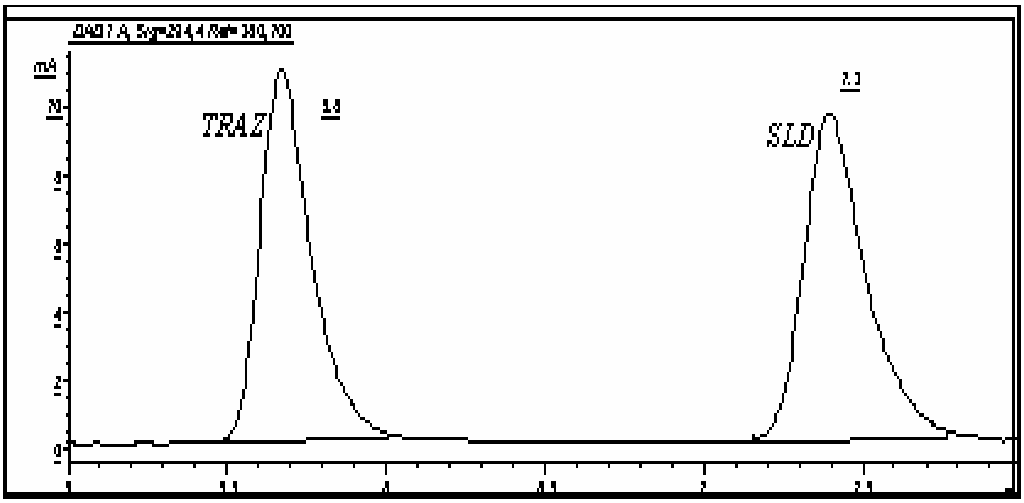


FIG. 2.7: Typical chromatogram of sildenafil and Trazodone.

In the procedures used during the experiments above the mobile phase composition was determined by the relevant low pressure solvent switching valves of the quaternary pump. Due to uncertainties inherent in the delivery of the relevant solutions by the switching valves, as well as possible incomplete mixing, especially at low delivery volumes, the above results could only be considered as preliminary indicators of the optimal values of the various mobile phase parameters which affect the quality of the chromatograms. Taking into account the results obtained, it was decided to use acetonitrile as organic modifier at a level of 30 to 35% v/v of the mobile phase and to prepare mobile phases for further optimisation as follows:

- To a volume of organic modifier (say 300 ml.) was added 700 ml. 0.05 M H_3PO_4.
- The resultant solution's pH was then adjusted dropwise with 5 M NaOH solution while stirring vigorously on a magnetic stirrer and monitoring the pH with a pH meter. In this manner a mobile phase with the required apparent pH could be produced very reproducibly.

Keeping acetonitrile constant at 30%, mobile phases at different pH values were prepared as described above and chromatograms obtained of sildenafil and trazodone. The effects of mobile phase pH on several parameters were calculated and are presented in the following tables and figures.

TABLE 2.4: Retention times at different pH values of mobile phase containing 30% acetonitrile.

Mobile phase pH	Retention time (min.)	
	SLD	TRZ
2.2	4.35	3.15
3	4.684	3.44
4	4.88	3.57
5	5.501	3.867
6	10.27	6.548
7	18.38	15.24

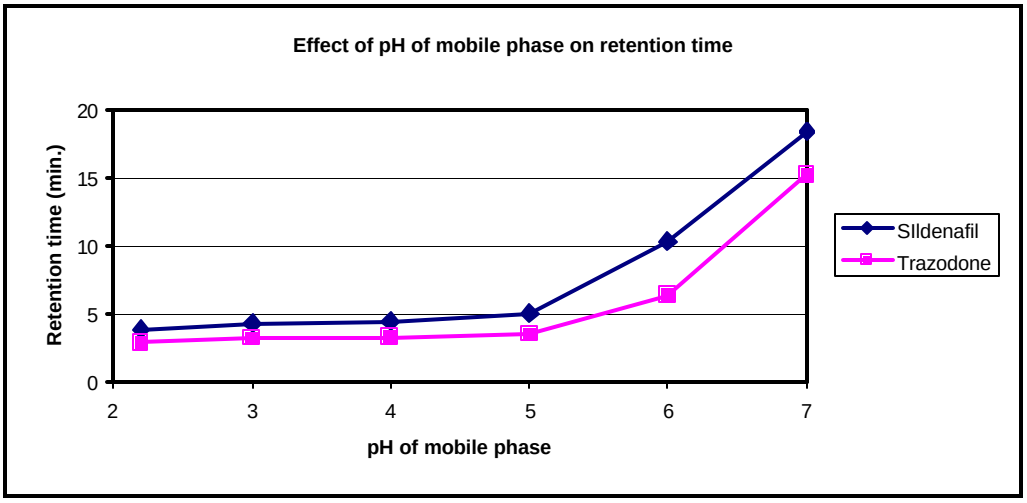


FIG. 2.8: Graphical presentation of the data in Table 2.4.

Effect of mobile phase pH on column efficiency (N) and resolution were calculated at different pH values using equation 2.1 and 2.2 respectively.

$$N = 16 \times \left(\frac{t_R}{W}\right)^2 \qquad (2.1)$$

$$R_S = \frac{t_{R2} - t_{R1}}{1.5(W_1 + W_2)} \qquad (2.2)$$

Where: t_{R1} and t_{R2} are retention times of the two peaks.

W_1 and W_2 are peak width of the two peaks.

R_s is peak resolution.

N is Number of theoretical plates (measure of efficiency).

TABLE 2.5: Effect of Mobile phase (acetonitrile 30 % and 0.05 M H_3PO_4) pH on column efficiency and resolution.

pH	Trazodone			Sildenafil			Resolution
	$_R$ (min.)	Width (min.)	Efficiency (N)	$_R$ (min.)	Width (min.)	Efficiency (N)	
2.2	3.15	0.16	6201.56	4.35	0.1835	8991.38	52.4
3	3.44	0.1683	6684.50	4.684	0.19	9724.04	56.38
4	3.57	0.151	8943.40	4.88	0.1918	10357.68	64.52
5	3.867	0.171	8182.31	5.501	0.2278	9330.30	76.59
6	6.548	0.279	8813.10	10.27	0.4131	9888.96	180.78

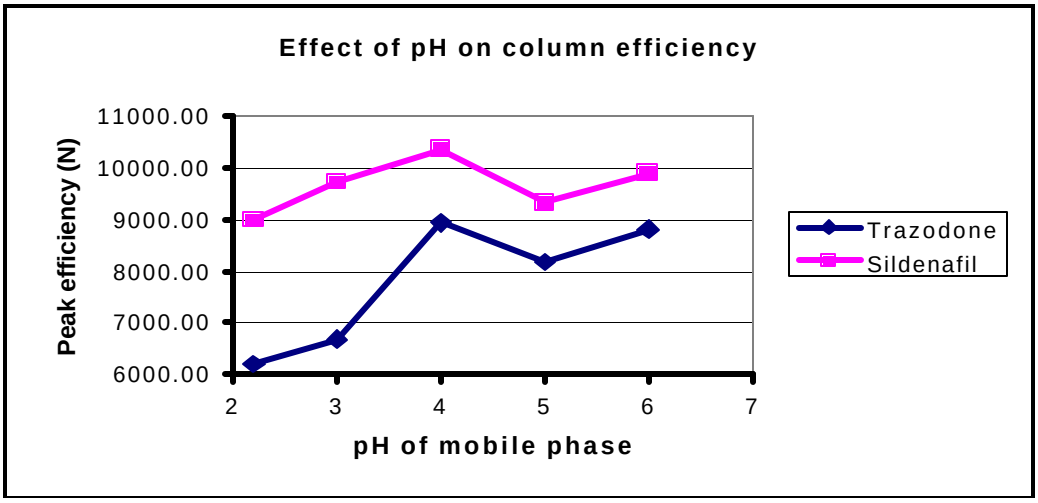


FIG. 2.9: Effect of mobile phase pH (acetonitrile 30 % & 0.05 M H_3PO_4 70 %) on column efficiency (N).

2.1.2 Extraction from plasma

Since the objective of this study was to develop a simple reliable method that would facilitate analysis of sildenafil in human body fluids in a large number of samples over a relatively short period of time (bioequivalence studies), in a cost effective manner, it was decided to investigate a number of different extraction procedures. The literature search revealed several extraction procedures for sildenafil from plasma and the simplest liquid/liquid extraction procedure appeared to be the diethyl ether extraction procedure published by Lee and Min (2001). It was therefore decided to investigate this procedure as a first approximation. To do this, a pool of blank human plasma was obtained and spiked with relevant concentrations of sildenafil before extraction.

2.1.2.1 Liquid-liquid extraction (LLE)

The extraction procedure described by Lee and Min (2001) was attempted with some variations.

- 1 ml. plasma in a screw-capped glass tube was spiked with 100 ng/ml sildenafil, and 100 ng/ml trazodone as internal standard.
- 300 µl of 0.01 M NaOH and 6 ml. of diethyl ether were added and the tube vortex mixed for 1 min. centrifuged at 2500 rpm for 1 min.
- The aqueous phase was frozen in an alcohol freezing bath at about -25 °C.
- The organic phase was decanted into a 5 ml. disposable glass ampoule.
- The solvent was evaporated under nitrogen and the residue reconstituted in 100 µl mobile phase.
- 10 µl of the reconstituted extract was injected onto the HPLC column.

The extracts resulting from this process indicated high recovery of both sildenafil and trazodone but were not clean (several potential interfering peaks) and formed a white emulsion when reconstituted with the mobile phase. Several extractions using various volumes (100 to 400 µl) of 0.01 M NaOH solution were attempted but none of them solved the problem. A back-extraction using 0.01 M HCl (100,150 or 200 µl) was tried but although this resulted in cleaner extracts, the recovery of sildenafil diminished considerably and trazodone did not extract at all.

The dirty extracts were subsequently shown to be the result of a small amount of non-frozen aqueous phase that was decanted accidentally together with the ether phase. This problem was solved by replacing the thick-walled screw-capped glass tubes with thin-walled disposable glass ampoules to perform the extractions in.

The final optimised extraction procedure was as follows:

Extraction procedure

- To 0.4 ml. plasma in a 10 ml. disposable glass ampoule was added 50 µl internal standard solution (35 µg/ml trazodone in methanol), 600 µl 0.01 M NaOH and 7 ml. diethyl ether.
- Vortex mixed for 1 min.
- Centrifuged for 10 min. at 2500 rpm.

- After freezing the aqueous phase in an alcohol freezing bath at about –25 °C, the organic layer was decanted into a 5 ml. disposable amber glass ampoule.
- The organic layer was evaporated on a Savant vacuum evaporator at about 60 °C. The residue was reconstituted in 100 µl mobile phase and 20 µl injected onto the HPLC column.

Chromatography

The mobile phase was 30 % acetonitrile in 0.05 M H_3PO_4 adjusted to an apparent pH of 6.0 with a flow rate of 0.3 ml/min. and detection at 230 nm. The chromatograms were acceptably clean with only small potential interfering peaks near the retention times of sildenafil (5.2 min.) and trazodone (3.8 min.) as can be seen in Fig. 2.10.

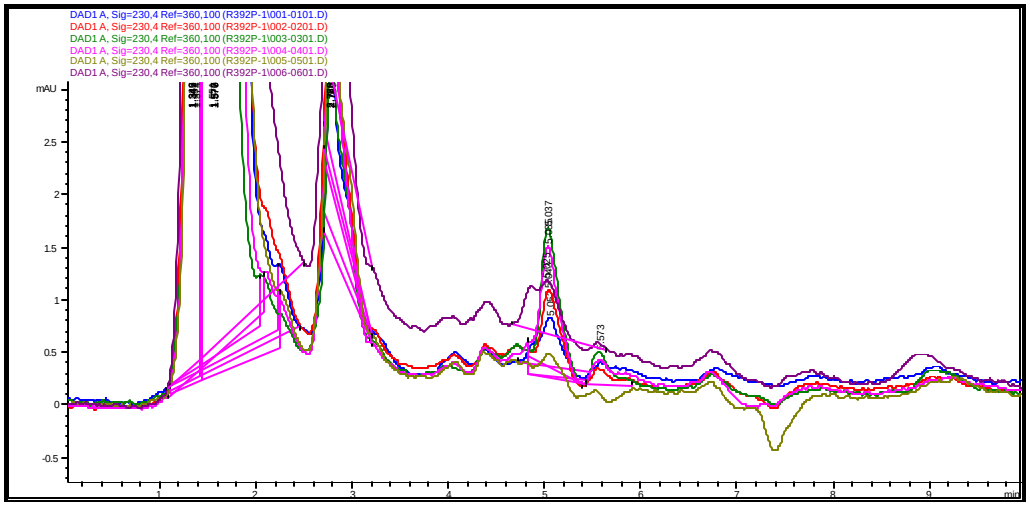


FIG. 2.10: Chromatograms of 0.4 ml. plasma extracts obtained with the optimised extraction procedure of 6 blank plasma pools collected on different dates.

The liquid/liquid extraction with diethyl ether and the chromatographic procedure using the phosphate buffer based mobile phase for UV-detection was now considered to be acceptable to proceed with the formal validation of the assay method. However, since the instruments were already set up for UV detection it was decided to first also investigate the chromatography using an acetate buffer based mobile phase for MS/MS detection.

A chromatogram of a blank plasma extract using the acetate buffer based mobile phase indicated cons iderable interference of endogenous components with sildenafil.

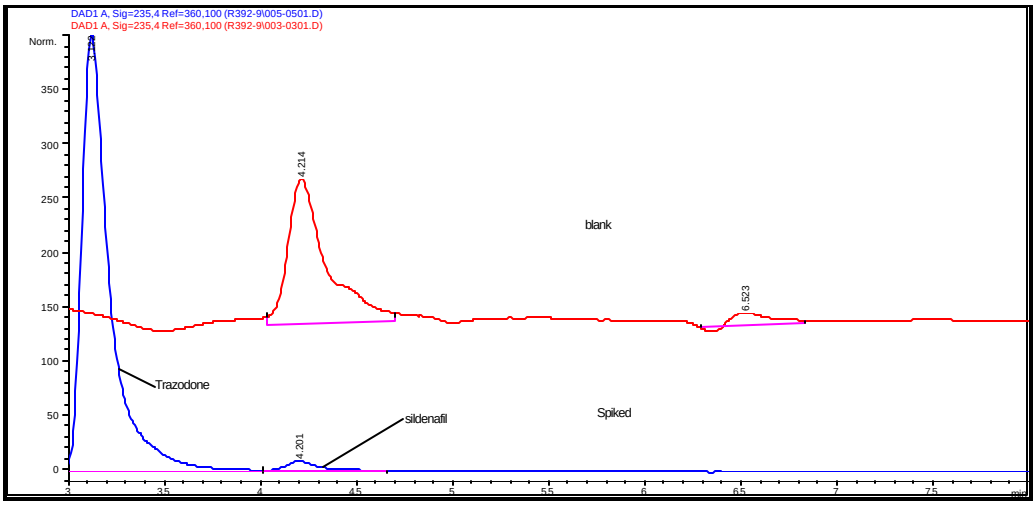


FIG. 2.11: 0.4 ml. Blank and spiked plasma (100 ng/ml sildenafil) alkalinised with 300 ml 0.01 M NaOH and extracted with 6 ml. diethyl ether. Mobile phase: 30% acetonitrile in 0.05 M ammonium acetate buffer pH 5.8. Detection wavelength = 235 nm.

Note the potential interfering peak, at the retention time of sildenafil, in the blank plasma extract.

Several experiments involving modifications of the extraction procedure such as changing the extraction solvents and modifications of the mobile phase pH were performed in attempts to minimise interference from the co-extracted endogenous components to no avail. The retention times of the interfering peaks and sildenafil moved in the same direction and to the same extent with changing pH of the mobile phase.

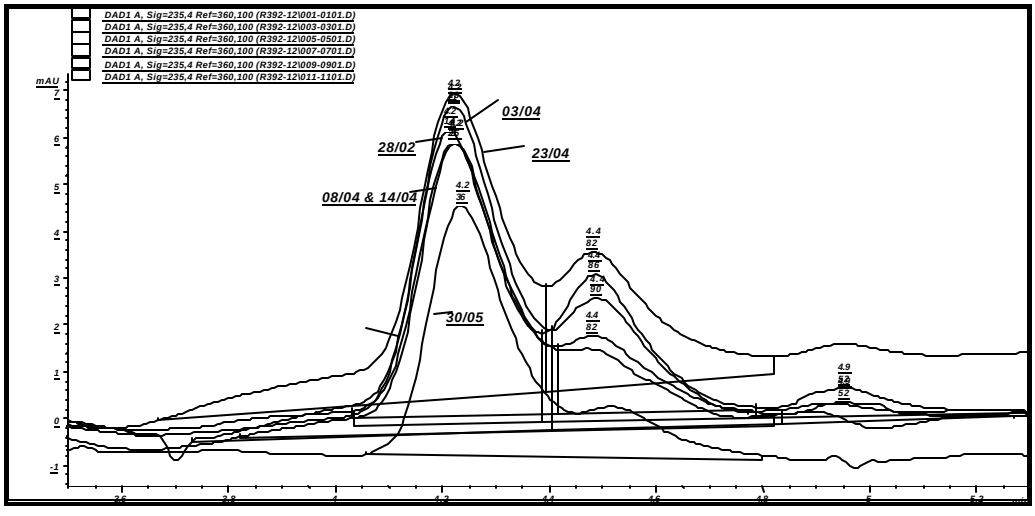


FIG. 2.12: 0.4 ml. Blank plasma pooled on different date alkalinised with 300 ml 0.01 M NaOH and extracted with 4 ml. diethyl ether. Mobile phase: 30% acetonitrile in 0.05 M ammonium acetate buffer pH 5.8. Detection wavelength = 235 nm.

The overlaid chromatograms (Fig. 2.12) of extracts of six different blank plasma pools confirmed that the interference arises from endogenous components.

The area of the peak at 4.2 minutes that interferes with sildenafil ranged from 58-83 mAu*s that is comparable with the area obtained from 400 ng/ml sildenafil stock solution (equivalent to the concentration of reconstituted extract of 100 ng/ml plasma sample). Changing the detection wavelength did not significantly change the interference's peak area relative to the peak area of sildenafil nor did changing the pH of the mobile phase achieve separation between sildenafil and the interfering peak.

Since the acetate buffer based mobile phase was investigated with the objective of developing an LC/MS/MS assay method, no further optimisation of the chromatographic process was considered necessary at this stage as the interfering peak would probably not play any role (except a possible matrix effect) if the very high potential specificity of MS/MS were to be employed.

2.1.2.1.1 Degradation of sildenafil and trazodone during LLE with diethyl ether

When extracting plasma samples spiked with sildenafil and trazodone with diethyl ether as described in the final optimised extraction procedure, two additional peaks with retention times of 3.85 and 5.5 minutes that were not present in the chromatograms of blank plasma extracts or directly injected aqueous solutions of the analytes, were observed.

Since these peaks were not present in aqueous solutions of the analytes injected directly onto the HPLC column, they were suspected to be artefacts produced in the extraction procedure. To confirm this suspicion, an aqueous solution containing sildenafil and trazodone was extracted with diethyl ether as described, and the extract, reconstituted in mobile phase, chromatographed with a mobile phase of 30% acetonitrile in 0.05 M phosphate buffer adjusted to an apparent pH of 6. This chromatogram A is compared with a chromatogram B of a solution obtained by spiking mobile phase directly with sildenafil and trazodone (Fig. 2.13).

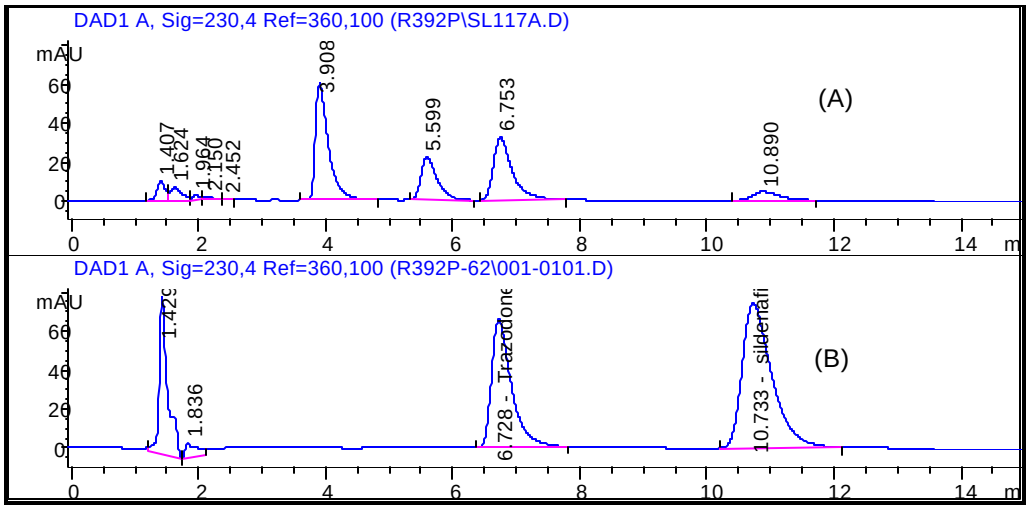


FIG. 2.13: (A). Isocratic chromatogram of diethyl ether extract of an aqueous solution of sildenafil and trazodone. (B) Isocratic chromatogram of sildenafil and trazodone spiked directly into mobile phase pH 6.

Comparison of the UV spectra (Fig. 2.14) of the four chromatographic peaks suggest that the component with retention time of 5.599 min. in chromatogram A originates from sildenafil (t_R = 10.89 min. in chromatogram A and 10.733 min. in chromatogram B) and that the component with retention time 3.908 min. in chromatogram A originates from trazodone (t_R = 6.753 min. in chromatogram A and 6.728 min. in chromatogram B).

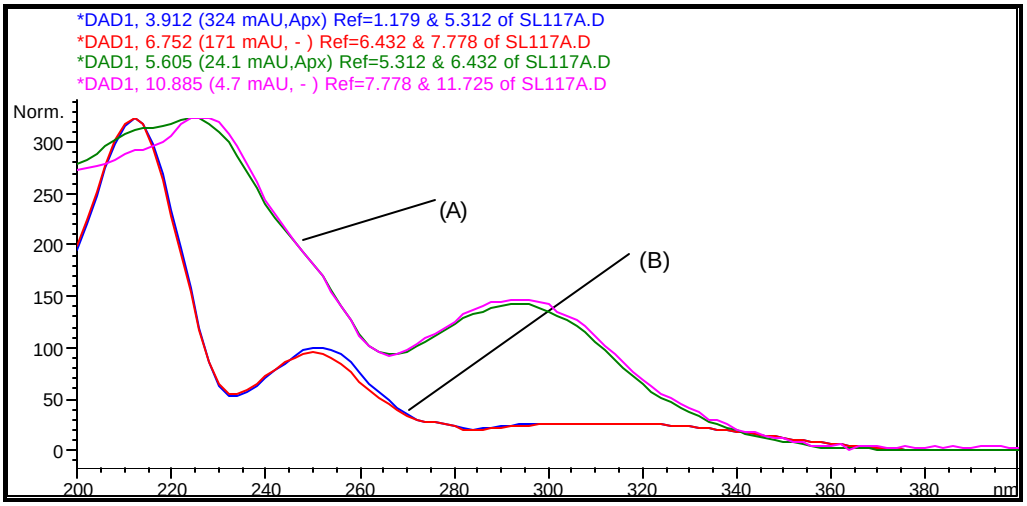


FIG. 2.14: (A) UV-Spectra of sildenafil and the peak at 5.6 min. (B) UV-Spectra of trazodone and the peak at 3.9 min.

Since the extraction procedure involved contact of the analytes only with diethyl ether, water and NaOH, the possibility of oxidation of the analytes by peroxide present in the diethyl ether was immediately suspected.

To investigate this possibility, a diethyl ether solution of sildenafil (0.17 µg/ml) and trazodone (0.86 µg/ml) was prepared from a methanol stock solution (501 µg/ml sildenafil and 200 µg/ml trazodone in methanol). The diethyl ether solution was divided into two; half of which was stored in the dark while the other half was stored exposed to laboratory light conditions. To obtain concentrated solutions for injection onto the HPLC column, 7ml. of each diethyl ether solution was evaporated and reconstituted in 100 µl of mobile phase pH 6 (30 % acetonitrile in 0.05 M phosphate buffer) after 0, 1, 2, 4, 6, 8, 10 and 24 hr storage at room temperature.

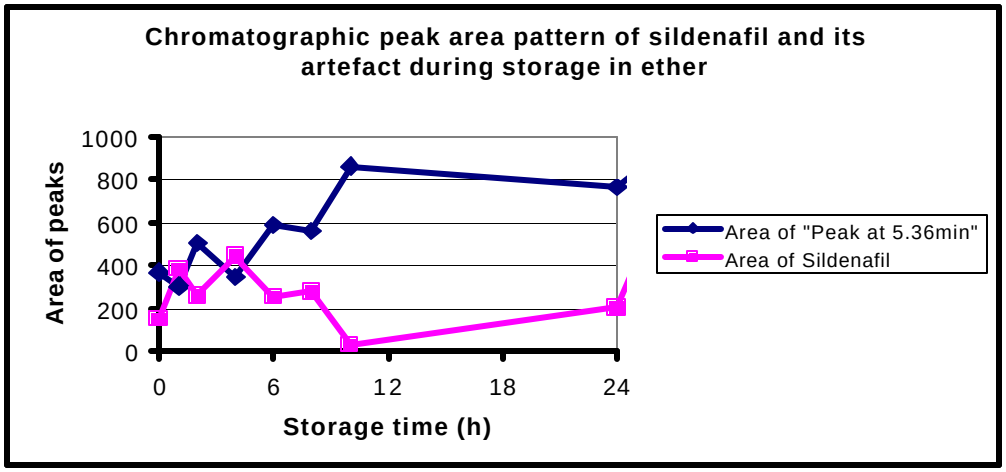


FIG. 2.15: Peak area pattern of sildenafil and its artefact during storage of sildenafil in ethyl ether solution.

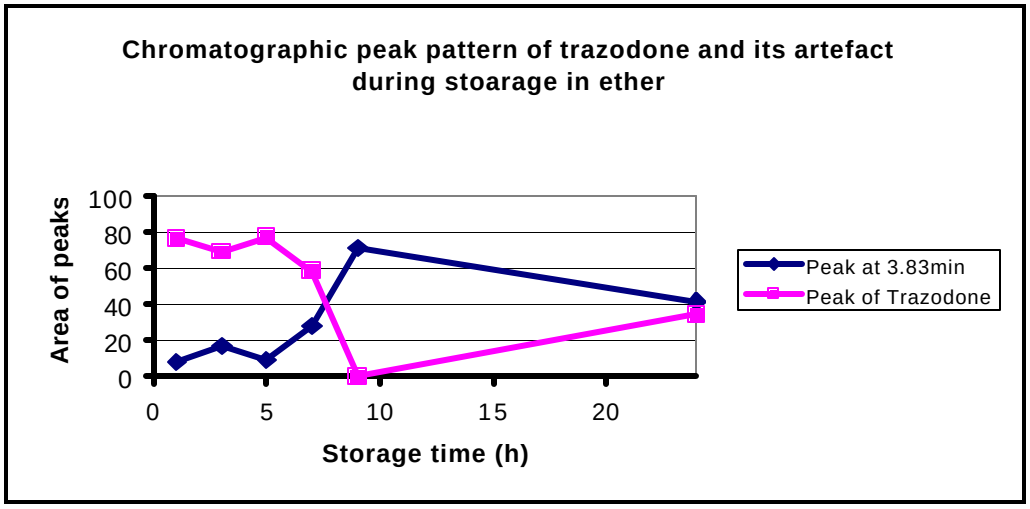


FIG. 2.16: Peak area pattern of trazodone and its artefact during storage of trazodone in diethyl ether solution.

The chromatograms obtained were basically the same as shown Fig. 2.13, confirming that artefacts resulted from the contact with diethyl ether. The peaks were present in solutions stored both in light and dark conditions. The peak areas of the sildenafil artefact appeared to increase with diethyl ether contact time (Fig. 2.15) but this was not very consistent probably pointing to some instability of the artefact.

To test the stability of the artefacts the reconstituted extracts that had been standing in the autosampler for 15 hours at room temperature were re-injected and the peak areas of the artefacts compared (Table 2.6 and Table 2.7).

TABLE 2.6: Comparison of the peak area pattern of sildenafil and its artefact after storing the mobile phase reconstituted solution for 15 hrs.

Sample	Peak area of the peak at min.			
	Fresh solution		Aged solution	
	Artefact	SLD	Artefact	SLD
A	369	151	401	144
B	303	386	343	370
C	[503]	[261]	[714]	[105]
D	346	444	378	437
E	589	254	648	214
F	563	279	598	272
G	863	31	902	19

TABLE 2.7: Comparison of the peak area pattern of trazodone and its artefact after storing the mobile phase reconstituted solution for 15 hrs.

Sample	Peak Area of the peak at min.			
	Fresh solution		Aged solution	
	Artefact	TRZ	Artefact	TRZ
A	815.9	675	876	696
B	560.2	1183	585	1184
C	822	988	1017	792
D	591	1281	608	1279
E	1061	782	1084	724
F	973	830	998	836
G	1542	78	1536	69

Except for sample C of sildenafil, the artefact of both sildenafil and trazodone appears to be quite stable on-instrument at room temperature and exposed to subdued laboratory lighting

conditions. In fact the peak areas of the artefact all increased slightly, relative to the peak area of sildenafil.

2.1.2.1.2 Conclusion

The formation of the artefacts during liquid/liquid extraction with diethyl ether obviously eliminates such an extraction procedure for sildenafil. It is interesting to speculate why the formation of this artefact was not observed by Lee and Min (2001). It is likely that the much smaller volume of diethyl ether used by them for the extraction yielded much smaller amounts of these artefacts that were then either not detected or considered to be endogenous components that on their chromatographic system did not interfere with the assay of sildenafil. Be that as it may, this calls into question the results obtained by Lee and Min since they will be influenced by the volume and quality of the diethyl ether used for extraction. At this stage it appeared quite certain that the artefacts would be oxidation products of sildenafil and trazodone; probably N-oxides. The nature of the artefact of sildenafil was studied in more detail at a later stage (Chapter 3) In the meantime another extraction procedure had to be developed.

2.1.2.2 Solid phase extraction (SPE)

Although the objective of this study was to develop a simple assay procedure involving a simple extraction procedure, the problems associated with interfering components in the liquid/liquid extractions using other extraction solvents precluded any further study of simple liquid/liquid extraction procedures and prompted an investigation into solid phase extraction procedures to obtain clean extracts for chromatography with UV detection.

2.1.2.2.1 Instruments and materials

100 mg. tC_{18}, Sep-Pak® Cartridges (Waters, Massachusetts, USA) were chosen and conditioning, washing and elution processes were performed on a SPEEDISK® 48 place positive pressure processor (Mallinckrodt Baker, Holland).

2.1.2.2.2 Chromatography

The chromatography set up which was optimised during the liquid/liquid extraction was retained as it is.

2.1.2.2.3 Optimisation of the SPE process

The parameters to be optimised in an SPE process are:

- Activation of the solid phase column.
- The loading solvent of the plasma.
- The washing solvent of the cartridge and its volume.
- The eluting solvent of the analyte and its volume.

General SPE procedure

The following general procedure was followed during optimisation:

- 0.5 ml. plasma in Eppendorf centrifuge tube was diluted with a suitable solvent.
- The resulting solution was vortexed for 10 seconds.
- The tC_{18} cartridge was conditioned with 1 ml. of methanol followed by 1 ml. of HPLC water.
- The diluted plasma solution was loaded onto the cartridge with the flow rate adjusted to about 1 ml/min on the SPEEDISK® 48.
- The cartridge was washed with suitable amount of solvent.

