Bioanalytical Method Development & Validation
Introduction
Bioanalytical chemistry is the qualitative and quantitative analysis of drug substances in biological fluids (mainly plasma and urine) or tissues. It plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. The main analytical phase that comprises bioanalytical service are method development, method validation and sample analysis.

Analytical methods are used for product research, product development, process control and chemical quality control purpose. Each of the technique used whether chromatographic or spectroscopic, they 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 is 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.

A full validation requires a high workload and should therefore starts 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 the validation studies are performed (Green, 1996). Method development clears the way for the further processes on the validation stage. It must be recognized that proper validation requires a lot of work. However, this effort is repaid by the time saved when running the method routinely during the sample analysis.

2.1.1 Method development

Method development involves evaluation and optimization 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 summarizing main starting points for future work is of primary importance.

Based on the information from this survey, following can be done.

1. Choice of the instrument that is suitable for analysis of analyte of interest. This includes the choice of column associated with instrument of choice, the detector, the mobile phase in HPLC.
2. Choice of internal standard, which is suitable for the study such that it has similar chromatographic properties to analyte.

3. Choice of extraction procedure, which is time economical, gives the highest possible recovery without interference at the elution time of 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 standardization. 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 the blank. Another important issue in method development stage is the choice of internal standard versus external standardization. The external standard calibration method is a simple but less precise method and should only be used when sample preparation is simple and small or no instrumental variations are observed. The method is not suitable for use with complicated matrices but is often used in pharmaceutical product analysis characterized by simple matrices and easy sample preparation. The internal standard method is more accurate method. The internal standard technique can compensate for both instrumental and sample preparation errors and variations (e.g. dilution and extraction). Sample preparations steps such as extraction often results in sample losses, and a proper internal standard should be chosen to mimic the variations in these steps. Thus both the accuracy and precision of quantitative data increases if an internal standard is included in the procedure. The internal standard should be similar but not identical to the analyte, and the two should be well resolved in the chromatographic step. The standard curves are obtained from standards of blank samples spiked with different known concentrations of the analyte of interest and addition of an internal standard at different concentration. Also to the unknown samples the same constant concentration of the internal standard is added. The standard samples are processed in parallel with the unknown samples. In the calibration curve, the ratios of analyte to internal standard peak area (or height) are plotted versus the concentration of the analyte. A proper internal standard in a bioanalytical chromatographic method should fulfill the
requirements like be well resolved from the compound of interest and other peaks, not be present in the sample, be similar to retention to analyte, be available in high purity (not contaminated with the analyte), be stable, should resemble the analyte in all sample preparation steps, be of similar structure to the analyte.

2.1.2 Sample preparation

The purpose of sample preparation is to create a processed sample that leads to better analytical results compared with the initial sample. The prepared sample should be an aliquot relatively free of interferences that is compatible with the HPLC method and that will not damage the column. The whole advanced analytical process can be wasted if an unsuitable preparation method has been employed before the sample reaches the instrument. In practice the choice of sample-preparation procedure is dependant on both the nature and size of the sample and on the selectivity of the separation and detection systems employed. Sample pre-treatment may include a large number of methodologies. Ideally, sample preparation techniques should be fast, easy to use and inexpensive.

2.1.3 Extraction

The main sample preparation techniques are liquid-liquid extraction and solid phase extraction. In these methods the analysts aims to separate the analyte of interest from sample matrix so that as few potentially interfering species as possible are carried through to the analytical separation stage. LLE of an analyte is based on its partition between an aqueous phase and an immiscible organic phase. The distribution of the analyte between the two phases is affected by the pH and ionic strength of the aqueous solution and also of the type of the organic solvent used. By adjusting the pH of the aqueous phase, the analytes may be extracted into the organic phase with good recovery. The distribution ratio can be increased by proper choice of the organic solvent. The volume ratio between the aqueous phase and the organic phase also affects the recovery. Solid phase extraction is an alternative to LLE. In SPE the analytes are partitioned between a solid and a liquid. Generally the interfering compounds are rinsed off the solid adsorbents and the analytes are then desorbed with an eluting solvent.
2.1.4 Chromatographic separation

Analyte in a mixture should be preferably be separated prior to detection. Chromatography in different forms is today the leading analytical method for separation of components in a mixture. The chromatographic procedure for the separation of substances is based on differences in rates of migration through the column arising from different partitions of the compounds between a stationary phase and a mobile phase transported through the system. Chromatographic methods can be classified according to the physical state of the mobile phase into the following basic categories: gas chromatography (GC), supercritical fluid chromatography (SFC) and liquid chromatography (LC). The stationary phases can be of widely different character and their main properties are often the basics for further classification of the methods. LC is today the main tool for analysis of various substances in different matrices. LC can be categorized on the basis of the mechanism of interaction of the solute with the stationary phase as: adsorption chromatography (liquid-solid chromatography), partition chromatography (liquid-liquid chromatography), ion-exchange chromatography (IEC), size exclusion chromatography (SEC) and affinity chromatography. Successful chromatography requires a proper balance of intermolecular forces between the three active parts in the separation process, the solute, the mobile phase, and the stationary phase; the polarities for these three parts should be carefully blended for a good separation to be realized in a reasonable time. The mobile phase composition is readily changed in HPLC separations and the retention is preferably adjusted by changing mobile phase composition or solvent strength.

2.1.5 Detection

There exist several different detectors suitable for detecting the analytes after the chromatographic separation. Some commercial detectors used in LC are: ultraviolet (UV) detectors, fluorescence detectors, electrochemical detectors, refractive index detectors, and mass spectrometry detectors. The choice of detectors depends on the sample and the purpose of the analysis. 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 the subsequent routine analysis. The differences found between individual instruments representing
similar models from the same manufacturer is not surprising and should be accounted
for.
The following two parameters must be determined at the method development stage as
they are benchmark for the further work.

2.1.6 Limits of detection and quantification  

The US pharmacopoeia defines the limit of detection as the lowest concentration of
analyte in a sample that can be detected but not necessarily quantitated. They also
defines the lowest limit of quantification 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.
The limits are commonly associated with the signal to noise (S/N). In the case of LLOD,
analyte 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
baseline 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 harmonization)
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
and the slope of the calibration curve at levels approaching the limits according to
equation below.

\[ \text{LLOD} = 3.3 \times (\delta/S) \]
\[ \text{LLOQ} = 10 \times (\delta/S) \]
The standard deviation of the response can be determined based on the standard
deviation of the blank, based on the residual standard deviation of 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.

2.1.7 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 relationship than would be for the linear relationship.

The difference observed 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. 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. 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 exist and a minimum of five non-zero standards remains in the curve.

1. Loss of sensitivity.
2. Poor chromatography.
3. Loss during sample processing.
4. 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 the concentration.
plot. A correlation coefficient of more than 0.999 is generally considered as evidence of an acceptable fit of the data to the regression line (Green, 1996).

2.1.8 Prevalidation

The quality of bioanalytical data is highly dependent on selection of an appropriate regression model for the calibration curves. In bioanalytical assays, variance normally increases with concentration. The use of traditional approach with heteroscedastic data will lead to impaired accuracy. To account heteroscedasticity, transformation and weighting approaches are generally used to generate the calibration curves. During 'pre-validation', the model to be used as calibration curve is identified and the quality of fit is assessed. The accuracy profile discriminates the acceptable and non acceptable regression models by using estimates of bias and standard deviation of the intermediate precision obtained from validation standards or back calculated concentrations of calibration standards analyzed in replicate series. During 'validation', the model is focused on the estimation of bias and precision of method, not on the calibration curve. A good regression model is foundation for accurate and reproducible quantification over the whole calibration range. Correlation coefficient $r^2$ alone is not adequate to demonstrate linearity since $r^2$ values above 0.999 can be achieved even when the data show signs of curvature. Standard curve fitting is determined by applying the simplest model that adequately describes the concentration–response relationship using appropriate weighting and statistical tests for goodness of fit. The most common approach to fit a calibration curve is by using ordinary (non-weighted) linear regression method (OLR). This traditional approach presupposes that each data point in the range has a constant absolute variance (i.e. homoscedastic data). Two approaches commonly used to make the variance homogeneous are weighted linear regression (WLR) and/or transformation. These models will normally generate a better curve fit (i.e. smaller sum of residuals and random scatter in residual plots) and increase accuracy over whole concentration range.

