T.C.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

ASSESSMENT OF THE RELATIONSHIP BETWEEN DRUG LEVEL AND CYTOCHROME P450 ENZYME ACTIVITIES IN PATIENTS DEMANDING GENETIC POLYMORPHISM AND ESCITALOPRAM MONITORIZATION

DOCTOR OF PHARMACEUTICAL TOXICOLOGY THESIS

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..21..09...2018 and numbered ..2018/16-01

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

10.09.2018

Signature

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LIST OF SYMBOLS AND ABBREVIATIONS

Bioavailability	B
Central Nervous System	CNS
Clearance	Cl
Cytochrome P450	CYP
Demethyl escitalopram	S-DCT
Didemethyl escitalopram	S-DDCT
Dimethyl Sulfoxide	DMSO
Dose interval	T
Drug concentration	c
Elimination half-time	t1/2
Escitalopram	S-CT
Extensive metabolizers	EM
Gas chromatography	GC,
High performance liquid chromatography	HPLC
Intermediate metabolizers	IM
Intramuscular	i.m
Intravenous	i.v
Liquid chromatography mass spectrometry	LC-MS / MS
Long	L
Lower Limit of Quantification	LOQ
Maximum plasma concentration	Cmax
Metabolic ratio	MR
Polymerase Chain Reaction	PCR
Poor metabolizers	PM
Quality control samples	QCs
Real-Time Polymerase Chain Reaction	Q-PCR
Serotonin (5-hydroxytryptamine)	5-HT
Serotonin transporter protein	5-HTT

Short	S
Single nucleotid polymorphism	SNP
SLC6A4	Solute Carrier Family 6 Member 4
Steady state concentration	Css
Therapeutic Drug Monitorization	TDM
Total clearance	tot Cl
Ultrarapid metabolizer	UM
Variable Number of Tandem Repeats	VNTR
Volume of distribution	Vd

ABSTRACT

Canbolat, F. (2018). Assessment of the relationship between drug level and cytochrome P450 enzyme activities in patients demanding genetic polymorphism and escitalopram monitorization. Yeditepe University, Institute of Health Science, Department of Pharmaceutical Toxicology, PhD Thesis, İstanbul.

Therapeutic Drug Monitorization (TDM) is used to determine the concentration of drug in plasma to adjust the dose of therapeutic drug. Selective and sensitive analytical methods are used to determine drug and metabolite levels for the successful application of TDM. The aim of the study was to develop and validate a new method using liquid chromatography mass spectrometry (LC-MS / MS) to analyze quantitative assay of escitalopram (S-CT) and its metabolites in human plasma and urine samples. Also, association between metabolic ratio (MR) of S-CT and its metabolites and cytochrome P450 enzyme activities (CYP2C19 and CYP2D6) of patients were assessed using this validated method in order to determine individual pharmacokinetic characteristics. It is thought that besides knowing the pharmacokinetic characteristics of patients, pharmacodynamics characteristics of individuals have an essential role in increasing the success of the treatment. Therefore, serotonin transporter protein (5-HTT) polymorphism distribution of the same patients was examined as well. With the study, it is aimed that it could help the physician provide a convenient and safe treatment dose for the patients.

In this study, plasma and urine samples collected from patients using escitalopram as part of their treatment in NPİstanbul Brain Hospital were analyzed by LC-MS / MS at Üsküdar University Clinical Pharmacogenetic Laboratory and the concentration of S-CT, dimethyl escitalopram (S-DCT) and didemethyl escitalopram (S-DDCT) were measured. In addition, a genotyping study was performed from whole blood samples taken to identify CYP2C19, CYP2D6 and pharmacodynamic (5-HTT) polymorphisms of the same patients and statistical evaluation was performed by looking at the relationship between blood level results and MR of patients with their genotyping results.

The mean S-DCT and S-DDCT concentrations of the 30 patients with mean plasma escitalopram concentration of 27.59 ng/mL were 85.52 ng/mL and 44.30 ng/mL,

respectively. The mean S-CT / S-DCT value of the S-CT, metabolized to the S-DCT by the CYP2C19 enzyme as the major pathway, was calculated to be 0.40. In this study, it is found that the mean MR of patients in the CYP2C19 EM and IM groups is different compared to the UM group. Therefore, it is thought that by considering the S-CT / S-DCT value for many patients without genotyping analysis, a pre-assessment can be made regarding the CYP2C19 enzyme activity. Also, it is found that the mean MR of patients in the CYP2D6 EM and CYP2D6 Het. EM group is different compared to the CYP2D6 IM group. When the response of 30 patients to drug treatment with 5-HTT polymorphism distribution of patients was examined, it was observed that the difference between the groups was statistically significant ($p < 0.05$). While a statistical significance ($p < 0.05$) was detected between LL (long:long) and SS (short:short) groups in reponse to treatment, no statistical significance in the response to treatment was found between the LS (long:short) group and the other groups ($p > 0.05$). Evaluation of the data obtained from our study by physicians is thought to be able to contribute treatment of patients in the application of individual drug treatment.

Key Words: Escitalopram; Therapeutic Drug Monitoring; LC-MS/MS, Polymorphism

The present work was supported by the Scientific Research Fund of Üsküdar University.

ABSTRACT (TURKISH)

Canbolat,F. (2018). Genetik polimorfizmi ve essitalopram monitorizasyonu talep edilen hastalarda ilaç düzeyi ile sitokrom P450 enzim aktiviteleri arasındaki ilişkinin değerlendirilmesi. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji ABD, Doktora Tezi, İstanbul.

Terapötik ilaç dozunun ayarlanması için kullanılan terapötik ilaç monitörizasyonu (TDM), plazmadaki ilacın konsantrasyonunu belirlemede kullanılır. TDM'nin başarılı bir şekilde uygulanması için ilaç ve metabolit seviyelerini belirlemede seçici ve duyarlı analitik yöntemler kullanılır.

Çalışmanın amacı, insan plazma ve idrar örneklerinde essitalopram (S-CT) ve metabolitlerinin kantitatif analizini yapmak için sıvı kromatografi kütle spektrometresi (LC-MS / MS) kullanarak yeni bir yöntem geliştirmek ve doğrulamaktır. Ayrıca, bireysel farmakokinetik özelliklerin belirlenmesi amacıyla, essitalopram ve metabolitlerinin metabolik oranı (MR) ile hastaların sitokrom P450 enzim aktiviteleri (CYP2C19 ve CYP2D6) arasındaki ilişki bu doğrulanmış yöntem kullanılarak değerlendirildi. Hastaların farmakokinetik özelliklerini bilmenin yanı sıra, bireylerin farmakodinamik özelliklerini bilmenin de tedavinin başarısını artırmada önemli bir rolü olduğu düşünülmektedir. Bu nedenle, aynı hastaların seratonin taşıyıcı protein (5-HTT) polimorfizim dağılımı da incelenmiştir. Bu çalışma ile hekimin hastalara uygun ve güvenli bir tedavi dozu sağlamasına yardımcı olması amaçlanmıştır.

Bu çalışmada, NPİstanbul Beyin Hastanesi'nde essitalopram kullanan hastalardan alınan plazma ve idrar örnekleri Üsküdar Üniversitesi Klinik Farmakogenetik Laboratuvarı'nda sıvı kromatografi kütle spektrometresi (LC-MS / MS) cihazı kullanılarak analiz edildi ve S-CT, dimetil essitalopram (S-DCT) ve didemetil essitalopram (S-DDCT) konsantrasyonları ölçüldü. Ek olarak, aynı hastaların CYP2C19, CYP2D6 ve farmakodinamik (5-HTT) polimorfizmlerini tanımlamak için alınan tüm kan örneklerinden bir genotipleme çalışması yapıldı. İstatistiksel değerlendirme, genotipleme sonuçları ile hastaların kan seviyesi sonuçları ve metabolik oranları arasındaki ilişkiye bakılarak yapıldı. Ortalama plazma S-CT konsantrasyonu

27.59 ng / mL olan 30 hastanın ortalama S-DCT ve S-DDCT konsantrasyonları sırasıyla 85.52 ng / mL ve 44.30 ng / mL idi.

Major yol olarak CYP2C19 enzimi tarafından S-DCT'ye metabolize olan essitalopramın ortalama S-CT / S-DCT değeri 0.40 olarak hesaplandı. Bu çalışmada, CYP2C19 EM ve CYP2C19 IM gruplarındaki hastaların ortalama metabolik oranı, UM grubuna kıyasla farklı olduğu bulunmuştur. Bu nedenle, genotipleme analizi yapılmayan bir çok hasta için S-CT / S-DCT değeri dikkate alınarak, CYP2C19 enzim aktivitesi ile ilgili bir ön değerlendirme yapılabileceği düşünülmektedir. Ayrıca, CYP2D6 EM ve CYP2D6 Het EM hastalarında ortalama metabolik oranın da CYP2D6 IM grubuna göre farklı olduğu bulunmuştur. Hastaların 5-HTT polimorfizim dağılımı ile ilaç tedavisine verdikleri yanıt incelendiğinde, gruplar arasındaki farkın istatistiksel olarak anlamlı olduğu gözlendi ($p <0.05$). Tedaviye yanıtta LL (uzun:uzun) ve SS (kısa:kısa) grupları arasında istatistiksel anlamlılık ($p <0.05$) saptanırken, LS (uzun:kısa) grubu ile diğer gruplar arasında tedaviye yanıtta istatistiksel anlamlılık bulunamadı ($p> 0.05$). Çalışmamızdan elde edilen verilerin hekimler tarafından değerlendirilmesinin, bireysel ilaç tedavisinin uygulanmasında hastaların tedavisine katkıda bulunabileceği düşünülmektedir.

Anahtar Kelimeler: Essitalopram, terapötik ilaç monitörizasyonu, LC-MS/MS, Polimorfizm

Bu çalışma, Üsküdar Üniversitesi Bilimsel Araştırma Projeleri Birimi tarafından desteklenmiştir.

1. INTRODUCTION AND PURPOSE

A drug is a chemical that has an impact on biological function. Drug effects, depending on the dose taken, can be either harmless or harmful or both (1). To obtain steady state concentration (C_{ss}) within a given therapeutic drug range, drugs are applied in a serious of repeated dose. Patient's indivudual differences may influence the degree of drug absorption, drug biotansformation and drug excretion, which affects Css. Therefore, knowledge of pharmacokinetic variability of patients, especially biotransformation, is important for drug therapy. Liver is the primary organ where drugs are metabolised, yet, less likely, in extrahepatic system including the intestinal tissue, lung or the brain tissue. Various activities of drug-metabolizing enzymes result in different plasma concentrations of drugs due to interpersonal varieties and even intra-individual differences. The majority of drugs undergo phase-1 metabolism, which is by and large metabolized by cytochrome P450 (CYP) enzymes.