- The analyte was eluted with a suitable amount of the desired solvent.
- The eluate (normally organic solvent) was evaporated on a SAVANT®vacuum drier.
- The residue was reconstituted with 100 µl of relevant mobile phase.

The first trial was done loading the plasma with 0.5 ml. NaOH (.01M) and 0.5 ml. of 0.05 M phosphate buffer (pH 5) and using 1ml. of water followed by 1ml. of mobile phase as washing solvent. The result of this extraction shows that all the sildenafil and trazodone spiked vanishes. The analyte is either lost in the washing process or is bound with the protein of the plasma. To identify the problems, the following runs were done using acetonitrile to remove the analyte from the plasma and washing only with water.

The precipitate from the acetonitrile treatment was made to settle by centrifuging it for 5 min. at 2500 rpm. From the results in the Table 2.8 it can be seen that the suitable washing solvent was water, but the analytes were not bind to the protein of the plasma as the plasma diluted with buffer gave a recovery not lesser than that diluted with acetonitrile.

TABLE 2.8: Optimisation of washing and diluting solvent. In all cases 0.5 ml. NaOH was added to 0.5 ml. of plasma, the pH of phosphate buffer (0.05 M) and mobile phase was 5. All eluted with 1 ml. methanol.

Type plasma	Dilution solvent (0.5 ml.)	Washing solvent	Result.
Spiked	Buffer (pH=5)	Water	Good recovery
Blank	Buffer (pH=5)	Water	Large interference to TRZ & unclear chromatography
Spiked	Buffer (pH=5)	Buffer (pH 5)	Both analytes vanished
Blank	Buffer (pH=5)	Buffer (pH 5)	Large interference to TRZ & unclear chromatography.

NB: The spiked plasma has a concentration of 100 ng/ml sildenafil.

When the washing solvent was pH 5 buffer, the analytes vanished which is reasonable as the retention time of both sildenafil and trazodone is low when pH of mobile phase is 5 and hence lost in the washing step. At neutral pH value of the washing solvent (water) the recovery of the analytes was found to be good.

At this stage optimising recovery became the main priority.

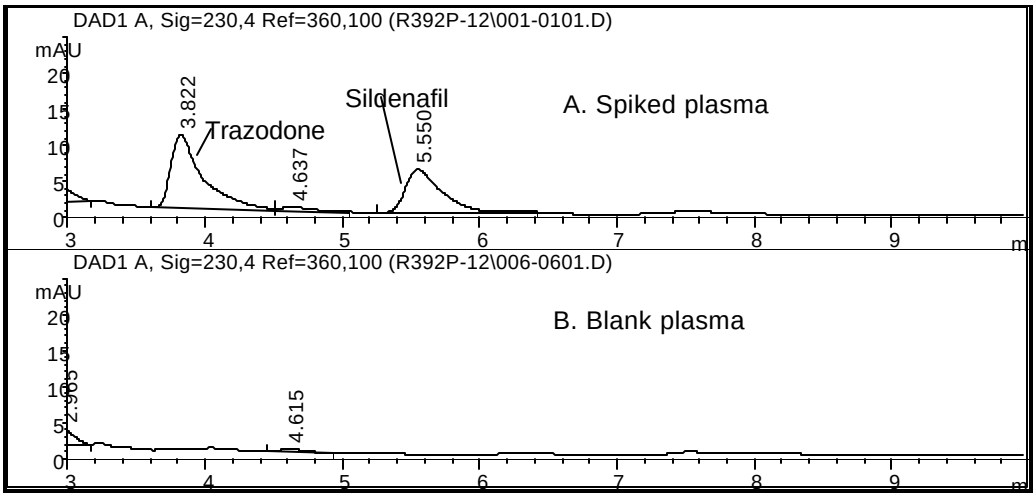


FIG. 2.17: (A) 0.5 ml. spiked plasma (100 ng/ml sildenafil and 2 mg/ml trazodone) (B) 0.5 ml. Blank plasma extracted SPE using 0.5 ml. NaOH (0 .01 M) as diluting solvent, mobile phase, acetonitrile : 0.05 M H3PO4 (35 : 65) adjusted to pH 5 with 5M NaOH.

Previous experience with the chromatography of sildenafil and trazodone during the optimisation of the liquid/liquid extraction procedure indicated that both analytes were fairly strongly retained on the chromatography column when the mobile phase had an apparent pH above 6. It was therefore decided to investigate the loading of spiked and blank plasma samples, diluted with mobile phase at various pHs, onto the activated SPE cartridges. The following SPE procedure was followed:

Experiment 1

- 0.5 ml. Plasma containing 100 ng/ml sildenafil in Eppendorf centrifuge tubes
- Added 100 µl trazodone solution (30 µg /ml in methanol).
- Diluted with 0.5 ml. of 30% acetonitrile in 0.05 M phosphate buffer based mobile phase adjusted to a range of apparent pH values (7, 8, 9, 10, 11).
- The resulting solution was vortexed for 10 seconds.
- The tC_{18} cartridge was conditioned with 1 ml. of methanol followed by 1 ml. of HPLC water.
- The diluted plasma solution was loaded onto the cartridge with the flow rate adjusted to about 1 ml/min. on the SPEEDISK® 48.
- The cartridge was washed with 1 ml. of deionised water.
- The analyte was eluted with 2 ml. methanol.

- The eluate was evaporated on a SAVANT® vacuum drier at about 60 °C.
- The residue was reconstituted with 100 µl of the relevant mobile phase and 20 µl injected onto the HPLC column.

Chromatography was performed with mobile phase (30% acetonitrile in 0.05 M phosphate buffer) adjusted to apparent pH 5.

The results of this experiment are summarised in Table 2.9.

TABLE 2.9: Effect of pH of the loading mobile phase on the extraction yield of sildenafil and trazodone in the SPE process described.

pH of mobile phase used for dilution	Retention (min.) of analytes		Interfering peaks		Peak area		Extraction yield %	
	TRZ	SLD	tR (min.)	Area	TRZ	SLD	TRZ	SLD
7	3.44	4.91	3.2 & 3.7	11 & 16	2268	123	100.18	108.85
8	3.59	5.17	3.2 & 3.8	8 & 4	2165	104	95.63	92.03
9	3.6	5.2	3.2 &3.57	7 & 2	2080	86	91.87	76.12
10	3.6	5.2	3.2 & 3.4	8 & 6	2249	86.6	99.34	76.6
11	3.62	5.2	3.2 & 3.4	11	2071	77.9	89.09	68.93
12	3.72	5.34	3.2 & 3.6	8 & 11	2169	74.6	95.80	66.10

Note: Area of 500 ng/ml sildenafil in methanol =113 mAu*S and area of 30 µg/ml trazodone in methanol = 2264 mAu*s.

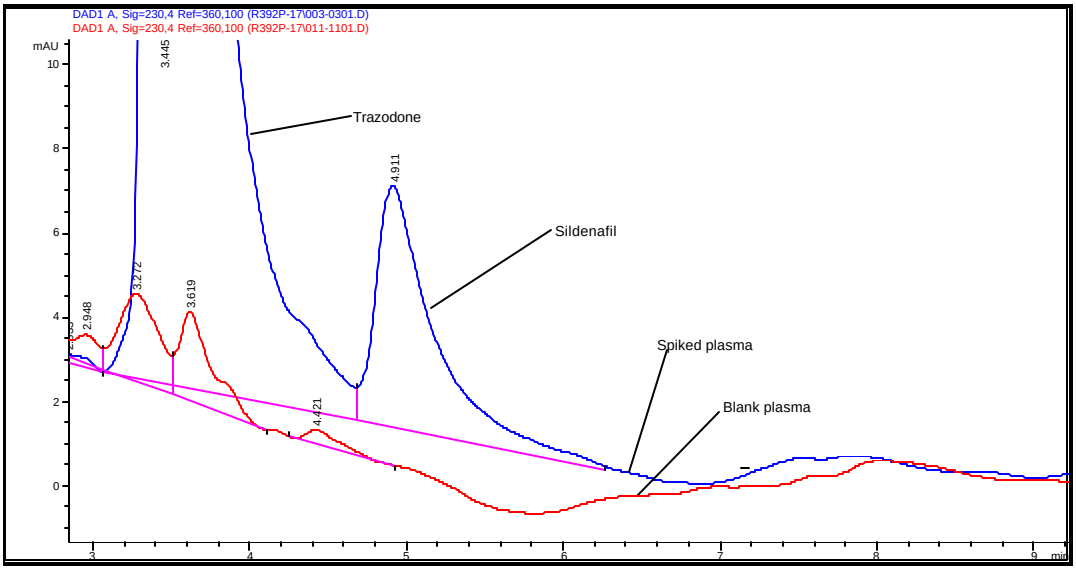


FIG. 2.18: Chromatograms of spiked and blank plasma extracts obtained by SPE as described in experiment 1 (retention time for sildenafil=4.91 min. and trazodone=3.44 min.).

As can be seen from the above Table 2.9 and a representative chromatogram Fig. 2.18, all of the mobile phase solutions used to load the plasma result in chromatograms have interfering peaks with trazodone. In all the chromatograms the peak for sildenafil was well resolved with no interfering peaks nearby. However, the recovery of sildenafil decreased with increasing pH of the loading solvent while the recovery of trazodone is not affected. This is to be expected as sildenafil has a weak acidic moiety. Thus increasing pH leads to its ionisation.

Experiment 2

Experiment 1 was repeated with 6 pools of unspiked blank plasma collected on different dates with only one modification; the plasma samples were all diluted with 0.01 M NaOH instead of the different pH mobile phases, before being loaded onto the cartridge. All the other SPE processing steps were identical to the previous experiment.

Chromatography was performed with mobile phase (30% acetonitrile in 0.05 M phosphate buffer) adjusted to apparent pH 5.

The results are conveniently summarised in Fig. 2.19.

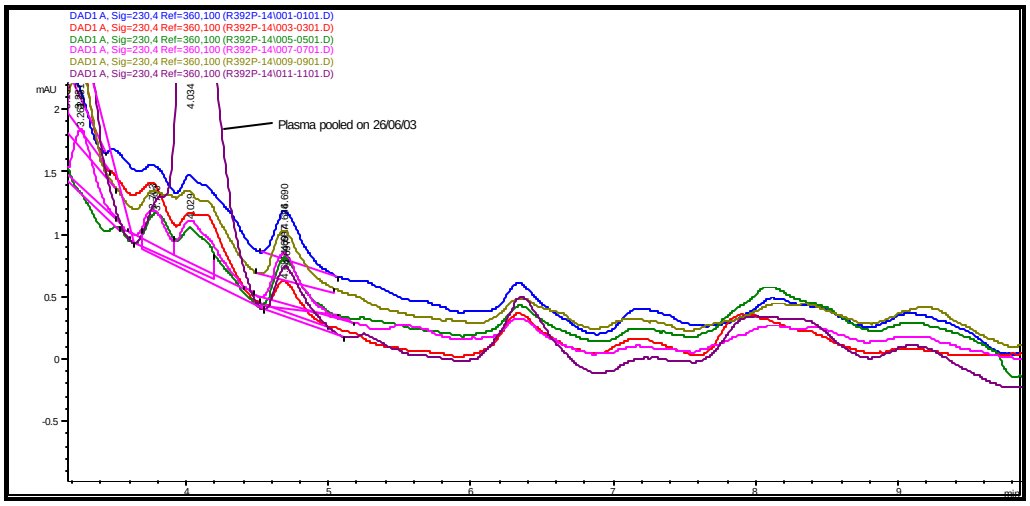


FIG. 2.19: Chromatograms of blank plasma pooled on different dates and obtained by SPE as described in experiment 2 (retention time for sildenafil=5.5 min. and trazodone=3.8 min.).

Several small peaks in the retention time region of trazodone were still discernable. However, except for one plasma pool (this appears to have been contaminated) their peak areas were very small (peak heights < 0.5 mAu*s) and within acceptance criteria.

The next parameters of the SPE to be optimised were the washing and eluting solvents.

Experiment 3

Experiment 2 was performed on several blank plasma pools changing the washing solvent to 0.05 M phosphate buffer with pH 7 and 9. The chromatograms obtained were no further improvement on those obtained in experiment 2. For the sake of simplicity of the extraction procedure it was therefore decided to retain water as the washing solvent.

Experiment 4

Finally the effect of the volume of washing solvent (water) and eluting solvent (methanol) was investigated. A blank plasma pool was spiked to contain 65 ng/ml sildenafil. 0.5 ml. Samples of this spiked plasma pool were then processed as described in experiment 1 (values of trazodone used was 50 µl of 20 µg/ml trazodone) while varying the volumes of washing and eluting solvents 1:1, 1:2, 1:3, 2:1, 2:2, 2:3, and 3:2. When the reconstituted extracts were injected onto the HPLC using three different mobile phases; 30% acetonitrile in 0.05 M phosphate buffer with the apparent pH of the mobile phases at pH 5, 5.5 and 6, they all displayed comparable chromatographic purity and recovery of analytes (Table 2.10).

TABLE 2.10: Optimisation of washing (water) and eluting (methanol) solvent amount.

pH of mobile phase	Area ratio of sildenafil to trazodone at given ratios of washing solvent (water) to eluting solvent (methanol)						
	1:1	**1:2**	**1:3**	**2:1**	**2:2**	**2:3**	**3:2**
5	0.00889	0.089	0.114	0.0849	0.109	0.0809	0.0867
5.5	0.102	0.15	0.138	0.112	0.117	0.0857	0.111
6	0.005	0.089	0.115	0.076	0.102	0.0785	0.0813

An overall inspection of the chromatograms and the data indicated that 1 ml. of water as washing solvent and 2 ml. of methanol as eluting solvent gave the best result.

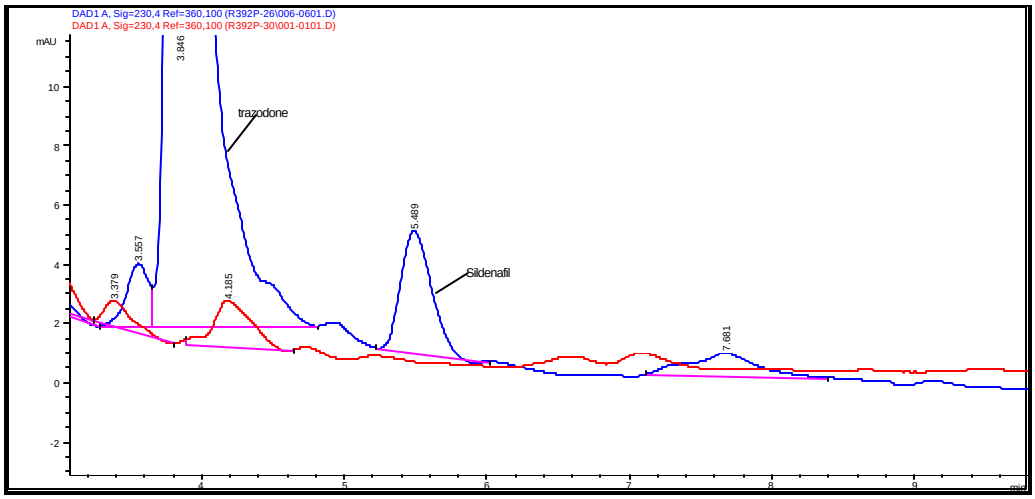


FIG. 2.20: Chromatograms of blank and spiked plasma (50 ng/ml sildenafil and 1000 ng/ml trazodone) extracts obtained by SPE in experiment 4. Mobile phase pH = 5; sildenafil.

A typical chromatogram obtained with the pH 5 mobile phase is presented in Fig. 2.20 above.

The final optimised SPE method can therefore be summarised as follows:

Preferred SPE procedure

- To 0.5 ml. plasma in an Eppendorf microfuge tube add 0.5 ml. NaOH (0.01 M) and 100 µl internal standard solution (10 µg/ml trazodone in methanol) (pH of 0.01 M NaOH=11.6, pH of plasma=7.4 and pH of final solution=9.7).
- Vortex mix for 10 seconds.
- Condition the tC_{18} cartridge with 1ml. of methanol followed by 1 ml. of deionised water.
- Load the diluted plasma solution at 1 ml/min.
- Washed with 1 ml. of deionised water.
- Elute the analyte with 2 ml. of methanol.
- Evaporate the organic solvent on a SAVANT® vacuum evaporator.
- Reconstitute the extract with 100 µl mobile phase.
- Inject 20 µl of the reconstituted extract onto the HPLC.

Preferred mobile phase

An important part of method development is integrating the optimisation of mobile phase with the optimisation of the extraction method to end up with a good assay method as a

whole. The turn-around time of a chromatographic procedure, i.e. the time elapsed between two sample injections in an automated sequence of injections by an autosampler in an automated assay method, is an important parameter if a large number of samples have to be assayed in a clinical trial it is therefore important to reduce chromatographic runtimes as much as possible without compromising separation integrity.

An inspection of the chromatograms (Fig. 2.20 & 2.21) and the data in Table 2.10 indicates that chromatograms obtained with the mobile phase pH 5 would be preferred to those obtained with the mobile phase pH 6 from the point of view of the turn-around time. In both cases sildenafil elutes in regions free of interference. Although in both cases there are still interfering components eluting together with trazodone, these interfering peaks are very small relative to the peak area of trazodone and will therefore have very little influence on the quantitation of sildenafil.

It was therefore decided to proceed with the formal validation of this assay method using the mobile phase 30% (acetonitrile in 0.05 M phosphate buffer) adjusted to apparent pH 5.

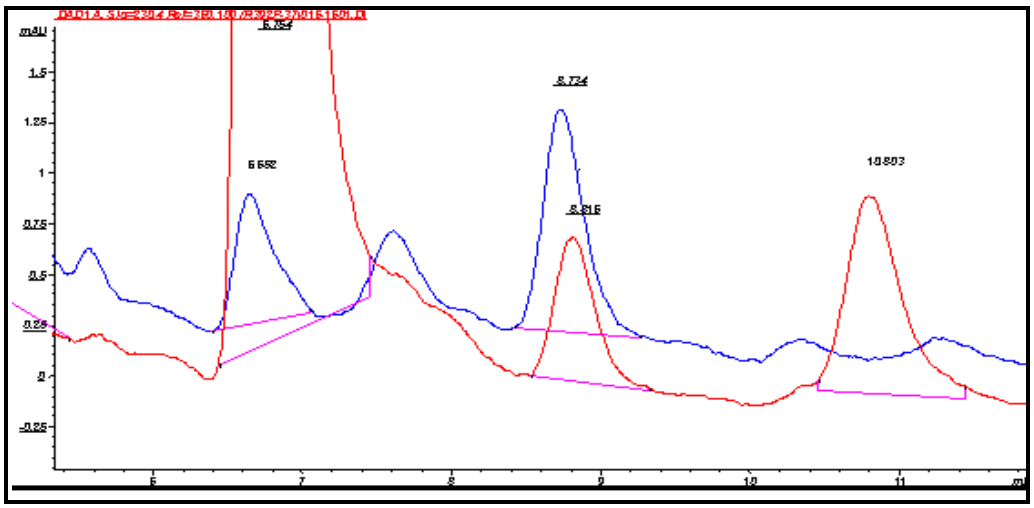


FIG. 2.21: Chromatograms of blank and spiked plasma (50 ng/ml sildenafil and 1000 ng/ml trazodone) extracts obtained by SPE in experiment 4. Mobile phase pH = 6 ($t_{R\ sildenafil}$ = about 10.8 min. and $t_{R\ trazodone}$ = about 5.5 min.

2.1.3 Pre-validation

A short pre-validation was performed before starting the final validation process to ensure that all procedures as set up yield the expected results. Pre-validation entailed the assaying of calibration standards and quality control samples, in a batch method to check that the

linearity of the calibration line and the reproducibility of assayed quality controls satisfy existing acceptance criteria.

Experience gained during the method development suggested that a sildenafil concentration of the order 50 ng/ml would probably be the LLOQ and according to the pharmacokinetic data surveyed, the maximum concentration in plasma of sildenafil expected after a single oral dose of 100 mg would be around 1500 ng/ml (Cmax).

Hence calibration standards spanning a range of 50 ng/ml (LLOQ) to 1600 ng/ml (Cmax) were prepared and quality controls with concentration falling within this range up to near the LLOQ were also prepared.

Blank plasma screened during the method validation was pooled together and 6 ml. of it was spiked with sildenafil stock solution (in methanol) to give a plasma concentration of 1600 ng/ml sildenafil and this was diluted serially 1:1 with blank plasma to obtain all the levels of the calibration range (1600,800,400,200,100 and 50 ng/ml). 10 ml. of the same pool of blank plasma was also spiked with 100 ng/ml sildenafil was to produce the quality control.

Extraction of the calibration standards in single-fold and the quality control was done according to the preferred SPE extraction procedure and chromatographed with the preferred mobile phase. The results obtained are presented in Table 2.11 and Fig. 2.22.

TABLE 2.11: Reproducibility of the assay of 0.5 ml. of plasma spiked with 100 ng/ml sildenafil.

No.	Peak area of sildenafil	Peak area of trazodone	Peak area ratio (SLD/TRZ)
1	91.63	1009	0.0908
2	101.51	1073	0.0946
3	92.60	1054	0.0879
4	104	1186	0.0877
5	96	1098	0.0874
Mean	97.15	1084	0.0897
RSD	5.44	65.66	0.0031
CV %	5.60	6.06	3.43

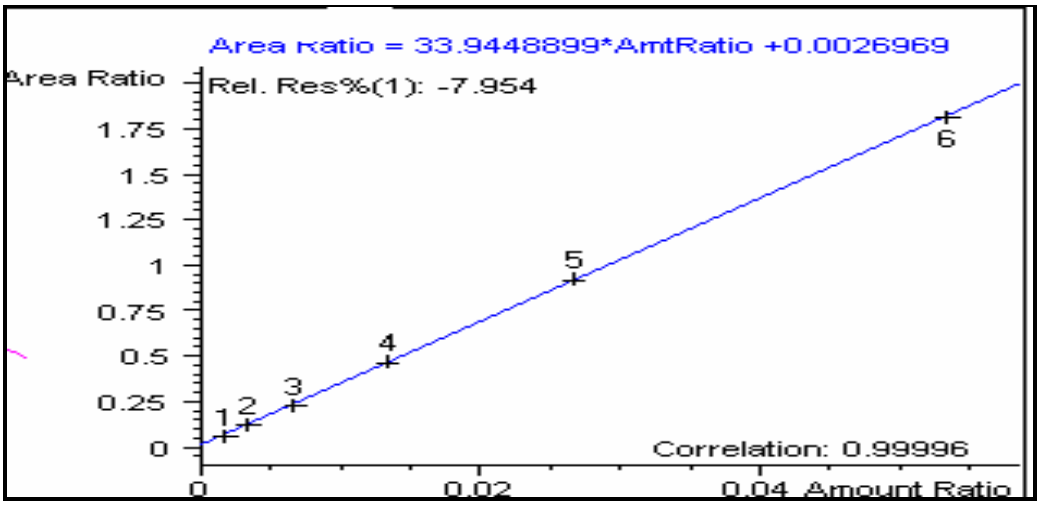


FIG. 2.22: Calibration curve representing plasma extraction by solid phase method at the range of 50 ng/ml to 1600 ng/ml sildenafil.

The results show that the extraction method has good linearity over the entire range with a correlation coefficient of 0.999.

The reproducibility of the assay was evaluated based on the coefficient of variance (CV%) of the area ratio of sildenafil/trazodone of the replicate assays of the 100 ng/ml quality control. The low CV value shows the reproducibility of the assay procedure is good. Table 2.11.

2.1.3.1 Stability of stock solution in glass and plastic containers

According to Lee and Min (2001), a significant loss of sildenafil was observed when aqueous stock solutions of sildenafil were stored in a plastic container. Since the SPE extraction process was to be performed in Eppendorf plastic tubes, the following experiment was done to compare the stability of stock solution when stored in plastic and glass containers.

An aqueous stock solution containing 9.93 µg/ml sildenafil and 20.95 µg/ml trazodone was prepared in water The solution was divided into three containers, one stored in glass at room temperature, one stored in plastic at room temperature and one stored in glass at -20 °C (assumed stable as a freshly prepared solution). 20 µl of each solution was injected onto the HPLC after storage for 0, 2, 4, 6, 24 hrs and the peak area ratios were compared.

TABLE 2.12: Comparison of stability of stock solution in glass and plastic containers.

Contact time (h)	Sildenafil peak area ratio with respect to sildenafil stored at –20 °C in glass	
	Solution stored in glass at room temp.	Solution stored in poly-propylene at room temp
0	1.000151	1.000151
2	1.051037	1.015949
4	1.103559	0.983051
24	1.102564	0.769776

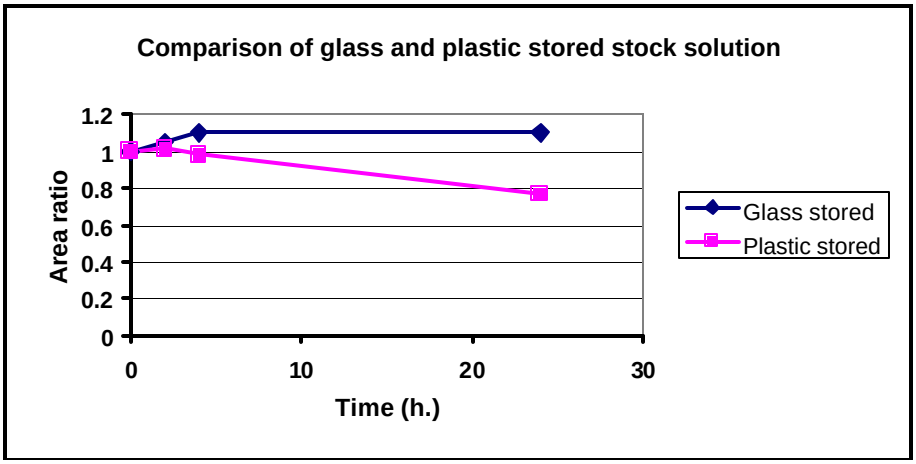


FIG. 2.23: Graphical presentation of data in Table 2.12.

As can be seen from the Fig. 2.23, sildenafil shows significant decrease in peak area ratio with time. During the extraction procedure contact time with plastic must be kept short to avoid loss of analyte.

2.1.4 HPLC-UV assay method validation

The results of the pre-validation indicated that the final method validation could be performed. The validation process was performed in the following sequence:

- Blank plasma screening.
- Planning of calibration (STD) and quality control (QC) standards.
- Preparation of calibration and quality control standards.
- Preparation of internal standard solution, Performance Verification Standard and mobile phase.
- Setting up the validation batch.
- Assaying the intra-day, inter-day-I and inter-day-II samples.

2.1.4.1 Blank plasma screening

Blank normal human plasma was obtained from healthy human volunteers participating in clinical trials. To ensure that the plasma obtained does not contain exogenous components that could interfere with the analytes of interest it needs to be analysed by the assay method before the calibration standards and quality controls are prepared. Most of the work on plasma screening was done during the method development phase and plasma pooled on 12/06/03, 18/06/03, 19/06/03, 23/06/03, 25/06/03 was selected to prepare a single combine blank plasma pool for further work. These normal plasmas were collected into one large polypropylene beaker. After removal of clotted fibrin by sieving and centrifugation, the relatively clot-free plasma was divided and stored in screw-capped 500ml. polypropylene containers and stored at -20 ^{o}C.

2.1.4.2 Planning of calibration (STD) and quality control (QC) standards

At this stage of validation, two parameters must be decided. The first parameter was the range of the calibration standards. According to Lee and Min (2001), the maximum concentration of sildenafil found in a pharmacokinetic study performed after administration of 100 mg of sildenafil with grapefruit juice was 1517 ng/ml. So the C_{max} of the calibration curve was chosen to be 1600 ng/ml. Several experiments during method development indicated that a signal to noise ratio (S/N) of about 100 could be achieved with the assay of samples with a concentration of 50 ng/ml. In pharmacokinetic studies the aim of an assay method is to be able to assay plasma samples over a period of about 4 to 5 half-lives after

dosing. This means that if a C_{max} of about 1600 ng/ml is expected, the assay method should have an LLOQ in the region of about 100 ng/ml. The S/N of about 100 observed for samples assayed at 50 ng/ml during the method development meant that, although it appeared possible to attain an LLOQ in the region of about 10 ng/ml, an LLOQ of 50 ng/ml should be easily attained. Since it is standard practice to validate assay method for phramacokinetic studies up to 2 x C_{max}, it was therefore decided to prepare calibration standards ranging from 50 ng/ml to 3200 ng/ml (2 x C_{max}).

Formal validation of assay methods that are used in clinical studies requires large volumes of blank plasma due to the many calibration standards and quality controls that need to be processed. To avoid wastage of scarce resources it is therefore important to carefully plan the preparation of the calibration standards and quality controls. As the calibration line is meant to show the relation between the concentration and the response of the instrument, it must encompass the whole range of concentrations over which the analyte is to be assayed. A calibration line with range from 50 ng/ml to 3200 ng/ml, by 1:1 dilution of the highest concentration (3200 ng/ml), requires at least seven calibration levels. As the purpose of quality control standards (QC) is to assess the performance of the assay procedure; it must also cover the whole range of calibration line. Hence six quality control standard levels need to be prepared.

TABLE 2.13: Calibration standards (STD) of sildenafil to be prepared.

Code	Concentration (ng/ml)
STD H (2 x C_{max})	3200
STD G	1600
STD F	800
STD E	400
STD D	200
STD C	100
STD B (LLOQ)	50

TABLE 2.14: Quality control (QC) standards of sildenafil to be prepared

Code	Concentration (ng/ml)
QC F (1.8 x C_{max})	2880
QC E	1440
QC D	720
QC C (1.3 x STD D	260
QC B (1.3 x STD C)	120
QC A (1.3 x LLOQ)	65

NB: The concentrations in Table 2.13 and Table 2.14 are concentrations to be aimed at but not the concentrations that might actually be obtained when preparing them using the Standard Operating Procedure (SOP) established in the laboratory in which this study was performed.

The following three phases in the validation require calibration and quality control standards:

- Intra-day validation.
- Inter-day-I validation.
- Inter-day-II validation.

Based on the number of calibration standards, the volume of plasma required for the validation was calculated with the aid of a calculation sheet set up in Excel. 2.15.

Each step of the validation will have the following set of calibration standards (STD) and quality control standards (QC).

TABLE 2.15: Calculated volume of plasma needed for preparation of STDs and QCs required in validation.

Phase	Sets of STDs (A)	Sets of QCs (B)	Levels STDs (C)	Levels QCs (D)	Replicates. STDs (E)	Replicates QCs(F)	Sample volume (G)	Volume (ml.)
Intra-day	1	1	7	6	2	6	0.8	40
Inter-day-I	1	1	7	6	1	6	0.8	34.4
Inter-day-II	1	1	7	6	1	6	0.8	34.4
							Total	108.8

Volume of plasma = A x C x E x G +B x D x F x G

2.1.4.3 Preparation of calibration and quality control samples

The calibration standards and quality control standards were prepared by two different analysts following an SOP of the laboratory.

A 99.8 % pure sildenafil citrate from SynFine Research was used as standard reference material to prepare the stock solution for both calibration and quality control standards.

All pipetters were calibrated prior to preparation of the stock solution using QC Pipette™ software for their accuracy and precision of their volumetric measurements.

All the volumetric measurements were done by weighing on a Mettler toleddo electronic microbalance, changing the specific volume to weight using its specific gravity of the stock solution solvents and plasma.