2.1.9 Method validation

When a method has been developed it is important to validate it to confirm that it is suitable for its intended purpose. The validation tells how good the methods are, specifically whether it is good enough for the intended application. The US Food and
Drug Administration (FDA) have edited draft guidelines with detailed recommendations for method validation of bioanalytical methods in the pharmaceutical industry. The International Conference on Harmonization (ICH) has provided definitions of validation issues included in "analytical procedures" for the fields of bioanalytical methodology, pharmaceutical and biotechnological procedures. Likewise the US Pharmacopoeia has published guidelines for method validation for analytical methods for pharmaceutical products. However, the guidelines from ICH and USP are not as detailed as those from the FDA. The most common validation parameters are briefly described below.

2.1.9.1 Precision and accuracy

The precision of an analytical method is the closeness of a series of individual measurements of an analyte when analytical procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. The precision is calculated as Coefficient of Variation (C.V), i.e. relative standard deviation (RSD). The measured RSD can be subdivided into three categories: repeatability (intra-day precision), intermediate precision (interday precision), and reproducibility (between laboratories precision). Repeatability is obtained when the analysis is performed in one laboratory by one analyst using the same equipment at the same day. Repeatability should be tested by the analysis of minimum of five determinations at three different concentrations (low, medium and high) in the range of expected concentrations according to FDA. For bioanalytical applications the precision values at each concentration level should be better than 15 % except for the lower limit of quantification (LLOQ) where it should not exceed 20 %. The intermediate precision shows the variation affected in day-to-day analysis, by different analysts, different instrument, etc. Reproducibility as above represents the precision obtained between different laboratories. 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

\[
\% \text{ Bias} = \frac{\text{measured value} - \text{true value}}{\text{true value}} \times 100.
\]

Some of the possible errors 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 the analysis of certified reference material or standard reference material. Accuracy should be measured using a minimum of five determinations for at least three concentrations (low, medium and high) in the range of expected concentrations. The mean value should be within 15% from the true value except for the LLOQ where it should be within 20%. Both precision and accuracy can be calculated from the same analytical experiments.

2.1.9.2 Selectivity / specificity

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 present in biological fluids) and exogenous sources (impurities in the 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.

The following practical approach may be used during method development to investigate the selectivity of analytical method.

- Processing blank samples from different sources will help to demonstrate lack of interference from the substances native to 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 potential interference with the analyte of interest.
• Processing of reagent blank in the absence of biological matrix is normally adequate to demonstrate the selectivity with regard to exogenous interference 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.

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

2.1.9.3 Limit of quantification and detection

A clear distinction should be made between the limit of detection (LOD) and lower limit of quantification (LLOQ). The LOD is defined as the concentration of analyte that results in a peak height three times the noise when injected into the chromatographic system i.e. the point at which a measured value is greater than uncertainty associated with it. LOD is lowest concentration of analyte in a sample that can be detected but not necessarily quantified. The LLOQ is the lowest concentration of the analyte in a sample that can be quantified with acceptable accuracy and precision. The LLOQ should have an accuracy of 80 – 120 % and a precision of maximum 20%.

2.1.9.4 Recovery

High recovery of the analyte(s) from the matrix is a desirable outcome of sample preparation, and is therefore an important characteristic of the extraction procedure. The absolute recovery is the ratio of response measured for a spiked sample (in matrix) treated according to the whole analytical procedure to that of a non-biological sample spiked (in aqueous solution) with the same quantity of the analyte substance and directly injected into the chromatographic system. The relative recovery is the ratio of the responses between extracted spiked samples (in matrix) and extracted spiked pure samples (in aqueous solution). The relative recovery can be used, together with the absolute recovery, to reveal whether sample losses in the extraction are due to matrix effects or to bad extraction. The recovery should be determined by a minimum of six determinations for at least three concentrations (low, medium and high) in the range of expected concentrations. The absolute recovery should preferably exceed 90 % and the
relative recovery 95%. However, sometimes it may be necessary to sacrifice high recovery in order to achieve better selectivity, but it is important that the sensitivity, precision and accuracy still are acceptable. The internal standard should have a similar recovery as the analyte(s) and it should be within 15% of that determined for the analyte.

2.1.9.5 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 (i.e. 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 corresponds to the concentration of the analyte at the time of sampling (Hartman 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 and Burnett, 1995).

According to the recommendations on the Washington conference report by Shah et al., 1992, the stability of the analyte in the 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 Dadgar and Brunett (1995) gave the following details to be investigated.

2.1.9.5.1 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 analyzed.

2.1.9.5.2 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.
2.1.9.5.3 Short term temperature stability
The stability of the analyte in matrix at ambient temperature should be evaluated. At least three aliquots of each of the low and high concentration should be investigated and it is sometimes called the "bench top stability".

2.1.9.5.4 Post preparative stability
The stability of the analyte in the final extract during the expected maximum analysis time, which for automatic injections can be up to 48 h, should be assessed.

2.1.9.5.5 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 the samples at high, medium and low concentration unassisted and allowing them to freeze again for at least 12-24 hrs. The cycle is repeated twice and the sample is processed at the end of 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).

2.1.9.6 Robustness
Robustness tests examine the effect that small changes in operational parameters, have on the analysis results. The robustness is not mentioned in the FDA guidelines and is not considered in most validation guidelines. However, for methods that are used for a long time period and in different laboratories, testing of the robustness is important. Only effects of small changes in the experimental conditions need to be tested. Some parameters that could be tested are slight changes in the pH of the buffer, in the concentration of the organic modifier in the mobile phase, in ambient temperature, and in the detection wavelength.

2.2.0 Quality Control
When the analytical method has been validated for the routine use, its accuracy and precision should be controlled regularly to ensure that the method continues to work
satisfactorily. For this purpose, a number of separately prepared (from different weighing than the ones used for the standard curve) quality control (QC) samples should be analyzed in each run. The QC samples are often duplicates at three concentrations (low, medium and high) within the range. At least four of the six QC samples should be within 20% of their respectively nominal value, and at least one at each concentration level. Also a standard curve should be processed during each run.
Selective serotonin reuptake inhibitors (SSRIs) are a class of antidepressants for treating depression, anxiety disorders and some personality disorders. These drugs are designed to allow the available neurotransmitter serotonin to be utilized more efficiently. A low level of utilization of serotonin is currently seen as one among several neurochemical symptoms of depression. Low level of serotonin in turn can be caused by an anxiety disorder, because serotonin is needed to metabolize stress hormones. Citalopram is a highly selective and potent serotonin (5-hydroxy tryptamine) reuptake inhibitor with minimal effects on the neuronal reuptake of norepinephrine and dopamine. The ability of citalopram to potentiate serotonergic activity in the central nervous system via inhibition of the neuronal reuptake of serotonin is thought to be responsible for its antidepressant action. Tolerance to the inhibition of serotonin reuptake is not induced by long term treatment of rats with citalopram. Citalopram has no or very little affinity for a series of receptors including serotonin 5-HT1A, 5-HT2, dopamine D1and D2, α1, α2, β-adrenergic, histamine H1, muscarinic, cholinergic, benzodiazepine, GABA and opioid receptors.

Citalopram hydrobromide is a racemic bicyclic phthalanederivative designated (±)-1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile,hydrobromide salt and its empirical chemical formula is C20H22BrFN20. The melting point of citalopram hydrobromide is 185 – 188°C. It is sparingly soluble in water, very slightly soluble in diethyl ether, soluble in ethanol and freely soluble in chloroform. It is having pH of 5.5 – 6.5 and pKa of 9.5 determined by microtitration and log P value of 1.57.

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. It is
catalyzed by group of enzymes known as the cytochrome P 450. Citalopram is metabolized in liver to demethylcitalopram (DCT), didemethylcitalopram (DDCT), citalopram N-oxide and a deaminated propionic acid derivative. In vitro studies show that DCT, DDCT, and citalopram N-oxide also inhibit neuronal reuptake of serotonin but are less selective and less potent than the parent compound and are of minor clinical importance. Unchanged citalopram is the predominant compound in plasma. In vitro studies indicated that the biotransformation of citalopram to its demethyl metabolite depends on both CYP2C19 and CYP3A4, with a small contribution from CYP2D6.