Particularly CYP isoenzymes, included drug-metabolysing enzymes, show genetic diversity. These diversities are known as genetic polymorphism and they are in general otosamal ressessive, which are genetic diversities seen more frequently than % 1 of the population (2). Polimorphism could be divided in two subcatagories as ultra rapid (UM) and poor metabolisers (PM). Genetic polymorphisms of drug-metabolizing enzymes may have clinical significance due to the fact that unforseen advers and toxic effect may appear in PM owing to the fact that drug concentration in plasma rose even more. Nonresponsiveness may exist in UM because drug concentration can not reach therapeutic concentration. Thus, the popularity of CYP genotyping methods are on the rise each and every day and for the use in clinical practises, guidelines have been released. Additionally, for an impressive number of drugs, the quantification of drug concentration in plasma through Therapeutic Drug Monitorization (TDM) has become clinically significant for dose adjustment (3). The objective of TDM is to optimize the pharmacotherapy of each patient and it is one of the main methods which could be used for personalized medicine in the treatment. There are more benefits of TDM in pharmacology. As mentioned in the study of Schütze and Schwarz (4) in 2016, as well as the phenotype method which is carried out by taking probe drug, TDM could be used as a phenotyping method concerning the the drug followed in analysis and is crosschecked with the the dosage taken daily. The essential issue in TDM in pharmacology is to measure the amount of drug and metabolite in plasma. As also

stated in the same study, metabolic ratio (MR) could be calculated by TDM results (3,4). If a drug is exposed to biotransformation by large through a responsible enzyme, the MR is the indication of the enzyme activity. Diverging distinctly from the required range, an MR is an indication of a polymorphism or an interaction eg pharmacokinetic (4).

The AGNP Consensus Guidelines published in 2011 included drug blood levels and phenotyping studies for psychotropic drugs (3). In this publication, it is stated that interpretation of drug and metabolite blood levels of some drugs by TDM analysis will facilitate the determination of the phenotype characteristics of the person without addressing to the phenotyping method. To illustrate, there is a proof of this case for the ratio of venlafaxine to O-desmethyl venlafaxine, and risperidone to paliperidone to concerning the CYP2D6 activity. Instead of a probe drug dextromethorphan uptake orally in order to determine CYP2D6 enzyme activity among the patients using risperidon and venlefaxine, only by evaluating these drugs based on drug blood levels provides a more easily applicable treatment than probe drug dextromethorphan. This is because the oral uptake of a second drug, a second blood intake or a long period of urine accumulation in phenotypic studies initiated by the use of probe drug are among the limitations of phenotype analysis. Besides, among psychotropic drugs, drugs that use the CYP2C19 enzyme pathway are also widely used besides those metabolized via the CYP2D6 enzyme pathway. Omeprazole is the most commonly used probe drug for CYP2C19 phenotype evaluation (5). Phenotype studies are performed by measuring the omeprazole / hydroxy omeprazole ratio in plasma three hours after administration of omeprazole 20 mg orally (6,7). However, regarding CYP2C19 enzyme, MR is yet to be researched and not yet standardized in routine TDM (3,4). To illustrate, escitalopram (S-CT) which is predominantly metabolised by CYP2C19 enzyme pathway is commonly prescribed in psycopharmacotherapy. According to the study of Zohar (8) in 2008 and other studies, S-CT is one of the selective serotonin reuptake inhibitors (SSRIs). Upon oral intake, it is absorbed, reaching maximum plasma concentration (C_{max}) within nearly 3-4 hours after single or multiple-dose administration (9,10). The elimination half-time ($t_{1/2}$) of S-CT takes around / nearly 27–33 hours. C_{ss} is reached between 7–10 days. The ratio of S-CT's binding to plasma protein is low (56%). It has a wide range of distribution in tissues (9,10). CYP2C19, CYP2D6 and CYP3A4

metabolizes S-CT (11–13). CYP2C19 seems to be instrumental in converting S-CT to demethyl escitalopram (S-DCT), whereas it is known that CYP2D6 has a role in converting S-DCT to didemethyl escitalopram (S-DDCT) (14). Additionally, clinical studies display no proof of influence of CYP3A4 strong inhibitors on S-CT concentration in plasma when taken together. This shows that CYP3A4 has a slightly partial role in escitalopram metabolization (15). As far as the efffects of S-CT in the body is concerned, it is seen in studies that the effect of S-CT begins when reaching a level higher than 80% serotonin transporter protein (5-HTT) occupancy (3,16). It is thought that besides knowing the pharmacokinetic characteristics of patients, pharmacodynamics of individuals has an essential role in increasing the success of the treatment. Recognition of the pharmacokinetic characteristics of patients, pharmacodynamics of individuals during the S-CT treatment will increase the expected response to the treatment and reduce the undesired effects.

Therefore, in this study, the concentration of S-CT, S-DCT and S-DDCT have been analysed in urine and plasma samples of patients treated by S-CT therapy. Meanwhile, MR for S-CT, S-DCT and S-DDCT has been calculated. Also, CYP2C19 polimorphism and 5-HTT pharmacodynamic polymorphism have been identified in the same patients. Eventually, the relationship between results of CYP2C19 genotype polymorphism and results of S-CT and metabolite concentration in urine and plasma, and MR has been evaluated.

Of the patients whose CYP2C19 polimorphism were identified, for the ones with the plasma concentration of S-CT and S-DCT below or above the expected level according to their CYP2C19 polimorphism, the CYP2D6 polimorphism have been also analysed. By taking this analysis into account, these patients have been reevaluated.

Evaluating the data at hand, as well as phenotyping method administered in order to identify CYP2C19 enzyme acvity by taking probe drug, it is thought that the current phenotype characteristics of individual could be identified with the analysis of drug plasma concentration.

Thanks to this method, it is thought that both taking the patients' S-CT drug concentration and MR of S-CT and its metabolites concentrations into consideration, the physician could administer the correct drug treatment therapy properly.

At the same time, when pharmacodynamic (5-HTT) polymorphism charactetistics and drug plasma concentration results of these patients whose pharmacokinetic polymorphism has been identified are evaluated together, as the required drug plasma concentration of each patient could be followed by the physician according to their 5-HTT activity, it is thought that the side effects of the drug could be reduced much more and the desired result could be increased.

2. LITERATURE REVİEW

2.1. Drug and Pharmacologic Properties

Affecting physiological function in a biochemical way, drugs are chemicals commonly having bounds with specific target sites (17). The initial step of a successful therapeutic intervention might be derived from a correct choice of drug therapy (18). For the qualitative decision in the drug therapy, quantitative aspects are required to be taken into consideration to make sure that the drug could reach the target sites so that they could show expected effect. Pharmacological properties of drug decide the amount and rate of drug reaching the target site.

2.2. Movement of Drug in The Body

In order to produce desired clinical effect, a drug must have an effective concentration in the target site. Both the administration dose and additionally other factors including administration routes, absorption, distribution, binding, metabolisation and excretion affect the drug concentration (19).

Providing that the drug is to be used locally, the method could be directly applying to the affected surface. In contrast, if the aim is to have an effect inside the body, the adminstration should be handled in a way which can reach the systematic circulation, transporting to the target site(s). As mentioned by Chillistone and Hardman (17) in 2017, the primary routes of drug administration are oral, injection, (namely intravenous, intramuscular, subcutaneous or intrathecal), rectal, inhalational and sublingual. Taken by this ways, in order to show an effect in the target site(s), all drugs must pass through cell membranes (19).

A drug could pass through a cell membrane by four main mechanisms, the primary of which diffuses passively. Means of carrier transport, filtrating through pores, and engulfing constitute the other three.

Passive diffusion: Most drugs cross cell membranes from high concentration passage to lower concentration passage by passive diffusion. It doesn't need cellular energy. The rate of passive diffusion is related directly to the size of the molecule,

molecule concentration and the ratio of ionization form, the lipid solubility and protein bounding. Small molecules can cross the cell more readily than larger molecules (17). There is a correlation between the rate of lipid solubility and the rate of transport accross membranes for such drugs. The higher is the drug's lipid solubility, the more easily it transports accross membranes. The drug's lipid solubility is calculated by proportioning the ratio of drug solubility in octanol to that of water and this ratio is expressed with log P. When the drug has positive log P value, it is expressed with high lipid solubility, but when it has negative log P, it is associated with water solubility. In general having low lipid solubility, the ionized form does not pass readily through the lipid domain of a membrane. However, only the nonionization form of drugs (e.g. nonpolar dissolving in lipids) can cross easily the cell membrane, because cell membrane consists of lipid molecules. The relationship between pKa and pH is given by the Henderson- Hasselbach equations (17).

$$pH = pK_a - \log \frac{[\text{Nonionized drug}]}{[\text{Ionized drug}]}$$

Besides, for the drug that has a highly affinitive protein bond, fraction of the bounding drug can't cross the cell because of the larger moleculer size, whereas free drug (unbounding drug) can easily cross the cell (17). Small molecules, soluble in water, transport accross through pores.

Transport through carrier: Some drugs are transported by this way. In facilitated diffusion, endogenous carrier protein converts to a different shape and releases the drug to the side of the membrane with the lower concentration. It does not need energy. Also, in active transport, some drugs, unable to cross with diffusion, cannot dissolve in the lipid layer or too large molecules are transported against a concentration gradient by carrier protein. It requires energy.

Endocytosis: It is a process in order to move the large molecules and particles into the cells by pinching of the newly formed vesicle. There are two main forms which are phagocytosis and pinocytosis.

2.2.1. Pharmacokinetic and Pharmacokinetic Interactions

Pharmacokinetic is the area of study which focuses on how drugs move within the body (18). It is a description of processes including absorption, distribution, elimination (biotransformation and excretion) of a drug and this process enables the administered drug to enter the body fluids, be distributed to different parts of the body and finally removed by elimination (20) (Figure 2.1) .