2.1.4.3.1 Calibration standards (STD)

The author of this dissertation prepared the calibration standards. 7.243mg of reference standard sildenafil citrate (equivalent to 5.16mg free base sildenafil) was dissolved in 8.15 g of methanol to give a stock solution **(stock A)** having concentration of 501.05 µg/ml. 250 µl of this stock solution was spiked into normal blank plasma to produce the highest concentration standard (STD H). The remaining calibration standards were prepared by 1:1 serial dilutions of STD H using normal blank plasma. Table 2.16 summarises the preparation of the calibration standards.

TABLE 2.16: Calculation of calibration standard concentrations.

Preparation of stock SA for spiking STD H.

Solvent Used	S G solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml.)	Volume spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.792	5.156	8.150	10.290	250	501.05

Preparation of calibration levels.

Sample Code & No.	Source Solution	A	B	C	D ng/ml
STD H	Stock SA	20.720	60.720		3195
STD G	STD H	20.652	40.654	60.677	1598
STD F	STD G	20.622	40.641	60.633	799
STD E	STD F	20.437	40.441	60.417	399
STD D	STD E	20.616	40.618	60.597	199
STD C	STD D	20.652	40.645	60.436	99.2
STD B	STD C	20.595	40.621	60.638	50

Note: Mass of biological fluid (g) is converted to volume (ml.). SG = 1.0269 for plasma

Analyte = sildenafil

Biological fluid = plasma.

Key:

A = Mass of empty container

B = Mass of empty container plus normal biological fluid.

C = Total mass of container plus spiked plasma

D = Concentration (ng/ml).

To make provision for unforeseen problems that might occur during the validation process, each set of STD and QC samples was prepared in triplicate. 0.8 ml. Of each sample of the calibration standards was aliquoted into 1.5ml. Eppendorf microfuge tubes and stored at about -20 ^{o}C in a temperature-monitored freezer until analysis. The samples not actually used during the validation were stored for the purpose of determining the long-term stability of sildenafil in plasma.

2.1.4.3.2 Quality control standards (QC)

Following an SOP of the laboratory, quality control standards were prepared by an analyst other than the author of this dissertation. 8.60 mg (equivalent to 6.123 mg free base) reference standard sildenafil citrate was dissolved in 6.12 g of methanol giving a stock solution **(stock B)** with concentration of 792.39 µg/ml. 500 µl of stock-B was spiked in a normal blank plasma to give the highest concentration of quality control standard (QC F) with concentration of 2901 ng/ml. Similar to calibration standard preparation, QC E was prepared by diluting QC F with normal blank plasma. QC D was prepared by diluting QC E to give the appropriate concentration as can be seen from the table below. The rest of the lower QC levels were prepared by 1:1 serial dilution of QC D using normal blank plasma.

TABLE 2.17: Calculation of quality control standard concentration.

Preparation of Stock Solution QA for Spiking QC F.

Solvent used	S G solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml.)	Volume spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.792	6.123	6.120	7.727	500	792.39

Preparation of quality control Standards.

Sample Code & No.	Source Solution	A	B	C	D ng/ml
QC F	**Stock SA**	45.637	185.363		2901
QC E	**QC F**	20.668	90.675	160.670	1450
QC D	**QC E**	20.617	110.622	160.638	518
QC C	**QC D**	20.690	90.689	160.700	259
QC B	**QC C**	20.517	90.514	160.532	130
QC A	***QC B***	***20.658***	***90.653***	***160.654***	***65***

Note: Mass of biological fluid (g) is converted to volume (ml.). SG = 1.0269 for plasma

Analyte = sildenafil

Biological fluid = plasma.

Key:

A = Mass of empty container.

B = Mass of empty container plus normal biological fluid.

C = Total mass of container plus spiked plasma.

D = Concentration (ng/ml).

0.8ml. Of each level of the quality control standards were aliquot into 1.5ml. Eppendorf microfuge tubes and stored at -20^{o}C until analysis.

2.1.4.4 Preparation of internal standard and system performance verification samples

Based on previous work done by Saisho *et al.* (2001), the internal standard for this assay method was chosen to be trazodone. A stock solution **(Stock C)** was prepared by dissolving reference standard trazodone HCl equivalent to 3.43 mg free base trazodone in 13.55 g of methanol (200 µg/ml trazodone base). During method development, it was confirmed that spiking 50 µl of a 20 µg/ml trazodone solution; i.e. 1000 ng of trazodone in the extraction process gave a peak height response equivalent to 75 % of the response of the $C_{max.}$ of sildenafil. However, to improve the accuracy and precision of the internal standard pipetting step, it was decided to add 100 µl of a 10 µg/ml solution of trazodone in methanol as internal standard in extracting 500 µl of spiked plasma during the validation process. Stock C was

therefore diluted 10x with methanol to obtain the desired concentration of the internal standard spiking solution.

System performance verification (SPV) samples are included at the beginning, middle and end of each batch of samples assayed to monitor and ensure reproducible performance of analytical system throughout its use during a particular study. It is used to indicate whether the instrument in use is working properly or not and to give a green light to proceed with the assaying of the next batch of samples. In this assay method, the mobile phase was used to make up system performance verification samples. The SPV solution was prepared to contain sildenafil at a concentration of 75 % of the final concentration of the reconstituted plasma extract of a sildenafil C_{max} sample and trazodone at a concentration equivalent to the final concentration of reconstituted plasma extract. Thus the final concentration of the performance verification samples was made up to contain 6 µg/ml sildenafil and 10 µg/ml trazodone in mobile phase.

2.1.4.5 Mobile phase preparation

The mobile phase was prepared by mixing acetonitrile with 0.05 M H_3PO_4 (3 : 7 v/v) and adjusting the apparent pH to 5.0 using 5 M NaOH solution. Each batch of mobile phase was degassed by sparging with helium for 1 minute.

2.1.4.6 Compiling the validation batches

Validation batches must contain:

- Extracts of **calibration standards** to obtain a calibration line.
- Extracts of **quality controls** to assess the accuracy and precision of the assay method.
- Extracts of the **highest QC diluted 2x** with blank plasma to assess the validity of diluting samples with concentrations higher than the highest calibration standard if such samples were to occur during the processing of study samples.
- Extracts of **blank plasma samples** to which no internal standard has been added to monitor possible carryover effects from previous injections and for the possible appearance of other interfering peaks.
- Extracts of **zero samples**; these are extracts of blank plasma samples spiked with the internal standard.

- Extracts of **on-instrument stability samples** to assess the stability of the analytes in the reconstituted extracts while the samples are being assayed.
- **Freeze and thaw stability samples** to assess the stability of the analyte in samples that have undergone more than one freeze and thaw cycle.
- **Bench-top stability samples** to assess the stability of the analytes in the thawed plasma samples while they are standing at room temperature on the bench-top awaiting extraction.
- System performance verification samples.

2.1.4.6.1 Intra-day validation batch

The intra-day validation batch consists of 1 set of STD samples at 7 concentration levels (STD H to STD B) in duplicate, 1 set of QC samples at 6 concentration levels (QC A to QC F), 6 QC F (dil) samples, 6 blank plasma samples, 2 zero samples (blank plasma with internal standard only), 8 on-instrument stability samples (1450 ng/ml), 5 freeze and thaw stability samples at -20 ºC (1450 ng/ml), 5 freeze and thaw stability samples at -20 ºC (259 ng/ml) and 3 performance verification samples.

2.1.4.6.2 Inter day validation batches

2.1.4.6.2.1 Inter-day-I validation batch

This batch consists of 1 set of STD samples at 5 concentration levels (STD H to STD D), 2 set of STD samples at 2 concentration levels (STD C to STD B), 6set of QC samples at 6 concentration levels (QC A to QC F), 6 blank plasma samples, 2 zero samples (blank plasma with internal standard only), 8 on-instrument stability samples (1450 ng/ml) and 3 performance verification samples.

2.1.4.6.2.2 Inter-day-II validation batch

This final step of the validation process also consists of 1 set of STD samples at 5 concentration levels (STD H to STD D), 2 sets of STD samples at 2 concentration levels (STD C to STD B), 6 sets of QC samples at 6 concentration levels (QC A to QC F), 6 blank plasma samples, 2 zero samples (blank plasma with internal standard only), 5 bench-top stability samples (1450 ng/ml), 5 bench-top stability samples (259 ng/ml) and 3 performance verification samples.

All reasonable care was taken from preparation of STD and QC samples up to execution of the different validation steps by storing the aliquots in temperature monitored freezers at

about -20 ^{o}C. To assess the decomposition of the analyte that may occur due to different reasons, the following stability test extracts were prepared.

On-instrument stability samples

- 16 **On-instrument stability samples** (1450 ng/ml, QC E) run during execution of Intra-day and Inter-day-I validation were extracted and the extracts were pooled together after reconstituting in mobile phase. The combined final extract was again divided into 16 aliquots for injection and placed in the autosampler.

Freeze and thaw stability samples

- **Freeze and thaw stability samples** were prepared as follows. Two sets of spiked plasma samples, five replicates of each (1450 ng/ml and 259 ng/ml) that were stored at -20 ^{o}C, were thawed completely unassisted at room temperature and refrozen immediately to -20 ^{o}C. This cycle was repeated twice with 20 hour intervals and the samples were extracted for injection during the Inter-day-I validation.

Bench-top stability samples

- **Two categories of bench-top stability samples** (259 ng/ml and 1450 ng/ml), each of replicate of 5 were thawed unassisted and kept at room temperature for 20 hrs. before extraction on the Inter-day-II validation.

2.1.4.1.7 Extraction of analyte from plasma

The preferred extraction procedure optimised during method development was used throughout the validation process:

- To 0.5 ml. plasma in an Eppendorf microfuge tube add 0.5 ml. NaOH (0.01 M) and 100 µl internal standard solution (10 µg/ml trazodone in methanol) (pH of 0.01 M NaOH = 11.6, pH of plasma = 7.4 and pH of final solution = 9.7).
- Vortex mix for 10 seconds.
- Condition the tC_{18} cartridge with 1ml. of methanol followed by 1 ml. of deionised water.
- Load the diluted plasma solution at 1 ml/min.
- Washed with 1 ml. of deionised water.
- Elute the analyte with 2 ml. of methanol.

- Evaporate the organic solvent on a SAVANT® vacuum evaporator.
- Reconstitute the extract with 100 µl mobile phase.
- Inject 20 µl of the reconstituted extract onto the HPLC.

2.1.5 Results and discussion

The method was validated to meet the acceptance criteria of the FDA Guidance for Industry, Bioanalytical Method Validation. Since the condition of the instrument is crucial for the whole process of validation, replicate performance verification samples (n = 4) were injected onto the HPLC column to assess the condition of the instrument before the full validation batch was processed. The CV % of the area ratio of sildenafil to the internal standard was found to be below 1 %, which was a promising result and allowed the continuation of batch validation.

Results were calculated using the PhIRSt (Phoenix International Life Sciences, Montreal, Canada) chromatographic data-reporting package. Peak heights and areas are electronically read automatically from report files generated by HPCHEM software. Data are automatically summarised, calibration curves calculated according to pre-set regression equations and concentrations interpolated by the program. Results are presented in printed ordered tables with performance statistics per batch and later summarised to give overall study statistics. This package has been validated in Canada by the manufacturer to FDA requirements.

Representative chromatograms of the validation done are given in the Appendix (Fig. A.1 to Fig. A.7).

2.1.5.1 Linearity.

The method developed was linear over the whole range of concentration from 50 - 3195 ng/ml for the three phases of the validation done. Using a linear regression equation weighted 1/concentration, all the calibration lines passed all the acceptance criteria. The results are presented in Table 2.18.

TABLE 2.18: Calibration line linearity results of the validation.

	Intra-day validation	Inter-day-I validation	Inter-day-II validation
Calibration standard	STD B – STD H	STD B – STD H	STD B – STD H
Calibration range	50 – 3195 ng/ml	50 – 3195 ng/ml	50 – 3195 ng/ml
Results			
Regression equation	1/Concentration linear.	1/Concentration linear.	1/Concentration linear.
Slope	0.001054	0.000986	0.001169
Intercept	0.013965	0.001464	0.006229
r^2	0.999	0.999	0.997

2.1.5.2 Accuracy and precision

With the PhIRSt data processing program a large number of regression equations can be fitted to the calibration data: PhIRSt uses the following regression algorithms.

TABLE 2.19: Regression algorithms used by PhIRSt program.

Type of regression	Regression equation
Linear	$Y = mx + b$
Linear (1/C)	$Y = mx + b$
Linear ($1/C^2$)	$Y = mx + b$
Log-Log	$Ln (Y) = m \ln (x) + c$
Wagner	$Ln (Y) = a (\ln (x)^2) + b \ln (x) + c$
Quadratic	$Y = a (x^2) + b (x) + c$
Quadratic (1/C)	$Y = a (x^2) + b (x) + c$
Quadratic ($1/C^2$)	$Y = a (x^2) + b (x) + c$

The values of the QCs are calculated using these regression equations. In this way the most suitable regression can be chosen to obtain the best results with the validation. However, the simplest regression giving acceptable results (i.e. passing all the acceptance criteria) should be chosen. In this validation the linear regression of: (sildenafil/trazodone peak height ratio) vs 1/(concentration of sildenafil) yielded excellent results. Summaries of the statistics for calculated concentrations of intra- and inter- day validation quality control standards based on peak height ratio are presented in Tables 2.20 to 2.22**.** For comparison data using the sildenafil/trazodone peak area ratio with and with out internal standard and sildenafil peak height with out internal standard are given in the Appendix (Table A.1 to Table A.9)

TABLE 2.20: Summary statistics for calculated concentrations of intra-day- validation quality control standards based on peak height ratio.

Code & Nominal Conc. / Replicates	QC F 2901.00 ng/ml	QC F (DIL) 1450.50 ng/ml	QC E 1450.00 ng/ml	QC D 518.00 ng/ml	QC C 259.00 ng/ml	QC B 130.00 ng/ml	QC A 65.00 ng/ml
1	[3417]	1548.82	1547.5	476.37	[323.92]	125.341	[104.191]
2	2558.844	1539.65	1650.77	555.25	233.839	134.081	65.78
3	2707.773	1234.45	1379.38	442.02	258.904	144.092	[100.112]
4	2761.542	1462.55	1405.77	504.17	247.239	137.079	74.268
5	2513.288	1553.67	1441.81	467.25	248.98	129.578	68.901
6	2808.215	1344.16	1329.75	472.94	246.191	127.238	68.346
MEAN	2669.93	1447.22	1459.16	486.33	247.03	132.90	69.32
%nom	92.0	99.8	100.6	93.9	95.4	102.2	106.7
CV%	4.8	9.1	8.2	8.1	3.6	5.3	5.1

Mean = mean value of area CV % = Precision (coefficient of variance)

% nom = Accuracy (mean bias from nominal concentration) [] = outlier not included in statistics

The MNR -ESD outlier test is used: The Maximum Normal Residual test modified by using a backwards elimination algorithm referred to as the Extreme Studentised Deviate in order to be able to detect multiple outliers. Hawkins (1980) has shown this to be an acceptable method for detecting multiple outliers, as the error is well defined and acceptably small.

TABLE. 2.21: Summary statistics for calculated concentrations of inter-day-I validation quality control standards based on peak height ratio.

Code & Nominal conc. / Replicates	**QC F 2901.00 ng/ml**	**QC E 1450.00 ng/ml**	**QC D 518.00 ng/ml**	**QC C 259.00 ng/ml**	**QC B 130.00 ng/ml**	**QC A 65.00 ng/ml**
1	3012.91	1417.42	501.51	288.79	123.506	81.05
2	3159.23	1339.35	450.85	293.03	144.812	61.84
3	3181.43	1299.39	509.06	274.47	142.75	B
4	3300.45	1303.12	496.04	292.34	147.115	58.176
5	B	1569.19	452.42	228.458	131.58	54.607
6	2295.377	1500.72	514.02	220.85	128.59	62.974
MEAN	2989.88	1404.87	487.32	266.32	136.39	63.73
%nom	103.1	96.9	94.1	102.8	104.9	98.0
CV%	13.4	7.9	5.8	12.4	7.2	16.0

B = Lost on the process.

TABLE 2.22: Summary statistics for calculated concentrations of inter-day-II validation quality control standards based on peak height ratio.

Code Nominal conc. / Replicates	QC F 2901.00 ng/ml	QC E 15450.00 ng/ml	QC D 518.00 ng/ml	QC C 259.00 ng/ml	QC B 130.00 ng/ml	QC A 65.00 ng/ml
1	2774.972	1456.787	526.175	260.488	122.093	54.409
2	2899.648	1454.003	562.313	236.693	131.089	61.482
3	2896.656	1589.589	539.465	252.037	135.311	63.451
4	2840.053	1491.429	538.759	258.354	125.231	65.879
5	2842.109	1484.336	535.935	259.374	137.956	64.859
6	2781.848	1472.713	527.95	272.471	131.158	67.628
MEAN	2839.21	1491.48	538.43	256.57	130.47	62.95
%nom	97.9	9.7	103.9	99.1	100.4	96.8
CV%	1.9	3.4	2.4	4.6	4.6	7.4

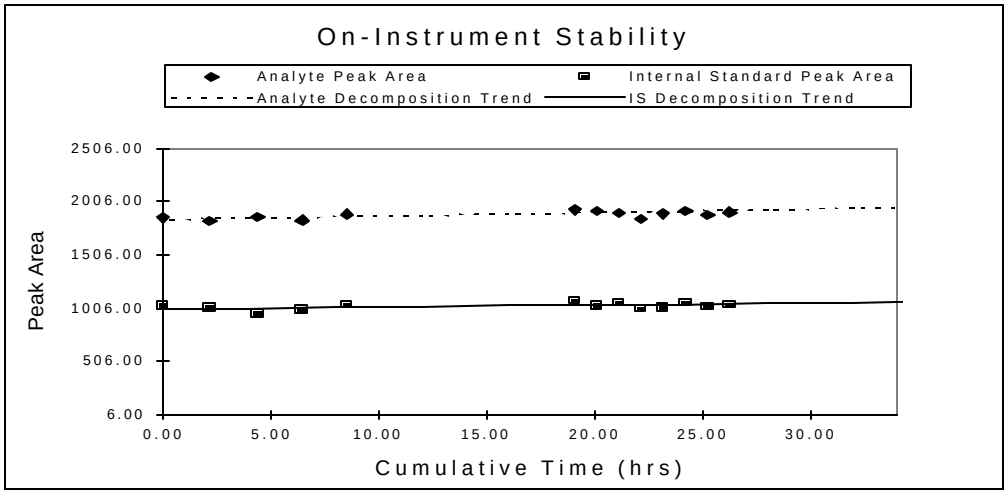
As shown in the results above, the accuracy and precision of the method developed were within the range of acceptance criteria recommended by international industry guidelines for bionalytical method validation. According to regulatory authorities, the CV % of LLOQ must be less than 20 %, the 16 % CV value of QC A on the Inter-day-I is still within the limit. The values in bracket are QC leve ls rejected because either they are outliers.

2.1.5.3 Analyte stability

In all the stability tests, the C_{max} concentration is represented by QC E (1450 ng/ml) while the 0.2 x C_{max} concentration is represented by QC C (259 ng/ml).

2.1.5.3.1 On-instrument stability

The on-instrument stability samples were QC E (1450 ng/ml) samples. 16 aliquots of QC E were extracted and were mixed together after reconstituting with mobile phase. They were finally divided into 16 aliquots and injected at time intervals over a period of 40 hrs while standing in the autosampler at room temperature. The results of this stability test are presented in Fig. 2.24.



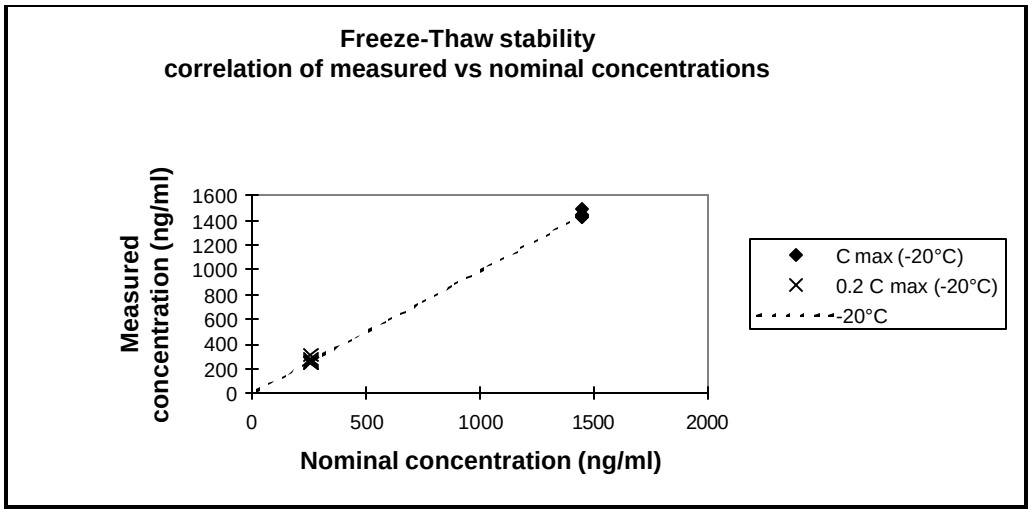
	Rate (area/h)	Intercept	%/hr	%/Batch	Max Batch Duration
Decomposition Trend (sildenafil):	3.2483	1838.69	0.18%	7.09%	84.9
Decomposition Trend (IS):	2.05222	998.738	0.21%	8.24%	73

FIG. 2.24: On-instrument stability of sildenafil and trazodone extracts when reconstituted in mobile phase and kept at room temperature.

The on-instrument stability data indicate a slight increase in the peak areas of both sildenafil and trazodone over a period of 41 hours. This indicates practically no decomposition of both analytes and is probably due to very slow evaporation of the solvent in which the extracts are reconstituted. According to the laboratory's acceptance criteria the analytes' chromatographic peak areas are allowed to change by 15% in a batch run. The maximum batch run times of sildenafil and trazodone would therefore be 84.9 and 73 hours respectively. For the sake of quality assurance (not on-instrument instability of the analytes), batch run times including re-injection of samples, should therefore not exceed these time limits.

2.1.5.3.2 Freeze and thaw stability:

Three freeze and thaw cycles between -20 ^{o}C and room temperature were performed on three QC E (1450 ng/ml) and three QC C (259 ng/ml) samples. The results are presented in Fig. 2.25.



Slope (-20°C) =	***0.99***

FIG. 2.25: Freeze and Thaw samples result for sildenafil.

As can be seen from the slope of the correlation of nominal and measured concentrations, sildenafil was stable in three freeze and thaw cycles. A slope equal to one indicates no decomposition.

2.1.5.3.3 Bench-top stability test:

This test assesses the short-term stability of the analyte in biological matrix at room temperature. Five samples of the two categories of spiked plasma QC E and QC C (1450 ng/ml and 259 ng/ml) were kept on the laboratory bench at room temperature for 20 hrs. before extraction. The results are presented in Table 2.23.

From the table below (Table 2.23.), it can be seen that the measured concentration of sildenafil compared with the nominal shows practically no deviation. This shows that sildenafil in plasma is stable for 20 hrs. when kept at room temperature. During the extraction process, the loss of analyte due to decomposition is minimal.

TABLE 2.23: Results of the assay of bench-top stability samples.

Nominal conc. (ng/ml)	Measured conc. (ng/ml)	Calculated % of nominal
1450	1425	98.28
1450	1508	104.00
1450	1475	101.72
1450	1427	98.41
1450	1439	99.24
Mean	1455	100.33
STD.	35.88	2.47
CV%	2.47	N/A
%Nom.	100.33	N/A

Nominal Conc. (ng/ml)	Measured conc. (ng/ml)	Calculated % of nominal
259.0	270.0	104.25
259.0	262.0	101.16
259.0	255.0	98.46
259.0	261.0	100.77
259.0	258.0	99.61
Mean	261.2	100.8
STD.	5.63	2.17
CV%	2.16	N/A
%Nom.	100.85	N/A

2.1.5.4 Analyte recovery:

The determination of analyte recovery assesses whether the method provides response for the entire analyte present in the plasma and is an assessment of the efficiency of the extraction method used. It is calculated by comparing the response of a reconstituted extract of a sample that has been subjected to the assay process with the response of a solution with a known concentration of the analyte The performance verification sample area response was used to calculate the absolute recovery of sildenafil at high concentration (1450 ng/ml), medium concentration (259 ng/ml) and low concentration (130 ng/ml) QC samples extracted in six-fold. The theoretical area response of sildenafil used to calculate the recovery was a mean of six replicate SPV sample area values.

TABLE 2.24: Absolute recovery of sildenafil using response factor areas.

Sample	Analyte ng/ml	Mean of peak areas		Absolute recovery (%)	CV (%)
		After extraction	Theoretical values		
High conc.	1450	1797	1839	97.7	8.4
Medium conc.	259	317	329	96.5	6.9
Low conc.	130	165	165	100.0	9.1

TABLE 2.25: Absolute recovery of internal standard using response factor area.

ISTD ng/ml	Mean of peak areas		Absolute recovery (%)	CV (%)
	After extraction	Theoretical values		
5000	932	1132	82.3	8.0

From the results above, it is clear that the solid phase extraction procedure is efficient.

2.1.6 Conclusion

A selective, sensitive and rapid HPLC - UV method for determination of sildenafil in human plasma was developed and validated. Using trazodone as internal standard, 500 µl of plasma buffered with 500 µl of 0.01 M NaOH was extracted by SPE on t-C_{18} cartridge. A C_{18} reversed phase liquid chromatography using 0.05 M H_3PO_4 : Acetonitrile (70 : 30), pH 5 as mobile phase interfaced with ultraviolet detector was used for analysis.

The calibration range was between 50 ng/ml to 3200 ng/ml, with LLOQ at 50 ng/ml (S/N = 100). It was decided to keep the LLOQ at 50 ng/ml in order to include the C_{max} value of 1517 ng/ml found in a previous study done by Lee and Min (2001) when sildenafil was administered with cytochrome CYP 3A4 inhibitor diets such as grapefruit juice. The calibration curve weighted at 1/concentration linear was linear over the whole range of 50 ng/ml to 3200 ng/ml (r2 = 0.999, 0.999 and 0.997 for intraday, interday-I and interday-II validations respectively).

The chromatographic procedure has an acceptably short turn-around time of about 8.5 min. The SPE procedure used was short and selective having a recovery of 97.7 %, 96.5 % and 100 % at high, medium and low concentrations respectively, while the recovery of trazodone was found to be 82.3 %. The reproducibility and repeatability of the assay method was confirmed by the good precision and accuracy results of the three step validations done using the QC standards at concentration levels of 2901 ng/ml, 1450 ng/ml, 518 ng/ml, 259 ng/ml, 130 ng/ml and 65 ng/ml in six-fold. The intraday precision (CV %) was between 3.6% to 9.1% and accuracy (%nom) between 92 % to 106 %. Precision (CV %) between 1.9 % to 7.4 % and accuracy between 96.8 % to 100 % was calculated for interday-I and the interday-II has got a precision value between 5.8 % to 16 % (at LLOQ) and accuracy between 94 % to 104.9 %.

The stability tests done on sildenafil at C_{max} (1450 ng/ml) and 0.2xC_{max} (259 ng/ml) shows that sildenafil is stable on-instrument (autosampler temperature was kept at room

temperature) in mobile phase injection solution for 84 hrs, stable in three freeze and thaw cycles at –20 ^{o}C and stable for 24 hrs on bench top (at room temperature). A stock solution stored on a glass container showed little change of response when stored at room temperature for 24 hrs.

2.2 LC–MS/MS ASSAY METHOD FOR SILDENAFIL

2.2.1 Method development

Method development involves evaluation and optimisation of the various stages of sample preparation, chromatographic separation, detection and quantification. Optimisation of various parameters were attempted in order to develop a selective and sensitive method for analysis of sildenafil on LC-MS / MS. A volatile mobile phase, selective extraction procedure and sensitive acquisition methods were optimised.

2.2.1.1 Instrumentation.

The LC-MS / MS system used was the Sciex API 2000 and Q-TRAP LC-MS / MS system (Applied Biosystems, Ontario, Canada) with electro spray ionisation in the positive ion mode. HPLC analysis was performed using an Agilent 1100 Series quaternary pump combined with an HP 1100 series autosampler, and an Agilent 1100 series vacuum degasser (Germany). The column used was Discovery® C_{18} bonded 5 µm silica, (15 cm x 2.1 mm) (Supelco, USA) with a mobile phase flow rate of 0.25 ml/min. An HP 1100 series thermostat column compartment (Germany) was used to control the temperature.

2.2.1.2 Mobile phase optimisation.

To prepare a mobile phase for the LC-MS / MS system, a volatile mobile phase is required, hence a buffer of ammonium acetate was prepared by adding 25 % ammonia to 0.05 M acetic acid until the pH of the solution was adjusted to 7. The pH at different percentages of acetic acid and the buffer was measured. This data was used to adjust the pH of the mobile phase. All the mobile phase optimisation was done on the HPLC-Photodiode Array Detector (DAD) at 230 nm and flow rate of 300 µl/min described in section 2.1.1.5.

Data specific to the optimisation of the current mobile phase is presented in the following tables and figures.

TABLE 2.26: The effect of acetic acid (AcOH) on the pH of the 0.05M acetate buffer.

Volume of 0.05M AcOH (ml.)	Volume of ammonium acetate buffer (ml.)	% of acid	pH
10	0	100	2.85
9	1	90	3.61
8	2	80	3.96
7	3	70	4.23
6	4	60	4.44
5	5	50	4.61
4	6	40	4.8
3	7	30	5
2	8	20	5.22
1	9	10	5.55
0	10	0	7

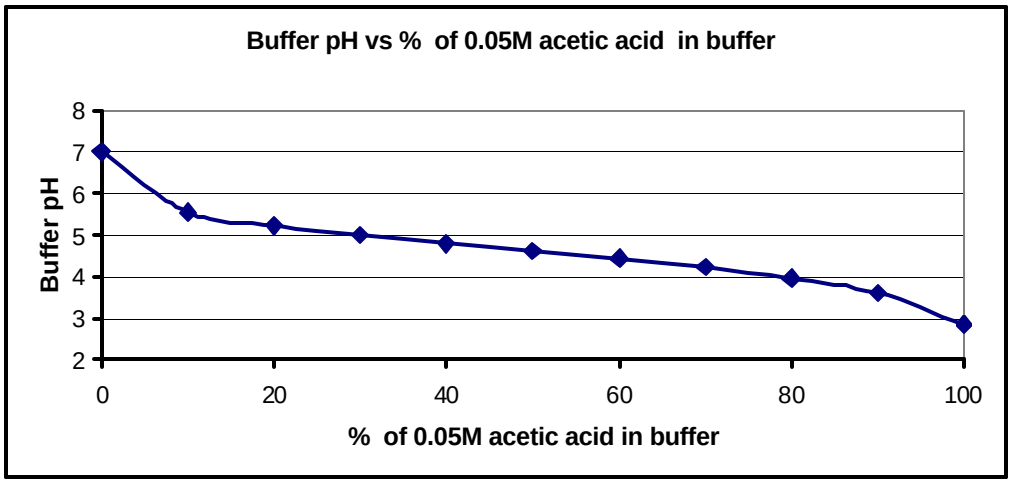


FIG. 2.26: Effect of acetic acid on pH of the 0.05M acetate buffer.

The effect of pH of the buffer on the retention of sildenafil, while acetonitrile was kept constant at 35% is presented in Table 2.27 and Fig. 2.27.

TABLE 2.27: Effect of pH of 0.05M acetate buffers on retention time of sildenafil (using 35% acetonitrile).