Demethyl citalopram
Didemethyl citalopram
Citalopram

N-oxide

Deaminated propionic acid derivative

2.2.3 Pharmacokinetics of Citalopram

Pharmacokinetics is the study of the way in which the body handles the administered drug. The major pharmacokinetic variables are absorption, volume of distribution, half life (t1/2), Cmax (maximum concentration) and tmax (time to attain maximum concentration), metabolism and clearance. Following the administration of a single oral dose of citalopram (40 mg) to healthy male volunteers, peak blood levels occur at about 4 hours. The absolute bioavailability of citalopram is about 80 % (range 52% to 93 %) relative to intravenous dose and absorption is not affected by food. After intravenous infusion in healthy male volunteers the apparent volume of distribution (Vd) is about
12L/kg, indicating a pronounced tissue distribution and after oral administration the apparent volume of distribution is 17L/kg. The binding of citalopram and its demethylated metabolite to plasma proteins is about 80%. The single and multiple dose pharmacokinetics of citalopram are linear and dose proportional in a dose range of 10 – 60 mg/day. Steady-state plasma levels are achieved in patients in 1-2 weeks. At a daily dose of 40 mg, the average plasma concentration is about 83 ng/ml (n = 114) with a range from 30 – 200ng/ml. Citalopram does not accumulate during long term treatment. The elimination half life of it is approximately 37 hours (ranged 30 – 42 hrs) which allows recommendation of once-daily dosing. The systemic citalopram clearance is 0.33 L/min. It is eliminated primarily via the liver (85 %) and the remainder via kidneys; approximately 12 % (range 6 – 12 %) of the daily dose is excreted as unchanged citalopram in urine.

2.2.4 Pharmacokinetic interactions of Citalopram

In vitro studies indicate that the primary isoenzymes involved in citalopram metabolism are cytochrome P450 3A4 (CYP3A4) and 2C19 (CYP 2C 19). Interaction with medications that inhibit CYP3A4, such as ketoconazole, itraconazole, fluconazole, and macrolide antibiotics or with medications that inhibit CYP 2C 19 such as omeprazole, should be considered.

Combination containing any of the following medication depending on the amount present may also interact with this medication.

- Alcohol – although studies indicate that the cognitive and motor effects of alcohol are not potentiated by citalopram, concurrent use is not recommended.
- Tricyclic antiderpressants – In subject taking 40 mg per day of citalopram, the plasma concentration of desipramine, the active metabolite of imipramine, was increased by 50 % after administration of a single 100 mg dose of imipramine; there was no effect on imipramine or citalopram concentration
- Aspirin or NSAIDS or other drugs affecting coagulation – Concurrent risk may potentiate risk of abnormal bleeding; patient should be cautioned.
- Carbamazepine – Although a short term pharmacokinetic study showed no change in plasma concentrations of carbamazepine or citalopram during
concurrent use, the long term enzyme-inducing effect of carbamazepine could lead to increased clearance of citalopram.

- CNS drugs – Caution should be used given the primary CNS effects of citalopram.
- Cimetidine – AUC and maximum plasma concentration of citalopram were increased by 41% to 43% and 39% respectively, by concomitant cimetidine use.
- Ketoconazole – Decrease in Cmax and AUC of ketoconazole, loss of efficacy may occur.
- Linezolid – Serotonin syndrome has been reported in two patients' concomitantly receiving linezolid which is reversible non-selective MAOI.
- Lithium – Caution should be exercised with concomitant use because lithium may enhance the serotonergic effects of citalopram; plasma lithium levels should be monitored with appropriate adjustment to the lithium dose.
- Metoprolol – plasma concentrations of metoprolol were increased twofold by concominant citalopram use; however, no clinically significant effects on heart rate and blood pressure were seen.
- MAO inhibitors including furazolidone, procarbazine, and selegiline – Serious and sometimes fatal reactions have occurred in patients receiving a serotonin reuptake inhibitor with an MAO inhibitor, reaction have included hyperthermia, rigidity, myoclonus, autonomic instability with rapid fluctuations of vital signs, and mental status changes including extreme agitation progressing to delirium and coma; some cases presented with features resembling neuroleptic malignant syndrome; concurrent use of an MAO inhibitor and citalopram is contraindicated; at least 14 days should elapse between discontinuation of one medication (MAO inhibitor or citalopram) and the initiation of other.
- Serotonergics or other medications or substances with serotonergic activity – Increased risk of developing the serotonin syndrome, a rare but potentially fatal hyperserotonergic state that may occur in patients receiving serotonergic medications such as citalopram, usually in combination; symptoms typically occur shortly after the addition of a serotonergic agent to a regimen that include
other serotonin-enhancing drugs or after an increase in dosage of a serotonergic agent; symptoms include agitation, diaphoresis, diarrhea, fever, hyperreflexia, incoordination, mental status changes (confusion, hypomania) myoclonus, shivering or tremor.

- Sumatriptan – Postmarketing reports describe patients with weakness, hyperreflexia, and incoordination following use of a SSRI and sumatriptan, if concomitant use is clinically warranted; appropriate observation of patients is advisable.

- Warfarin – Although citalopram did not alter warfarin pharmacokinetics in one study, prothrombin time was increased by 5 %.

2.2.5 Side effects of Citalopram

Citalopram is contradicted within 14 days of monoamine oxidase inhibitor therapy. It should be used with caution in patients with a history of hyponatremia, seizure disorders, and suicide ideation. It should also be used with caution in patients with hepatic or renal dysfunction. The most common adverse effects associated with citalopram include dry mouth, nausea, somnolence, insomnia, increased sweating, tremor, diarrhea and sexual dysfunction.
# Literature survey

## Citalopram

**For estimation in biological fluids**

**By HPLC**

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**By GC-MS**

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### By LC with fluorescence detection

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<td>15.</td>
<td>Spectrofluorometric determination of citalopram in pharmaceutical preparations and spiked human plasma using organized media. J.AOAC. Int. 2006. 89(5), 1288-95</td>
<td>El-Sherbiny DT</td>
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**By HPLC-Electrospray tandem mass spectrometry**

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<th>Sr. No.</th>
<th>Reference</th>
<th>Author(s)</th>
</tr>
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</table>

**Detailed literature survey for estimation of Citalopram in biological fluids**

**Rapid determination of citalopram in human plasma by high-performance liquid chromatography**

Macek et al (2001) 09 have reported rapid high-performance liquid chromatographic method for the quantitation of citalopram in human plasma. The sample preparation involved liquid–liquid extraction of citalopram with hexane–isoamyl alcohol (98:2 v/v) and back-extraction of the drug to 0.02 M hydrochloric acid. Liquid chromatography was performed on a cyano column (45×4.6 mm, 5 μm particles), the mobile phase consisted of an acetonitrile–phosphate buffer, pH 6.0 (50:50, v/v). The run time was 2.6 min. The fluorimetric detector was set at an excitation wavelength of 236 nm and an emission wavelength of 306 nm. Verapamil was used as the internal standard. The limit of quantitation was 0.96 ng/ml using 1 ml of plasma.

**Simultaneous determination of citalopram, monodesmethylcitalopram and didesmethylcitalopram in plasma by high-performance liquid chromatography after column extraction**

Abstract not available.

**Determination of the antidepressant agent citalopram and metabolites in plasma by liquid chromatography with fluorescence detection**

Oeyehaug et al (1982) 13 have reported high-performance liquid chromatographic for the determination of citalopram and its two main metabolites (the methylamino and amino derivatives). The compounds were extracted from alkaline plasma with diethyl ether. The combined ether layers were evaporated after addition of 50 μl of 0.1 N HCl. The residual extracts were purified with diethyl ether and 20 μl were injected into a
Spherisorb ODS 5-µm column with acetonitrile-0.6% phosphate buffer pH 3 (55:45, v/v) as the mobile phase. Using a fluorescence detector the detection limits are 1 ng/ml of plasma for citalopram and the methylamino metabolite and 0.5 ng/ml for the amino metabolite.

**Determination of citalopram, amitriptyline and clomipramine in plasma by reversed-phase high performance liquid chromatography**

Rop et al (1985) 14 have reported determination of citalopram, amitriptyline, clomipramine and their desmethyl metabolites after alkaline diethyl ether extraction from plasma by high-performance liquid chromatography using two internal standards and µBondapak C18 as stationary phase. Elution is carried out isocratically at 0.5 or 1 ml/min with mixture of acetonitrile- potassium dihydrogen phosphate-distilled water (45:50:5). Detection is monitored by absorption at 254 nm. The detection limit is less than 5 ng/ml for each compound.

**Solid phase microextraction using poly (pyrrole) film and liquid chromatography with UV detection for analysis of antidepressants in plasma samples.**

Chaves et al (2009) 02 have reported evaluation of polypyrrole film by analyzing new-generation antidepressants (mirtazapine, citalopram, paroxetine, duloxetine, fluoxetine, and sertraline) in plasma sample by SPME and liquid chromatography with UV detection (LC-UV). The SPME-PPY/LC method showed to be linear in concentrations ranging from the limit of quantification (LOQ) to 1200 ng/ ml The LOQ values range from 16 to 25 ng/ ml. The inter-day precision of the SPME-PPY/LC method presented coefficient of variation (CV) lower than 15%.