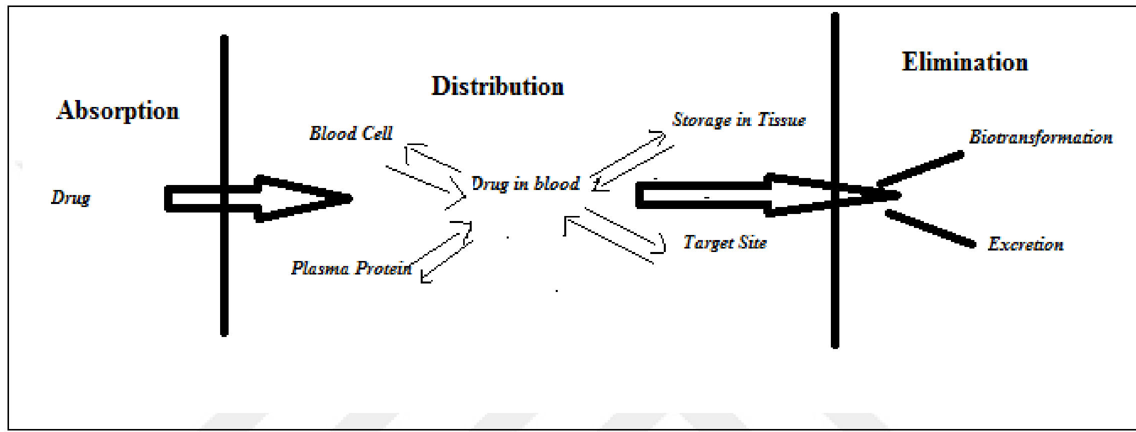

Figure 2.1. Pharmacokinetic Parameter of Drug

Pharmacokinetic is also described as the association of drug concentration rates with one another in various parts of the body. Both the proportion of administered drug in body fluids and the proportion of the drug reaching target site are important for bioavailability.

However, the concentration of drugs in the body could change depending on drug-drug and / or drug-food interaction. An alteration in the pharmacokinetic process of the other drug caused by one drug or food leads to pharmacokinetic interactions (21). In fact, by and large, occurrance of an overlap of the spheres of activity of two drugs causes a drug interaction and therefore the action caused by one drug affect the behavior of another (21).

As stated by Corrie and Hardman (21) in 2017, pharmacokinetic intereactions might occur through various stages including administration, absorption, distribution, biotransformation or excretion of a drug (or drugs). Also, in another study by Brewer and Williams (22) in 2012, it has been mentioned that due to pharmacokinetic interactions, the concentration rate of drugs in the body could change. It was mentioned

by Kennedy et al. in 2016 that the majority of the pharmacokinetic interactions affect drug elimination process (23).

2.2.1.1. Absorption

Absorption is the process by which drugs transport across body membranes and enter the bloodstream following the drug's site of administration (17). Besides, in some cases, direct administration of drug (e.g. pomade, eyedrop, nasaldrop) to the effect site makes absorption into bloodstream unnecessary in order to reveal the drug's therapeutic effect.

Injection (intravenous (i.v), intramuscular (i.m), subcutaneous or intrathecal), oral, rectal, sublingual and inhalational are primary routes of administration.

While intravenous administration requires no absorption due to rapidly entering into the bloodstream, the factors such as where the injection is applied and blood flow could change the rate of intramuscular and subcutaneous absorption. Oral administration, on the other side, is the primary route for drug administration. In stomach, acidic drugs are largely non ionized because stomach has low pH. However, stomach does not have a key role due to small surface area, whereas as an absorption site, the small intestine is primary due to its large surface area.

As Chillistone and Hardman (17) mentioned in their study in 2017, absorption plays a key role for bioavailability (B)*, which is defined as the proportion of a drug orally entering the systemic body circulation in comparison with the same dose administered intravenously.

Another route is sublingual in which oral mucose with blood abundance bypasses the portal circulation. Thus, under circumstances in which a rapid drug effect is required, especially for the drugs, getting unstable when affected at gastric pH, and rapidly metabolised by the liver, sublingual route is preferred.

Drugs taken by inhalation can be used for therapeutic effects (either local or sytemic). Once drug particles reach the alveoli, systemic absorption occurs and the particle size is

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*The ratio of the areas under the concentration- time curves for the same dose given orally and i.v. is taken into consideration to determine oral bioavailability (17).

significant (17). Having a large surface area and high blood flow enables adjustment of drug concentration in plasma rapidly.

In spite of the fact that alteration of pH or to a drug which binds to another substance in the stomach could change the process of drug absorption, in general drug absorption and interactions occur in the small intestine owing to an alteration of blood flow or motility or a change in the bacteria in the intestine (23). Some examples of drug interaction during absorption could be as follows:

By changing the pH of the stomach, antacids, histamine H2 antagonists, proton pump inhibitors affect the absorbtion rate and the absorption ratio of other drugs absorbed in the stomach. The drugs affecting gastrointestinal motility (e.g anticholinergics) can also affect the absorption rate and absorption rate of the drugs which are co-administered.

2.2.1.2. Distribution

Drug distribution process is referred as the motion of a drug from the absorption area to the other areas in the body. When drugs are absorbed into the blood stream, they are required to reach their effect sites to show their clinical effects. Solubility of drug, rate of blood flow, drug uptake into tissue and how easily the chemical crosses the local the cell membrane are factors which affect distribution to tissues (17,19). The body fluids which are effective in distribution process are divided into four compartments as interstitial (accounting for 15% of the total body weight), plasma (accounting for 5% of the body weight) intracellular (accounting for 40% of the body weight), and transcellular (accounting for 1% of the body weight) (Figure 2.2) (17).

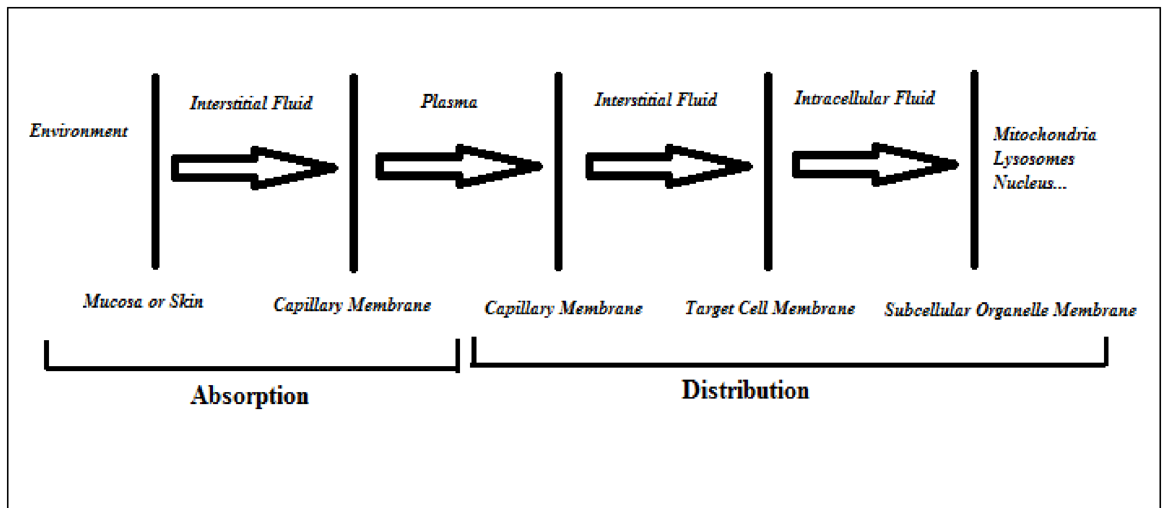


Figure 2.2 Drug distribution process in the body fluids

The drug which is available in the interstitial fluid can move by mainly penetrating into local tissue, the blood circulating system and the lymphatic system. Once the drug has entered into the blood stream, it is present in the blood partially bound or partially free. Plasma proteins, especially albumin, also alpha 1- acid glycoprotein can not only bind normal endogen compounds in the body but also a number of xenobiotic, such as drugs (19). Drugs binding to plasma proteins are not available for distribution to the extravascular region. Knowing that plasma contains unbound fraction of drug and bound fraction of drug in equilibrium, only free drug is able to pass through the capillary membranes. Therefore, the unbound concentration rather than the total concentration of drug in plasma is more decisive in the pharmacological effect of drug. Also, this sitution prolongs the elimination half time of the drug within the body.

Volume of distribution (V_d)[†]of a drug is expressed as the quantity of the distribution of a drug to the plasma and the other parts of the body after oral or parenteral dosing determines how extensively a drug is distributed in the body fluids. While the very high appearance of V_d is an indication of the drug's distribution to a particular tissue or storage like adipose tissue, the low V_d may indicate that the drug is

[†] The V_d can be calculated by the formula; V_d: dose (mg)/ plasma concentration (mg/L).

more likely to distrubute to plasma. When a drug enters the blood stream, it may be stored, redistributed, biotransformed, excreted.

The distribution of drugs into the Central Nervous System (CNS) from the blood is unique as the capillary endothelial cells in the brain are tight junctions, there appears very few or no pores between the cells and there are not enough vesicles in these cells, which reduces their transport ability. Lipid solubility is decisive in the penetration of the drugs into the brain. By and large, while nonionized, unbound and lipophilic drugs are easily uptaken by brain, some other drugs may penetrate into brain by specific uptake tranporters.

During the process of drug distribution, drug-drug interaction may affect especially the ratio of binding of drug's to plasma proteins and the the rate of blood flow (17). Significant drug interections might be caused by competition for binding sites on plasma proteins.

As a matter of fact, a number of drugs bind commonly to plasma proteins, e.g. albumin and therefore can prevent each other from reaching their site of action due to competing with one another since the binding affinity of some is greater than others, which increases the concentration of free drug and change the expected effect. To illustrate, as Brewer and Williams (22) mentioned in their study in 2012, ''as diazepam has a much greater binding affinity in comparison with phenytoin, it replaces phenytoin in plasma proteins and increases plasma free phenytoin concentration, rising the adverse drug effect''(22,23). Further, drug interaction including plasma protein binding can change the volume of distribution of drug, elimination half time, and clearance of drug.
Changes in blood flow resulted from, for instance, the reduction of cardiac output or vasoconstriction could have an impact on drug distribution (23).

2.2.1.3. Drug Elimination

Through drug elimination process, the active drug is removed from the body and consist of biotransformation and excretion.

2.2.1.3.A. Biotransformation (Metabolism)

Drug metabolism is known as biotransformation process conducted by enzymatic system in the body (21). The liver is the primary, in general, unit to handle

the drug metabolism, while but rarely, drugs are metabolised in extrahepatic tissues including the lung, stomach, intestine, kidneys or brain (24). The activity of drugs are often reduced by metabolism (e.g. alprazolam), yet in certain cases, it plays a role to convert one pharmacologically active subtstance to one other active substance (e.g risperidon, clozapine). Also it plays a role to convert inactive drug (prodrug) to an active drug. There are three types of metabolism.