% of 0.05M AcOH added to buffer pH7	Resultant pH of mobile phase buffer	Retention time of sildenafil (min.)
10	5.55	9.5
20	5.22	7.311
30	5	5.728
40	4.8	5.605
50	4.61	5.153
60	4.41	4.805
70	4.23	4.478
80	3.96	4.25
90	3.61	4.032

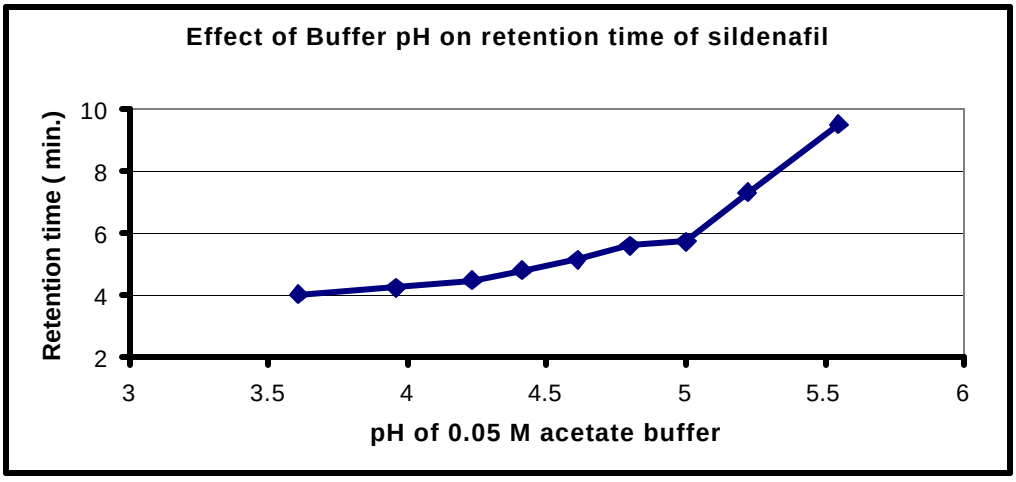


FIG. 2.27: Graphical presentation of the data in Table 2.27.

When initially a mobile phase composed of acetonitrile and acetate buffer (50:50) was used to chromatograph a 500 µg/ml solution of sildenafil (in ethanol), a split peak appeared at 3.50 min. Several parameters were changed such as the nature of the injection solvent, pH of the buffer and also mobile phase composition but the splitting of the sildenafil as well as the trazodone peak persisted. When the problem persisted even when the acetate buffer based mobile phase was replaced with the original phosphate buffer based mobile phase, it was realised that the problem lay with the column.

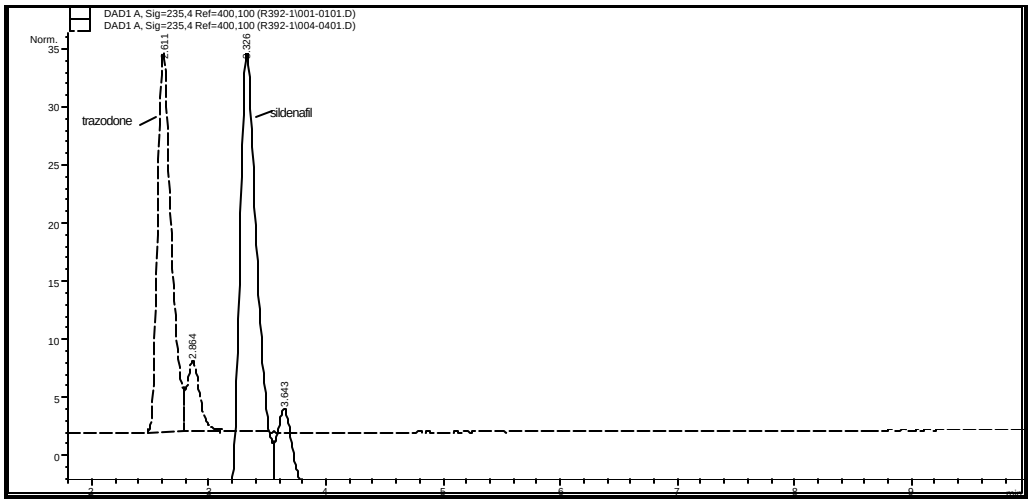


FIG. 2.28: Overlaid chromatograms of sildenafil and trazodone illustrating peak splitting caused by some defect in the chromatographic column.

The problem was resolved when the defective column was replaced with a new one.

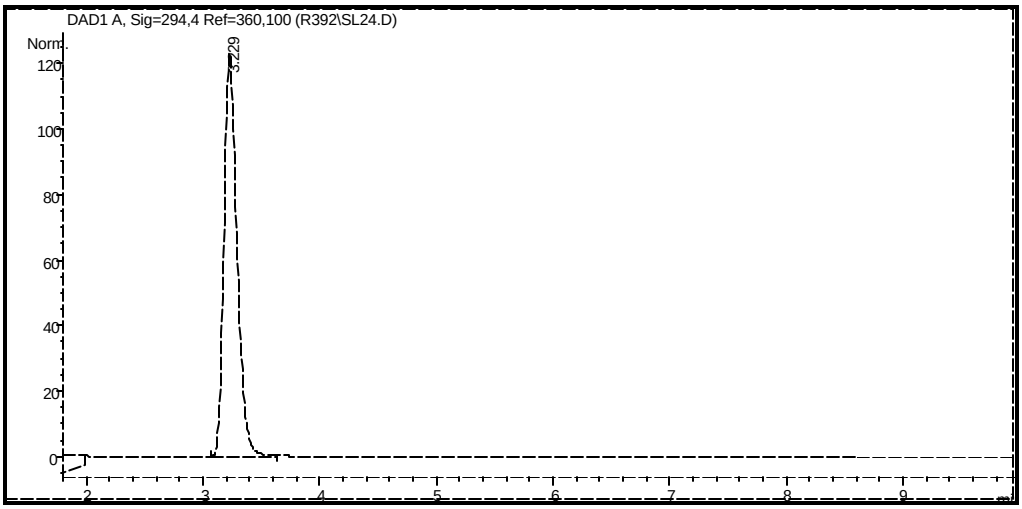


FIG. 2.29: Chromatogram of sildenafil on replacing the defective column with a new one.

In the procedures used during the experiments above the mobile phase composition was determined by the relevant low -pressure solvent switching valves of the quaternary pump. Due to uncertainties inherent in the delivery of the relevant solutions by the switching valves, as well as possible incomplete mixing, especially at low delivery volumes, the above results could only be considered as preliminary indicators of the optimal values of the various mobile phase parameters which affect the quality of the chromatograms. Taking into account the results obtained, it was decided to use acetonitrile as organic modifier at a level of 35% v/v of the mobile phase and to prepare mobile phases for further optimisation as follows:

- To a volume of organic modifier (say 300 ml.) was added 700 ml. 0.05 M Acetic acid.

- The resultant solution's pH was then adjusted dropwise with 5 M NaOH solution while stirring vigorously on a magnetic stirrer and monitoring the pH with a pH meter. In this manner a mobile phase with the required apparent pH could be produced very reproducibly.

Keeping acetonitrile constant at 35%, mobile phases at different pH values were prepared as described above and chromatograms obtained of sildenafil and trazodone. The effects of mobile phase pH on several parameters were calculated and are presented in the following tables and figures.

TABLE 2.28: Effect of mobile phase (acetonitrile 35% and 0.05M acetic acid 65%) pH on retentio n time, column efficiency and resolution.

pH	Trazodone			Sildenafil			Resolution
	t_R (min.)	Width (min.)	Efficiency (N)	t_R (min.)	Width (min.)	Efficiency (N)	
4	2.37	0.1906	2473	2.74	0.1932	3218	26
4.5	2.6	0.1483	4918	3.08	0.167	5442	17.3
5	2.76	0.1759	3939	3.41	0.1982	4736	21.44
5.5	3.05	0.3352	1324	4.04	0.2959	2982	22.24
6	3.78	0.3236	2183	5.37	0.392	3002	40.69

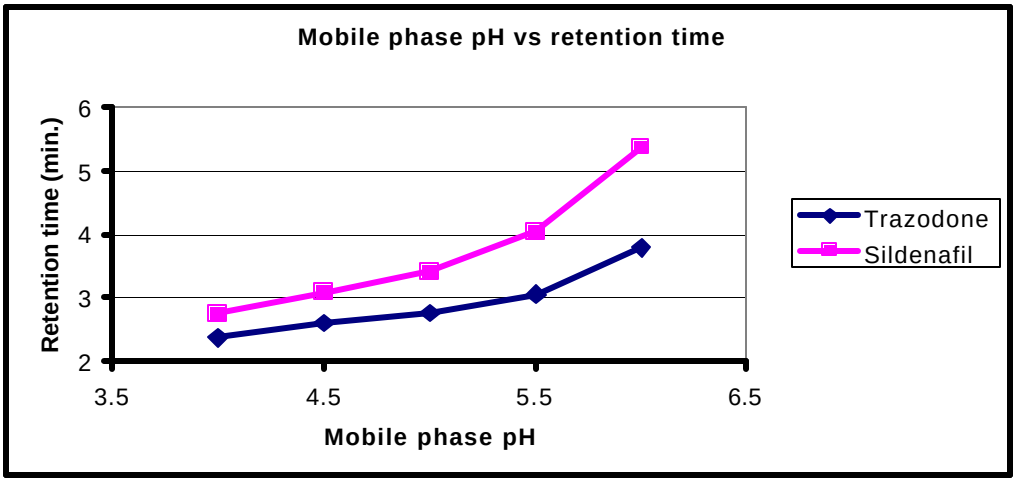


FIG. 2.30: Graphical presentation of data in Table 2.28.

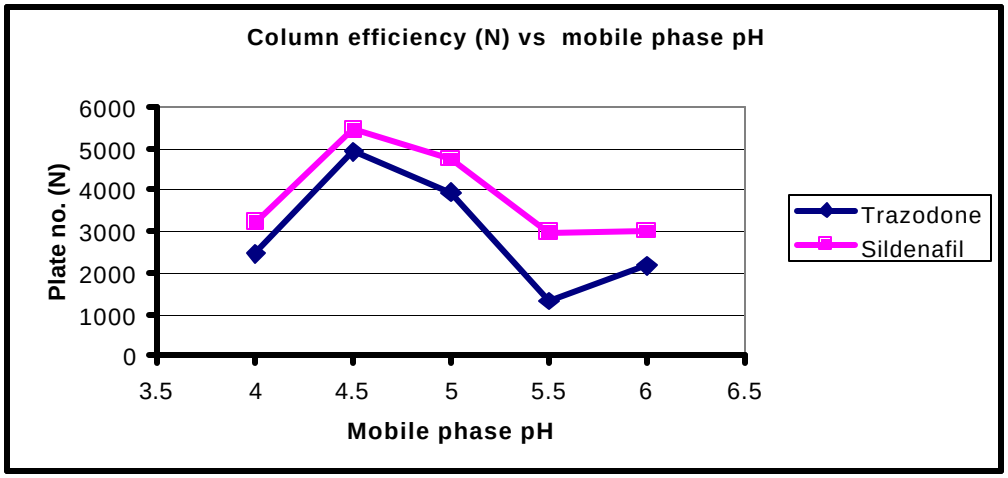


FIG. 2.31: Graphical presentation of data in Table 2.28.

2.2.1.2.1 Optimisation of an alternative mobile phase

Since electrospray ionisation is more efficient the higher the concentration of the organic modifier in the mobile phase, a second mobile phase was developed using methanol as organic solvent. Since methanol is more polar with lesser eluting power than acetonitrile in reversed phase chromatography, the amount of methanol in the mobile phase needs be more than is the case with acetonitrile to get shorter retention times. Since the objective of this study was to develop a simple reliable method that would facilitate analysis of sildenafil in human body fluids in a large number of samples over a relatively short period of time (bioequivalence studies), in a cost effective manner the suitability of a mobile phase with 60 % methanol as organic modifier was investigated.

The results of these investigations are presented in Table 2.29, Fig. 2.31 and Fig. 2.32.

TABLE 2.29: Effect of pH of mobile phase (Methanol 60% and 0.05M acetic acid 40%) on retention time, column efficiency (N) and resolution.

pH	Trazodone			Sildenafil			Resolution
	t_R (min.)	Width (min.)	Efficiency (N)	t_R (min)	Width (min.)	Efficiency (N)	
4	1.6	-	-	2.33	0.1361	4689	-
4.5	2.09	0.1226	4649	2.89	0.1775	4241	25.55
5	2.36	0.2152	1924	3.37	0.2317	3384	25.9
5.5	2.93	0.3244	1305	4.2	0.3191	2771	28.14
6	4.18	0.436	1470	5.14	0.3073	4476	24.07

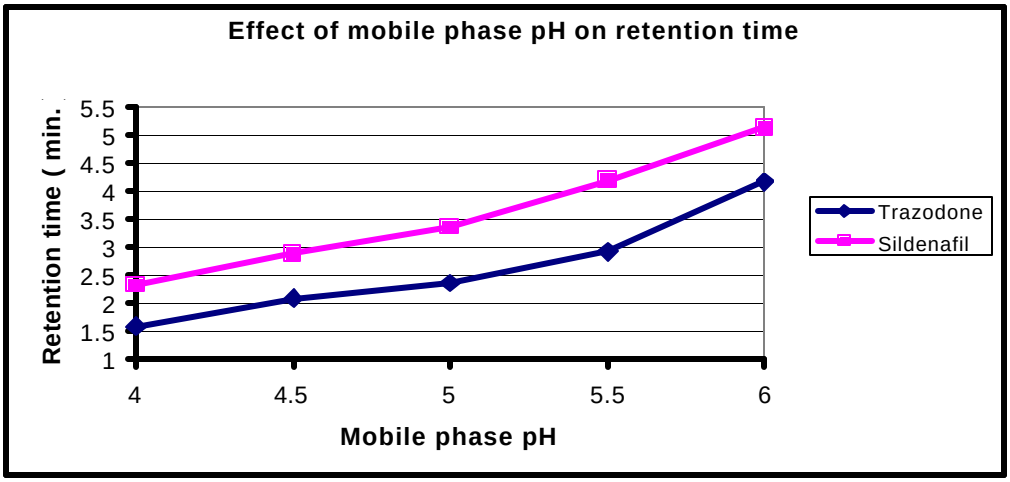


FIG. 2.32: Graphical presentation of data in Table 2.29.

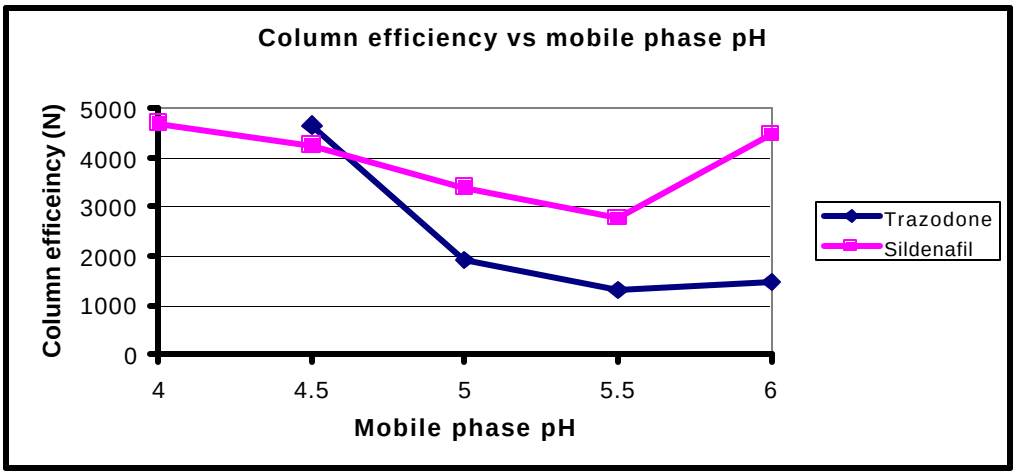


FIG. 2.33: Graphical presentation of data in Table 2.29.

Column efficiency and resolution were calculated using equations 1.1 and 1.2

The mobile phase optimisation up to this stage was done with HPLC - photodiode-array detector. Sildenafil and trazodone have short retention times, good column efficiency and good resolution of the two peaks in chromatograms obtained with mobile phase (60% methanol in 0.05 M Acetic acid) adjusted to apparent pH 4.5, so it was decided to proceed with the validation of the assay method on the LC-MS/MS system using this mobile phase at a flow rate of 200 µl/min.

2.2.1.3 Optimisation of the LC-MS/MS system

MS/MS is a much more specific and selective method of detection than UV. Interference by co-eluting components is not considered as significant a problem as with a UV detection system although the so-called "matrix effect" needs to be tested for. For this reason the whole

method development process was focused on mobile phase and extraction process optimisation.

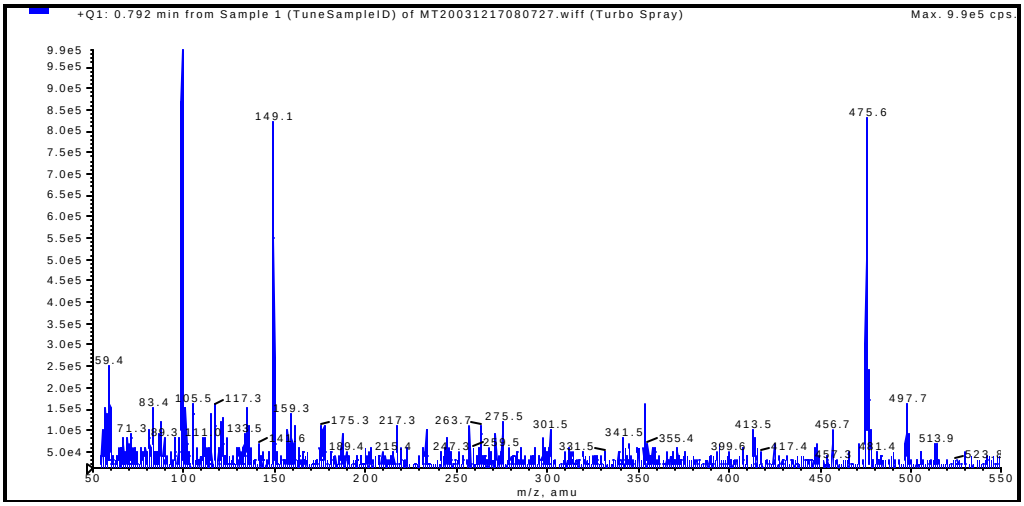
The Q-trap MS / MS instrument was calibrated with polypropylene glycol (PPG) standard in positive and negative ionisation mode. Infusion was done using 500 ng/ml sildenafil and trazodone in mobile phase. Using the spectra of the infused sildenafil solution, mass spectrometer parameters were optimised.

- Nitrogen as nebulizer, turboIonSpray and curtain gas was set at 70, 70 and 15.
- The heated nebulizer temperature was set to 400 °C.
- The declustering and the ion spray potentials were set at 71 V and 4800 V respectively.
- The collision activated dissociation (CAD) gas flow was optimised at 5.

Preferred MS/MS detection conditions

An Applied Biosystem API 2000 LC-MS/MS detector at unit resolution in the multiple reaction monitoring (MRM) mode monitoring the transition of the protonated molecular ions m/z 475 and m/z 372 to the product ions m/z 58 and m/z 176 for the analyte and internal standard respectively. TurboIonSpray ionisation (ESI) was used for ion production. The instrument was interfaced to a computer running Applied Biosystems Analyst Version 1.2 software.

(A)



(B)

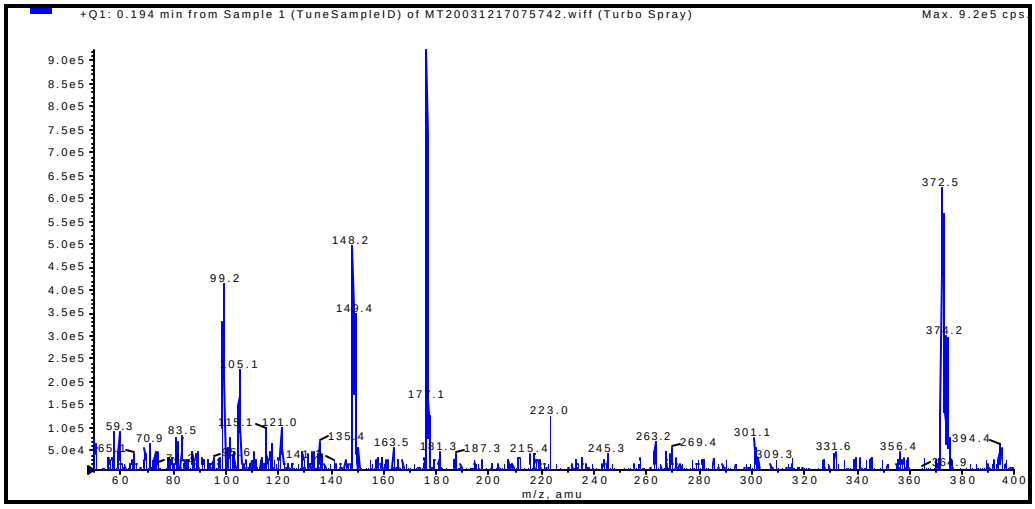
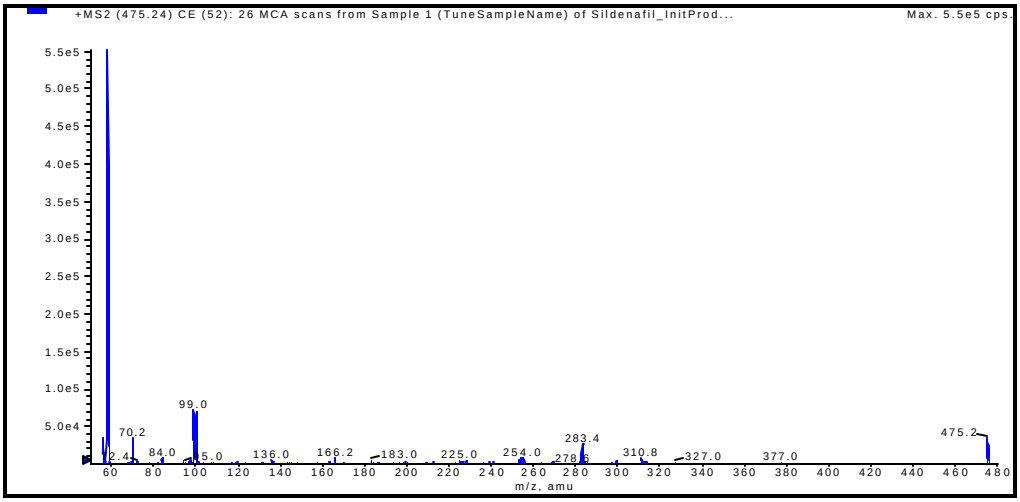


FIG. 2.34: Full scan mass spectra of (A) sildenafil (B) Trazodone after infusion of pure solution (~ 500 ng /ml in mobile phase). The protonated molecular ion [M + 1] ions at m/z 475 and m/z 372 are shown.

(A)



(B)

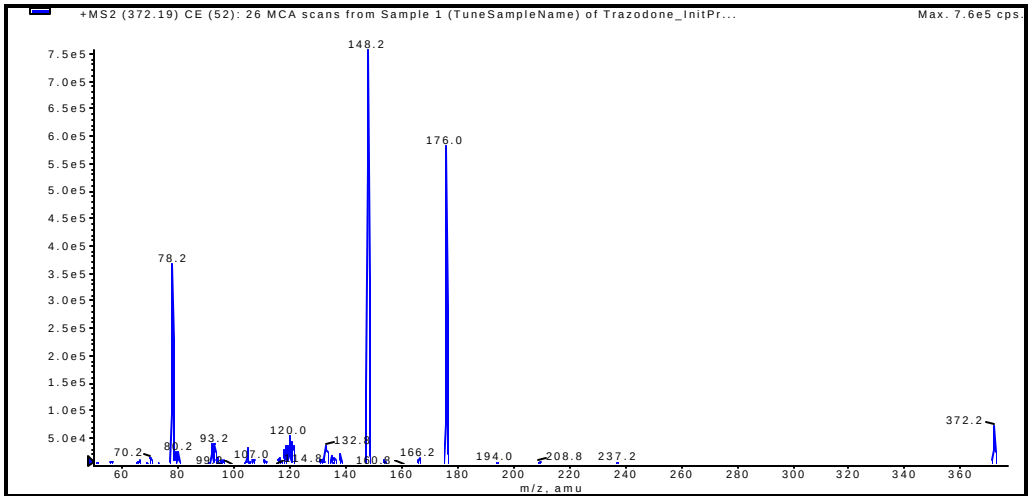


FIG. 2.35: Full product ion spectra of sildenafil (m/z = 475) (A) and trazodone (m/z = 372) (B) after collision, showing the product ions at m/z 58 and m/z 176 respectively.

To assess the stability of the instrument response, a system performance verification sample (SPV) containing 100 ng/ml sildenafil and 100 ng/ml trazodone in mobile phase was prepared. The SPV sample was injected 100 times overnight and the CV% of the response area was below 2 % that indicates that the instrument was very stable. It was also found that the ionisation pattern of trazodone (internal standard) followed that of sildenafil.

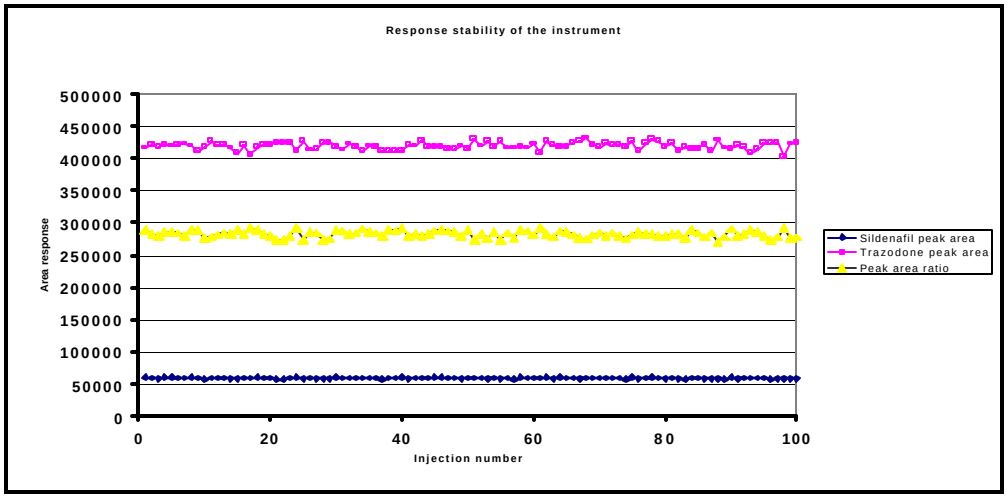


FIG. 2.36: Graph showing the response stability of the instrument.

2.2.1.4 Extraction from plasma

The SPE procedure used in the HPLC-UV assay method is costly and time consuming but was necessary to ensure freedom of interference from endogenous components with the analytes of interest. However since the selectivity of MS/MS detection is much higher than

UV detection and since the objective of this study was to develop a simple reliable method that would facilitate analysis of sildenafil in human body fluids in a large number of samples over a relatively short period of time (bioequivalence studies), in a cost effective manner the suitability of an alternative, simpler extraction procedure was also investigated.

2.2.1.4.1 Protein precipitation

Plasma protein precipitation is probably the simplest of all the sample preparation procedures for chromatography. The standard protein precipitation procedure with acetonitrile involves the addition of two volumes of acetonitrile to one volume of plasma followed by a short period of vigorous shaking (e.g. vortex mixing) whereafter the sample is centrifuged hard and the clear supernatant is injected onto the chromatography column. This standard procedure with trazodone as internal standard dissolved in the acetonitrile was therefore investigated first:

- To 200 µl plasma containing sildenafil (100 ng/ml) in a 1.5 ml. Eppendorf microfuge tube, was added 400 µl acetonitrile containing trazodone (100 ng/ml).
- The solution was vortex mixed vigorously for 1min.
- Centrifuged at 11000 rpm for 5 min.
- 10 µl of the supernatant solution was injected onto the chromatography column at a flow rate of 200 µl/min. The strong eluting power of acetonitrile over the mobile phase resulted in distorted peaks of both analytes (Fig. 2.37).

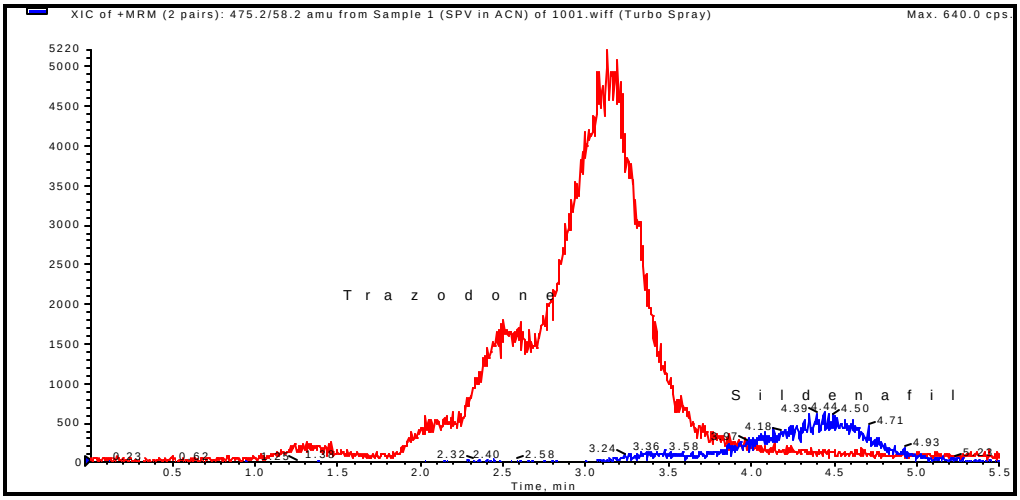


FIG. 2.37: Chromatography of 100ng/ml sildenafil and trazodone in mobile phase when precipitated with acetonitrile (1:2) and injecting the supernatant solution.

In an attempt to improve the peak shape 300 µl plasma was precipitated with 600 µl acetonitrile, the supernatant decanted into a 10 ml. glass ampoule and evaporated at 60 ^{o}C under nitrogen. The residue was reconstituted in 300 µl mobile phase and 10 µl was injected to the HPLC.

This procedure yielded peaks with less tailing for sildenafil and trazodone (Fig. 2.38).

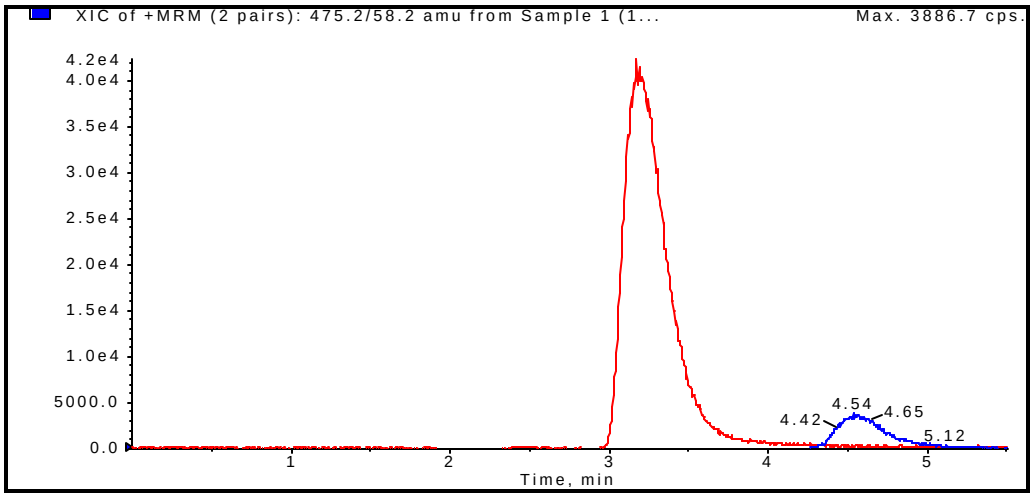


FIG. 2.38: Chromatography of 100ng/ml sildenafil and trazodone in mobile phase when precipitated with acetonitrile (1:2) and supernatant evaporated before injection.