**Polydimethylsiloxane/polypyrrole stir bar sorptive extraction and liquid chromatography analysis of antidepressants in plasma samples.**

Melo et al (2009) 12 have reported a stir bar sorptive extraction (SBSE) of antidepressants (mirtazapine, citalopram, paroxetine, duloxetine, fluoxetine and sertraline) from plasma samples, followed by liquid chromatography analysis (SBSE/LC-UV). The extractions were based on both adsorption (PPY) and sorption (PDMS) mechanisms. The PDMS/PPY coated stir bar showed high extraction efficiency (sensitivity and selectivity) toward the target analytes. The quantification limits (LOQ) of
the SBSE/LC-UV method ranged from 20 ng/ml to 50 ng/ml, and the linear range was from LOQ to 500 ng/ml, with a determination coefficient higher than 0.99.

Stir bar sorptive extraction and liquid chromatography with UV detection for determination of antidepressants in plasma samples.

Chaves et al (2007) have reported a sensitive and reproducible stir bar sorptive extraction and liquid chromatography (SBSE/LC-UV) method for the determination of sertraline, mirtazapine, fluoxetine, citalopram, paroxetine, imipramine, nortriptyline, amitriptyline, and desipramine in plasma samples. The SBSE/LC-UV method showed to be linear in a concentration ranging from the limit of quantification (LOQ) to 1000.0 ng mL(-1). The LOQ values ranged from 10.0 ng/ml to 40.0 ng/ml.

Determination of plasma levels of citalopram and its demethylated and deaminated metabolites by gas chromatography and gas chromatography-mass spectrometry

Reymond et al (1993) have reported sensitive and specific methods based on gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS) for the determination of levels of citalopram, desmethylcitalopram and didesmethylcitalopram in the plasma of patients treated with citalopram are presented, as well as a GC—MS procedure for the assay of the citalopram propionic acid derivative. After addition of a separate internal standard for each drug, liquid—solvent extraction is used to separate the basic compounds from the acid compounds. The limits of quantification were 1 ng/ml for citalopram and desmethylcitalopram and 2 ng/ml for the other metabolites. The correlation coefficients for the calibration curves (range 1–500 ng/ml) were 0.999 for all compounds, whether determined by GC or GC—MS.

Comparison of electron and chemical ionization modes by validation of a quantitative gas chromatographic mass spectrometric assay of new generation antidepressants and their active metabolite in plasma.

Wille et al (2007) have reported gas chromatographic-mass spectrometric method (GC-MS) for the simultaneous determination of the 'new' antidepressants (mirtazapine, viloxazine, venlafaxine, trazodone, citalopram, mianserin, reboxetine, fluoxetine, fluvoxamine, sertraline, maprotiline, melitracen, paroxetine) and their active metabolites (desmethylmirtazapine, O-desmethylvenlafaxine, m-chlorophenylpiperazine,
Literature survey
desmethylcitalopram, didesmethyleitalopram, desmethylmianserin, desmethylfluoxetine, desmethylsertraline, desmethylmaprotiline) in plasma using different ionization modes was developed and validated. Sample preparation consisted of a strong cation exchange mechanism and derivatisation with heptafluorobutyrylimidazole. The GC separation was performed in 24.8 min. Identification and quantification was based on selected ion monitoring in electron (El) and chemical ionization (Cl) modes. Calibration by linear and quadratic regression for electron and chemical ionization, respectively, utilized deuterated internal standards and a weighing factor 1/ x². Limits of quantitation were established between 5 and 12.5 ng/ml in El and positive ionization Cl (PICl), and 1 and 6.25 ng/ml in negative ionization Cl (NICl).

Simultaneous analysis of citalopram and desmethylcitalopram by liquid chromatography with fluorescence detection after solid phase extraction
Quin et al (2005) ¹⁶ have reported analysis of citalopram and desmethylcitalopram by liquid chromatography with fluorescence detection. The calibration was linear over the concentration range of 12–1600 ng/mL for CIT and 6–800 ng/mL for DCIT. The lower limit of quantification was 12 ng/ml for CIT and 6 ng/ml for DCIT.

Simultaneous determination of citalopram, fluoxetine, paroxetine and their metabolites in plasma and whole blood by high-performance liquid chromatography with ultraviolet and fluorescence detection
Kristoffersen et al (1999) ⁰⁸ have reported method for the simultaneous determination of the three selective serotonin reuptake inhibitors (SSRIs) citalopram, fluoxetine, paroxetine and their metabolites in whole blood and plasma. The method was validated for the concentration range 0.050–5.0 µmol/l with fluorescence detection and 0.12–5.0 µmol/l with ultraviolet detection. The limits of quantitation were 0.025 µmol/l for citalopram and paroxetine, 0.050 µmol/l for desmethyl citalopram, di-desmethyl citalopram and citalopram -N-oxide, 0.12 µmol/l for the paroxetine metabolites by fluorescence detection, and 0.10 µmol/l for fluoxetine and norfluoxetine by ultraviolet detection.
Simultaneous determination of citalopram and its metabolites by high-performance liquid chromatography with column switching and fluorescence detection by direct plasma injection

Matsui et al (1995) 10 have reported high-performance liquid chromatography with a successive column-switching technique for simultaneous determination of citalopram and its four metabolites in plasma. Plasma samples were injected directly, and the target compounds were purified and concentrated with an inexpensive commercial octadecyl guard column. Then, the six-port valve was switched, and the compounds retained in the column were eluted by the back-flush method using 20 mM phosphate buffer (pH 4.6)-acetonitrile (70:30, v/v) containing 0.1% diethylamine and separated with an ODS column. The compounds were assayed with a fluorescence detector at an excitation wavelength of 249 nm and an emission wavelength of 302 nm. The limits of quantitation of this method were 2.0 ng/ml for citalopram, desmethylcitalopram, didesmethylcitalopram, citalopram propionic acid and citalopram N-oxide.


Millan et al (2008) 11 have reported rapid and sensitive HPLC enantioselective method with fluorescence detection to determine (-)-(R) and (+)-(S) enantiomers of the metabolites of citalopram, demethyl- and didemethyl-citalopram in plasma and brain tissue. Linearity was obtained over the concentration range 5-1000 ng/ml and 100-10,000 ng/g for spiked drug-free plasma and brain tissue, respectively, with detection limits lower than 2.1 ng/ml and 42.8 ng/g.

Spectrofluorometric determination of citalopram in pharmaceutical preparations and spiked human plasma using organized media.

El-Sherbiny DT (2006) 05 have reported spectrofluorometric method for determination of citalopram in pharmaceutical preparations and spiked human plasma using organized media. The native fluorescence of citalopram (CIT) was obtained in citrate buffer of pH 6.5 with and without beta-cyclodextrin (beta-CD) or sodium dodecyl sulfate (SDS) as fluorescence enhancers at 305 nm using 242 nm for excitation. Organized media-enhanced spectrofluorometric methods were developed for the determination of CIT, in
pure form as well as in pharmaceutical preparations. The fluorescence intensity-concentration plots were rectilinear over the ranges 0.06 to 0.64, 0.04 to 0.40, and 0.02 to 0.26 microg/mL with lower detection limits of 0.02, 0.01, and 0.007 μg/ml, either in citrate buffer only or in beta-CD and SDS as organized media, respectively.

**Simultaneous determination of fluoxetine, citalopram, paroxetine, venlafaxine in plasma by high performance liquid chromatography-electrospray ionization mass spectrometry**

Juan et al (2005) have reported rapid and sensitive HPLC–MS/ESI method for simultaneous determination and screening of the four most commonly prescribed nontricyclic antidepressants: fluoxetine, citalopram, paroxetine and venlafaxine in human plasma. The analytes in plasma were extracted by solid-phase-extraction column after samples had been alkalnized. The HPLC separation of the analytes was performed on a MACHEREY-NAGEL C18 (250 mm × 4.6 mm, 5 μm, Germany) column, using water (formic acid 0.6%, ammonium acetate: 30 mmol/l)–acetonitrile (35:65, v/v) as mobile phase, with a flow-rate of 0.85 ml/min. The calibration curves were linear in the 5.0–1000.0 ng/ml range for all compounds, all of them with coefficients of determination above 0.9900. The limits of detection (LODs) were 0.5, 0.3, 0.3 and 0.1 ng/ml for fluoxetine, citalopram, paroxetine and venlafaxine, respectively.