Phase 1 consists of oxidation, reduction and hydrolysis. Oxidation is the primary one. CYP, which is present in the endoplasmic reticulum causes many oxidative phase 1 reactions. Besides, Chillistone and Hardman (25) in their study in 2017 indicated that ‘‘other enzyme types in the phase 1 metabolism are flavin monooxygenases, monoamine oxidase, alcohol dehydrogenase, peroxidase, aldehyde dehydrogenase, NADPH-cytochrome p450 reductase, amidases, oxydoreductase, reduced cytochrome P450’’.

Some of the phase 1 processes occur in various sites, either in the plasma or other tissues.

- Cocaine is converted to ecgonine methylester in the plasma by cholinesterase (26).
- Benzo(a) pyrene is metabolized in the lung by oxidation.
- Cyclosporine is metabolized in the intestine by oxidation.

Peng and Zhong (27) mentioned in 2015 that ‘‘phase 2 contains conjugation reactions by which small molecular group (e.g. amino, hdroxyl, thiol, sulphate, glutamate, acetate, methyl, and most commonly glucuronide) is chemically bounded to drug by transferase enzymes, such as UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), N-acetyltransferases (NATs), thiopurine methyltransferase (TPMT), catechol-O-methyl transferase (COMT). In phase-III, the uptake and excretion process of drugs and their metabolites take place via transporters. While conjugation reactions occuring in the liver, intestine, lung, kidney and etc. are the main reactions of phase-II, phase-III reactions occur in kidney’’. Certain drugs and other subtances can catalyze induce or inhibite the enzymes which are involved in drug metabolism, which can contribute to the existance of clinically vital drug interactions causing therapeutic failure, drug toxicity or tolerance.

2.2.1.3.A.1. Cytochrome P450 (CYP) Enzymes and Polymorphism

CYP enzymes diversely distributed in different tissues are essential for the biotransformation of many drugs and endogenous substances and such enzymes are especially found in the liver tissue, but at the same they reside in the extrahepatic tissues including, lungs, brain, intestine, kidneys (28–30). The reason why these enzymes are called CYP is that they are binding with membranes in cyto cell and containing heme pigment which absorb lights at a wavelength of 450 nm under carbon monoxide (28). CYPs play a significant role in oxidative reactions in that they take an atom from molecular oxygen and then insert it into a substrate as well as acting in reduction reactions (29). There are 57 active CYP genes, and 58 pseudogenes. Also, there are a number of substrates of CYP enzymes. Generally, CYP families including 1 to 3 are responsible for phase 1 reaction for exogenous substrates such as drugs while CYP families including greater than 3 have high affinity for endogenous substrates such as steroids, fatty acids, prostaglandins, leukotrienes, and biogene amines. Most drugs are predominantly metabolized by CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4.

CYP1A2, which is predominantly 10 to 15% of total CYP in the liver, has an essential role not only in biotransformation of several drugs, but also endogenous substrates (Table 2.1) . CYP1A2 substrates could be affected by drug interactions resulted from the inhibition of the enzyme by xeonobiotics (e.g. fluvoxamine) or induction of enzyme by xeonobiotics (e.g. smoking) (29).

CYP2C9, which is mainly about 20 % of total P450 in the liver has an essential role, not only in biotransformation of some drugs but endogenous substrates as well (Table 2.1). The variety in terms of interindiviuality is great in CYP2C9 acvity which leads to variations in drug response and adverse effects (29,31).

CYP2C19 metabolizes clinically important drugs including omeprazole, pantoprazole, clopidogrel, and several antidepressants drugs including citalopram, escitalopram, sertralin. Besides, CYP2C19 metabolizes endogenous substrates including progesterone and melatonin (29,31).

CYP2D6 is not only present in the liver, but, though less in amount, in some extrahepatic systems as well. CYP2D6 has important role in several drug metabolism (Table 2.1). Except for CYP2D6, CYPs could be induced (29,31,32).

CYP3A4 is not only the most abundant CYP450 enzyme in the liver, but also highly expressed in some extrahepatic tissues including intestine. A number of drugs and endogenous substances are metabolised by CYP3A4 (Table 2.1). When one drug, substrate of CYP3A4, is combined with a strong inhibitor of CYP3A4, its metabolisation slows down and therefore may lead to an increase in drug concentration in plasma as high as 10 to 20 times and result in adverse or toxic effect. Similarly, when combined with an inducer, this drug is metabolized more rapidly, so the drug concentration in plasma may decrease to only 5 to 10 % of its expected concentration, which may result in therapeutic failure (29).

TABLE 2.1. Substrates of Major Cytochrome P450 (CYP) Enzymes		
Enzyme	Drug	Endogenous
CYP1A2	Phenacetin, Acetaminophen, Olanzapine, Lidocaine, Duloxetine, Clozapine, Theophyline	Oestrogens, Retinoic acid Melatonin Prostaglandins Arashidonic acid,
CYP2C9	Warfarin, Losartan, Tolbutamide, Glibenclamide Candesartan, Valproic acid, Phenytoin, Carvedilol, Ibuprofen	Linolenic acid Arachidonic acid
CYP2D6	Propafenone, Flecainide, Mexiletine, Paroxetine, Amitriptyline, Venlafaxin, Risperidone, Aripiprazole, Metroprolol, Tamoxifen, Tramadol Codeine, Donepezil, Haloperidol, Metoprolol,	5-methoxytryptamine (5-MT) 5-methoxy-N,N-dimethytryptamine (5-MDMT)
CYP2C19	Pantoprazole, Omeprazole, Clopidogrel, Escitalopram, Citalopram, Sertralin, Phenobarbital, Phenytoin	Melatonin Progesterone
CYP3A4	Tacrolimus, Cyclosparine, Erythromycin, Tamoxifen, Alprazolam, Diazepam, Simvastatin, Zolpidem	Progesterone, Testosterone, Androstenedione Bile acids. Cortisol

While sometimes one drug may be metabolised into variety of products by different CYP enzymes, sometimes many drugs can be metabolized by the same CYP enzymes (29). Besides, CYP polymorphisms are primarily the reason for the alteration of drug metabolism, the substrates for these certain enzymes, which likely causes differences in response as well as adverse drug reactions (33). Single nucleotid polymorphism (SNP) is common in CYP polimorphisms (33). All human beings inherit

one allele from both their mothers anf fathers. Alleles are known as “wild type” or “ variant” , having wild type which occur widely in the general population (28). The majority of CYP family members, polymorphic and allelic variants, and this variation in CYP genes causes phenotypes, typically known as extensive (EM), poor (PM), intermediate (IM) and ultrarapid (UM) metabolizers (33,34). An EM, referred to normal activity of CYP enzyme (also expressed as *1), contains two copies of wildtype alleles (34). Polymorphism takes place once one or both wildtype alleles is substtituted by a variant allele, usually encoding a CYP enzyme with reduced, no activity or deletion of entire gene. Whereas the EM is on account of one or two alleles which have normal function, the existence of two nonfunctional (null) alleles or deletion of entire gene is the reason of the PM. Generally, IM carries one null allele and one other allle which has reduced function. On the other hand, UMs have more than one extra functional gene (Figure 2.3) (33).

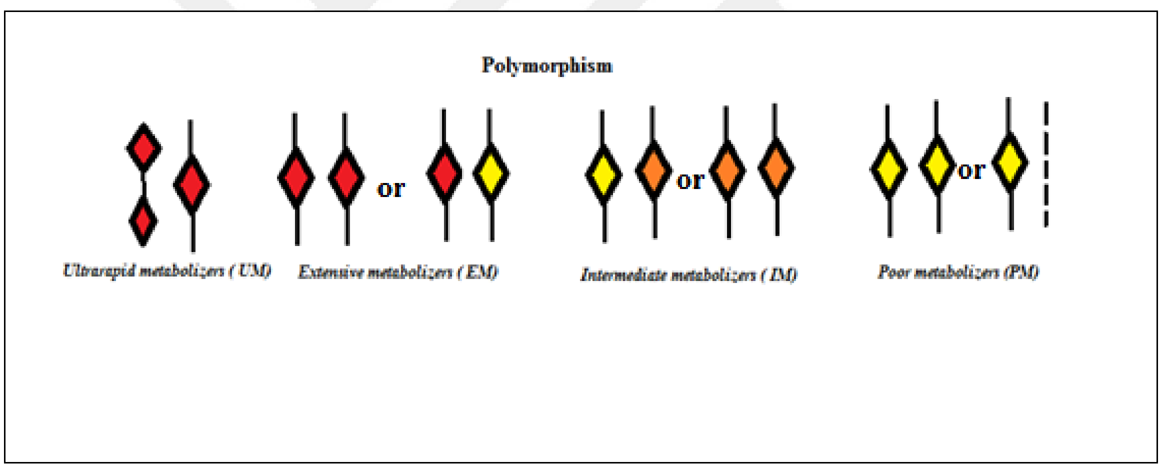


Figure 2.3 Polimorphism of cytochrome P450. Red: normal function alleles, yellow; nonfunctional (null) alleles, orange; reduced functional alleles, dashed-line; deleted alleles.

Pharmacogenetics is the field studying the different responses of individuals to drugs used in their treatment due to individual genetic polymorphism. The identified genetic polymorphism of CYP enzymes enable to adjust the most appropirate drug dosage with least possible adverse effect and the type of rational drug treatment accordance with individual genetic differences (33).

2.2.1.3.B. Renal Excretion

In drug's disposal from the body, the kidneys have a significant role. A drug or metabolite which is both water soluble (polar) and small, having a weak bound to proteins in the bloodstream is widely excreted in the urine (25). Following the access of the drug into the renal artery and renal circulation, the extent that the drug undergoes renal excretion is determined by mainly three ways, namely glomerular filtration, tubular secretion, and tubular reabsorption in kidney.

Glomerular filtration: Glomerular filtration is knowns as the process whereby when a drug goes through the renal afferent arteriole, it travels through Bowmen's capsule and the glomerulus for the removal of excess wastes and fluids. In this proccess, small molecules pass into the pores within membrane while proteins (e.g. albumin) and blood cells as they are too large can not pass through the pores. Also, excessively large drugs to penetrate into the membrane cannot be filtered. On average, the kidneys of an adult human make 170 liter of a filtrate a day, but the large amount of filtrate, approximately 99 %, is reabsorbed into the blood, the remainder of filtrate is excreted as urine.