To decrease the solvent evaporation time, a 5:1 precipitation ratio of acetonitrile to plasma was investigated. The reasoning for this step was that acetonitrile and water form a low boiling azeotrope containing 81% acetonitrile and 19 % water which would aid in the evaporation of the water residue which was always present at the end of the evaporation of the acetonitrile in the 2:1 precipitation process. Thus, 200 µl of plasma containing 50 ng/ml sildenafil was precipitated with 1000 µl acetonitrile containing 100 ng/ml trazodone, the supernatant evaporated and the residue reconstituted in 200 µl of mobile phase. The result showed that both supernatants evaporated at an almost equal length of time (35 - 40 min.).

Recovery

To assess and compare the recovery of sildenafil and trazodone of the two protein precipitation procedures, the following experiment was performed in four fold.

Protein precipitation procedure I

- 300 µl plasma containing 50 ng /ml sildenafil.
- Added 600 µl acetonitrile containing 16.7 ng/ml trazodone.

- Vortex mixed for 1 minute.
- Centrifuged at 11000 rpm for 5 minute.
- Decanted and evaporated supernatant.
- Reconstituted residue in 300 µl mobile phase.
- Injected 10 µl onto HPLC column (autosampler was kept at 4 ^{o}C).

Protein precipitation procedure II

- 200 µl plasma containing 50 ng /ml sildenafil.
- Added 1000 µl acetonitrile containing 10 ng/ml trazodone.
- Vortex mixed for 1 minute.
- Centrifuged at 11000 rpm for 5 minute.
- Decanted and evaporated supernatant.
- Reconstituted residue in 200 µl mobile phase.
- Injected 10 µl onto HPLC column (autosampler was kept at 4 ^{o}C).

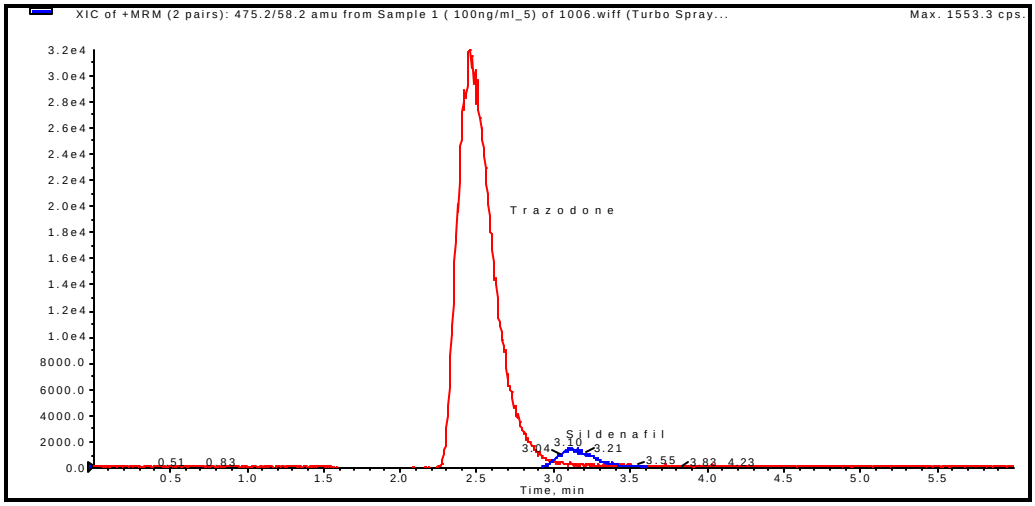
The results are presented in Table 2.30.

TABLE 2.30: CoResult for extract of plasma of procedure I and procedure II above. SPV solution contains 100ng/ml sildenafil and 100ng/ml trazodone. (n = 4).

	Sildenafil			Trazodone		
	SPV	**2:1**	**5:1**	**SPV**	**2:1**	**5:1**
Average peak area	54000	23366.67	18700	334500	96066.67	141666.7
Recovery		86.54%	69.26%		86.16%	84.70%

To try to further improve the peak shapes, a weak eluting power reconstituting solvent was tried. 0.29 % and 2.9 % acetic acid were used as reconstituting solvents, but none of them improved the peak shapes. Mobile phase therefore remained the preferred reconstituting and injection solvent. The retention time was longer than obtained on HPLC-DAD because the flow rate was decreased from 300 µl/min to 200 µl/min to reduce the amount of eluent that might reach the mass spectrometer and keep it clean. Further peak shape improvement and useful shorter retention times were obtained by increasing the flow rate to 250 µl/min and the column temperature to 45 ^{o}C (Fig. 2.39).

(A)



(B)

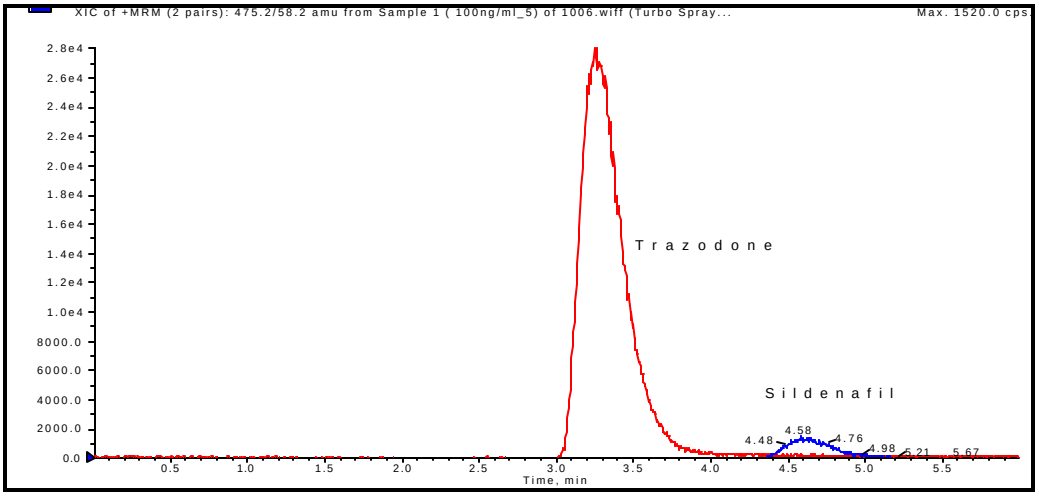


FIG. 2.39: Chromatogram of 50 ng/ml sildenafil and 100 ng/ml trazodone in mobile phase (A) flow rate 250 ml/ml and column temperature 45° C. (B) Flow rate 200 ml/ml and column temperature 25 °C.

The final optimised method was set to mobile phase (methanol : 0.05M acetic acid, 60 : 40) pH adjusted to 4.5 by 25% ammonia, flow rate (250 µl/ml) and column temperature 45°C.

2.2.1.4.2 Reproducibility of the precipitation method.

To assess the reproducibility of the precipitation method plasma spiked with 50 ng/ml of sildenafil and 100 ng/ml trazodone was precipitated in 10-fold and injected onto the LC-MS/MS system. The results are presented in Table 2.31.

TABLE 2.31: Reproducibility of precipitating 300 ml of plasma spiked with 50 ng/ml sildenafil and 100 ng/ml trazodone (n = 10).

	Peak area of SLD	Peak area of TRZ	Peak area ratio SLD/TRZ
Average	56320	422600	0.1335
STD	1073	21009	0.0059
CV %	1.9	4.97	4.39

The low CV % of the peak areas and peak area ratio indicates that the precipitation procedure used was reproducible.

2.2.1.4.3 Effect of injection volume

The process of quantification at very low concentrations is a competition between signal and noise. In order to keep the instrument clean the amount of material flowing into the mass spectrometer should be kept as low as possible. This means that only very pure solvents should be used for the mobile phase and the amount of extract injected should be as small as possible, i.e. a small injection volume is preferable. A comparison was made between injecting 10 µl and 5 µl of a reconstituted extract of sildenafil from a 3 ng/ml plasma sample. Since in both cases the sildenafil peak had a similar signal to noise ratio (S/N = 14) it was decided that the injection volume of extracts should be fixed at 5 µl.

Preferred extraction procedure

- To 300 µl of plasma sample in a 1.5 ml Eppendorf microfuge tube, add 600 µl acetonitrile containing trazodone (50 ng/ml).
- Vortex mix for 1 min. and centrifuged at 11000 rpm for 5 min.
- Decant supernatant solution into 10 ml glass ampoule.
- Evaporated solvent at 60 ^{o}C under nitrogen.
- Reconstituted extract in 300 µl mobile phase.
- Inject 5µl of reconstituted extract onto the HPLC (autosampler was kept at 4 ^{o}C).

2.2.1.5 Matrix effect

It has been noted that coeluting, undetected endogenous matrix components may reduce the ion intensity of the analyte and adversely affect the reproducibility and accuracy of the LC-MS/MS assay (Matuszewski *et al.*, 1998). In order to determine whether this effect (called the Matrix Effect) is present or not, 10 different plasma pools were extracted and then spiked

with a known concentration of analyte. These samples were injected and peak areas compared. The reproducibility of the peak areas is an indication of the presence or absence of the matrix effect.

Samples of plasma pooled on 26/09/03, 12/11/ 03, 13/11/03, 24/11/03, 25/11/03, 01/12/03, 05/12/03, 08/12/03, 09/12/03, 12/12/03 were thawed and extracted using the following procedure:

- To 300 µl of each blank plasma (induplicate) in a 1.5 ml. Eppenforf microfuge tube was added 600 µl acetonitrile.
- The samples were vortex mixed for 1 min. and centrifuged at 11000 rpm for 5 min.
- The supernatant was decanted into 10 ml. glass ampoules and evaporated at 60 ^{o}C under nitrogen.
- One set of ten extracts was reconstituted with 300 µl mobile phase solution containing sildenafil at C_{max} (800 ng/ml) and trazodone (100 ng/ml) while the second set of extracts was reconstituted with 300 µl mobile phase containing sildenafil at 0.2 x C_{max} (100 ng/ml) and trazodone (100 ng/ml).
- 10 µl of each reconstituted solution was injected onto the HPLC (autosampler was kept at 4 ^{o}C).

The lower CV % value (Table 2.32) shows that the response of the analytes was stable which also implied that ionisation pattern of both analytes and internal standard was independent of any endogenous materials within the blank plasma. So the precipitation method developed had no matrix effect from ten of the blank plasma pooled on different days.

TABLE 2.32: Matrix effect (A) at 0.2 x C_{max} and (B) at C_{max}.

Plasma pooling date	Plasma conc. = 0.2xCmax			Plasma conc. = Cmax		
	Peak area of SLD	Peak area of TRZ	Peak area Ratio	Peak area of SLD	Peak area of TRZ	Peak area Ratio
12/11/03	86500	562000	0.154	379000	497000	0.763
13/11/03	76600	518000	0.148	356000	461000	0.772
24/11/03	75000	493000	0.152	355000	448000	0.792
25/11/03	77400	516000	0.150	399000	512000	0.779
01/12/03	72400	490000	0.148	396000	508000	0.780
05/12/03	71900	487000	0.148	383000	482000	0.795
8/12/03	69900	483000	0.145	376000	477000	0.788
09/12/03	69800	479000	0.146	370000	465000	0.796
12/12/03	70700	478000	0.148	369000	469000	0.787
26/09/03	74600	488000	0.153	412000	500000	0.824
Mean	74480	499400	0.149	379500	481900	0.788
STD	5010.39	26026.5	0.003	18518.6	21615.1	0.017
CV %	6.727	5.212	2.060	4.88	4.49	2.10

2.2.1.6 Pre-validation

Experience gained during the method development suggested that a sildenafil concentration of the order of 3 ng/ml would probably be the LLOQ and according to the pharmacokinetic data surveyed, the maximum concentration in plasma of sildenafil expected after a single oral dose of 100 mg would be around 1500 ng/ml (C_{max}).

Hence calibration standards spanning a range of 3.17 ng/ml (LLOQ) to 1600 ng/ml (Cmax) were prepared. Using the above precipitation procedure, a series of plasma from 1600 ng/ml to 3.17 ng/ml were precipitated and injected onto the HPLC. The calibration curve was linear over the whole range with r = 0. 9993 at weighting factor of 1/concentration (Fig. 2.40). The LLOQ was therefore found to be about 3 ng/ml with signal to noise ratio the lowest calibration standard being 14.

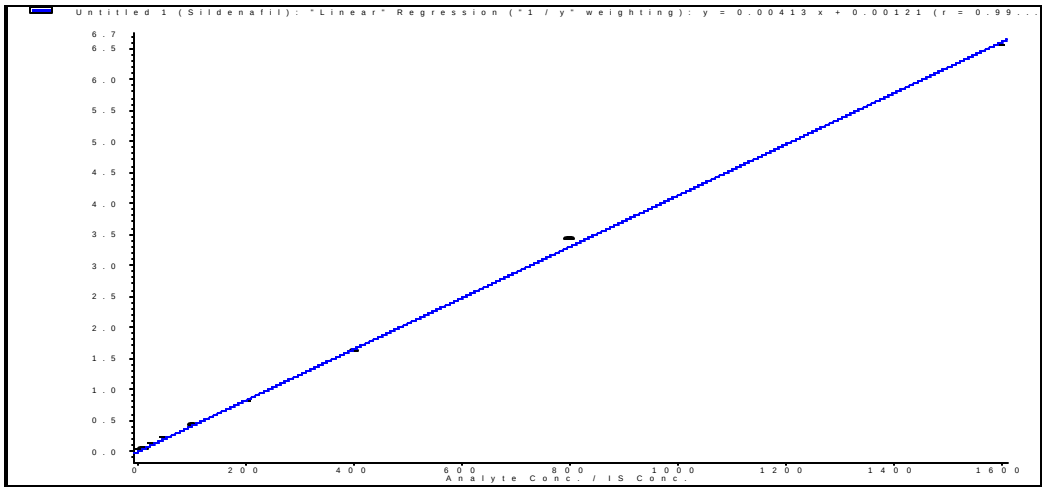


FIG. 2.40: Calibration curve for the prevalidation done.

2.2.2 LC-MS/MS Method validation

The results of the pre-validation indicated that the final method validation could be performed. The validation process was performed in the following sequence:

- Blank plasma screening.
- Planning of calibration (STD) and quality control (QC) standards.
- Preparation of calibration and quality control standards.
- Preparation of internal standard solution, Performance Verification Standard and mobile phase.
- Setting up the validation batch.
- Assaying the intra-day, inter-day-I and inter-day-II samples.

2.2.2.1 Blank plasma screening

Since sample interference is not a major problem of concern in LC-MS / MS systems as it is with HPLC-UV system, the main concern in plasma screening is to assess the matrix effect on the ionisation of the analyte. This was done during method development (section 2.2.1.5) and gave a good result with no effect on ionisation of sildenafil and no interference from the plasma with the peaks of the analyte. Blank plasma pooled on 12/11/03, 13/11/03, 24/11/03, 25/11/03, 01/12/03, 05/12/03, 08/12/03, 09/12/03 and 12/12/03 was selected to prepare a single combined blank plasma pool for further work. These normal plasmas were collected into one large polypropylene beaker. After removal of precipitated cryo protein by sieving and centrifugation, the relatively clot-free plasma was divided and stored in screw-capped 500ml. polypropylene containers at -20 ºC.

2.2.2.2 Planning of calibration and quality control standards

The main parameters to be set at this level were the range of calibration curve and the number of calibration standards and quality control standards. Unlike the HPLC assay method the LLOQ of the method was set to detect lower concentrations at 3 ng/ml with S/N of 14, while the maximum concentration was set at 800 ng/ml (C_{max}). This was decided upon because the LC-MS/MS assay method was bound to be used in clinical studies with lower doses of sildenafil. A calibration line with a range from 3 ng/ml to 1600 ng/ml (2 x C_{max}), by 1:1 dilution of the highest concentration (1600 ng/ml), requires at least ten calibration levels. As the purpose of quality control standards (QC) is to assess the performance of the assay

procedure; it must also cover the whole range of calibration line. Hence nine quality control standard levels needed to be prepared.

TABLE 2.33: Calibration standards (STD) of sildenafil to be prepared.

Code	Concentration (ng/ml.)
STD K (2 x C_{max})	1600
STD J	800
STD I	400
STD H	200
STD G	100
STD F	50
STD E	25
STD D	12.5
STD C	6.25
STD B (LLOQ)	3.125

TABLE 2.34: Quality control (QC) standards of sildenafil to be prepared

Code	Concentration(ng/ml)
QC I (1.8x C_{max})	1440
QC H	720
QC G	360
QC F	180
QC E	60
QC D	30
QC C (1.3x STD D	15
QC B (1.3 x STD C)	7.5
QC A (1.3 x LLOQ)	3.75

NB: The concentrations in Table 2.33 and Table 2.34 are concentrations to be aimed at but not the concentrations that might actually be obtained when preparing them using the Standard Operating Procedure (SOP) established in the laboratory in which this study was performed.

The following three phases in the validation require calibration and quality control standards:

- Intra-day validation.
- Inter-day-I validation.
- Inter-day-II validation.

Based on the number of calibration standards, the volume of plasma required for the validation was calculated with the aid of a calculation sheet set up in Excel. Table 2.35.

Each step of the validation will have the following set of calibration standards (STD) and quality control standards (QC).

TABLE 2.35: Calculated volume of plasma needed for preparation of STDs and QCs required in validation.

Phase	Sets of STDs (A)	Sets of QCs (B)	Levels STDs (C)	Levels QCs (D)	Replicates. STDs(E)	Replicates QCs(F)	Sample volume (G)	Volume (ml.)
Intra-day	1	1	10	9	2	6	0.8	59.2
Inter-day-I	1	1	10	9	1	6	0.8	51.2
Inter-day-II	1	1	10	9	1	6	0.8	51.2
							Total	161.6

Volume of plasma = A x C x E x G +B x D x F x G

2.2.2.3 Preparation of calibration standards and quality control standards.

The calibration standards and quality control standards were prepared by two different analysts following an SOP of the laboratory.

A 99.8 % pure sildenafil citrate from SynFine Research was used as standard reference material to prepare the stock solution for both calibration and quality control standards.

All pipetters were calibrated prior to preparation of the stock solution using QC Pipette™ software for their accuracy and precision of their volumetric measurements.

All the volumetric measurements were done by weighing on a Mettler toledo electronic microbalance, changing the specific volume to weight using its specific gravity of the stock solution solvents and plasma.

2.2.2.3.1 Calibration standards (STD)

The author of this dissertation prepared the calibration standards. 5.33 mg of reference standard sildenafil citrate (equivalent to 3.79 mg free base sildenafil) was dissolved in 19.94 g of methanol to give a stock solution **(stock A)** having concentration of 501.05 µg/ml. 250 µl of this stock solution was spiked into normal blank plasma to produce the highest concentration standard (STD K). The remaining calibration standards were prepared by 1:1 serial dilutions of STD K using normal blank plasma. Table 2.36 summarises the preparation of the calibration standards.

TABLE 2.36: Calculation of calibration standard concentrations.

Preparation of Stock Solution SA for Spiking STD K.

Solvent used	S G solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml.)	Volume spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.791	3.790	15.770	19.937	250	190.10

Preparation of calibration standards.

Sample Code & No.	Source Solution	A	B	C	D ng/ml
STD K (2 x C_{max})	**Stock SA**	20.620	50.630		1612
STD J (C_{max})	**STD K**	20.650	35.660	50.680	806
STD I	**STD J**	20.680	35.680	50.660	403
STD H	**STD I**	20.580	35.590	50.600	201
STD G	**STD H**	20.560	35.560	50.570	101
STD F	**STD G**	20.650	35.710	50.710	50.3
STD E	**STD F**	20.510	35.510	50.560	25.2
STD D	**STD E**	20.610	35.620	50.630	12.6
STD C	**STD D**	20.650	35.670	50.670	6.29
STD B (LLOQ)	**STD C**	20.640	35.480	50.550	3.17

Note: Mass of biological fluid (g) is converted to volume (ml.). SG = 1.0269 for plasma

Analyte = sildenafil

Biological fluid = plasma.

Key:

A = Mass of empty container

B = Mass of empty container plus normal biological fluid.

C = Total mass of container plus spiked plasma

D = Concentration (ng/ml).

0.5 ml. Of each levels were aliquot to 1.5ml. microfuge tubes and stored at $-20\ ^{o}C$ until extraction.

2.2.2.3.2 Preparation of quality control standards

Following an SOP of the laboratory, quality control standards were prepared by an analyst other than the author of this dissertation. 4.15 mg (equivalent to 2.950 mg free base) reference standard sildenafil citrate was dissolved in 10.216 g of methanol giving a stock solution **(stock B)** with concentration of 228.41 µg/ml. 250 µl of stock-B was spiked in a normal blank plasma to give the highest concentration of quality control standard (QC F)

with concentration of 1457 ng/ml. Similar to calibration standard preparation, QC E was prepared by diluting QC F with normal blank plasma. QC D was prepared by diluting QC E to give the appropriate concentration as can be seen from the table below. The rest of the lower QC levels were prepared by 1:1 serial dilution of QC D using normal blank plasma.

TABLE 2.37: Calculation of quality control standard concentrations.

Preparation of Stock Solution S B for Spiking QC I.

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml.)	Volume spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.791	2.950	10.216	12.915	250	228.41

Preparation of quality control standards.

Sample Code & No.	Source Solution	A	B	C	D ng/ml
QC I (1.8xC_{max})	**Stock QA**	20.610	60.610		1457
QC H	**QC I**	20.730	40.730	60.720	728
QC G	**QC H**	20.540	40.540	60.490	364
QC F	**QC G**	20.540	40.550	60.540	182
QC E	**QC F**	20.610	46.610	60.630	63.7
QC D	**QC E**	20.610	40.610	60.610	31.8
QC C (1.3xSTD-D)	**QC D**	20.490	40.500	60.520	15.9
QC B (1.3xSTD-C)	**QC C**	20.590	40.600	60.620	7.96
QC A (1.3xLLOQ)	**QC B**	20.630	40.640	60.640	3.98

Note: Mass of biological fluid (g) is converted to volume (ml.). SG = 1.0269 for plasma

Analyte = sildenafil.

Biological fluid = plasma.

Key:

A = Mass of empty container.

B = Mass of empty container plus normal biological fluid.

C = Total mass of container plus spiked plasma.

D = Concentration (ng/ml).

0.5 ml. Of each levels were aliquot to 1.5ml. microfuge tubes and stored at -20 ^{o}C until extraction.

NB: The variation of some of levels from the planned one is due to changes made during weighing.

2.2.2.4 Preparation of System performance verification samples and mobile phase.

The composition of the mobile phase was determined during method development. In order to have a consistent result throughout the validation process, a large volume of mobile phase was prepared by adding 1200 ml. of methanol to 800 ml. of 0.05 M acetic acid and adjusting the apparent pH to 4.50 using 25 % ammonia solution.

System performance verification (SPV) samples are included at the beginning, middle and end of each batch of samples assayed to monitor and ensure reproducible performance of analytical system throughout its use during a particular study. It is used to indicate whether the instrument in use is working properly or not and to give a green light to proceed with the assaying of the next batch of samples. In this assay method, the mobile phase was used to make up system performance verification samples. The SPV solution was prepared to contain sildenafil at a concentration of 100ng/ml and trazodone at 100 ng/ml.

Before every validation step, 20 injections of this sample were done to stabilise the signal of the LC-MS / MS system.

2.2.2.5 Compiling the validation batch

Validation batches must contain:

- Extracts of **calibration standards** to obtain a calibration line.
- Extracts of **quality controls** to assess the accuracy and precision of the assay method.
- Extracts of the **highest QC diluted 2x** with blank plasma to assess the validity of diluting samples with concentrations higher than the highest calibration standard if such samples were to occur during the processing of study samples.
- Extracts of **blank plasma samples** to which no internal standard has been added to monitor possible carryover effects from previous injections and for the possible appearance of other interfering peaks.
- Extracts of **zero samples**; these are extracts of blank plasma samples spiked with the internal standard.
- Extracts of **on-instrument stability samples** to assess the stability of the analytes in the reconstituted extracts while the samples are being assayed.

- **Long-term stability samples** to assess whether the analyte is stable in the plasma matrix under the sample storage conditions for the time period required for the samples generated in a clinical study to be analysed.
- System performance verification samples.

2.2.2.5.1 Intra-day validation batch

The intra-day validation batch consisted of 1 set of STD samples at 10 concentration levels (STD K to STD B) in duplicate, 6 sets of QC samples at 9 concentration levels (QC A to QC I), 6 QC I (dil) samples, 6 blank plasma samples, 2 zero samples (blank plasma with internal standard only), 8 on-instrument stability samples (1450 ng/ml), and 3 performance verification samples.

2.2.2.5.2 Inter day validation batches

Two additional inter-day validations were performed. Each consisted of 1 set of STD samples at 8 concentration levels (STD K to STD D), two sets of STD samples at 2 concentration levels (STD C to STD B), 6 sets of QC samples at 9 concentration levels (QC A to QC I), 6 blank plasma samples, 2 zero samples (blank plasma with internal standard only), 8 on-instrument stability samples (806 ng/ml) and 3 performance verification samples.

In addition, the second inter-day validation batch contained 5 long term matrix stability samples stored at –70 °C and at –20 °C each at a concentration of 1450 ng/ml and 5 long term stability samples stored at -70 °C and -20 °C each at concentration of 259 ng/ml.

The long-term stability samples were stored at the respective temperatures from 03/10/03 to 08/01/04 (i.e. for a period of about 3 months). The on-instrument stability test was also repeated because the injection solutions on HPLC and LC-MS / MS systems are different. However, the rest of the stability tests done during the HPLC-UV method validation apply equally to this validation process.

2.2.2.6 Extraction of analyte from plasma

The preferred extraction procedure optimised during method development was used throughout the validation process:

- To 300 µl of plasma sample in a 1.5 ml. Eppendorf microfuge tube, add 600 µl acetonitrile containing trazodone (50 ng/ml).
- Vortex mix for 1 min.

- Centrifuged at 11000 rpm for 5 min.
- Decant supernatant solution into 10 ml. glass ampoule.
- Evaporated solvent at 60 ^{o}C under nitrogen.
- Reconstituted extract in 300 µl mobile phase.
- Inject 5µl of reconstituted extract onto the HPLC (autosampler was kept at 4 ^{o}C).

2.2.3 Results and discussion

The method was validated to meet the acceptance criteria of the FDA Guidance for Industry, Bioanalytical Method Validation. Since the condition of the instrument is crucial for the whole process of validation, replicate performance verification samples were injected 20x onto the HPLC column to assess the condition of the instrument before the full validation batch was processed. The CV % of the area ratio of sildenafil to the internal standard was found to be below 1 %, which was a promising result and allowed the continuation of batch validation.

Results were calculated using the PhIRSt (Phoenix International Life Sciences, Montreal, Canada) chromatographic data-reporting package. Peak areas and heights are electronically read automatically from report files generated by Analyst Version 1.3 software. Data are automatically summarised, calibration curves calculated according to pre-set regression equations and concentrations interpolated by the program. Results are presented in printed order tables with performance statistics per batch and later summarised to give overall study statistics. This package has been validated in Canada by the manufacturer to FDA requirements. A representative chromatogram of the validation done is given in the Appendix (Fig.A.8 to Fig.A.14).

2.2.3.1 Linearity

The method developed was linear over the whole range of concentrations from 3.17 -1612 ng/ml for the three phases of the validation done. Using a linear regression equation weighted 1/concentration, all the calibration lines passed all the acceptance criteria. The results are presented in Table 2.38.

TABLE 2.38: Calibration line linearity results of the validation.

	Intra-day validation	Inter-day-I validation	Inter-day-II validation
Calibration standard	**STD B – STD K**	**STD B – STD K**	**STD B – STD K**
Calibration range	3.17 – 1612 ng/ml	3.17 – 1612 ng/ml	3.17 – 1612 ng/ml
Results			
Regression equation	1/Concentration^2 linear	1/Concentration^2 linear	1/Concentration^2 linear
Slope	0.001775	0.002066	0.001969
Intercept	0.000742	0.000273	0.000016
r^2	0.995	0.994	0.995

All the data points of all calibration curves of the three validation phases were within the acceptable limit, i.e. the % deviation of the back-calculated concentration values were below 15 %.

2.2.3.2 Accuracy and precision

With the PhIRSt data processing program a large number of regression equations can be fitted to the calibration data: PhIRSt uses the following regression algorithms.

TABLE 2.39: Regression algorithms used by PhIRSt program.

Type of regression	Regression equation
Linear	$Y = mx + b$
Linear (1/C)	$Y = mx + b$
Linear ($1/C^2$)	$Y = mx + b$
Log-Log	$Ln (Y) = m \ln (x) + c$
Wagner	$Ln (Y) = a (\ln (x)^2) + b \ln (x) + c$
Quadratic	$Y = a (x^2) + b (x) + c$
Quadratic (1/C)	$Y = a (x^2) + b (x) + c$
Quadratic ($1/C^2$)	$Y = a (x^2) + b (x) + c$

The values of the QCs calculated using the regression equations. In this way the most suitable regression can be chosen to obtain the best results with the validation. However, the simplest regression giving acceptable results (i.e. passing all the acceptance criteria) should be chosen. In this validation the linear regression of: (sildenafil/trazodone peak area ratio) vs 1/(concentration of sildenafil)^2 yielded excellent results. A summary of the statistics for calculated concentrations of intra- and inter- day validation quality control standards based on peak area ratio is presented in Tables 2.40 to 2.42.

TABLE 2.40: Summary statistics for calculated concentrations of intra-day- validation quality control standards based on peak area ratio.

Code & nominal / Replicates	QC I 1457 ng/ml	QC I (dil) 1457 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC F 182 ng/ml	QC E 63.7 ng/ml	QC D 31.8 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1349.490	1374.336	659.242	341.964	175.549	57.112	32.538	16.392	7.170	4.408
2	1380.192	1370.564	726.470	342.685	161.332	64.019	32.501	15.721	7.629	4.042
3	1453.766	1486.482	711.493	358.534	182.667	71.075	30.165	15.667	6.889	5.012
4	1540.651	1533.490	745.073	376.547	175.186	63.441	32.112	15.718	9.560	4.164
5	1453.358	1442.543	737.799	360.260	178.694	60.980	28.955	14.952	8.436	3.802
6	1464.398	1476.081	756.086	360.876	175.582	64.410	29.888	15.163	7.672	3.788
MEAN	1440.309	1447.249	722.694	356.811	174.835	63.506	31.027	15.602	7.893	4.203
%Nom.	98.9	99.3	99.3	98.0	96.1	99.7	97.6	98.1	99.2	105.6
CV%	4.7	4.5	4.8	3.6	4.1	7.2	5.0	3.2	12.3	10.9

Mean = mean value of area CV % = Precision (coefficient of variance)

% nom = Accuracy (mean bias from nominal concentration)

TABLE 2.41: Summary statistics for calculated concentrations of inter-day I validation quality control standards based on peak area ratio.

Code & nominal / Replicates	QC I 1457 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC F 182 ng/ml	QC E 63.7 ng/ml	QC D 31.8 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1311.739	691.319	350.254	171.293	61.653	30.939	13.845	7.773	3.276
2	1432.620	709.110	327.764	172.015	61.161	30.261	15.999	8.862	3.637
3	B	706.860	347.386	180.265	61.059	28.293	16.137	10.918	F
4	1450.861	694.495	358.049	175.455	64.055	30.462	12.922	7.777	3.510
5	1535.709	732.817	369.035	191.964	64.604	34.645	13.667	6.828	4.042
6	1481.497	711.644	364.802	182.502	66.137	33.648	[17.906]	8.081	4.625
MEAN	1442.485	707.708	352.882	178.916	63.112	31.375	14.514	8.373	3.818
%nom	99.0	97.2	96.9	98.3	99.1	98.7	91.3	105.2	95.9
CV%	5.7	2.1	4.2	4.3	3.4	7.5	10.1	16.8	13.9

B = Lost on the process.