**Liquid chromatography-positive ion electrospray mass spectrometry method for the quantification of citalopram in human plasma**

Pistos et al (2004) have reported rapid, sensitive and novel narrow-bore liquid chromatography–mass spectrometric method for the quantification of citalopram in human plasma. The analyte and internal standard (imipramine) were extracted by liquid–liquid extraction with a mixture of hexane–heptane–isopropanol (88:10:2, v/v/v). The use of a Hypersil BDS C8 micro-bore column (250 mm × 2.1 mm i.d.; 3.5 μm particle size), results in substantial reduction in solvent consumption. The mobile phase consisted of 10 mM ammonium formate–formic acid (pH 4.5) and acetonitrile (30:70, v/v), pumped at a flow rate of 0.15 ml min⁻¹. The method had a chromatographic run time of 10.0 min and a linear calibration curve over the range 0.50–250 ng ml⁻¹ (r² > 0.996). The limit of quantitation was 0.50 ng ml⁻¹.
Determination of citalopram in human plasma with LC-MS/MS and its bioequivalent evaluation

Cao et al (2007) have reported a sensitive and selective LC-MS/MS method for determination of citalopram in human plasma to study the bioequivalence of different formulations containing citalopram. The samples were simply pretreated by protein precipitation using acetonitrile, and then analyzed on a Zorbax Extend C8 column. The mobile phase consisted of acetonitrile-water-formic acid (60:40:0.2), at a flow-rate of 0.5 mL x min(-1). A Thermo Finnigan TSQ Quantum Ultra tandem mass spectrometer equipped with electrospray ionization source as detector and operated in the positive ion mode. The linear calibration curves were obtained in the concentration range of 0.10-100 µg/ L. The lower limit of quantification was 0.10 µg/ L.

Stereospecific determination of citalopram and desmethylcitalopram by capillary electrophoresis and liquid-phase microextraction

Andersen et al (2003) have reported chiral capillary electrophoresis (CE) system allowing simultaneous enantiomer determination of citalopram (CIT) and its pharmacologically active metabolite desmethylcitalopram (DCIT). Excellent chiral separation was obtained using 1% sulfated-β-cyclodextrin (S-β-CD) as chiral selector in combination with 12% ACN in 25 mM phosphate pH 2.5. The limit of quantification (S/N=10) was <11.2 ng/ml, intra-day precision was <12.8% RSD, and inter-day precision was <14.5% RSD, for all enantiomers.
Aim of work
After performing detail literature survey, following conclusions were made

1. HPLC method reported by Macek et al (2001) involved back extraction step.

2. HPLC method reported by Oeyehaug et al (1982) involved sample preparation procedure which is not simple.


6. HPLC method reported by Kristoffersen et al (1999) had drawback of poor sensitivity with respect to UV studies.

7. HPLC method reported by Matsui et al (1995) had longer retention times for all the compounds estimated.


All above mentioned reported methods, involved tedious extraction steps, long run time, sophisticated instrumentation, this may not be suitable for routine monitoring of citalopram. Therefore, a simpler assay method which involves simpler instrumentation, simpler extraction procedure and which is rapid is highly desirable.

Therefore present study describes a new simple LC method for the determination of citalopram in plasma and urine using UV detection. The main features of this method include simple mobile phase consisting of methanol and buffer only, making it more cost effective, single step sample pretreatment procedure using liquid-liquid extraction technique, low plasma and urine volumes required for analytical runs, shorter analytical run time, lower limit of quantification with proposed method and simultaneous estimation of citalopram in plasma and urine samples using same developed method.
Experimental
4.1 Apparatus
The liquid chromatographic system consisted of Shimadzu HPLC model (VP series) containing LC-10AT (VP series) pump, variable wavelength programmable UV/VIS detector SPD-10AVP and Rheodyne injector (7725i) with 20 μl fixed loop. Chromatographic analysis was performed on a Phenomenex Gemini C18 column with 250 X 4.6mm i.d. and 5 μm particle size. Shimadzu electronic balance (AX 200) was used for weighing purpose. Vortexing of biological samples was performed on Remi Cyclomixer CM101 and centrifugation was performed on Plastocrafts refrigerated centrifuge machine. All samples were injected onto HPLC system by Hamilton hypodermic syringe needle.

4.2 Materials
Analytical pure sample of citalopram hydrobromide (purity 99.9%) and carbamazepine (purity 99.9%), were procured as gift sample from Torrent Pharmaceutical Ltd, Gujarat. Methanol (HPLC grade, purity 99.9%) was procured from E.Merck (India). HPLC grade water was obtained by double distillation and purification through milli-Q water purification system. Potassium dihydrogen orthophosphate (A.R.grade, purity 99.5%) was procured from Qualigens. Dichloromethane used for extraction was obtained from Merck (Mumbai, India). Drug free (blank) human plasma was obtained from Indian Red Cross Society (Anand Branch, India) and was stored at -35°C prior to use.

4.3 Preparation of Mobile Phase
Potassium dihydrogen orthophosphate was weighed (2.38 g) and dissolved in 350 ml of water. This solution was mixed with 650 ml of methanol. The solution was sonicated for 10 minutes and filtered using Whatman filter paper (No.1).

4.4 Preparation of Standard Stock and Working stock solutions
The standard stock solutions of 1mg/ml for citalopram and carbamazepine (IS) were prepared by dissolving their requisite amount in methanol. These stock solutions were further diluted appropriately to get an intermediate concentration of 10 μg/ml for citalopram and carbamazepine respectively. Working standards of citalopram of different concentrations i.e. 0.1, 0.2, 0.4, 0.8, 1.6, 2.0, 3.0, 4.0 μg/ml required for spiking blood and urine calibration, and quality control samples were subsequently prepared using the standards and intermediate stock solutions. Carbamazepine working solution
of 1.0 μg/ml was prepared using the intermediate stock of 10 μg/ml and was used as internal standard in blood and urine samples preparation. All of the standard stock, intermediate stock, and working stock solutions were prepared and stored at 2 – 8 °C until use. Drug free plasma was withdrawn from plasma freezer and allowed to get completely thawed before use. Five percent spiking with working stock solution of citalopram was done in blank plasma and urine to achieve the desired concentration of citalopram for calibration and quality control (QC) samples. The spiked QC samples were stored at -70°C for stability studies.

4.5 Chromatographic Conditions
A reverse phase C-18 column equilibrated with mobile phase methanol: 0.05 M potassium dihydrogen orthophosphate (65:35, v/v) was used. Mobile phase flow rate was maintained at 1 ml/min and effluents were monitored at 239 nm. The sample was injected using a 20 μl fixed loop, and the total run time was 8 min.

4.6 Calibration curve
Blank plasma and urine sample was screened prior to spiking to ensure it free from endogenous interferences at retention times of citalopram and carbamazepine. An eight point standard curve of citalopram was prepared by spiking the blank plasma and urine sample with appropriate amount of citalopram. The calibration curve ranged from 5.0 – 200.0 ng/ml. For both plasma and urine samples, peak area ratios of the analyte versus internal standard were used for quantitation.

4.7 Pre-validation
A short pre-validation was performed to ensure that all procedures as set up yields expected results. The standard stock solutions of 1mg/ml for citalopram and carbamazepine (IS) were prepared by dissolving their requisite amount in methanol. These stock solutions were further diluted appropriately to get an intermediate concentration of 10 μg/ml for citalopram and carbamazepine respectively. Working standards of citalopram of different concentrations i.e. 0.1, 0.2, 0.4, 0.8, 1.6, 2.0, 3.0, 4.0 μg/ml required for spiking blood calibration, and quality control samples were subsequently prepared using the standards and intermediate stock solutions. Carbamazepine working solution of 1.0 μg/ml was prepared using the intermediate stock of 10 μg/ml and was used as internal standard in blood samples preparation. All
of the standard stock, intermediate stock, and working stock solutions were prepared and stored at 2 – 8 °C until use. Drug free plasma was withdrawn from plasma freezer and allowed to get completely thawed before use. Five percent spiking with working stock solution of citalopram was done in blank plasma and urine to achieve the desired concentration of citalopram (5, 10, 20, 40, 80, 100, 150, 200 ng/ ml) for calibration and quality control (QC) samples (10, 80, 180 ng/ ml). The spiked QC samples were stored at -35°C for stability studies. Extraction of calibration standards in single fold and the quality control was done according to the developed liquid-liquid extraction procedure and chromatographed with the preferred mobile phase.

Pre-validation batches prepared were as follows
1. 6 replicates of each calibration standard over the proposed range
2. 6 replicates of quality control standards.
3. 6 replicates of extracted blank matrix without internal standard.
4. One blank with internal standard added.