Tubular secretion: Tubular secretion occuring in the proximal tubule of the nephron is responsible for the transport of some molecules out of the blood and into the urine including potasium ions, hydrogen ions, and some xenobiotics. Secretion is an active transport process, and therefore it needs energy. Active secretion occurs by means of a carrier including basic carriers transporting basic drug and acidic carriers transporting acidic drug.

Tubular reabsorption: Reabsorption occuring mainly in the proximal tangled tubule of nephron is primarily passive diffusion process. pH of the urine affects reabsorption and excretion because nonionized form of the drug readily passes through the membrane. The drug which has acidic properties is more ionized in alkaline urine, and thus excreted to a great extent while this drug is less ionized in acidic urine, so nonionized form of this drug can be reabsorbed and thus re-enters the blood circulation.

The factors, namely diet, kidney disorders and drugs may affect urine pH, which as a result can alter the ratio of the excretion of several drugs.

Additionally, some drugs with high solubility in lipid can be readily reabsorbed, and reenter blood circulation. Under this circumstance, their elimination half time in the body may prolong, and can arise drug toxicity.

2.2.1.3.C. Biliary Excretion

High molecular weight drugs are excreted through the bile produced by the liver. This process primarily contains active secretion rather than passive diffusion. Due to active secretion into the biliary canaliculus, proccess can be inhibition and competition. The drugs removed from the body may be unchanged or conjugated by this way. When in the digestive tract, the reabsorbtion of drugs with high lipid solubility maybe in unchanged form and bacterial glucuronidase may hydrolyse glucuronide conjugates of drugs and therefore reabsorbed. The process known as enterohepatic circulation, the excretion into the intestinal tract through the bile reabsorbtion and return to the liver by the portal circulation prolongs the half time of drug in the body as well the efficacy or toxicity of drug.

2.2.1.3.D. Other Routes of Excretion

Some anaesthetics are primarily removed through lungs. As far as blood gases are concerned, the excretion happens through passive diffusion from the blood into the alveols. Gases which have a low solubility in blood are excreted more rapidly when compared to other gases with a high solubility. The other removal routes, namely, tears, skin, hair, saliva, sweat and breast milk may have drug and metabolites. Non-ionized, lipid-soluble drugs are excreted by passive diffusion as well (25).

2.3. Individual Specific Drug Treatment Methods

As is known, pharmacokinetic studies of a drug are usually performed on those who are EMs for that drug. In these individuals, genes encoding a number of enzymes which are present in the biotransformation of drugs are found in most of the population.

However, in some patients, drugs may not be effective at standard doses due to polymorphisms. A physician-recommended drug may not reach the standard effective doses of the drug as a UMs for that drug will metabolize faster than EMs. Alternatively, the effect expected from the drug may not be achieved at standard doses, since a drug used as a prodrug will turn a PMs into a drug that is slower than EMs. On the other hand, those who are PMs or UMs when the drug itself or the metabolites have serious side effects may have different effects than those of EMs. Therefore, individual treatment, especially in cases of multiple genes, such as psychiatric disorders, cancer, and diseases with environmental influences, has special precaution.

Determining the appropriate individual drug and dose regimen by taking individual differences into consideration, severity of side effects and interactions, and possible adverse drug reactions decrease but efficacy of treatment increases (6). In addition to the commonly used phenotyping and genotyping methods for determining polymorphisms, TDM that has been widely used in the world is one of the methods that can be used with the purpose of personalized treatment. While genotyping methods are used to determine polimorphisms in enzymes, carrier proteins and receptors, the activities of the enzymes playing a role in biotransformation of drug are determined by phenotyping and level of drug in body fluids (eg plasma, blood, urine) have been evaluated by TDM (6).

2.3.1. Genotyping

Drugs interact with a number of proteins in the organism until excreted. This interaction may be at the level of pharmacokinetics (drug absorption, metabolism, distribution, excretion) or pharmacodynamics (ion channels, receptors, enzymes). This interaction between the drug and the organism occurs between proteins, which are gene products, and drug molecules. Genotyping analyzes provide the comparison of drugs that can be used in the treatment of the patient with the genetic makeup of the individual and the application of the most effective drug with the least side effect at the right dose (35). Genotyping analysis have a key role in polygenic complex diseases such as psychiatric disorders where pharmacotherapies are inadequate and / or expensive.

2.3.2. Phenotyping

Phenotype analysis; as it reflects the sum of the genetic and environmental effects on the activity of enzyme, use in clinical practice is highly informative (6). By phenotyping; functional predisposition of polymorphisms, prediction of drug / food interactions, drug dose and dose interval to be used in first and recurrent drug applications can be determined. These methods are divided directly and indirectly into two. Today, indirect analyzes by probe drug applications are widely used to decide the activities of enzymes playing a role in drug biotransformation (36), because of limitations in the use of direct enzyme assays (such as lack of enzymes at sufficient levels in cells). In general, enzyme activity with probe drugs can be determined by using chromotographic methods; it is performed by measuring not only probe drug but also its metabolite concentration in body fluid after probe drug administration.

The MR parameter is often used to determine enzyme activity. The MR based on analysis of probe drug and its metabolites in body fluids at specific time points. An ideal probe should have the following properties:

- ✓ Elimination must be fully dependent on metabolism,
- ✓ With linear pharmacokinetic properties,
- ✓ Metabolism should be minimally affected by the rate of binding to blood plasma and plasma proteins,
- ✓ Metabolism pathway and enzymes are known,
- ✓ With the single probe drug administration, the activities of different enzymes and polymorphic pathways can be determined simultaneously with the detection of specific metabolites,
- ✓ Prophylactic drug excretion, urinary flow and renal clearance are not affected by effective factors on excretion,
- ✓ If it is to be administered orally, it should be absorbed completely and rapidly,
- ✓ Should not be toxic in both healthy and liver disease individuals,
- ✓ Not affected by other enzyme systems,
- ✓ There should be no significant pharmacological effects at the administration dose,
- ✓ Probe drugs and / or metabolites may be measured in biological fluids,
- ✓ Interaction with chemical and environmental factors, or not at all,

- ✓ The effects of the methods used on individuals should be very low and easily applicable.

2.3.3. Therapeutic Drug Monitorization (TDM)

TDM helps know a phenotyping of person and dose adjustment for the prescribed drugs by measuring drug and metabolite. So as to titrate the dose of individual patients, TDM in pharmacotherapy measures the drug and its metabolite in body fluids including plasma, urine, milk and oral fluid in order to obtain a desired drug concentration in treatment. Therefore, for a number of drugs, it is advised that the quantification of drug and its metabolite in plasma be a clinical routine to adjust drug dose (3,4).

For a successful TDM, it is important that the method used in the quantitative analysis of drug and its metabolite be selective and sensitive. The methods to be used in biological matrix for quantitative measurement of drugs is required to be validated so that it could be a valid and reliable method. The primary validation parameters for bioanalytical methods include accuracy, precision, selectivity, sensitivity, stability and matrix effect. For numerous drugs, chromatographic methods inluding gas chromatography (GC), high-performance liquid chromatography (HPLC) and LC-MS/MS are preferred, but HPLC-UV or HPLC-FLD demonstrates a major shortcoming, the potential interference of the analysis by unknown signals. On the other hand, such problems occuring in HPLC are not an issue of concern for LC-MS/MS due to its high accuracy and senstivity. Additionally, having notably reduced runtimes and reduced sample volumes are the advantages of LC-MS/MS over other chromatographic methods (3,4,37).

TDM in pharmacology offers some other uses as follows:

- ✓ TDM is used to reduce the risk of toxication.
- ✓ The interaction of multidrug use
- ✓ inappropriate use of prescribed drugs
- ✓ Reduction of relapses during treatment
- ✓ Genetic polymorphism in drug biotransformation

- ✓ Pregnancy or breast-feeding
- ✓ Person below18 years old and above 65.
- ✓ Mentally retarded patients.
- ✓ Forensic patients

2.3.3.1. Relationship Between Drug Dose and Drug Concentration in Plasma

In cases where TDM is generally used, consecutive dose to obtain a C_{ss} is of vital significance. In general, drugs reach C_{ss} in plasma, approximately after 4-5 times of the $t_{1/2}$, when drug intake ratio is in equlibrium with the ratio of drug loss (Figure 2.4). In the case where the dose interval (T), the clearance (Cl) and the bioavailability (B) is known, the assessment could be made regarding the dose necessary to obtain C_{ss} in plasma (3,4).

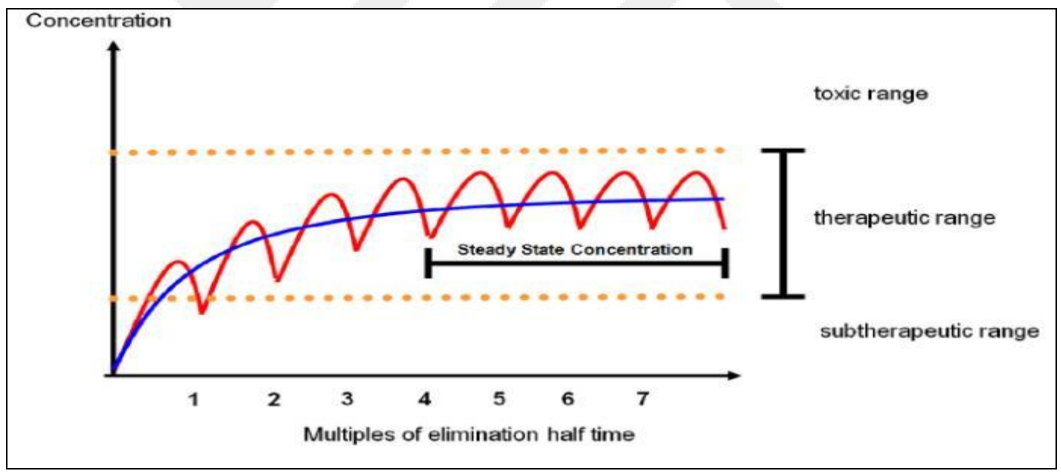


Figure 2.4 Representing C_{ss} of a certain drug. C_{ss} is reached in approximately 4-5 half-times

There is a correlation between the drug dose in the steady state (mg/day) of the drug (Dss) and blood drug concentration (c) and total clearance ($_{tot}Cl$) (3,4).

$c = Dss / {}_{tot}Cl$

Based on the given formula above, it could be concluded that the administration of a high dose results in a high concentration while a low dose results in a low concentration, which makes it significant to know if the drug concentration is in accordance with the dose or not (3,4).