F = outside of the range.

TABLE 2.42: Summary statistics for calculated concentrations of inter-day II validation quality control standards based on peak area ratio.

Code & nominal / Replicates	QC I 1457 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC E 63.7 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1250.987	659.477	371.933	70.146	15.132	7.158	4.351
2	1291.872	685.950	387.387	70.098	17.122	7.065	3.783
3	1308.092	674.022	386.311	77.146	16.751	7.271	4.244
4	1330.661	708.231	406.851	72.070	16.938	7.123	4.314
5	1475.275	781.578	417.018	71.703	20.324	7.340	3.965
6	1430.548	793.598	405.781	62.019	18.147	8.030	3.941
MEAN	1347.906	717.143	395.880	70.530	17.402	7.331	4.100
%nom	92.5	98.5	108.8	110.7	109.4	92.1	103.0
CV%	6.4	7.9	4.2	7.0	9.9	4.9	5.7

As shown in the results above, the accuracy and precision of the method developed were within the range of acceptance criteria recommended by international industry guidelines for bioanalytical method validation. For comparison, data of peak area without internal standard and peak height ratio with internal standard and peak height without internal standards are given in the Appendix (Table A.10 to Table A.18).

2.2.3.3 Analyte stability

Since the bench top, freeze and thaw and the stock solution stability tests were performed during the HPLC-UV assay method validation for sildenafil; only the long term matrix stability and on-instrument stability tests were performed during this validation.

2.2.3.3.1 Long term matrix stability test:

This is done to assess whether the analyte is stable in the plasma matrix under the sample storage conditions for the time period required for the samples generated in a clinical study to be analysed. Two categories of stability samples at concentration of 1450 ng/ml and 259 ng/ml (QC E and QC C prepared during the validation of the HPLC-UV assay method) were stored at temperatures of $-70\ ^{o}C$ and $-20\ ^{o}C$ for 3 months (from 03/10/03 to 08/01/04). Five samples of each category (a total of 20 samples) were assayed in the inter-day II validation batch. The results are presented in Table 2.43.

TABLE 2.43: Long term matrix stability results at –70 °C and –20 °C.

Cmax	Measured conc. (ng/ml)	
Nominal conc.	**Cmax (-20°C)**	**Cmax (-70°C)**
1450.00	1486.000	1358.000
1450.00	1303.000	1384.000
1450.00	1329.000	1455.000
1450.00	1543.000	1259.000
1450.00	B	1427.000
Mean	1415.25	1376.60
STD.	117.42	75.69
CV%	8.30	5.50
%Nom.	97.60	94.94

0.2Cmax	Measured conc. (ng/ml)	
Nominal conc.	**0.2Cmax (-20°C)**	**0.2Cmax (-70°C)**
259.00	265.000	268.000
259.00	262.000	230.000
259.00	223.000	225.000
259.00	233.000	230.000
259.00	253.000	B
Mean	247.20	238.25
STD.	18.42	19.97
CV%	7.45	8.38
%Nom.	95.44	91.99

Note: B = samples lost in process.

The data in Table 2.45 indicate, within experimental error, that sildenafil is stable in plasma stored at -20 °C and at -70 °C for 98 days.

2.2.3.3.2 On-instrument stability:

The on-instrument stability samples that were extracted were STD J (806 ng/ml) samples prepared during the validation of the assay method. 16 STD J samples were extracted according to the procedure and were mixed together after reconstituting with mobile phase. They were finally divided into 16 aliquots and injected at time intervals over a period of 33 hrs while standing in the autosampler at 4°C. The results of this stability test are presented in Table 2.44.

TABLE 2.44: On-instrument stability of Sildenafil at 4°C when the injection solution is in acetate buffer mobile phase.

Replicates	Injection time	Time difference	Cumulative time (hr.)	SLD peak area	TZD peak area	Peak area ratio
1	04:30		0.00	131000	95000	1.379
2	06:03	01:33:00	1.55	132000.00	79400	1.662
3	07:44	01:41:00	3.23	133000.00	87800	1.515
4	09:19	01:35:00	4.82	122000.00	79400	1.537
5	11:06	01:47:00	6.60	120000.00	80300	1.494
6	12:54	01:48:00	8.40	120000.00	81100	1.480
7	14:48	01:54:00	10.30	115000.00	78300	1.469
8	14:55	00:07:00	10.42	117000.00	77900	1.502
9	06:40	15:45:00	26.17	106000.00	62500	1.696
10	07:43	01:03:00	27.22	102000.00	60000	1.700
11	08:51	01:08:00	28.35	101000.00	60400	1.672
12	09:54	01:03:00	29.40	96600.00	56600	1.707
13	11:02	01:08:00	30.53	92300.00	52700	1.751
14	12:10	01:08:00	31.67	88000.00	49400	1.781
15	13:34	01:24:00	33.07	91000.00	51100	1.781
16	13:39	00:05:00	33.15	87400.00	49900	1.752
			Mean	109643.75	68862.50	1.62
			STD	16082.25	14991.77	0.13
			CV	14.67%	21.77%	8.12%

10-20 SPV samples were injected before each validation batch to stabilise the response of the MS / MS detector. This usually overcomes the initial detector sensitivity drift which is often encountered when a batch of samples is processed on the MS/MS detector but as can be seen from the results obtained (Table 2.46), there was a clear linear decreasing trend (Fig. 2.41) in response for both sildenafil and trazodone over the period of 33 hours during which the samples stood on the autosampler awaiting injection. However, the trend of the peak area ratio (Fig. 2.42) which runs almost parallel to the X axis suggests that the trends of the peak areas of sildenafil and trazodone do not indicate instability of the analytes but rather a steady decrease in detector sensitivity. The slight upward trend of the peak area ratio line could be an indication of some on-instrument instability of trazodone relative to sildenafil. To prove that this is not merely speculation, the procedure for testing on-instrument stability should be modified to include a few (say 3) freshly prepared extracts at the end of the validation batch

to be run without interruption of the batch processing. To avoid having to do the fresh extractions at some inconvenient time such as one o'clock in the morning, the start of a batch run should be planned to occur at a carefully pre-determined time taking the sample turn-around time into consideration.

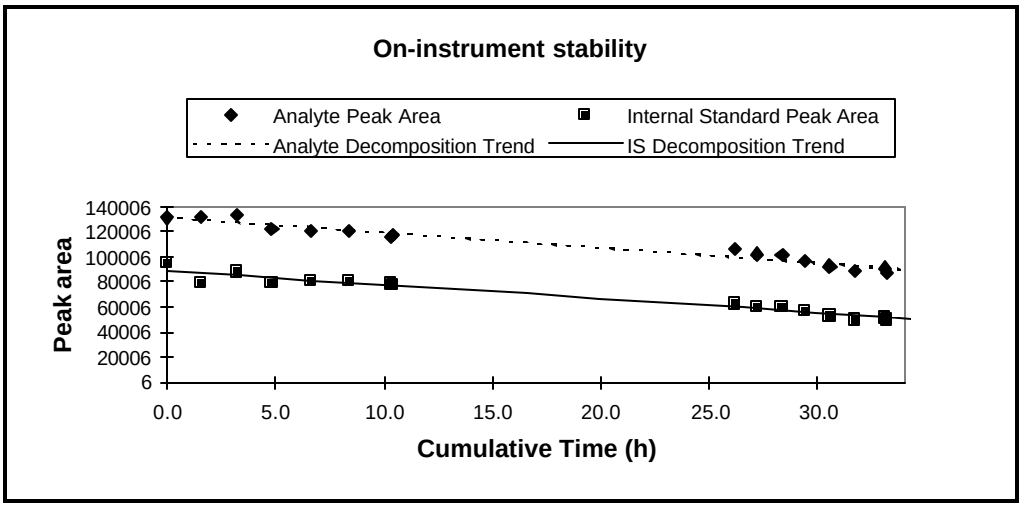


FIG. 2.41: Graphical presentation of data in Table 2.46.

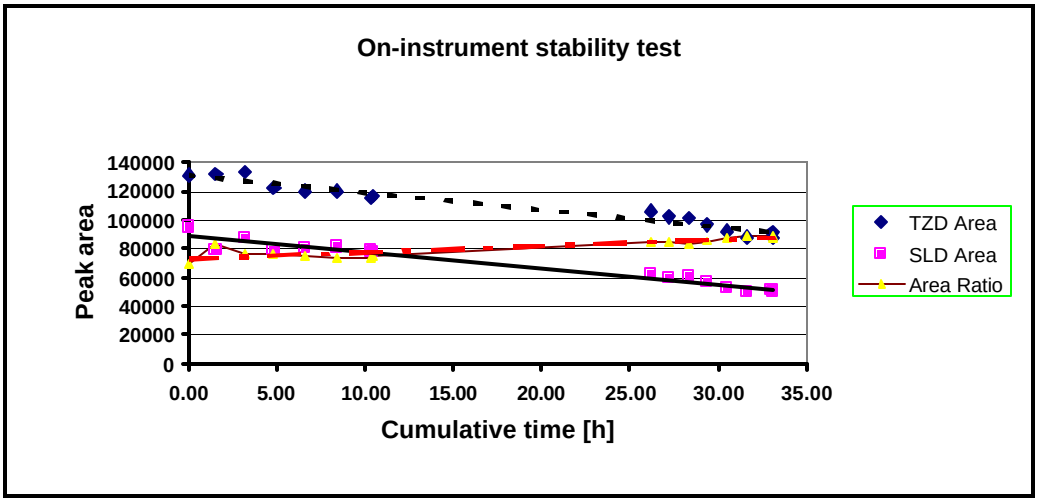


FIG. 2.42: Graphical presentation of data in Table 2.46 including the trendline for peak area ratio.

2.2.4.4 Analyte recovery.

The determination of analyte recovery assesses whether the method provides response for the entire analyte present in the plasma and is an assessment of the efficiency of the extraction method used. It is calculated by comparing the response of a reconstituted extract of a sample that has been subjected to the assay process with the response of a solution with a known concentration of the analyte The performance verification sample area response was used to

calculate the absolute recovery of sildenafil at high concentration (728 ng/ml), medium concentration (182 ng/ml) and low concentration (63.7 ng/ml) QC samples extracted in six-fold. The theoretical area response of sildenafil used to calculate the recovery is a mean of six replicate SPV sample area values.

The results in the Table 2.45 show that the precipitation method has a good absolute recovery for both sildenafil and trazodone.

TABLE 2.45: Absolute recovery of sildenafil and trazodone when extracted by precipitation.

(A) Sildenafil.

Sample type	Analyte ng/ml	Mean of peak areas		Absolute recovery(%)	CV (%)
		After extraction	Theoretical values		
High Conc.	728	87744.44	103060.53	86.65	6.17
Medium Conc.	182	24291.67	29666	82.10	7.29
Low Conc.	63.7	7535.56	9017.80	83.97	14.62

(B) Trazodone.

Sample type	Analyte ng/ml	Mean of peak areas		Absolute recovery(%)	CV (%)
		After extraction	Theoretical Values		
Internal Std.	50	71633.33	77750	91.50	8.60

2.2.5 Conclusion

A selective, sensitive and rapid LC-MS-MS method for the determination of sildenafil in human plasma was developed and validated using the Applied Biosystem Q-Trap LC-MS / MS system. Ionisation was by TurboIonSspray in positive mode. The detector was coupled with reversed phase HPLC system consisting of a quaternary solvent delivery system, a thermostated autosampler (4 ^{o}C) and a thermostated column (40 ^{o}C). The mobile phase used was 0.05 M acetic acid 40 % in methanol adjusted to apparent pH 4.5. The precipitation procedure developed using acetonitrile was short and no matrix effect was observed.

The response was linear over the whole range 3.17 ng/ml to 1612 ng/ml (r^2 = 0.995, 0.994, and 0.995 for intraday, interday-I and interday-II validations) using a 1/ concentration^2 weighted linear regression equation. The recoveries of the extraction procedure at high (728 ng/ml), medium (182 ng/ml) and low (63.7 ng/ml) concentrations of sildenafil were found to be 86.6 %, 82.1 % and 83.97 % respectively while the recovery of trazodone was 91.5 %. To

confirm the acceptance of the method by international guidelines for bioanalytical laboratories, the precision (CV %) and accuracy (nom %) of the QC standards at 1457 ng/ml, 728 ng/ml, 364 ng/ml, 182 ng/ml, 63.7 ng/ml, 31.8 ng/ml, 15.9 ng/ml, 7.96 ng/ml and 3.98 ng/ml concentration levels were analysed in replicates (n = 6). The precision and accuracy for intra-day was found to be on the between 3.6 % to 12.3 % and 96.1 % to 105.6 % respectively, for inter-day–I between 2.1 % to 1.9 % and 91.3 % to 99.1 % respectively while the inter-day-II had a precision between 4.2 % to 9.9 % and accuracy between 92.6 % to 110 %.

Sildenafil was found to be stable in plasma for 98 days when stored at both −20 °C and −70 °C. This was confirmed by the long-term matrix stability test done at concentration levels of 1450 ng/ml and 259 ng/ml. The responses of the extracts of aged samples and extracts of newly prepared samples were indistinguishable with low variability. Sildenafil was found to be very stable on-instrument in the acetic acid buffer mobile phase.

3 IDENTIFICATION OF THE MAIN METABOLITE OF SILDENAFIL.

3.1 Sildenafil Metabolite from Plasma Sample.

Sildenafil is metabolised predominantly by cytochrome P450 namely CYP3A4 in the liver and is converted to an active metabolite, N-demethyl-sildenafil, that has approximately 50 % of the efficacy of the parent compound. In quantifying sildenafil from human plasma it is important to confirm that the chromatographic peak of sildenafil is well resolved from its metabolite peak.

Since MS/MS detectors are highly selective, interference of the metabolite with sildenafil is not a problem in an LC-MS/MS assay method, but in an HPLC–UV assay method, which is not very selective, especially at low wavelengths, there might be interference from the metabolite, and resolution of the metabolite from the parent drug must be confirmed.

3.1.1 Administration of Viagra Ô

Blood samples (10 ml.) were collected from a volunteer before administration of a 50 mg Viagra™ tablet and at 30, 60, 90, 120, 180, 240, 300 and 360 min after ingestion of the tablet. Plasma (subsequently labelled “dosed plasma” for convenience) was harvested after centrifuging the blood, and stored at –20°C until analysis.

3.1.2 Identification of the metabolite in dosed plasma.

Before starting to analyse the separation of sildenafil from its metabolite in the HPLC-UV assay method, it must be confirmed that the N-demethyl metabolite is indeed present in the dosed plasma sample. Blank and dosed plasma samples were extracted using SPE (section

2.1.2.2), reconstituted in mobile phase (methanol : 05M acetic acid, 60 : 40) and injected onto the LC-MS/MS system (Micromass Quattro Ultima, Manchester, UK) under the chromatographic conditions described at the end of section 2.2.1.2.1. Two extra peaks that were not present in the chromatogram of the blank plasma extract were detected (Fig. 3.1 to Fig. 3.4) in the chromatogram of the dosed plasma extract. The mass and product ion spectra of the peaks at 2.78 min. and 2.94. min were scanned and compared with the spectra of sildenafil. The peak at 2.94 min. was identified as sildenafil since it has a molecular ion of m/z 475 and the same mass and product ion spectrum as sildenafil (Fig. 3.5).

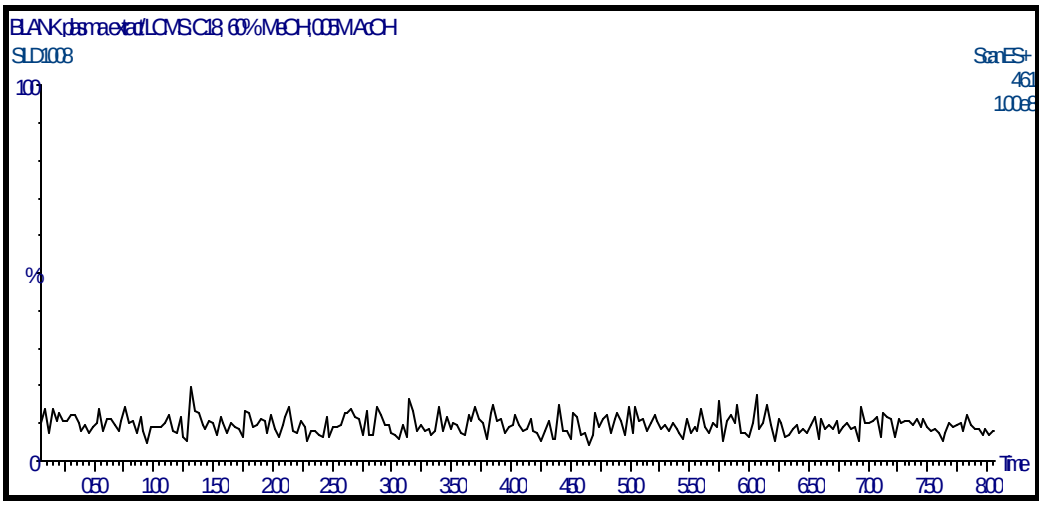


Fig. 3.1: Extracted ion (m/z = 461) chromatogram of blank plasma extract.

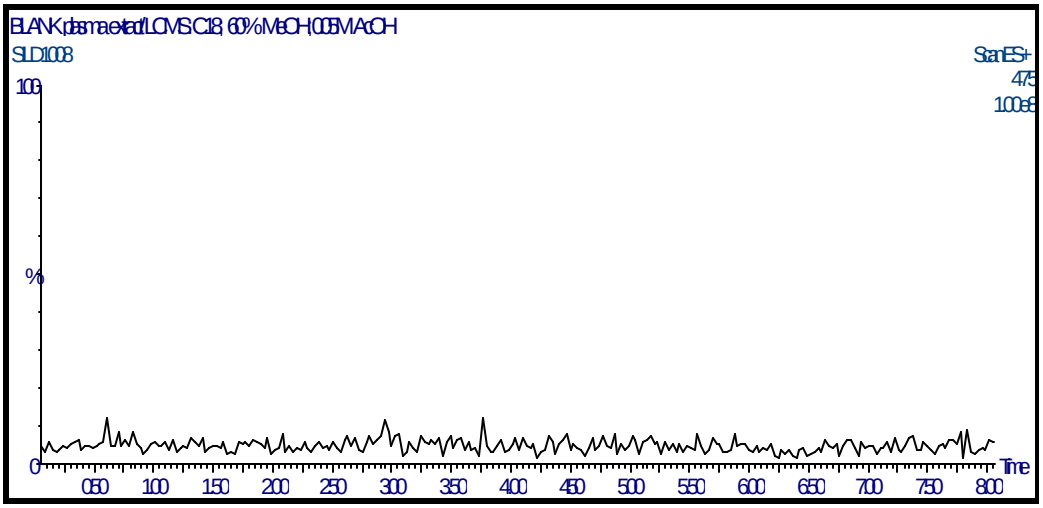


Fig. 3.2: Extracted ion (m/z = 475) chromatogram of blank plasma extract.

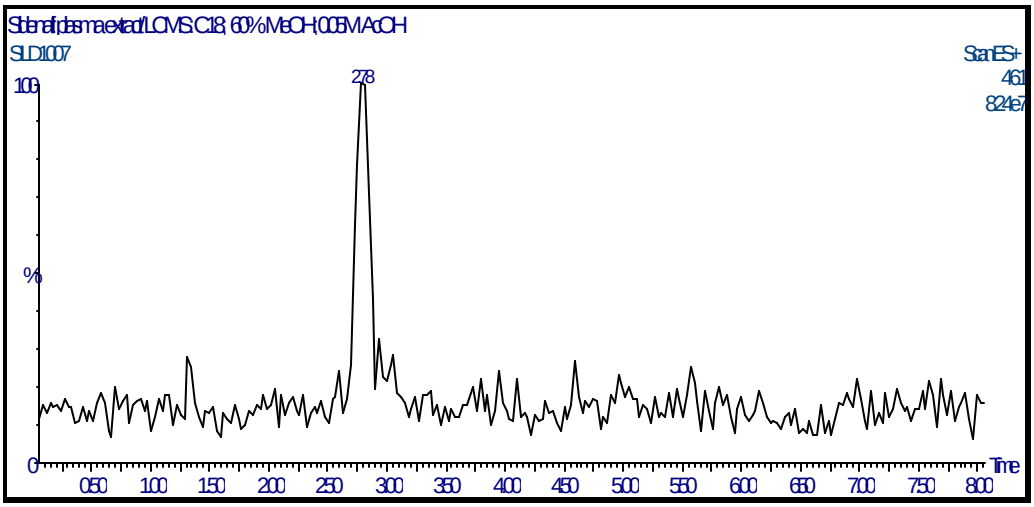


Fig. 3.3: Extracted ion (m/z = 461) chromatogram of dosed plasma extract.

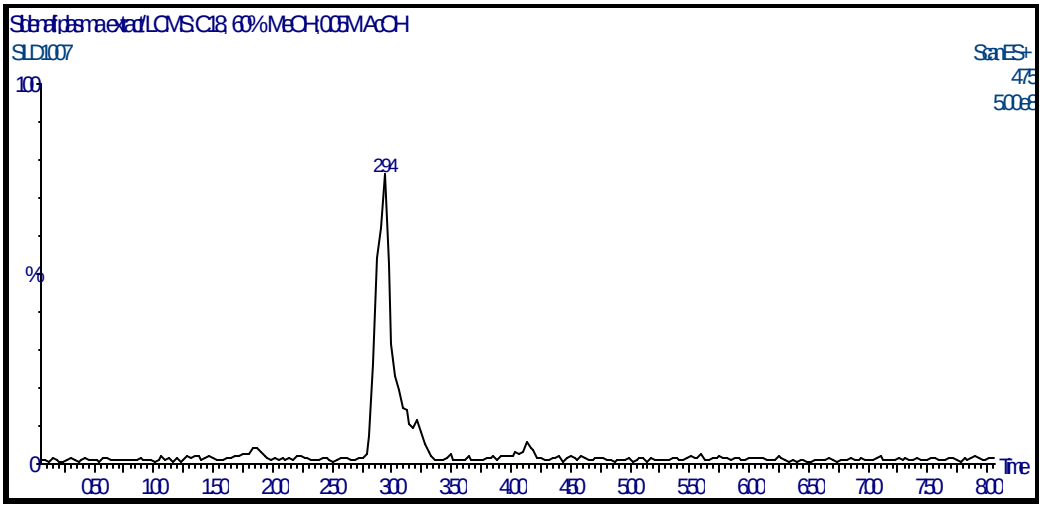


Fig. 3.4: Extracted ion (m/z = 475) chromatogram of dosed plasma extract.

The peak at 2.78 min. was identified as the demethyl metabolite of sildenafil on the basis of the molecular ion of m/z 461 and one of the main fragment ions with m/z = 85 which is 14 amu lower than a corresponding ion (m/z = 99) in the MS/MS spectrum of sildenafil while the fragment ions between m/z = 254 and 311 are similar in both compounds. The proposed plausible fragmentation patterns for the two compounds are presented in figures. 3.7 and 3.8

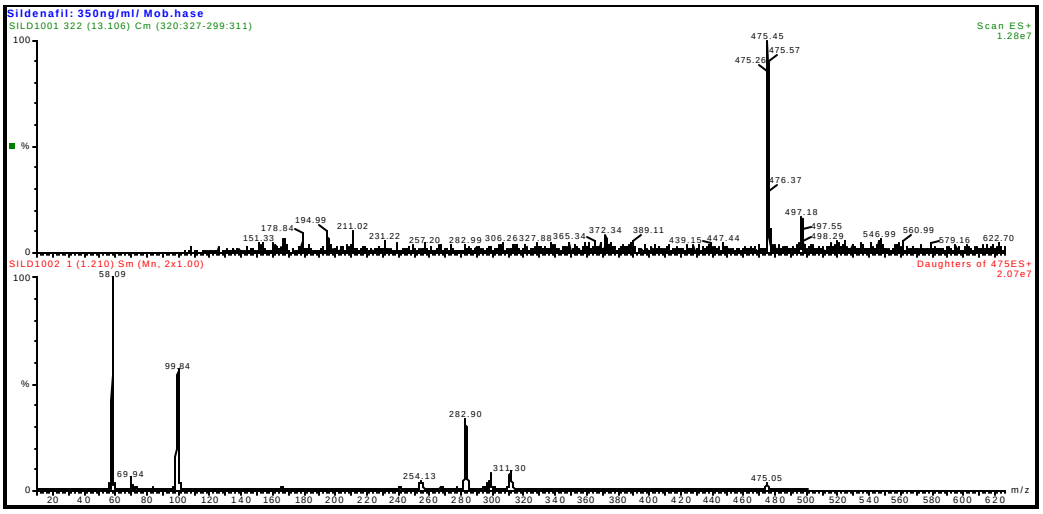


FIG. 3.5: Full scan mass spectra (Upper) and Full scan product ion spectra, of standard sildenafil solution showing the product ions at m/z 99.

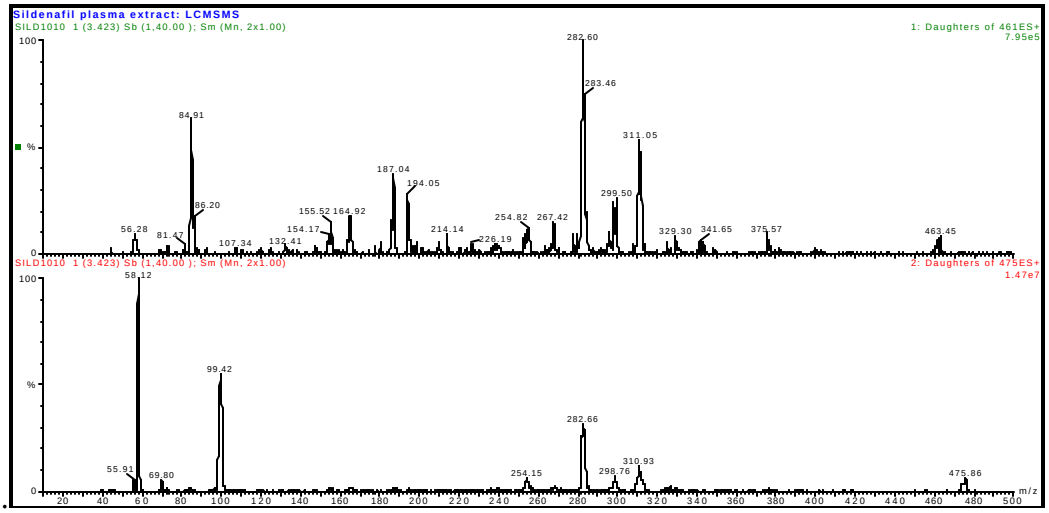


Fig. 3.6: Product ion spectra of dosed plasma extract, showing sildenafil t_R = 2.94 min. (Parent ion m/z = 475) (lower) and metabolite peak at t_R = 2.78 min. (Parent ion m/z = 461) (upper).

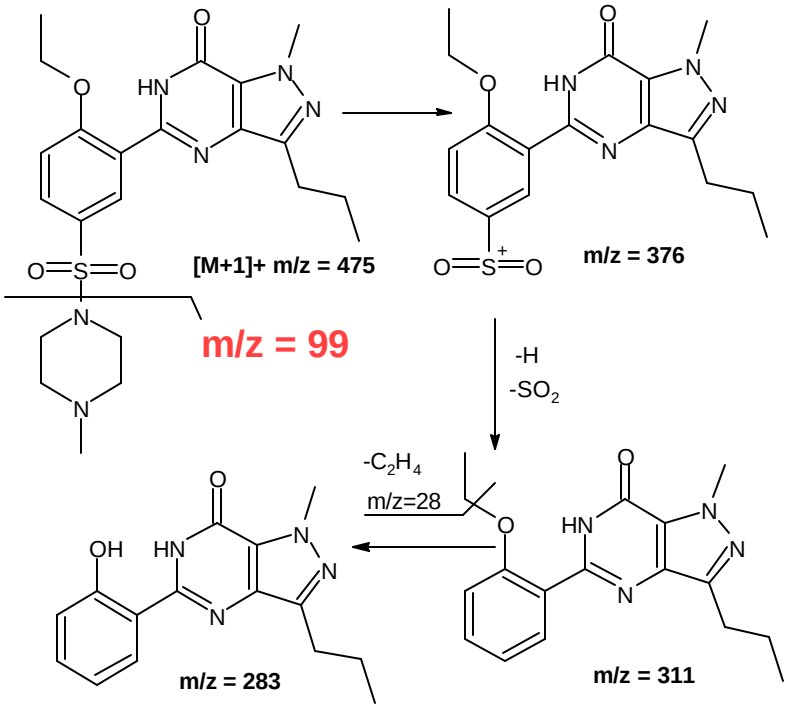


Fig. 3.7: Fragmentation pattern of sildenafil, peak with t_R = 2.78 min.

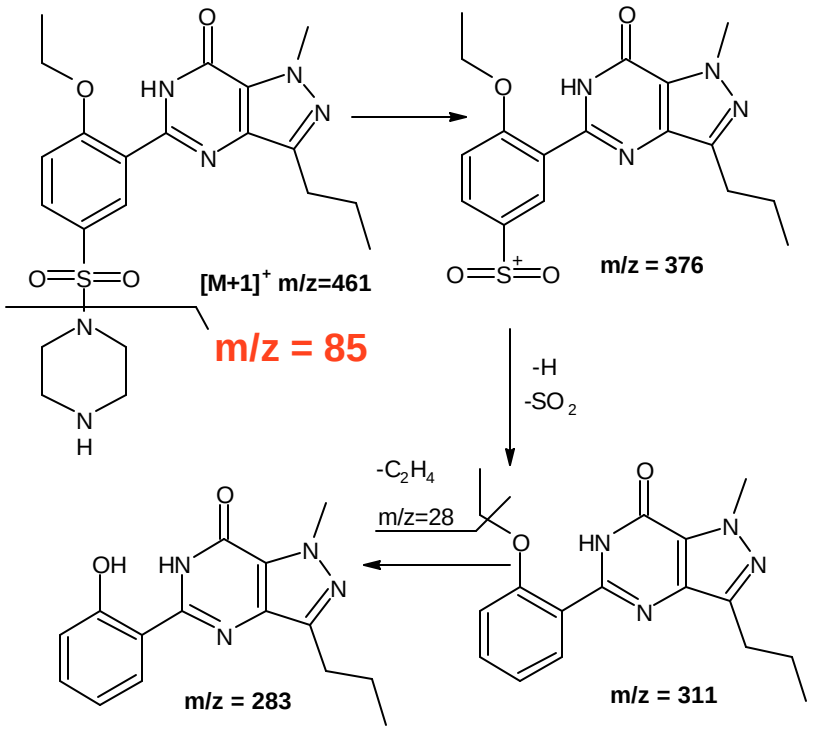


Fig. 3.8: Fragmentation pattern of sildenafil metabolite; peak with t_R = 2.94 min.

3.1.3 Chromatographic condition of metabolite in HPLC-UV.

HPLC-UV assay methods are not mass selective like mass spectrometry detectors, so the resolution of the metabolite peak from sildenafil is a big concern. The two peaks must be well resolved so that the quantification of sildenafil is reproducible and independent from its metabolite.

Having established that the demethyl metabolite of sildenafil is indeed present in the dosed plasma at a sufficient concentration to be detectable with the UV detector, 1.5 ml. of blank and dosed plasma were extracted using SPE (section 2.1.2.2) and reconstituted in 100µl of mobile phase (30 % 0.05M H_3PO_4 and 70 % acetonitrile, pH 5). 20 µl Of the reconstituted solutions and standard sildenafil (3 µg/ml in mobile phase) were injected onto the HPLC-UV system using the chromatography conditions established for the assay. A comparison of the three chromatograms showed that the metabolite is well resolved from sildenafil (Fig. 3.9). The metabolite and sildenafil have retention times of 4.07 min. and 5.01min. respectively. On can therefore conclude that the HPLC-UV assay method developed can be applied for pharmacokinetic studies successfully without any problem of interference from the metabolite.