4.7.1 Parameter estimation

Standard curves were constructed using different regression models. Back calculated concentrations ($x_{\text{new}}$) for the different regression models were calculated using equation:

$$x_{\text{new}} = \frac{y - a}{b}$$

Where ‘y’ is the citalopram/ carbamazepine peak area ratio response, ‘a’ is y-intercept and ‘b’ is the slope.

All regression models were fitted to data by manual calculation in Windows Excel® 2003 (Microsoft Corporation).
4.8 Validation of assay method

4.8.1 Selectivity
Chromatographic interferences from endogenous plasma and urine components were investigated using six blank plasma and urine samples from different sources.

4.8.2 Sensitivity
Sensitivity was determined in terms of LLOQ (lower limit of quantification) where the response of LLOQ was at least five times greater than the response of interference in blank matrix at the retention time of the analyte.

4.8.3 Linearity
Plasma samples were spiked in replicates of five at concentration ranging from 5.0 – 200 ng/ ml with addition of carbamazepine standard of 50 ng/ ml. The linearity of the method was determined by the analysis of standard plot associated with an eight point standard calibration curve. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of citalopram was calculated from the simple linear equation using regression analysis of spiked plasma and urine calibration standard with the reciprocity of the drug concentration as a weighing factor (1/concentration, i.e. 1/x^2); y = mx + c.

4.8.4 Precision and accuracy
The intra-assay precision and accuracy was calculated for five replicates at each LLOQ, LQC, MQC and HQC levels each on the same analytical run, and inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs.

4.8.5 Recovery
The percentage recovery of citalopram was determined by comparing the mean area of five replicates each of extracted quality control samples; low quality control (LQC = 10 ng/ ml), middle quality control (MQC = 80 ng/ ml) and high quality control (HQC = 180 ng/ ml) samples with mean area of freshly prepared un-extracted LQC, MQC and HQC samples (i.e. proportionate spiking of QC’s and IS in extracted blank plasma and urine reconstituted solution to get the concentration equivalent to the extracted QC concentration.)
4.8.6 Stability
The chemical stability of the analytes in human plasma and urine samples were evaluated under specific conditions for given time intervals.

4.8.6.1 Freeze-thaw stability
For freeze thaw stability testing, the concentrations of the QC i.e. LQC, MQC and HQC in triplicate after three freeze and thaw cycles were determined and compared to the freshly prepared samples.

4.8.6.2 Short term stability
The short term stability of citalopram plasma and urine samples was assessed by spiking citalopram solution into plasma and urine samples at LQC, MQC and HQC at triplicate. These samples were then stored at room temperature for 6 hours and extracted following the sample preparation procedure to get the reconstitution solution. The reconstituted solutions were analyzed on HPLC as described above.

4.8.6.3 Long term stability
The long term stability of citalopram plasma and urine samples was assessed by spiking QC samples into blank plasma and urine at triplicate and then storing plasma and urine samples for 10 days at – 35°C. Then after 10 days samples were extracted following the sample preparation procedure to get the reconstitution solution. The reconstitution solutions were analyzed on HPLC as described above.

4.8.6.4 Post preparative stability
Post preparative stability was assessed by spiking citalopram QC samples into blank plasma and urine in triplicate. The samples were then extracted to get the reconstitution solutions. These solutions were maintained for 0, 24 and 48 hours. After, they were analyzed on HPLC as described above.

4.8.6.5 Stock solution stability
For the evaluation of stock solution stability, three solutions of citalopram and carbamazepine were prepared in methanol : water (70:30, v/v) at triplicate and they were analyzed after remaining 24 hours and 48 hours at room temperature.
Result & Discussion
5.1 Spectroscopic measurement
In order to determine the wavelength at which the HPLC detector system must operate, UV spectra of 10 µg/ml of citalopram hydrobromide and 10 µg/ml of carbamazepine in methanol solution were measured using UV-Vis spectrophotometer (Shimadzu), over a wavelength range of 196 – 380 nm. The absorbance maximum for citalopram was found to lie at 239 nm. Then experiments were undertaken to see whether UV spectra of citalopram and carbamazepine are pH dependant or not. This was performed by taking 10 µg/ml citalopram in water solution, adding 200 µl of 10 M NaOH or 200 µl of concentrated HCl and recording the spectra. We found that there was no significant change in the spectra of both the drugs.

5.2 Optimization of mobile phase at the initial stage
Optimization of mobile phase was performed based on resolution, asymmetric factor and peak area obtained. Satisfactory separation, well resolved and good symmetrical peaks were obtained with the mobile phase methanol: 0.05 M potassium dihydrogen orthophosphate (70: 30 v/v; pH 6.2) as developed earlier while analyzing drug and internal standard in dosage forms. Resolution between citalopram hydrobromide and carbamazepine was found to be 4.76, which indicates good separation of both the compounds. The retention time of citalopram hydrobromide was found to be 3.8 min and that of carbamazepine was found to be 5.4 min respectively. The asymmetric factor for citalopram hydrobromide was 1.22. So it was decided to proceed with the mobile phase so selected for further chromatographic studies.
Blank samples of plasma and urine were tested to ensure that peaks of analyte and internal standard can be obtained free from background interferences and with good resolution between them. But analyzing blank plasma and urine samples, (Figure 1 and 2) it came to notice that endogenous interferences from blank plasma and urine samples showed peak at retention time of 3.3 which was the retention time of citalopram with mobile phase consisting of methanol: 0.05M potassium dihydrogen orthophosphate (70:30, v/v). Hence it was decided to further modify mobile phase so that retention time of drug and internal standard shifts to higher value maintaining the satisfactory separation, well resolved and good asymmetrical peaks. Again combinations of acetonitrile, methanol and buffer were tried like 40:60 (acetonitrile: }
Results and discussion

0.025 M potassium hydrogen orthophosphate), 50:50 (Methanol: 0.025 M potassium hydrogen orthophosphate), 60:40 (Methanol: 0.025 M potassium hydrogen orthophosphate), 65:35 (Methanol: 0.025 M potassium hydrogen orthophosphate) etc. Finally it was concluded that 65:35 (Methanol: 0.05 M potassium hydrogen orthophosphate) would be a good mobile phase which gives satisfactory separation with Rt of 4.3 with citalopram and Rt of 6.3 with carbamazepine (Figure 3 and 4)

5.3 Optimization of the method for extraction of drug from plasma.

Since the objective of this study was to develop a simple reliable method that would facilitate analysis of citalopram in biological matrices 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. Extraction of drug from biological matrix mainly involves three procedures i.e. Liquid-liquid extraction, Solid phase extraction and Protein precipitation. Of all the three procedures, liquid-liquid extraction is simple, provided it gives best results. Hence it was decided to proceed with liquid-liquid extraction step as first approximation. To do this, a pool of blank human plasma was obtained and spiked with relevant concentrations of citalopram before extraction.

It is known that citalopram is freely soluble in chloroform, and hence it was decided to use solvents mainly chloroform, dichloromethane, and to use to some extents diethyl ether, hexane, ethyl acetate, iso-amyl alcohol etc as extracting solvent.

Many different extracting procedures were tried for extraction of drugs (citalopram and carbamazepine) and the following procedure was concluded satisfactory at the end of the optimization step..

1. 475 μl of blank plasma was taken in 15 ml of stoppered centrifuge tube, spiked with 200 ng/ml of citalopram and 50ng/ml of carbamazepine as internal standard.

2. 50 μl of 0.01M NaOH solution was added and 4 ml of different solvents (dichloromethane, chloroform, diethyl ether, hexane, and binary mixture of these solvents in different proportions) were added and tube was vortexed for 2 min and further centrifuged in refrigerated centrifuge (Plastocrafts) for 10 min at 3000 rpm (12ºC).
3. After centrifugation for 10 mins, 3 ml of said solvents were removed and taken in another tube meant for evaporation.

4. Above solvents were evaporated at ambient temperature under nitrogen and residue so obtained was reconstituted in 100 µl of mobile phase.

5. 20 µl of the reconstituted extract was injected onto the HPLC column.

Optimization steps using above procedure is as follows

5.3.1 Choice of an extracting solvent: Using above procedure different solvents were tried as extracting solvent and gave following results

1. As with diethyl ether and mixture of hexane and isoamyl alcohol many procedures are reported in the literature and hence it was decided to try with some other solvent for better results.

2. As reported in the literature, citalopram is freely soluble in chloroform and hence it was decided to use chloroform, dichloromethane and carbon tetrachloride as extracting solvent. Again use of chloroform and carbon tetrachloride is limited due to its carcinogenic properties and hence it was decided to try for dichloromethane or combination of dichloromethane, diethyl ether and hexane in different proportions as extracting solvent.