In addition, for drug dose calculations, Habits, diets, individual diseases, physiological state and other environmental factors are important to be taken into consideration. TDM is analyzed at the point when the drug reaches the steady state concentration and blood collection could be made at trough level which is the level where drug is at its lowest concentration in the plasma (a common sense is that plasma sampling should be after 12 hours or just before the new dose intake) (3,4).

2.3.3.2. Therapeutic Drug Range in Plasma

TDM is also used to determine whether the drug concentration in plasma is within the accepted therapeutic drug range and this value shows highest effectiveness and safety. That the drug concentration remains within this accepted range is expected for a desired clinical response. Provided that the obtained value is below this range, there might occur an unresponsiveness (risk for loss of action) in drug treatment, whereas if the obtained value is over the this range, the risk of toxicity might increase (Figure 2.4) (3,4). Additionally, pharmacokinetic interactions or pharmacogenetic abnormalities can also lead to toxic blood concentration, in which cases TDM is still the significant clue.

2.3.3.3. Metabolic Ratio (MR) in TDM

The analysis of drug and its metabolite concentration is required as well so as to identify pharmacokinetic abnormalities. MR expressed as the ratio of the metabolite plasma concentration to the drug plasma concentration helps identify the metabolism ratio of drug.

Providing that a substential reduction of a drug through one enzyme, responsible for metabolization of that drug, occurs, the MR hints a clue about the activity of this enzyme. An MR deviating notably from the expected range indicates that there is a genetic diversity or a change in the pharmacokinetic process. (3,4).

O-desmethylvenlafaxine to venlafaxine, and paliperidone to risperidone regarding the CYP2D6 activity are proven examples of this condition (Table 2.2) (3,4).

TABLE 2.2. Ranges of Metabolite-to- Drug Concentration Ratios for Drugs

Drug	Metabolite	Enzyme	Concentration ratio of metabolite to drug in plasma
Risperidone	Paliperidone	CYP2D6	EM or IM: 1.5–10.0 PM: ≤ 1
Venlafaxine	O-Desmethylvenlafaxine	CYP2D6	EM or IM: 0.3 – 5.2 PM: ≤ 0.3 UM: > 5.2
Aripiprazole	Dehydroaripirazole	CYP2D6	PM: 0.2

2.4. Effect of Serotonin System on Physiological Events of Brain.

Even though one of the monoamine neurotransmitters is Serotonin (5-hydroxytryptamine, 5-HT) regulating a number of significant physiological incidents, ranging from sleep to motor activities, 5-HT plays a key role on modulating mood and behavior. There is a two-step syntesis process of 5-HT from amino acid tryptophan in humans. Owing to the fact that 5-HT itself cannot cross the blood-brain barrier, a transport protein transports the tryptophan to the central nervous system, where tryptophan is then converted to 5-HT by enzymes (Figure 2.5). Following biosynthesis process, 5-HT is packaged to be protected and stored from metabolism in presynaptic vesicles. Serotonergic signal transduction regulates serotonin receptors. After the depolarization process, 5-HT is allowed to leave to where it is able to bind to a postsynaptic 5-HT receptor to induce potency or to inhibit the feedback of 5-HT release to presynaptic receptors. 5-HT receptor systems complexly interact with many other neurotransmitter systems and coexist with other neurotransmitters in some neurons. The subtyping of serotonin receptors is based on several measures such as genetic, pharmacological and second messenger pairings. At present there are 14 known subtypes of serotonin receptors. However, only a subset of these receptor subtypes are involved in brain physiological events. Serotonin subtypes having a key role in depression and related disorders are 5-HT1A-B, 5-HT2A, 5-HT3. It is assumed that 5-HT1A receptors play an important role in the etiology of depression and anxiety based on their anxiolytic and antidepressant properties of these receptor agonists. 5-HT1A receptors are postsynaptic receptors located in the target neurons in the cortex and

subcortical areas at the end of the serotonergic system, while they act as receptors in soma and dendrites of serotonergic neurons. Thus, reduced 5-HT1A autoreceptor functions cause an increase in serotonin transmission; a decrease in function in target neurons leads to a decrease in the effects of 5-HT1A mediated neural transport. 5-HT1B receptors are known having a significant role in impulse-aggressive and sexual behavior regulation, alcohol and cocaine intake. 5-HT1D is a "terminal" autoreceptor, that is, located at the axon terminals and actively blocks 5-HT release by the presence of serotonin in the synaptic range. It controls the release of serotonin in this way. 5-HT2A is a postsynaptic regulator receptor. It is generally expressed as 5-HT2 and is one of the most important of the 5-HT receptors. The brain cortex and the caudate nucleus are places where the 5-HT2 receptors are most dense. Very few have been detected in other regions of the brain. It is stimulated by serotonin. Following this stimulation, serotonin activates the postsynaptic cell conduct systems, that is, the second messenger systems e.g phosphatidylinositol. These enable the transcription factors (transcription) to be produced in the cell to elicit the desired effect. These receptors depolarize in the membranes and cause vessel contraction, lymphocyte shape change, muscle twitches, back contractions. Stimulation of 5-HT2 receptors also cause psychological states (e.g. agitation, akathisia, anxiety) and physiological states such as sexual disorders. The stimulation of 5-HT3 receptors, with effects on the gut, causes nausea, headache, gastrointestinal system complaints and diarrhea, brain stem vomiting center and hypothalamic tracts. They are generally found in peripheral tissues. It is thought to help regulate acetylcholine and dopamine release in the central nervous system (38). The synaptic space is where relased 5-HT remains up to either reuptaken into the presynaptic neuron or metabolized by monoamine oxidase. A transporter located on the presynaptic neuron, 5-HTT, of which responsibility is to reuptake the neurotransmitter, plays a primary role in termination of serotonergic neurotransmission. 5-HT, reuptaken by 5-HTT, is restored into synaptic vesicles and reused by which 5-HTT controls the magnitude and duration of postsynaptic signaling of 5-HT. Monoamine oxidase modulates inactivation of any 5-HT escaping reuptake by 5-HTT and both of these processes take place concurrently (39). The changes in serotonin system are known to cause depression, SSRIs re-adjust the level serotonin in the brain, and therefore they have been preferred in the treatment by physicians for decades (40).

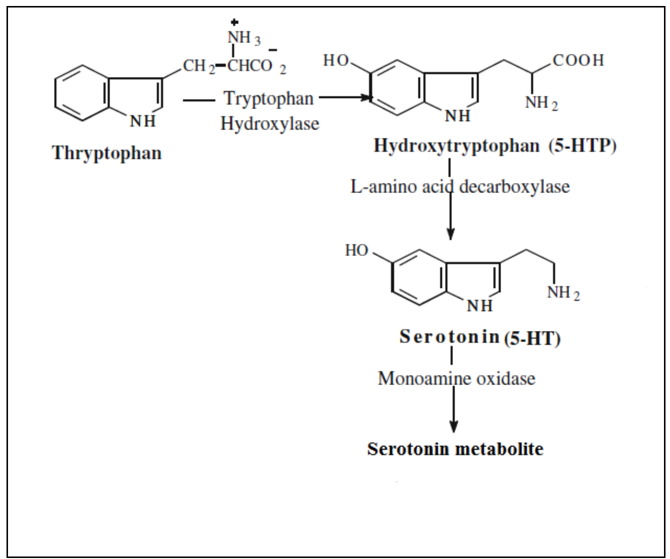


Figure 2.5 Synthesis and biotronsformation of Serotonin (41)

2.5. Escitalopram and Pharmacokinetic Properties

S-CT is one of the most commonly utilized SSRIs in depression and generalized anxiety treatment (9,42). S-CT shows antidepressant effect because it increase serotonin level in the brain which is caused by the inhibition of the serotonin transporter (10,43) (Figure 2.6). Waugh and Goa (44) mentioned in 2003 that ‘‘escitalopram with rare affinity for a wide range of other recoptors, namely dopamine, histamine, α- and β-adrenergic, benzodiazepine and muscarinic along with not binding or having rare affinity for Na^+, K^+, Cl^- and Ca^{2+} ion channels. Mostly mild and transient adverse events occured’’. Escitalopram has proven to be effective in order to treat depression and anxiety disorder after administered with the oral dose of 10-20 mg daily (9) .