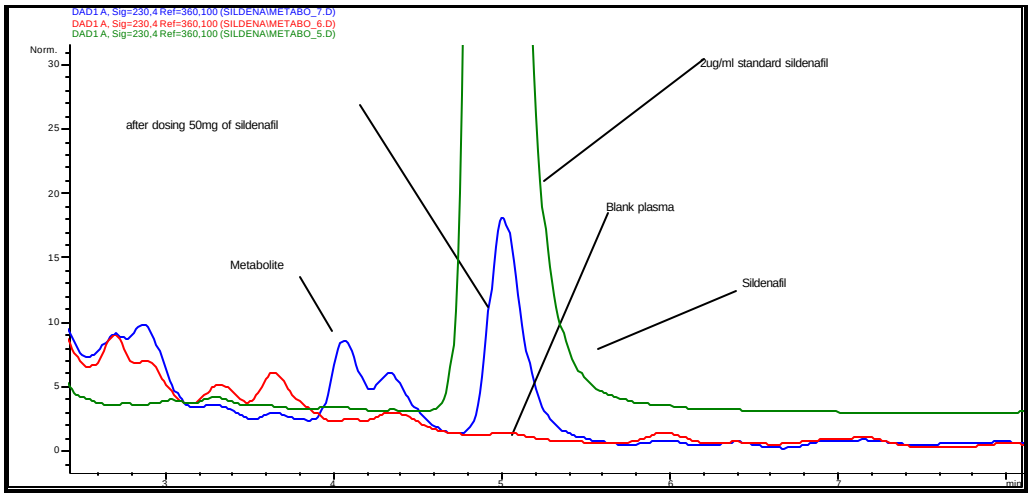


Fig. 3.9: Overlaid chromatograms of blank plasma extract, dosed plasma extract and standard solution of sildenafil (in mobile phase).

3.2 Identification Of The Artefact From Diethyl ether Treated Sildenafil Solution.

On trying to extract sildenafil from human plasma using a liquid– liquid extraction procedure with diethyl ether, artefacts were formed by both sildenafil and trazodone. Some preliminary studies were reported in section 2.1.2.1.1. In this section identification of the artefact produced by peroxide oxidation of sildenafil is addressed.

3.2.1 Preparation of the sildenafil artefact.

Since it was suspected that the artefact arose through oxidation of sildenafil by peroxides present in the diethyl ether used during the extraction process the following experiment was performed. A Viagra™ tablet containing 50 mg of sildenafil was ground fine in a porcelain mortar and added to 50 ml. of 0.01M NaOH in a 500 ml. Erlenmeyer flask. 250 ml. of diethyl ether was added and the mixture stirred vigorously on a magnetic stirrer. While stirring the solution, 10 ml. of H_2O_2 (30 %, 200 µl every 5min.) was added to facilitate the conversion of sildenafil to the artefact. 1 ml. Of the diethyl ether phase was retrieved at half an hour time intervals and the diethyl ether evaporated. The residue was reconstituted in 1 ml. mobile phase (40 % 0.05 M acetic acid and 60 % methanol, pH 5.5) and 20 µl was injected onto the HPLC-UV chromatography system to monitor the conversion of sildenafil to the artefact. It was interesting to observe that only about 50% conversion to a single artefact could be attained before an additional compound was also being formed and that the conversion to the main artefact could not be further increased. The following chromatograms illustrate the conversion to the artefacts.

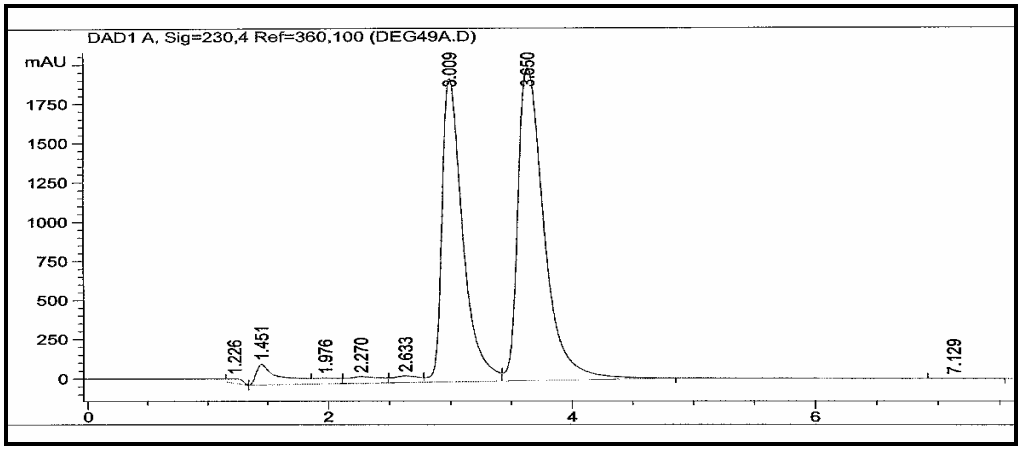


Fig. 3.10 : Chromatogram of sildenafil (t_R = 3.65) and main artefact (t_R = 3.09) during H_2O_2 oxidation after addition of 2.75 ml. H_2O_2

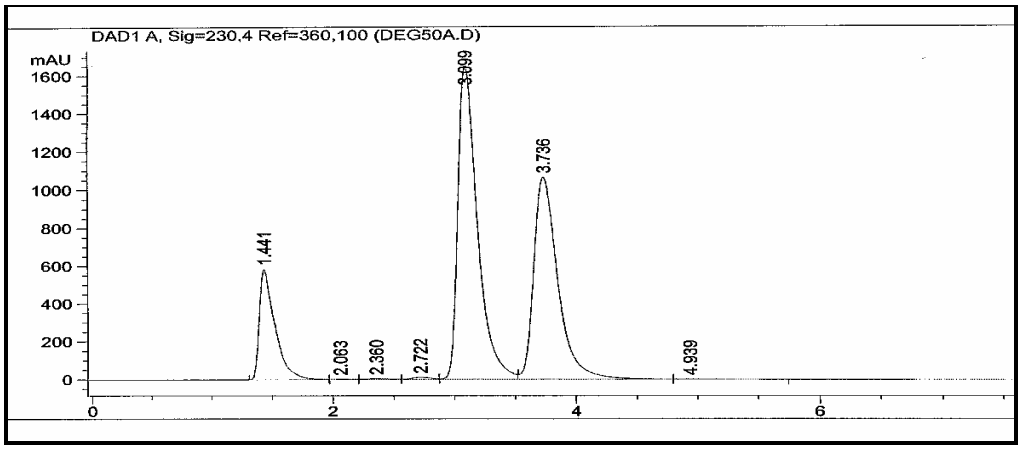


Fig. 3.11: Chromatogram of sildenafil (t_R = 3.74) and main artefact (t_R = 3.09) during H_2O_2 oxidation after addition of 4.75 ml. H_2O_2.

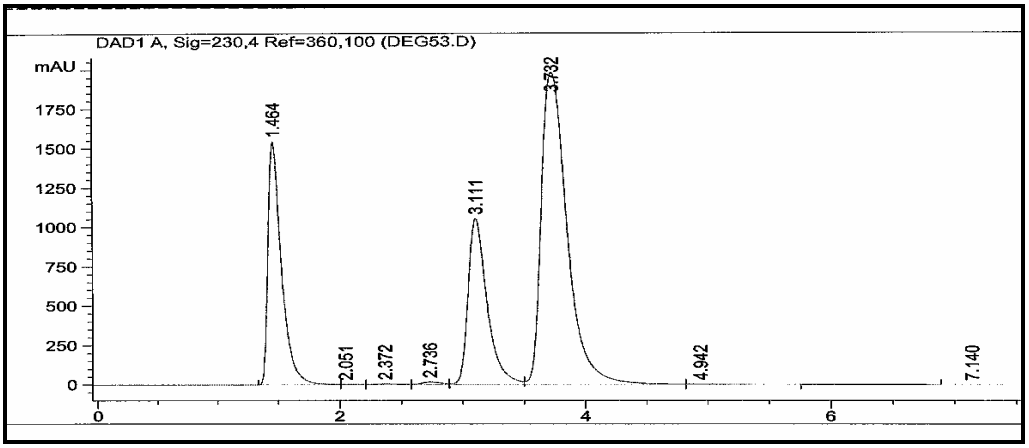


Fig. 3.12: Chromatogram of sildenafil (t_R = 3.73) and main artefact (t_R = 3.11) during H_2O_2 oxidation after addition of 10.75 ml. H_2O_2.

3.2.2 Mass spectrometric study of the main sildenafil artefact.

To try to elucidate the structure of the main artefact, the reconstituted extract was investigated mass spectrometrically. The solution (in pH 4.5 mobile phase), injected onto the LC/MS system, (Micromass Quattro Ultima, Manchester, UK) gave two peaks with retention times of 2.91 and 3.39 min. and molecular ions $[M+1]^+$ with m/z = 475 and 491 respectively (Fig. 3.13 and Fig. 3.14). The artefact therefore had a molecular mass of 16 a.m.u. higher than sildenafil which could only result from addition of one oxygen atom to sildenafil. The full mass and full product ion spectra of the two peaks are depicted in Fig. 3.15 and Fig. 3.16 respectively. It is likely that the main artefact is an N-Oxide of sildenafil and further structural elucidation of the peroxide oxidation of sildenafil and trazodone is recommended.

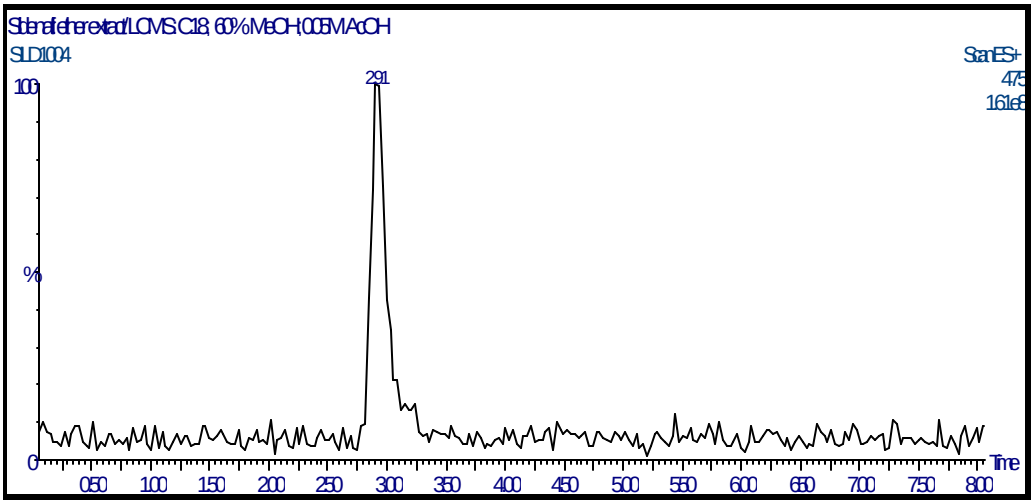


FIG. 3.13: Extracted ion (m/z = 475) chromatogram of diethyl ether treated solution of sildenafil.

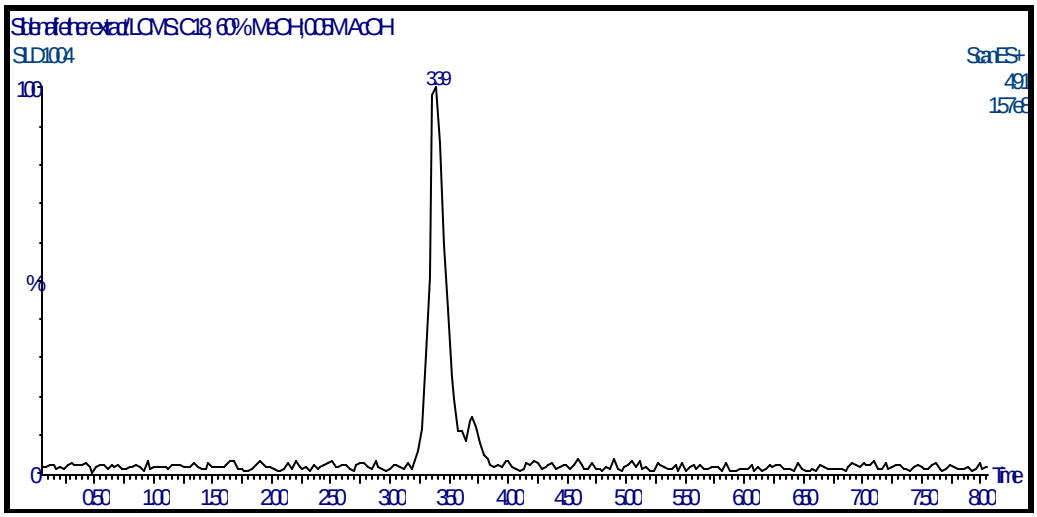


FIG. 3.14: Extracted ion (m/z = 491) chromatogram of diethyl ether treated solution of sildenafil.

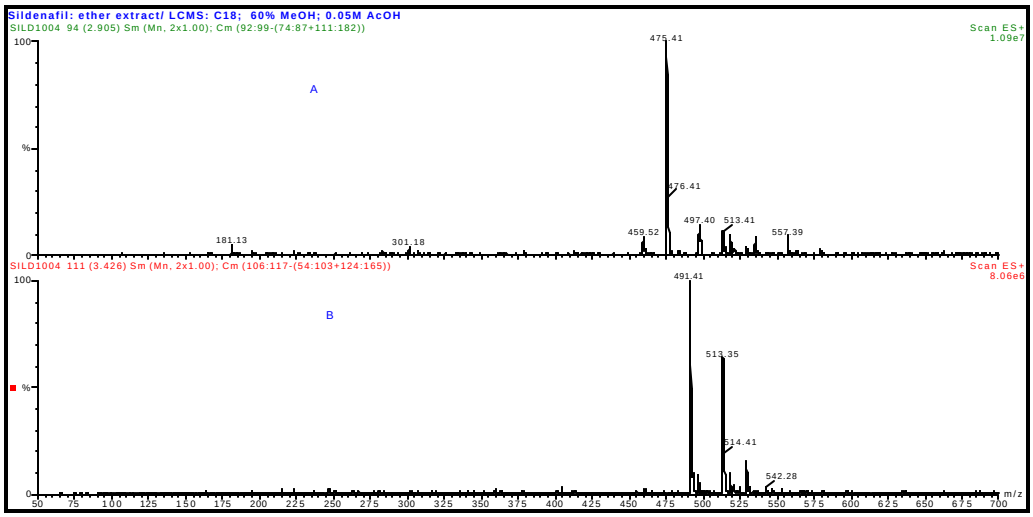


Fig. 3.15: Full mass spectra of (A) sildenafil (t_R = 2.91), and B the main artefact (t_R = 3.39).

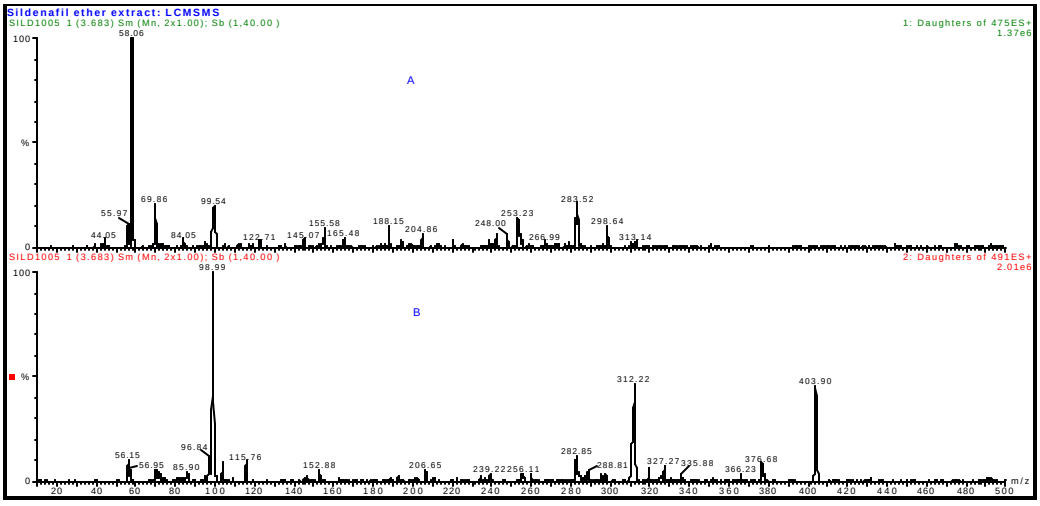


Fig. 3.16: Full product ion spectra of (A) sildenafil (parent ion m/z = 475) and (B) the main artefact (parent ion m/z = 491).

4 SUMMARY AND DISCUSSION.

The development and validation of bioanalytical assay methods suitable for the quantification of drugs in biological matrices is discussed. Relevant literature sources were consulted to understand the different parameters that must be included in method development and validation, to identify what constitutes a good assay method and to know the international regulations pertaining to bioanalytics that determine whether a developed assay method is acceptable or not. Further literature search was done to collect information on assay methods of sildenafil. The different aspects of these assay methods (extraction, instrumentation, total turn-around time and others) were assessed and an objective was set to develop selective, sensitive and rapid HPLC-UV and LC-MS/MS assay methods for quantification of sildenafil in human plasma.

The HPLC-UV assay method uses a SPE procedure. To 500 µl of plasma and 500 µl of 0.01 M NaOH in 1.5ml. Eppendorf tube (final pH 9.7) is added 100 µl internal standard solution (10µg/ml trazodone in methanol) and the mixture vortex mixed for 30 seconds. The solution is loaded onto a tC_{18} cartridge conditioned with 1 ml. of methanol followed by 1 ml. of HPLC water. After washing with 2 ml. of water, the analyte is eluted with 2ml. of methanol. The organic solvent is evaporated on a vacuum evaporator, reconstituted in 100 µl of mobile phase (30% acetonitrile in 0.05 M H_3PO_4 buffer, pH 5), and 20 µl injected onto the HPLC column. Sildenafil and trazodone elute at retention times of about 5.4 minutes and 3.8 minutes respectively and the total turn-around time is about 8.5 minutes. The recoveries of the extraction procedure at high, medium and low concentrations of sildenafil were 97.7 %, 96.5 % and 100 % respectively, while the recovery of trazodone was 82.3 %. The precision and accuracy of the intraday, inter-day-I and inter-day –II are within the accepted limits of

the international guideline for bioanalytical validations over the whole range between 50 ng/ml to 3200 ng/ml.

The extraction procedure used in the LC-MS/MS assay method is a simple protein precipitation. 300 µl Of plasma is precipitated with 600 µl of acetonitrile (containing 50 ng/ml trazodone). The supernatant is evaporated, the residue reconstituted in 300 µl mobile phase (60 % methanol in 0.05 M acetic acid, mobile phase adjusted to an apparent pH 4.5), and 5 µl injected onto the column. The absolute recoveries of the method at high, medium and low plasma concentration levels were 86.6 %, 82.1 % and 83.97 % respectively while the recovery of trazodone was 91.5 %. The precision and accuracy results of the validation lie within the accepted limits of the international guideline for bioanalytical method validation. The total turn-around time of the method is 4.5 min.

Since the methods developed will be used for pharmacokinetic studies of plasma samples in bioanalytical laboratories, the economical and time aspects of the assay methods were primarily given attention.

Various HPLC-UV assay methods have been reported for determination of sildenafil in biological samples. Even though Cooper *et al.* (1997) and Jeong *et al.*, (2001) developed assay methods having short turn-around times, the automated extraction procedure they use takes a long time and needs specialised instrumental setup and specialised software. Rapid SPE and LLE methods with good recoveries were developed by Jung *et al.* (2001) and J Lee & Min (2001) respectively, but the retention time of sildenafil is long (about 12 min.) which is very long compared with the new method developed. The LLE method by Bhoir *et al.* (2000) is simple but the repeated vortex mixing time makes the extraction process long and tedious. The mobile phase used also resulted in a long turn-around time.

Apart from the possibility of the so-called matrix effect, LC-MS/MS methods are far less prone to suffer from co-extracted analyte interference than the HPLC-UV assay methods are, and retention times and consequently also turn-around times are usually much shorter. Although no matrix effect was observed with the ten different plasma pools tested, this does not preclude a latent matrix effect developing during the processing of large numbers of samples injected in sequence with a short turn-around time. The latent matrix effect has been shown to develop as the result of strongly retained components that eventually elute from the chromatography column during extended batch analyses. This latent matrix effect can only be reduced by using clean extracts or eliminated by gradient elution to ensure that all strongly retained components have been eluted before the next sample injection. The occurrence of

the latent matrix effect in this study appears to be present as illustrated by the on-instrument stability data obtained during the intra-day validation batch run.

An interesting coincidental finding of the present study is the facile oxidation of both sildenafil and trazodone (probably to N-oxides) by peroxide present in diethyl ether when it is used as an extraction solvent. This calls into question any LLE procedure using solvents that could contain peroxides (e.g. the method published by Lee and Min (2000)).

5 APPENDIX

5.1 HPLC –UV assay method validation results

TABLE A.1: QC summary of intra-day batch (peak height).

Code & nom. con. / Replicates	QC F 2901 ng/ml	QC F(DIL) 1450 ng/ml	QC E 1450 ng/ml	QC D 518.0 ng/ml	QC C 259.0 ng/ml	QC B 130.00 ng/ml	QC A 65.00 ng/ml
1	[3698.468]	1435.745	1760.119	502.184	[398.769]	119.369	[156.45]
2	2415.948	1130.566	1769.816	595.106	259.056	162.873	87.615
3	2595.692	1305.742	1461.450	419.083	192.797	148.720	78.926
4	2468.341	1349.485	1430.654	478.994	294.258	138.762	75.242
5	2627.334	1002.256	1451.840	449.692	246.733	127.161	75.951
6	2693.581	1274.922	1310.472	500.068	271.180	135.775	71.993
MEAN	2560.179	1249.786	1530.725	490.855	252.805	138.777	77.945
%nom	88.3	86.2	105.6	94.8	97.6	106.8	119.9
CV%	4.5	12.6	12.4	12.2	15.0	11.2	7.6

TABLE A.2: QC summary of intra-day batch (peak area ratio).

Code & nom. con. / Replicates	QC F 2901 ng/ml	QC F(DIL) 1450 ng/ml	QC E 1450 ng/ml	QC D 518 ng/ml	QC C 259 ng/ml	QC B 130 ng/ml	QC A 65 ng/ml
1	2196.248	1367.792	1143.582	442.623	242.750	154.553	87.667
2	2852.137	1739.819	1220.425	413.091	258.360	124.861	77.232
3	2693.303	1377.374	1406.694	538.812	258.245	145.851	97.582
4	2990.399	1492.224	1297.749	449.888	234.861	136.850	68.689
5	2521.341	1378.293	1364.900	412.419	229.764	123.624	67.640
6	2582.018	1270.595	1347.723	435.053	223.453	121.015	69.709
MEAN	2639.241	1437.683	1296.846	448.648	241.239	134.459	78.087
%nom	91.0	99.2	89.4	86.6	93.1	103.4	120.1
CV%	10.5	11.4	7.6	10.4	6.1	10.1	15.6

TABLE A.3: QC summary of intra-day batch (peak area).

Code & nom. con. / Replicates	QC F 2901 ng/ml	QC F(DIL) 1450 ng/ml	QC E 1450 ng/ml	QC D 518.0 ng/ml	QC C 259.0 ng/ml	QC B 130.00 ng/ml	QC A 65.00 ng/ml
1	2784.587	1207.375	1460.617	507.225	293.421	122.586	[113.42]
2	2634.271	1275.498	1463.579	495.667	269.618	140.800	82.991
3	2691.287	1518.374	1591.681	513.994	179.591	140.732	65.852
4	2803.099	1509.489	1527.260	487.426	302.133	136.107	63.045
5	2923.056	1114.815	1707.195	479.189	252.711	126.366	67.409
6	3196.291	1454.693	1605.750	568.260	283.319	139.896	66.631
MEAN	2838.765	1346.707	1559.347	508.627	263.466	134.415	69.186
%nom	97.9	92.9	107.5	98.2	101.7	103.4	106.4
CV%	7.1	12.7	6.1	6.3	17.0	5.9	11.4

TABLE A.4: QC summary of inter-day-I batch (peak height).

Code & nom. con. / Replicates	QC F 2901 ng/ml	QC E 1450 ng/ml	QC E 518 ng/ml	QC C 259.0 ng/ml	QC B 130.0 ng/ml	QC A 65.00 ng/ml
1	3634.791	1900.307	570.609	380.436	132.931	46.288
2	3442.734	1662.650	669.834	289.772	159.289	58.854
3	3138.257	2126.717	613.365	281.101	151.529	50.246
4	3989.249	2115.093	736.858	419.548	172.106	68.436
5	3661.996	1928.581	715.873	321.768	170.001	77.452
6	3954.237	2129.324	663.252	328.585	167.284	71.012
MEAN	3636.877	1977.112	661.632	336.868	158.857	62.048
%nom	125.4	136.4	127.7	130.1	122.2	95.5
CV%	8.8	9.4	9.4	15.9	9.3	19.8

TABLE A.5: QC summary of inter-day-I batch (peak area ratio).

Code & nom. Con. / Replicates	QC F 2901 ng/ml	QC E 1450 ng/ml	QC E 518 ng/ml	QC C 259.0 ng/ml	QC B 130.0 ng/ml	QC A 65.00 ng/ml
1	3140.290	1444.445	510.822	238.168	128.637	65.840
2	3281.986	1368.161	469.260	281.504	152.256	99.036
3	3274.174	1364.894	516.644	280.675	147.727	55.659
4	3384.525	1376.062	502.388	288.074	153.998	69.309
5	2220.021	1605.572	444.628	232.653	131.621	95.661
6	2436.064	1535.160	525.968	225.531	122.934	61.885
MEAN	2956.177	1449.049	494.952	257.768	139.529	74.565
%nom	101.9	99.9	95.6	99.5	107.3	114.7
CV%	16.8	7.0	6.3	11.1	9.6	24.5

TBLE A.6: QC summary of inter-day-I batch (peak area).

Code & nom. Con. / Replicates	QC F 2901 ng/ml	QC E 1450 ng/ml	QC E 518 ng/ml	QC C 259.0 ng/ml	QC B 130.0 ng/ml	QC A 65.00 ng/ml
1	3629.948	1898.873	568.138	387.745	138.270	57.980
2	3506.881	1653.756	679.275	275.002	166.484	64.760
3	3117.338	2126.700	614.593	284.674	155.571	60.165
4	3959.482	2121.615	734.807	405.187	178.657	82.331
5	3613.674	1905.993	687.479	322.686	168.266	79.602
6	3965.585	2119.580	652.827	328.336	155.710	75.214
MEAN	3632.151	1971.086	656.187	333.938	160.493	70.009
%nom	125.2	135.9	126.7	128.9	123.5	107.7
CV%	8.7	9.6	8.9	15.9	8.7	14.8

TABLE A.7: QC summary of inter-day-II batch (peak height).

Code & Nom. con. / Replicate	QC F 2901 ng/ml	QC E 1450 ng/ml	QC E 518 ng/ml	QC C 259.0 ng/ml	QC B 130.0 ng/ml	QC A 65.00 ng/ml
1	2911.422	1398.920	516.101	244.617	117.963	54.558
2	2887.479	1490.169	510.147	232.625	128.149	63.366
3	2863.064	1472.611	520.347	247.415	137.559	66.656
4	2790.434	1348.754	584.355	250.886	115.353	56.336
5	3039.252	1546.331	557.228	269.965	143.842	69.563
6	2862.820	1554.006	533.387	231.248	125.294	68.171
MEAN	2892.412	1468.465	536.928	246.126	128.027	63.108
%nom	99.7	101.3	103.7	95.0	98.5	97.1
CV%	2.9	5.5	5.3	5.7	8.6	10.0

TABLE A.8: QC summary of inter-day-II batch (peak area ratio).

Code & nom. con.	QC F 2901	QC E 1450	QC E 518	QC C 259.0	QC B 130.0	QC A 65.00
Replicates	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
1	2797.220	1486.670	550.611	292.120	131.041	43.020
2	3009.974	1485.164	576.964	247.435	132.035	59.009
3	2982.544	1419.657	537.272	254.823	139.155	61.462
4	2886.913	1506.844	557.311	257.565	128.036	63.274
5	2910.371	1514.027	548.773	269.649	141.414	67.586
6	2856.577	1476.528	531.356	285.000	129.754	64.499
MEAN	2907.267	1481.482	550.381	267.765	133.573	59.808
%nom	100.2	102.2	106.3	103.4	102.7	92.0
CV%	2.7	2.3	2.9	6.6	4.1	14.6

TABLE A.9: QC summary of inter-day-II batch (peak are a).

Code & Nom. Con.	QC F 2901	QC E 1450	QC E 518	QC C 259.0	QC B 130.0	QC A 65.00
Replicates	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
1	2939.174	1429.993	534.075	268.063	121.132	36.158
2	2951.220	1531.523	527.104	238.540	123.861	52.939
3	2964.127	1434.295	526.846	251.663	139.087	59.440
4	2935.733	1420.529	620.569	255.890	116.553	56.395
5	3315.179	1678.655	611.586	294.969	151.308	71.678
6	3205.905	1707.049	584.426	258.241	131.341	66.286
MEAN	3051.890	1533.674	567.434	261.228	130.547	57.149
%nom	105.2	105.8	109.5	100.9	100.4	87.9
CV%	5.4	8.5	7.7	7.3	9.9	21.6

5.1.1 Representative chromatograms of HPLC-UV assay method.

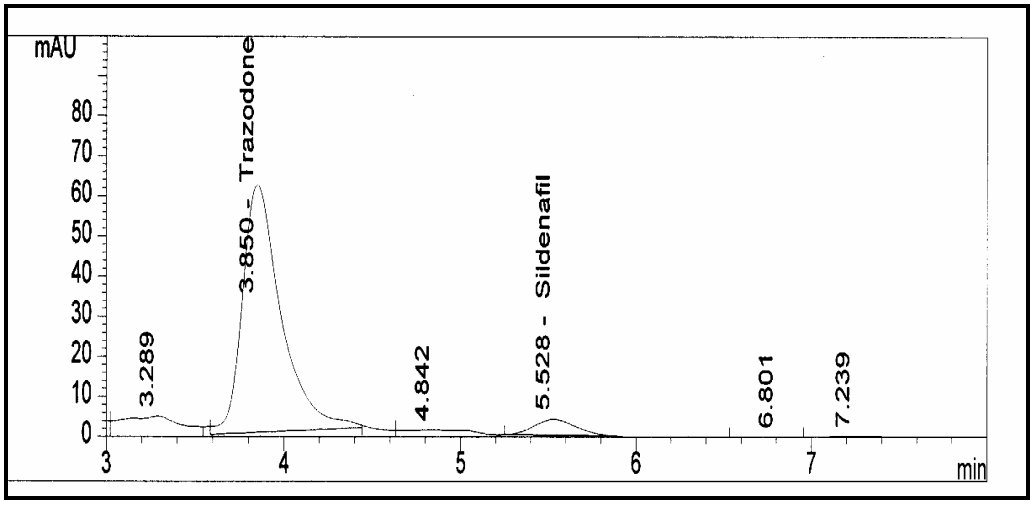


FIG. A.1: Low quality control sample extract. QC A (65 ng/ml).