3. The thing to be taken into consideration while selecting extracting solvent is recovery of drug and internal standard which should be consistent. Based on trial and error procedure following combinations of extracting solvent were tried taking concentration of citalopram as 200 ng/ml and that of carbamazepine as 50 ng/ml (n = 6)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Solvents taken</th>
<th>% recovery of citalopram obtained ± S.D</th>
<th>% recovery of carbamazepine obtained ± S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ethyl acetate*</td>
<td>40% ± 7.42</td>
<td>70% ± 4.52</td>
</tr>
<tr>
<td>2.</td>
<td>Diethyl ether + Dichloromethane (8:2)</td>
<td>60% ± 6.65</td>
<td>70% ± 3.33</td>
</tr>
<tr>
<td>3.</td>
<td>Diethyl ether + hexane (70:30)</td>
<td>70% ± 3.35</td>
<td>80% ± 2.56</td>
</tr>
<tr>
<td>4.</td>
<td>Dichloromethane</td>
<td>88% ± 1.85</td>
<td>92% ± 1.21</td>
</tr>
</tbody>
</table>
Results and discussion

Using ethyl acetate as extracting solvent, difficulty was faced as both aqueous and organic solvents were difficult to be isolated and extracts obtained were not clear. Taking dichloromethane as an extracting solvent, recovery of citalopram and carbamazepine obtained was highest among other solvent and combination of the solvents used and more important the recovery was consistent.

5.3.2 Choice of volume of 0.01M NaOH solution:

As the stability of citalopram in 25 ml solution of 0.01 M NaOH was determined experimentally and as it was found to be stable, double the amount of base i.e. 50 μl of 0.01M NaOH solution was decided to be added during the sample preparation step.

So finally extraction procedure was set to the following:

1. 475 μl of blank plasma was taken in 15 ml of stoppered centrifuge tube, spiked with 200 ng/ml of citalopram and 50ng/ml of carbamazepine as internal standard.
2. 50 μl of 0.01M NaOH solution was added and 4 ml of dichloromethane was added and tube was vortexed for 5 min and further centrifuged in refrigerated centrifuge for 10 min at 3000 rpm (12°C).
3. After centrifugation for 10 min, 3 ml of dichloromethane were removed and taken in another tube meant for evaporation.
4. It was evaporated at ambient temperature under nitrogen and residue so obtained was reconstituted in 100 μl of mobile phase.
5. 20 μl of the reconstituted extract was injected onto the HPLC column.

So finally method development phase which consists sample preparation phase was set to following mentioned procedure:

Procedure for Plasma samples – Blank plasma was taken out from – 35°C and kept at room temperature for thawing. Using micropipette, 475 μl of plasma was transferred into 15 ml capacity centrifuge tube and spiked with appropriate working standard solutions of citalopram and carbamazepine. The mixture was made alkaline with 50 μl of 0.01M NaOH and 4 ml of dichloromethane was added. The resulting mixture was then vortex mixed for 5 min. The tubes were centrifuged at 3000 rpm for 10 min and the organic layer was then transferred to fresh tubes and evaporated to dryness on the
water bath at temperature 40°C. The dried residue was reconstituted with 100 μl of mobile phase and 20 μl of it was injected onto the HPLC system.

**Procedure for Urine samples** - Using micropipette, 475 μl of urine was transferred into 15 ml capacity centrifuge tube and spiked with appropriate working standard solutions of citalopram and carbamazepine. The mixture was made alkaline with 50 μl of 0.01M NaOH and 4 ml of dichloromethane was added. The resulting mixture was then vortex mixed for 5 min. The tubes were centrifuged at 3000 rpm for 10 min and the organic layer was then transferred to fresh tubes and evaporated to dryness on the water bath at temperature 40°C. The dried residue was reconstituted with 100 μl of mobile phase and 20 μl of it was injected onto the HPLC system.

5.4 Prevalidation

Homoscedasticity was visually examined by plotting percentage of relative error (relative residual) against concentration. The graph depicted scattering of data at low level in OLR and was corrected by $1/x^2$ weighted regression model respectively.

5.4.1 Traditional approach and other regression models

The six replicates of calibrators were fitted to the regression models individually and as a mean of the replicates. The back calculated concentrations were calculated using various models. It is obvious from the results that the higher value of variance was found to be reduced by application of weights.

5.5 HPLC-UV assay method validation

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

- Blank plasma screening.
- Planning of Calibration and Quality control standards.
- Preparation of Calibration and Quality control standards
- Preparation of internal standard solution and mobile phase
- Setting up the validation batch
- Assaying intra-day, inter-day-I, inter-day II and stability studies samples.
5.5.1 Blank Plasma screening:
Blank normal human plasma was obtained from Indian Red Cross Society. To ensure that the plasma obtained does not contain any exogenous components that could interfere with the analytes of interest it was again analysed by the developed assay method before the preparation of calibration standards and quality control standards. The results revealed that it was free of interferences at the peaks of analyte and internal standard. This plasma was stored at -35°C until further use.

5.5.2 Planning of Calibration and Quality control standards
In for pharmacokinetic studies, usually the aim of an assay method is to be able to assay plasma samples of drug over a period of about 4 to 5 half-lives after dosing. As searched in the literature, maximum C_{max} achieved after administration of 40 mg dose of citalopram, is 83 ng/ ml. This means that if a C_{max} of about 83 ng/ ml is expected, the assay method should have an LLOQ in the region of about 5 ng/ ml. During method development, it was found that the concentration of 5 ng/ ml could be easily achieved accurately and precisely. So it was decided to set LLOQ at 5 ng/ ml. Since it is standard practice to validate the assay method for pharmacokinetic studies upto 2 × C_{max}, it was therefore decided to prepare the calibration standards ranging from 5 ng/ ml (LLOQ) to 200 ng/ ml (ULOQ).

Now for the quality control samples, guidelines say that LQC should be approx 3 × LOQ, MQC should be somewhat in the middle of the range, and HQC should be 90% of ULOQ. So following the guidelines, LQC was set to 10 ng/ ml, MQC to 80 ng/ ml and HQC to 180 ng/ ml.

5.5.3 Compiling the validation batches
Validation batches 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 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.

The method was validated to meet the acceptance criteria of the FDA Guidance for industry, Bioanalytical Method Validation.

5.5.4 Linearity
The method developed was linear over the whole range of concentration from 5.0 - 200.0 ng/ml for both plasma and urine samples. Using a linear regression equation weighted $1/(\text{concentration})^2$, all the calibration lines passed all the acceptance criteria. The calibration curves were plotted between response factor and concentration of the standard solutions. The linearity range using above developed method was found to be in the range of 5.0 - 200.0 ng/ml with regression equation i.e. $y = (0.0126 \pm 0.048) x + (0.0586 \pm 0.0209) (r = 0.999 \pm 0.0038)$ for plasma samples and $y = (0.0125 \pm 0.052) x + (0.0391 \pm 0.0105) (r = 0.998 \pm 0.0028)$ for urine samples where 'x' is the concentration of citalopram and 'y' is citalopram to carbamazepine peak area ratio. The result indicated no significant interday variability of slopes and intercepts over the optimized concentration range. The observed mean back calculated concentrations with accuracy (%) and precision (%CV) of five lineairities in plasma and urine are given in Table 1 and 2.

5.5.5 Accuracy and Precision studies
The values of the QC's were calculated using regression equations. In this way the most suitable regression can be chosen to obtain the best results with 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 (citalopram/ carbamazepine peak area ratio) vs $1/(\text{concentration of citalopram})^2$ yielded excellent results. Summaries of the statistics for calculated concentrations of intra and interday validation quality control standards based on peak area ratio in plasma and urine are presented in Table 3 and 4.

5.5.6 Selectivity and Sensitivity
The above rapid method of extraction gave very good selectivity and sensitivity for the analysis of citalopram and IS in blank plasma and urine. This method gave clean chromatograms free from background interference. The area observed at the elution
time of citalopram was 7% or less relative to the LLOQ (5 ng/ml) in six blank samples tested, whereas no area was observed at the elution time of IS. The % CV calculated for the citalopram area of LLOQ and area of IS were found to be 5.12 and 2.33, respectively.

5.5.7 Recovery
Using above mentioned extraction method overall mean recovery for citalopram at LQC, MQC and HQC was 88.5, 88.2 and 88.0% respectively for plasma samples and 92.3, 92.6, 93.6% for urine samples respectively having less variability within their replicates. The recovery of IS was found to be 92.3% with CV of 2.98%. Thus, the consistency in quantitative recoveries of citalopram and IS authenticates the extraction procedure for its application to routine sample analysis.