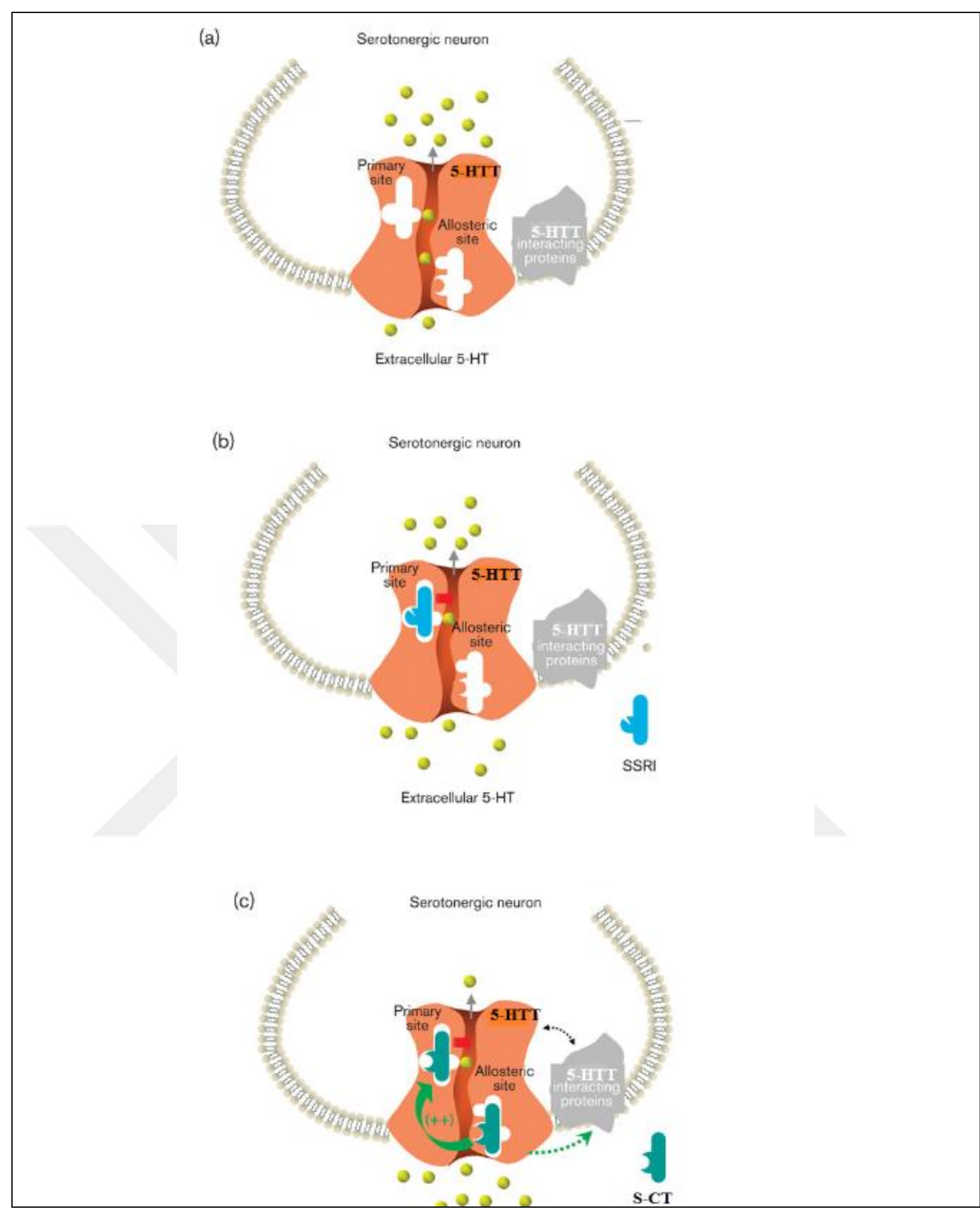


Figure 2.6. Represantation of antidepressant effect of escitalopram in the brain. 5-HT, reuptaken by 5-HTT, is restored into synaptic vesicles (a). SSRIs increase serotonin levels in the presynaptic area by inhibiting 5-HTT primary site (b). S-CT increase serotonin levels in the presynaptic area by inhibiting 5-HTT primary site and allosteric site (c).

Absorption

S-CT is easily absorbed upon oral intake. Rao (9) mentioned in single and multiple escitalopram dose study in 2007 that ‘‘once single dose is administered,

absorbtion of escitalopram is rapid, reaching Cmax in plasma of 18.8 ± 4.5 ng/mL in 3.0 ± 1.5 hours. Once a multiple dose of 10 and 30 mg/day of escitalopram was given to healthy male female volunteers for a long period of time, Cmax of S-CT in plasma, was found to be around 21 and 64 ng/mL, respectively''. Single and multiple dose studies showed similar elimination half-time values and C_{ss} were reached following an administration of approximately 7–10 days (9). When administered orally as opposed to the administration intravenously, the estimated bioavailability of escitalopram was found to be approxiametely 80%.

Distribution

S-CT exhibits a wide distribution in the body following oral uptake with a great volume ((V(z)/F) of about 1100L). S-CT has a property of low protein binding (56 % on average) and for this reason it has a low interaction in plasma protein in terms of drug displacement (9).

Metabolism

The metabolisation of S-CT mainly takes place in the liver and then the drug and its metabolite with less lipophilic compounds are removed easily in urine. There are primarily three enzymes in the biotransformation of S-CT, which are, with a major role, CYP2C19 and CYP2D6, and with a minor role, CYP3A4. S-CT converts to S-DCTand S-DDCT through oxidative metabolism with N-demethylation (15). CYP2C19 is known to have a significant role in converting S-CT to S-DCT, whereas CYP2D6 catalyses the conversion of S-DCT to S-DDCT (15) ,and some other phase 1 and phase 2 reaction could play a role in biotransformation of S-CT (e.g. deamination and dehydrogenation to a propionic acid derivative, as well as N-oxidation and glucuronide conjugation) (9).

$$\textbf{Escitalopram} \xrightarrow{CYP2C19} \textbf{Demethylescitalopram} \xrightarrow{CYP2D6} \textbf{Didemethylescitalopram}$$

Polymorphisms have proven to be affective on biotransformation of numerous drugs. After the multiple dose intake of S-CT, when compared to extensive metabolizers, poor metabolizers of CYPC19, resulted in higher plasma S-CT and lower

concentrations of S-DCT (9). Besides, when the multiple dose intake of S-CT, when compared to extensive metabolizers, poor metabolizers of CYP2D6, demethylating of S-DCT to S-DDCT, no comprehensive differences were found in poor metabolizers (9).

Excretion

The removal of the principles of S-CT and its metabolites takes place in renal, a small part of which is emptied with feces. The mean t1/2 of S-CT and S-DCT exhibits a figure around 27-33 hours and 59 hours respectively. The unchanged form of drug present in urine is 8–10% of the dose taken. When 20 mg S-CT was taken as a single dose, renal clearance of S-CT and S-DCT was found to be around 2.7 L/hour (9).

Drug-Drug Interactions

In depression treatment, it is common to see multiple drug use among patients. Therefore, it is a significant attention point for physicians prescribing antidepressants as there is a possibility of drug-drug interactions. Almost all antidepressants including SSRIs could inhibit the metabolizing CYP isoenzymes of most drugs used in clinical practice and therefore this sitiuation is of particular importance. As known, fluoxetine and fluvoxamine, which are in the group of SSRIs, show strong inhibitory effects on CYP2C19 and CYP2D6, so co-administration of S-CT with fluoxetine or fluvoxamine resulted in a higher plasma S-CT concentration. As well as the minimal interactions between S-CT and a few group of drugs such as Tricyclic antideppressants (TCA), lithium, warfarin, theophyline, carbamazepine (9).

2.6. Effect of Polimorphism on Escitalopram Treatment

During S-CT treatment, the knowledge of the genotypic characteristics of individuals, particularly those associated with 5-HTT and CYP enzymes (CYP2C19, CYP2D6), may be effective in determining therapeutic targets (39). Polymorphism is able to change the gene expression or gene activity where they are present.

SNP, a single nucleotide (A, T, C or G) in the DNA sequence, is the simplest genomic differences that occur every 1000 cases. Some change the genetic activity. It has predominantly influences on susceptibility to diseases and response to drugs. SNPs are

important in the way that individuals are sensitive to drugs and side effects. By knowing the polymorphisms of the patients during treatment, individual treatment options can be developed according to the genotype of the patient and new therapeutic targets can be determined.

2.6.1. 5-HTT Polymorphism on Escitalopram Treatment

The 5-HTT gene is composed of 630 amino acids at a weight of 68,000 daltons and encodes the serotonin transporter protein and is mapped to the chromosome 17q11.1-q12 by the gene coding for SLC6A4 (Solute Carrier Family 6 Member 4). The 5-HTT gene is 31 kb in length and contains 14 exons (45). There are two basic polymorphisms for this gene which are indicated to be effective in the regulation of serotonin-related behaviors, particularly in some psychiatric and psychosomatic disorders The first of the two polymorphisms with respect to the 5-HTT gene is the polymorphism of Variable Number of Tandem Repeats (VNTR) repeating in the 15-18 bp part of the second intron at 7 (~ 315 bp), 9 (~ 345 bp), 10 (~ 360 bp) or 12 (~ 390 bp) intervals. According to this polymorphism, genotypes are evaluated as 12/12, 12/10, 10/10, 12/9, 10/9, 9/9, 12/7, 10/7, 9/7 and 7/7. The second polymorphism exists in the transcriptional control region of the 5-HTT gene where a 44 bp GC (Guanine, Cytosine) consisting of 20-22 bp double repeats occurs depending on the repetitions of a rich sequence of insertions / deletions (5-HTTLPR). Bp long (L: L) form consisting of 16 repeats resulting from the insertion of the 44 bp repeat sequence; In the case of the deletion, however, the allele that is called as bp short (S) form consisting of 14 repeats occurs (Figure 2. 7).

S- homozygous (S/S), S/L heterozygous, or L-homozygous (L/L) are phenotype characteristics seen in 5-HTT (39). The long (L) and short (S) variants of 5-HTTLPR have been identified in a variety of studies showing different transcriptional effects (46) . The short ("S") variant is associated with the lower transcriptional activity of the promoter when compared to the long ("L") allele. In comparison of cells homozygous for the L allele (L/L/) with S allele regarding the production level of 5-HTT mRNA, it is evident that L allele (L/L) is 1.4 to 1.7 times greater (39,45). 5-HT from the synaptic cleft occurs at a of rate 1.9–2.2 times greater when compared to S/S and S/L variants. Thus, the L-homozygous variant increases transcriptional activity of the 5-HTT

promoter which results in a rise of 5-HTT expression and 5-HT reuptake from the synapse relatively more than those of S variant (39,43).

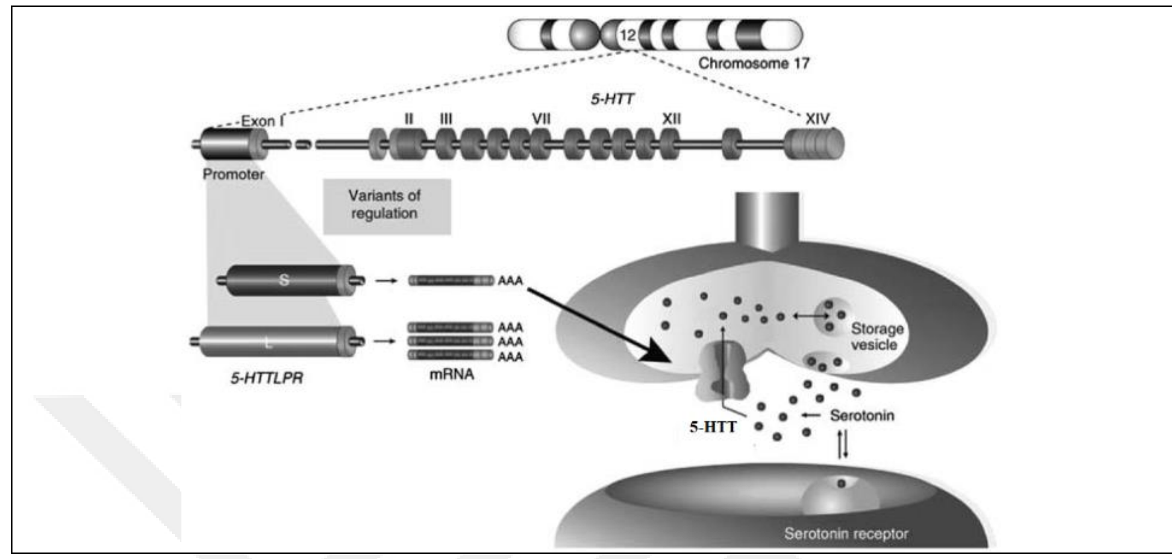


Figure 2.7 Represantation of L/S polymorhism of the 5-HTT and recycling of serotonin in neurons(47)

2.6.2. CYP2C19 Polymorphism on Escitalopram Treatment

The CYP2C19 enzyme has a role in the biotransformation of several of different classes of drugs such as the anticonvulsant S-mefenitoin, proton pump inhibitor omeprazole, antiplatelet clopidogrel, anxiolytic diazepam and serotonin reuptake inhibitor S-CT. CYP2C19 has also been claimed has a role in cyclophosphamide bioactivation. The polymorphic feature of CYP2C19 gene is high. More than 30 variant domains are thought to be linked to reduced, increased or unchanged enzyme activity. Polymorphisms in the CYP2C19 enzyme have been grouped with individuals with different enzyme activity rates, such as the PM, EM, IM and UM. The CYP2C19 * 1 allele is associated with functional CYP2C19. The alleles which cause the loss of function and are thought to cause the formation of PM phenotype are CYP2C19 * 2 (681G> A, rs4244285) and CYP2C19 * 3 (636G> A, rs4986893) (48,49). CYP2C19 * 2 is characterized by the formation of a defective splice site change of 681G → A in exon 5.