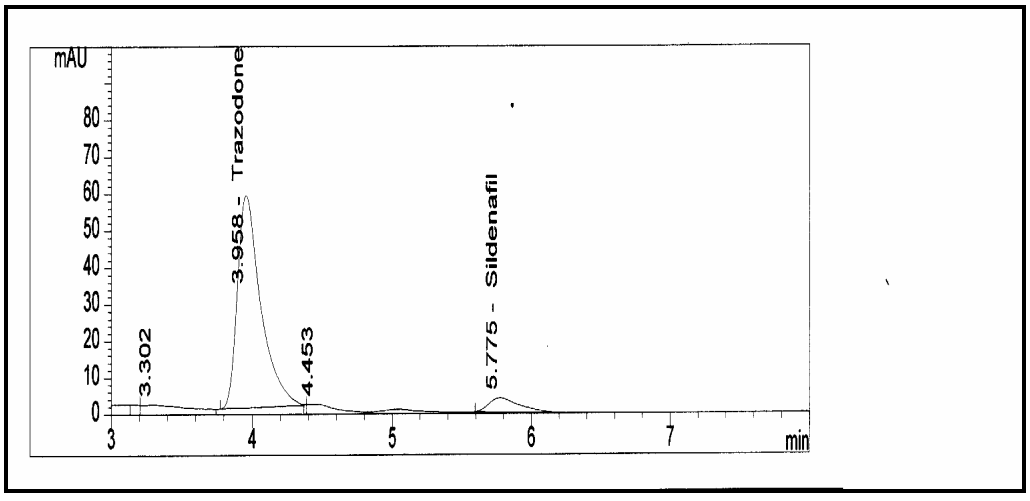


FIG. A.2: Low standard sample extract. STD B (49 ng /ml).

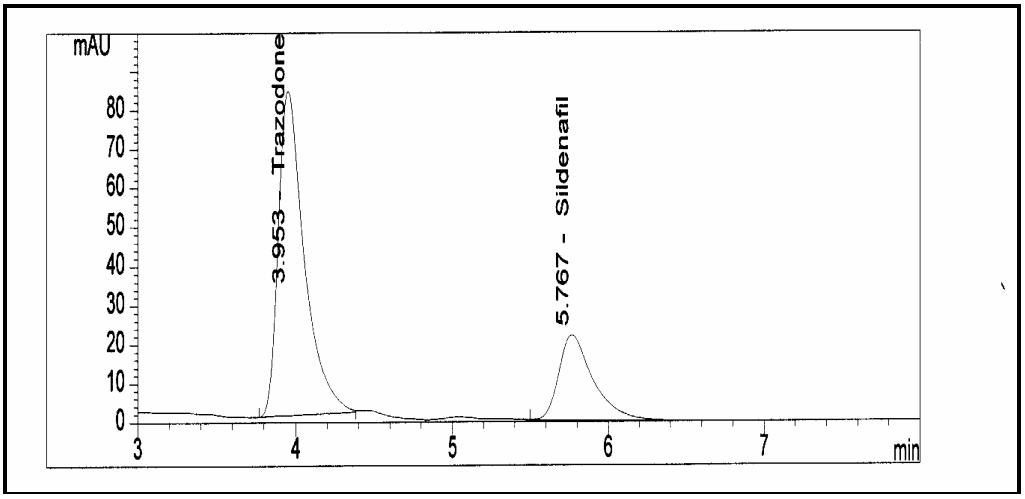


FIG. A.3: Medium quality control sample extract. QC C (259 ng/ml).

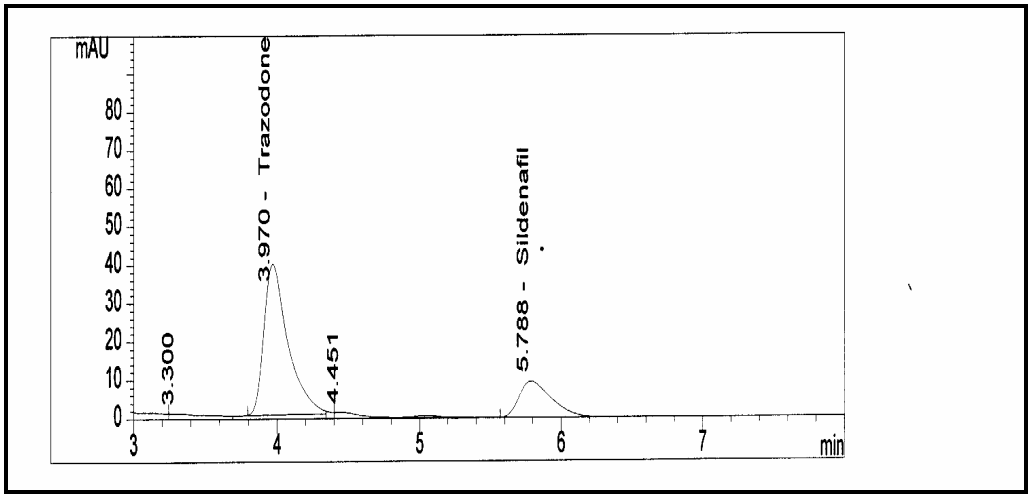


FIG. A.4: Medium calibration standard sample extract. STD D (199 ng/ml).

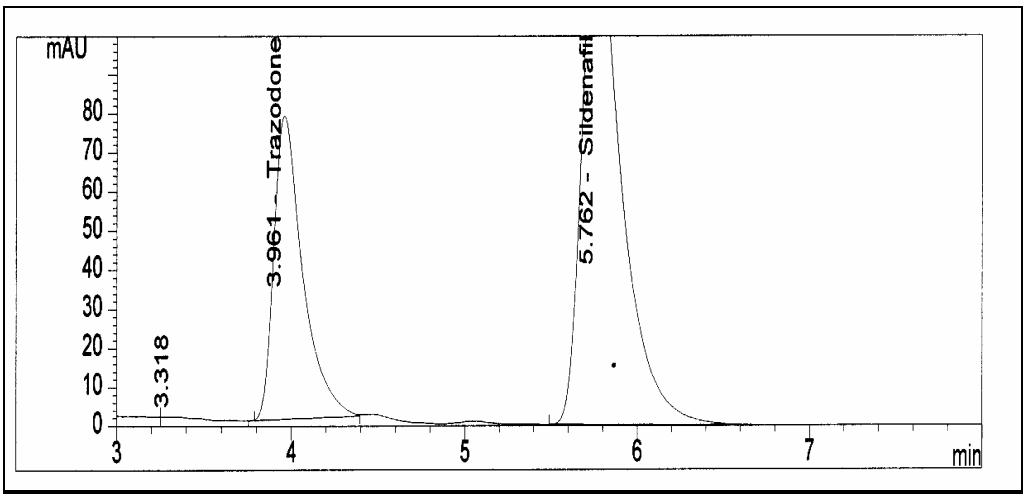


FIG. A.5: High quality control sample extract. QC E (1450 ng/ml).

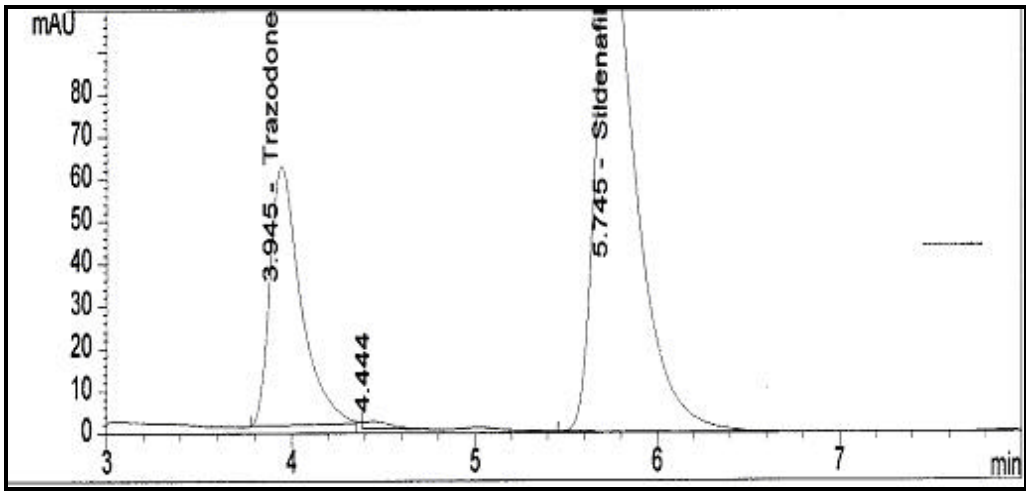


FIG. A.6: High standard sample extract. STD G (1598 ng/ml).

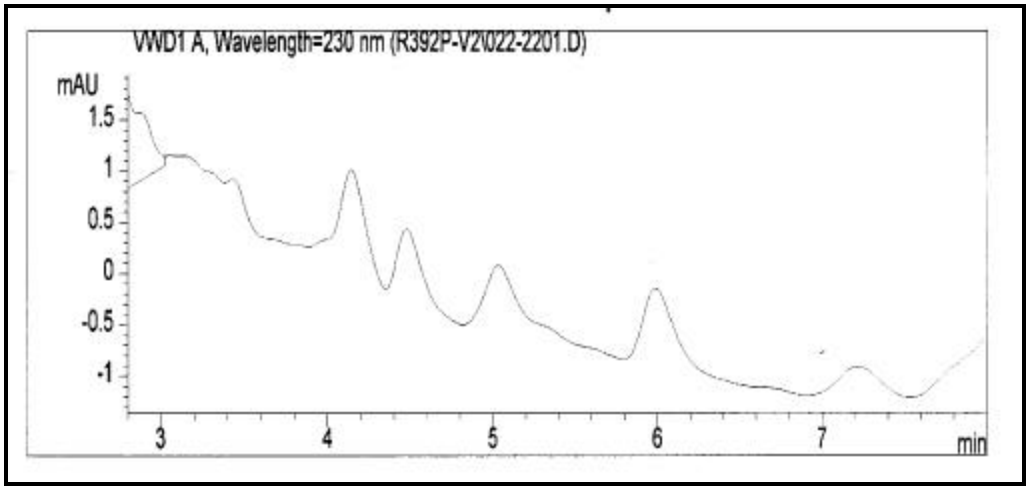


FIG. A.7: Blank plasma extract.

5.2 LC-MS/MS assay method validationresults

TABLE A.10: QC summary of intra-day batch (peak area).

Code & nom. con. / Replicates	QC I 1455 ng/ml	QC I(DIL) 1455 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC F 182 ng/ml	QC E 63.7 ng/ml	QC D 31.8 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1505.25	1485.815	736.392	336.324	187.764	65.920	34.188	17.182	8.255	*5.095*
2	1459.641	1459.641	755.939	358.844	189.068	65.920	34.188	17.377	8.320	*4.593*
3	1498.736	1498.736	749.423	366.296	186.461	70.481	30.213	14.641	7.278	*4.991*
4	1361.905	1329.437	690.781	349.355	163.004	58.166	30.474	14.836	8.385	*4.085*
5	1355. 389	1342.468	671.234	331.763	161.701	57.775	27.086	14.901	8.385	*4.020*
6	1394.484	1368.531	671.234	335.672	165.611	56.789	28.259	14.510	7.147	*3.837*
MEAN	1429.235	1414.105	712.501	346.376	175.602	62.509	30.735	15.575	7.962	*4.437*
%nom	98.2	97.2	97.9	95.2	96.5	98.1	96.6	98.0	100.0	*111.5*
CV%	*4.7*	*5.4*	*5.5*	*4.1*	*7.6*	*9.1*	*9.6*	*8.5*	*7.3*	*12.0*

TABLE A.11: QC summary of intra-day batch (peak height ratio).

Code & nom. con. / Replicates	QC I 1455 ng/ml	QC I (dil) 1455 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC F 182 ng/ml	QC E 63.7 ng/ml	QC D 31.8 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1334.081	1291.879	644.644	343.225	164.721	56.620	34.519	14.402	6.837	4.531
2	1355.235	1327.076	687.517	327.631	153.465	62.460	36.921	13.938	8.439	4.139
3	1421.964	1403.318	693.766	355.822	173.782	70.727	32.063	14.031	8.586	5.345
4	1493.182	1463.973	698.369	359.769	169.318	61.917	29.474	15.390	9.787	4.350
5	1432.672	1398.816	696.141	355.867	172.352	59.818	30.474	15.096	7.960	5.488
6	1439.276	1398.482	729.801	340.548	178.299	58.814	31.289	16.236	7.960	4.054
MEAN	1412.735	1380.591	691.706	347.144	168.656	61.726	32.457	14.849	8.262	4.651
%nom	97.1	94.9	95.0	95.4	92.7	96.9	102.1	93.4	103.8	116.9
CV%	4.1	4.4	4.0	3.5	5.2	7.9	8.6	6.0	11.7	13.3

TABLE A.12: QC summary of intra-day batch (peak height).

Code & nom. Con. / Replicates	QC I 1455 ng/ml	QC I (dil) 1455 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC F 182 ng/ml	QC E 63.7 ng/ml	QC D 31.8 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1489.224	1431.248	718.816	348.510	178.254	64.715	35.771	15.021	8.030	5.423
2	1425.378	1444.017	720.945	369.792	177.190	64.928	38.219	15.660	9.211	4.625
3	1457.301	1431.248	733.714	361.279	181.447	70.355	32.260	13.106	8.945	5.328
4	1340.250	1256.736	670.932	333.613	159.100	57.373	28.003	14.596	8.562	4.434
5	1329.609	1288.659	640.073	326.164	156.972	55.989	28.429	15.021	9.073	5.328
6	1361.532	1318.454	642.201	317.651	166.549	51.839	29.812	15.341	7.403	3.934
MEAN	1400.549	1361.727	687.780	342.835	169.919	60.867	32.082	14.791	8.537	4.845
%nom	96.3	93.6	94.5	94.2	93.4	95.6	100.9	93.0	107.3	121.7
CV%	4.7	6.1	6.1	6.0	6.2	11.4	13.0	6.1	8.2	12.5

TABLE A.13: QC summary of inter-day-I batch (peak area).

Code & nom. Con. / Replicates	QC I 1455 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC F 182 ng/ml	QC E 63.7 ng/ml	QC D 31.8 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1413.193	670.019	383.578	177.969	69.042	32.001	15.207	9.008	4.479
2	1475.978	702.195	358.466	183.462	63.628	32.943	16.384	9.478	4.370
3	B	719.460	358.466	181.108	60.645	29.098	15.992	11.362	3.381
4	1405.349	662.956	342.720	161.488	60.253	29.882	12.931	7.909	3.860
5	1248.395	612.731	303.532	163.843	56.565	30.275	12.068	6.638	4.158
6	1209.156	613.516	289.406	155.210	53.269	28.548	15.207	7.030	4.691
MEAN	1350.414	663.480	339.361	170.513	60.567	30.458	14.632	8.571	4.157
%nom	92.8	91.1	93.2	93.7	95.1	95.8	92.0	107.7	104.4
CV%	8.5	6.7	10.6	6.9	9.1	5.6	11.9	20.4	11.4

TABLE A.14: QC summary of inter-day-I batch (peak height ratio).

Code & nom. Con. / Replicates	QC I 1455 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC F 182 ng/ml	QC E 63.7 ng/ml	QC D 31.8 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1284.610	684.995	333.787	176.249	63.275	28.076	13.127	8.186	F
2	1403.726	717.340	324.121	175.402	62.747	29.687	14.461	9.276	F
3	B	686.733	342.875	186.210	64.027	29.076	16.748	12.331	F
4	1433.840	686.759	346.753	177.642	59.136	29.731	13.366	6.237	3.324
5	1543.901	725.515	356.314	188.437	64.068	37.391	14.900	7.738	3.957
6	1433.310	705.266	346.249	180.755	67.509	31.402	17.444	7.761	4.804
MEAN	1419.877	701.101	341.683	180.783	63.460	30.894	15.008	8.588	4.028
%nom	97.6	96.3	93.9	99.3	99.6	97.2	94.4	107.9	101.2
CV%	6.5	2.5	3.3	3.0	4.2	10.9	11.7	24.2	18.4

TABLE A.15: QC summary of inter-day-I batch (peak height).

Code & nom. Con. / Replicates	QC I 1455 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC F 182 ng/ml	QC E 63.7 ng/ml	QC D 31.8 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1381.705	668.438	367.367	178.714	70.044	30.375	14.869	9.532	4.519
2	1485.077	698.158	351.862	186.467	65.263	32.571	16.032	9.946	4.028
3	B	694.281	350.570	185.174	64.100	30.375	16.549	12.931	3.059
4	1394.627	673.608	316.974	163.208	56.218	28.842	13.448	6.431	3.718
5	1246.029	607.707	301.468	164.500	56.218	32.701	12.802	7.581	4.054
6	1189.175	596.078	283.378	154.163	52.600	28.403	15.127	6.858	4.945
MEAN	1339.323	656.378	328.603	172.038	60.741	30.545	14.805	8.070	4.054
%nom	92.0	90.2	90.3	94.5	95.4	96.1	93.1	101.4	101.9
CV%	8.9	6.7	10.0	7.7	11.1	5.9	9.8	19.6	16.0

TABLE A.16: QC summary of inter-day-II batch (peak area).

Code & nom. Con. / Replicates	QC I 1455 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC E 63.7 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1408.470	705.481	358.375	68.551	15.092	9.346	5.930
2	1371.266	694.312	347.127	36.686	17.292	9.249	5.076
3	1361.966	670.109	346.189	70.605	16.665	8.738	4.941
4	1334.067	664.523	300.193	62.277	15.932	8.160	5.054
5	1231.782	603.055	302.072	53.214	15.512	7.602	4.097
6	1213.187	617.960	288.911	44.186	14.143	8.313	4.155
MEAN	1320.123	659.240	323.811	55.920	15.773	8.568	4.876
%nom	90.7	90.6	89.0	87.8	99.2	107.6	122.5
CV%	6.0	6.2	9.3	24.4	7.1	7.9	14.0

TABLE A.17: QC summary of inter-day-II batch (peak height ratio).

Code & Nom. con. / Replicates	QC I 1455 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC E 63.7 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1173.519	634.183	370.879	66.396	17.259	5.570	1.909
2	1264.060	671.135	377.011	67.184	16.478	6.709	2.836
3	1229.684	653.409	380.211	77.628	17.643	7.281	3.079
4	1252.932	700.950	385.803	72.799	15.525	6.512	4.112
5	1414.194	716.388	406.747	74.892	20.032	8.752	3.827
6	1348.428	766.404	411.351	62.589	21.948	7.309	4.007
MEAN	1280.470	690.412	388.667	70.248	18.148	7.022	3.295
%nom	88.0	94.8	106.8	110.3	114.1	88.2	82.8
CV%	6.8	6.9	4.3	8.2	13.2	15.1	25.9

TABLE A.18: QC summary of inter-day-II batch (peak height).

Code & nom. Con. / Replicates	QC I 1455 ng/ml	QC H 728 ng/ml	QC G 364 ng/ml	QC E 63.7 ng/ml	QC C 15.9 ng/ml	QC B 7.96 ng/ml	QC A 3.98 ng/ml
1	1390.847	725.478	384.084	68.773	17.288	7.552	3.429
2	1335.166	701.333	355.700	33.709	15.974	8.604	4.358
3	1335.166	672.578	354.119	74.441	16.446	8.556	3.914
4	1264.895	669.546	314.472	65.599	13.764	7.472	4.898
5	1197.150	591.870	312.881	56.754	14.548	8.364	3.975
6	1181.244	642.210	306.511	43.390	15.482	7.393	4.251
MEAN	1284.078	667.169	337.961	57.111	15.584	7.990	4.138
%nom	88.3	91.6	92.8	89.7	98.0	100.4	104.0
CV%	6.5	7.0	9.2	27.6	8.2	7.2	11.9

5.2.1 Representative chromatograms of LC-MS/MS validation.

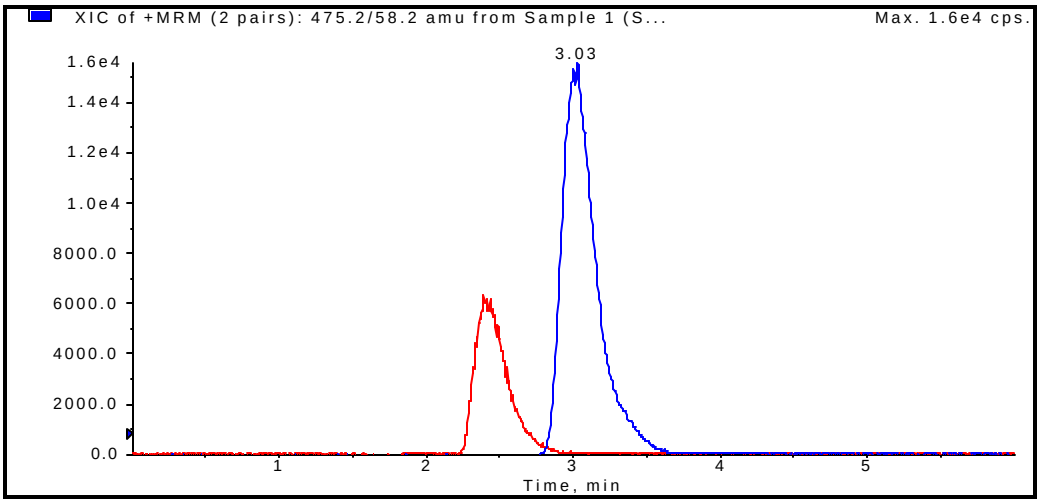


FIG. A.8: High standard sample extract (1600 ng/ml sildenafil) (STD K).

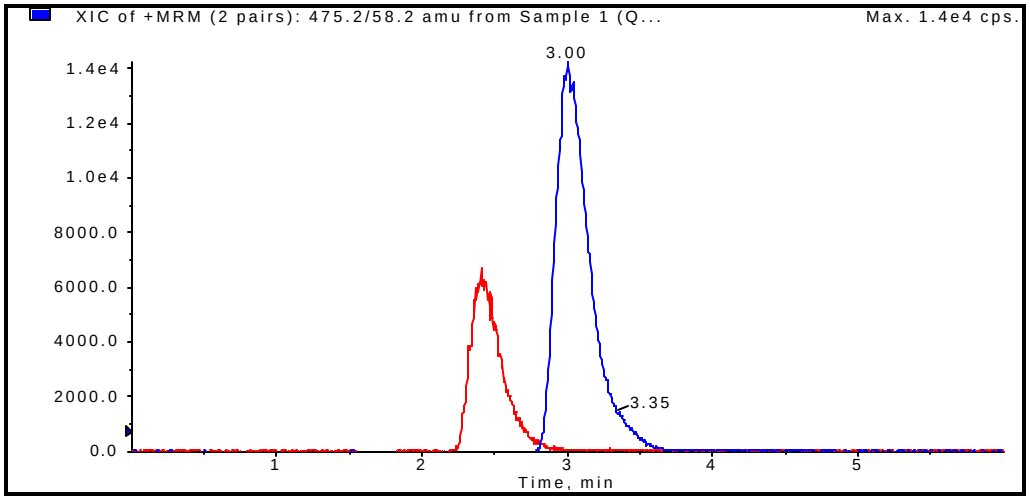


FIG. A.9: High Quality control sample extract (1440 ng/ml sildenafil) (QC I).

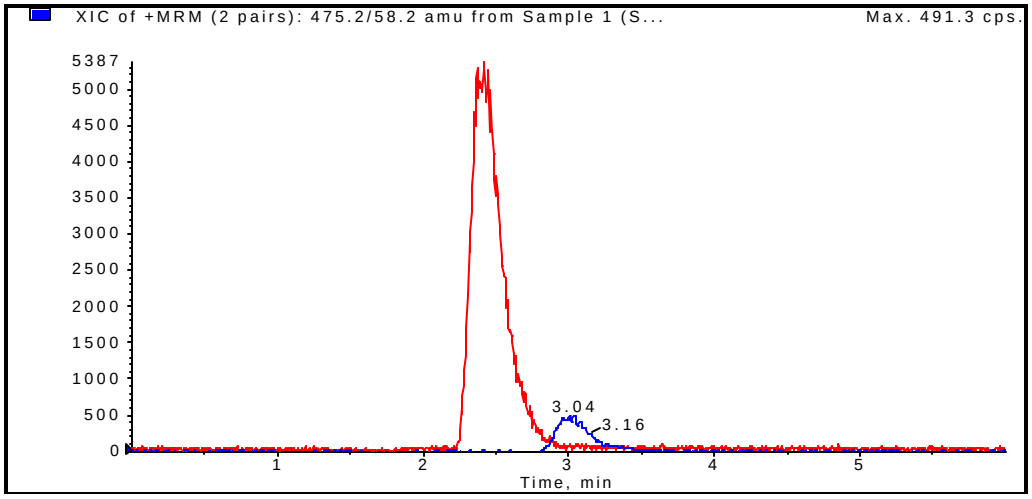


FIG. A.10: Medium standard extract (50 ng/ml sildenafil) (STD F).

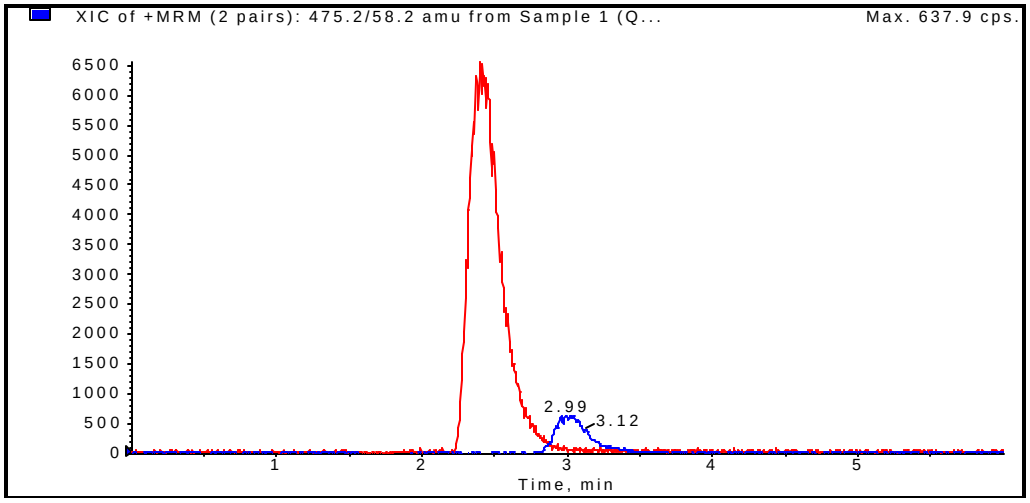


FIG. A.11: Medium quality control sample extract (90 ng/ml sildenafil) (QC E).

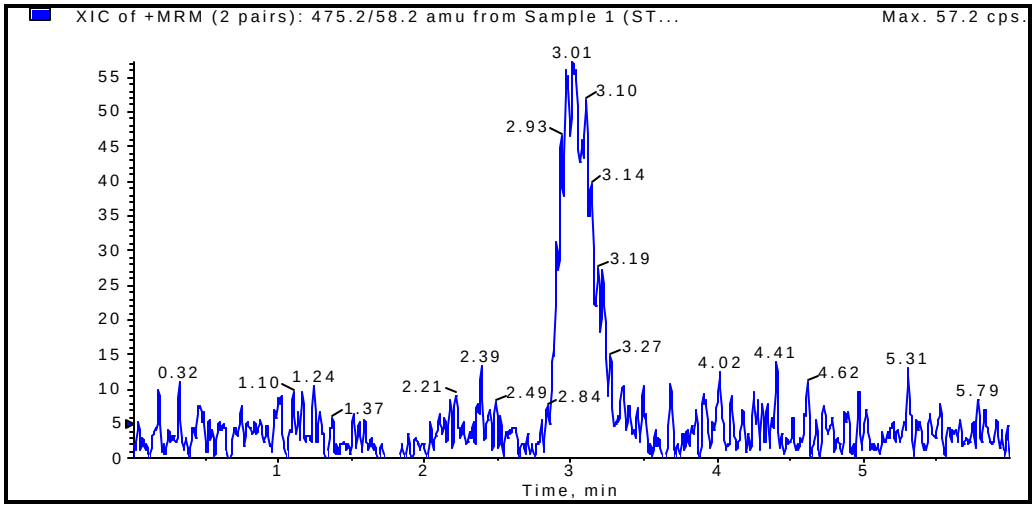


FIG. A.12: Low standard extract (6.25 ng/ml sildenafil) (STD C).

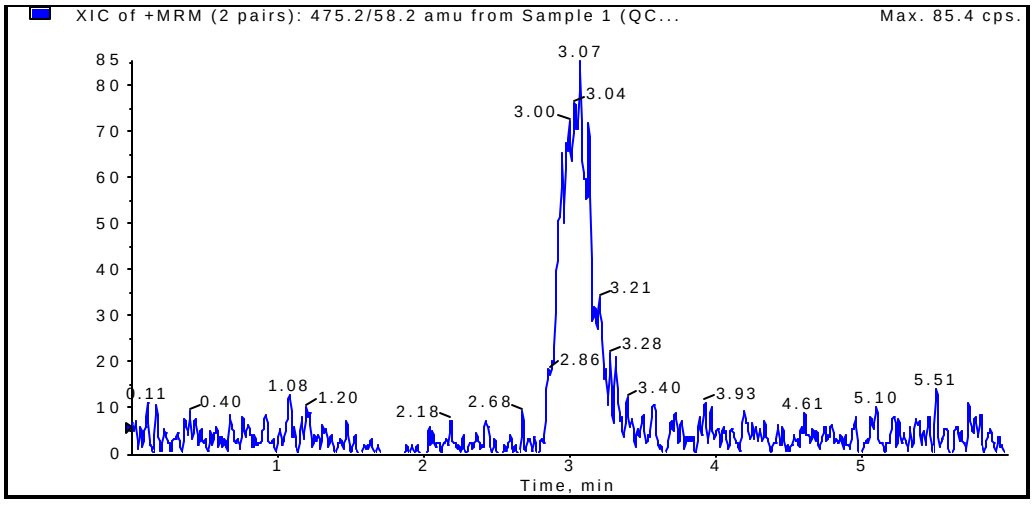


FIG. A.13: Low quality control sample extract (8.125 ng/ml sildenafil) (QCB).

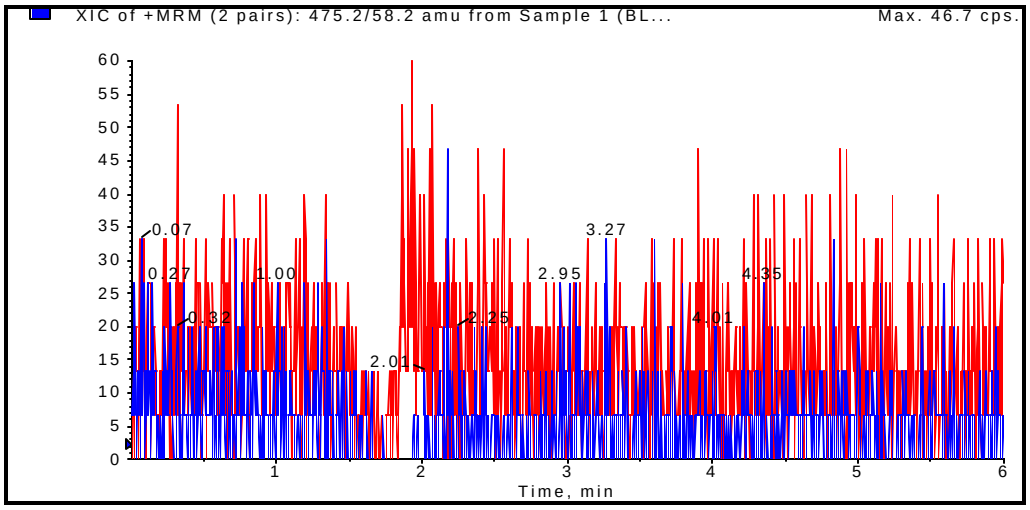


FIG. A.14: Blank plasma extract.

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