5.5.8 Stability
For the stability studies, the mean concentration of the stability samples was compared to the theoretical concentrations. The results obtained for the stability studies were well within the acceptable limit. Citalopram was found to be stable in plasma and urine for three freeze and thaw cycles with % RSD of less than 4%. Citalopram in control human plasma and urine was stable for 6 hr at least with % RSD of 1.2% in plasma and 1.5% in urine respectively. Citalopram plasma and urine samples for long term stability studies were found to be stable with % RSD < 3.77% for both the cases. For post preparative studies, citalopram was found to be stable in plasma and urine samples with % RSD of 1.2% and 1.5% respectively. Stock solutions of citalopram and IS were stable at room temperature for 24 hr and for 48 hr and % RSD was found to be within ± 5% for both.
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Fig 1: Blank spectra of plasma obtained in the mobile phase consisting of Methanol: 0.05 M buffer (65:35, v/v).

Fig 2: Blank spectra of urine obtained in the mobile phase consisting of Methanol: 0.05 M buffer (65:35, v/v).
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Fig 3: Spectra of citalopram (200 µg/ml) and carbamazepine (50 µg/ml) in mobile phase consisting of 65:35 (methanol: 0.05M potassium hydrogen orthophosphate, v/v) in plasma.

Fig 4: Spectra of citalopram (200 µg/ml) and carbamazepine (50 µg/ml) in mobile phase consisting of 65:35 (Methanol: 0.05M potassium hydrogen orthophosphate, v/v) in urine.
### TABLE 1 BACK CALCULATED CONCENTRATION OF CALIBRATION STANDARDS (CS) FROM EACH CALIBRATION CURVE OF CITALOPRAM IN PLASMA

<table>
<thead>
<tr>
<th>Linearity</th>
<th>Concentration found (ng/ml)</th>
<th>5a</th>
<th>10a</th>
<th>20a</th>
<th>40a</th>
<th>80a</th>
<th>100a</th>
<th>150a</th>
<th>200a</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>4.82</td>
<td>9.81</td>
<td>20.03</td>
<td>38.47</td>
<td>76.52</td>
<td>92.92</td>
<td>142.63</td>
<td>211.22</td>
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<tr>
<td>2</td>
<td></td>
<td>5.10</td>
<td>10.23</td>
<td>19.79</td>
<td>41.10</td>
<td>77.47</td>
<td>95.09</td>
<td>147.52</td>
<td>207.32</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>4.91</td>
<td>10.84</td>
<td>21.02</td>
<td>39.84</td>
<td>78.77</td>
<td>92.36</td>
<td>149.66</td>
<td>204.05</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>4.69</td>
<td>10.56</td>
<td>20.59</td>
<td>42.10</td>
<td>76.59</td>
<td>97.39</td>
<td>143.20</td>
<td>201.96</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>4.86</td>
<td>10.01</td>
<td>20.54</td>
<td>39.20</td>
<td>71.38</td>
<td>96.81</td>
<td>149.33</td>
<td>212.47</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>4.87</td>
<td>10.29</td>
<td>20.39</td>
<td>39.98</td>
<td>76.14</td>
<td>94.91</td>
<td>146.26</td>
<td>207.40</td>
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<td>Accuracy</td>
<td></td>
<td>97.4</td>
<td>102.9</td>
<td>101.9</td>
<td>99.9</td>
<td>95.17</td>
<td>94.9</td>
<td>97.50</td>
<td>103.72</td>
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<tr>
<td>S.D</td>
<td></td>
<td>0.149</td>
<td>0.414</td>
<td>0.487</td>
<td>1.214</td>
<td>2.814</td>
<td>2.250</td>
<td>3.16</td>
<td>4.50</td>
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<tr>
<td>CV (%)</td>
<td></td>
<td>3.05</td>
<td>4.03</td>
<td>2.38</td>
<td>3.30</td>
<td>3.69</td>
<td>2.37</td>
<td>2.16</td>
<td>2.16</td>
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</table>

* Added concentration (ng/ml)
# Results and discussion

## TABLE 2 BACK CALCULATED CONCENTRATION OF CALIBRATION STANDARDS (CS) FROM EACH CALIBRATION CURVE OF CITALOPRAM IN URINE

<table>
<thead>
<tr>
<th>Linearity</th>
<th>Concentration found (ng/ ml)</th>
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<tbody>
<tr>
<td></td>
<td>CS-1</td>
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<tr>
<td>5\textsuperscript{a}</td>
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</tr>
<tr>
<td>10\textsuperscript{a}</td>
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<tr>
<td>100\textsuperscript{a}</td>
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<tr>
<td>150\textsuperscript{a}</td>
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<tr>
<td>200\textsuperscript{a}</td>
<td>1</td>
</tr>
<tr>
<td>Mean</td>
<td>4.87</td>
</tr>
<tr>
<td>Accuracy</td>
<td>95.60</td>
</tr>
<tr>
<td>S.D</td>
<td>0.146</td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.99</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Added concentration (ng/ ml)
### TABLE 3 INTRA-ASSAY AND INTER-ASSAY PRECISION AND ACCURACY OF CITALOPRAM IN PLASMA

<table>
<thead>
<tr>
<th>Quality control samples</th>
<th>Conc. added (ng/ml)</th>
<th>Intra-assay</th>
<th></th>
<th></th>
<th></th>
<th>Inter-assay</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean conc (ng/ml)</td>
<td>RE (%)</td>
<td>S.D</td>
<td>CV (%)</td>
<td>Mean conc (ng/ml)</td>
<td>RE (%)</td>
<td>S.D</td>
<td>CV (%)</td>
<td></td>
</tr>
<tr>
<td>LLOQ</td>
<td>5 4.75 -5.0</td>
<td>0.147</td>
<td>3.05</td>
<td>4.66</td>
<td>-6.8</td>
<td>0.14</td>
<td>3.11</td>
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</tr>
<tr>
<td>LQC</td>
<td>10 10.72 7.2</td>
<td>0.256</td>
<td>2.61</td>
<td>9.98</td>
<td>-0.2</td>
<td>0.24</td>
<td>2.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MQC</td>
<td>80 79.86 -0.17</td>
<td>1.12</td>
<td>1.40</td>
<td>80.12</td>
<td>0.15</td>
<td>1.09</td>
<td>1.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HQC</td>
<td>180 179.56 -0.24</td>
<td>7.10</td>
<td>3.64</td>
<td>181.23</td>
<td>0.68</td>
<td>6.99</td>
<td>3.85</td>
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<td></td>
</tr>
</tbody>
</table>

S.D., standard deviation, RE., relative error, CV., coefficient of variation

a Mean of five replicate observations at each concentration

b Mean of 15 observations recorded over three different analytical runs (5 replicates/run)
Results and discussion

TABLE 4 INTRA-ASSAY AND INTER-ASSAY PRECISION AND ACCURACY OF CITALOPRAM IN URINE

<table>
<thead>
<tr>
<th>Quality control samples</th>
<th>Conc. added (ng/ml)</th>
<th>Intra-assay</th>
<th></th>
<th></th>
<th></th>
<th>Inter-assay</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean conc (ng/ml)</td>
<td>RE (%)</td>
<td>S.D (%)</td>
<td>CV (%)</td>
<td>Mean conc (ng/ml)</td>
<td>RE (%)</td>
<td>S.D (%)</td>
<td>CV (%)</td>
<td></td>
</tr>
<tr>
<td>LLOQ</td>
<td>5</td>
<td>5.10</td>
<td>2.0</td>
<td>0.156</td>
<td>2.85</td>
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<td>0.135</td>
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<td>LQC</td>
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<td>10.25</td>
<td>2.5</td>
<td>0.278</td>
<td>3.02</td>
<td>9.98</td>
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<td>MQC</td>
<td>80</td>
<td>78.86</td>
<td>-1.42</td>
<td>1.52</td>
<td>2.52</td>
<td>81.12</td>
<td>1.4</td>
<td>1.09</td>
<td>2.50</td>
</tr>
<tr>
<td>HQC</td>
<td>180</td>
<td>181.52</td>
<td>0.84</td>
<td>7.45</td>
<td>3.78</td>
<td>179.25</td>
<td>-0.41</td>
<td>8.25</td>
<td>3.22</td>
</tr>
</tbody>
</table>

S.D., standard deviation, RE., relative error, CV., coefficient of variation

a Mean of five replicate observations at each concentration

b Mean of 15 observations recorded over three different analytical runs (5 replicates/run)