This results in incomplete nonfunction protein formation (50,51). As for CYP2C19 * 3, it results in the formation of a point mutation ending premature end codon in exon 4 (51,52). On the other hand, CYP2C19 * 17 alleles were mentioned to be associated with increased enzyme activity (UM) as a result of increased gene transcription due to -806C> T and -3402C> T variation (15,53,54). However, the severity of this effect is thought to be less than that of the * 2 and * 3 alleles (15,53).

The CYP2C19 polymorphism has a notable influence on the metabolism of escitalopram (55). Lately, it has been found out in a study that patients (n=166) having escitalopram treatment who carry the CYP2C19*17 allele (UM phenotype) displayed lower plasma escitalopram concentrations than the patients homozygous for CYP2C19*1 and highlighted the need of adjusting dose (29,56). It is likely that CYP2C19 PMs will exhibit poor tolerance to several TCAs metabolized by CYP2C19 as well as to citalopram, escitalopram and sertraline (32).

2.6.3. CYP2D6 Polymorphism on Escitalopram Treatment

CYP2D6 is quite influential on the the biotransformations of approximately 50 % of drugs in clinical use. Effectiveness and cost of drug treatment can be influenced by CYP2D6 polymorphism. The estimation is that predictive CYP2D6 genotyping will be helpful for around 30 % to 50 % of CYP2D6 drug substrates (57,58).

As there is no other non-inducible enzyme by drugs except CYP2D6, the state of genetic polymorphism of CYP2D6 has great contributions to enzyme activity. There is a variety in the CYP2D6 gene variants which have been identified and they can be categorized depending on whether to remove, reduce, leave normal, increase or change the catalytic activity qualitatively. Among ethnic groups, most well-known variants are CYP2D6*2, CYP2D6*3, CYP2D6*4, CYP2D6*5, CYP2D6*6, CYP2D6*10, CYP2D6*17, and CYP2D6*41(29). Whereas the CYP2D6*3, CYP2D6*4, CYP2D6*5, alleles are associated with non functional CYP2D6, the CYP2D6*10, and CYP2D6*41 alleles are associated with reduced functional CYP2D6 (59,60).

3. METHOD

Plasma and urine samples collected from patients using S-CT as part of their treatment in NPİstanbul Brain Hospital were analyzed by LC-MS / MS at Üsküdar University Clinical Pharmacogenetic Laboratory and the levels of S-CT, S-DCT and S-DDCT were measured. In addition, a genotyping study was performed from whole blood samples taken to identify CYP2C19 and pharmacodynamic (5-HTT) polymorphisms of the same patients and statistical evaluation was performed by ANOVA method by looking at the relationship between blood-urine level results and metabolic ratios of patients with their genotyping results. Among patients whose CYP2C19 polymorphisms were identified, those whose plasma levels were out of expected in comparison to their polymorphisms, were reevaluated as a result of studying their CYP2D6 polymorphisms.

3.1. Reference Standards and Chemicals

Escitalopram oxalate, desipramine hydrochloride and beta glucuronidase were purchased from Sigma (Sigma Aldrich, USA). Demethyl escitalopram hydrochloride, didemethyl escitalopram hydrochloride were purchased from LGC standards (LGC standards, UK). Also, HPLC grade methanol, HPLC grade acetonitrile, formic acid, ammonium formate, amonium acetate, dimethyl sulfoxide (DMSO) were purchased from Merck, USA. Genomic Lysis/Binding buffer, wash buffer 1, wah buffer 2, elution buffer, proteinase K, RNase A, forward and reverse primers (to and fro), Taq polymerase enzyme were purchased from Invitrogen, Germany. Master mix were purchased from Thermofisher, USA.

3.2. Sample Selection

Between the dates of June 1, 2017 and June 1, 2018, blood, plasma and urine samples of 30 patients (males and females) using 20 mg S-CT between the ages of 18-65 were analyzed in Üsküdar University Clinical Pharmacogenetic Laboratory with

approval of Üsküdar University Ethics Committee to determine the monitoring of S-CT and its metabolites and genetic polymorphism (Appendix-A). There are no drugs or foods that affect (inhibit or induce) the S-CT metabolic pathway in drug treatment of selected patients. Patients with liver or multiple organ failure, diabetes mellitus are excluded from the study. Samples were selected from patients sent to the laboratory within 12 months. Samples were taken 12 hours after the last dose to determine the plasma and urine drug levels of the patients' S-CT and its metabolites S-DCT, S-DDCT. The blood, plasma and urine samples sent to the laboratory are kept at -20 ^{0}C until analysis.

3.3. Validation Study for Quantitative Determination Method of Esitalopram (S-CT) and Its Metabolites (Demethyl escitalopram (S-DCT), Didemethyl escitalopram (S-DDCT)) in Plasma and Urine by LC-MS/MS

Quantitative determination methods have been developed for plasma and urine samples for S-CT and metabolites (S-DCT, S-DDCT) considering the publications of bioanalytical method validation, and in these validation studies, selectivity, carryover, lower limit of quantification (LOQ), calibration curve, accuracy and precision, matrix effect, dilution, re-injection, stability parameters were studied (61–65). There are chromatografic and MRM conditions in Table 3.2-3.5.

TABLE 3.1. Equipment List

Equipment	Brand name
LC-MS/MS	Agilent 6470
Column	ACE-3 C 8 (3 µm, 3.0 mm × 150 mm)
Centrifuge	Hettich Rotofix
Vortex	Heidolph
Scales	Mettler Toledo AL
Real Time Quantstudio 3	Thermofisher
Thermal Cycler T100	BIO-RAD
Fusion FX	Vilber Lourmat
Microfuge16 centrifuge	Beckman Coulter

TABLE 3.2. Chromatographic Conditions

Mobile Phase A	1000 ml deionized water: 4 ml formic acid: 0.4 ml 5M ammonium formate[‡]: 30 ml methanol
Mobil Phase B	500 ml methanol: 500 ml acetonitrile: 4 ml formic acid: 0.4 ml 5M ammonium formate
Column Temperature	45 C
Flow rate	0.5 mL / min.
Run time	8 min.
Retention time (RT)	S-CT = about 2.67 min, S-DCT = about 2.67 min, S-DDCT = about 2.63 min and desipramine (as internal standard (IS)) = about 3.92 min.
Optimum injection volume	5 µl

[‡] Preparation of 5 M ammonium formate: 3.15 grams of ammonium formate is dissolved by water in a 10 ml volumetric flask.

TABLE 3.3. Mobile Phase Conditions			
Time (min)	**Mobile Phase A**	**Mobile Phase-B**	**Flow (ml)**
0	50	50	0.5
2	50	50	0.5
2.10	10	90	0.5
5	10	90	0.5
5.10	50	50	0.5
8	50	50	0.5

TABLE 3.4. MRM Conditions					
Molecule	**Precursor Ion**	**Product Ion**	**Fragmentor Voltage**	**Collision Energy**	**Polarity**
S-CT (66)	325.1	109.1	140	30	ESI (+)
S-DCT (66)	311.2	109.1	140	30	
S-DDCT (66)	297.0	109.1	120	30	
Desipramine (IS)(66)	267.0	72.0	120	15	

TABLE 3.5. MS / MS Source Conditions	
Gas temperature	250 C
Gas flow	5 ml
Nebulizer	45psi
Sheath gas temperature	325 C
Sheath gas flow	11ml

3.3.1. Validation Studies in Plasma Samples

3.3.1.1. Plasma Sample Preparation Method

- For blood samples taken from patients, samples are centrifuged prior to analysis to separate the plasma fraction.
- For each 500 µL plasma sample (patient sample, calibration and quality control samples), by adding 100 µL IS desipramine working solution and 400 µL 4%

zinc sulfate solution, it is vortexed for 30 sec. and centrifuged at 15000 rpm for 8 minutes. The clear part is transferred to the system.

- To prepare a blank plasma sample, add 100 µL of deionized water and 400 µL of 4% zinc sulfate solution to a 500 µL blank plasma sample without analytes and it is vortexed for 30 seconds and centrifuged at 15000 rpm for 8 minutes. The clear part is transferred to the system.
- To prepare a zero plasma sample, add 500 µL of blank plasma without analytes to a 100 µL IS desipramine working solution and 400 µL of 4% zinc sulfate solution and it is vortexed for 30 sec. and centrifuged at 15000 rpm for 8 minutes. The clear part is transferred to the system.
- The samples are placed on the device in the automatic sampler as described in the work list (Table 3.8).
- Optimum amount (5 µL) is injected into the system.

3.3.1.2. Preparation of Solutions

Preparation of 4% Zinc Sulfate Solution

7.04 grams of zinc sulfate hepta hydrate is dissolved by 70 % methanol in a 100 ml volumetric flask.

Preparation of Reference Standard Stock and Dilue Stock Solution (DS)

Stock standard solutions were prepared by dissolving 12 mg of escitalopram oxalate, 1 mg of demethyl escitalopram hydrochloride and 1 mg of didemethyl escitalopram hydrochloride in methanol ($c_{s\text{-}ct}$: 0.92, $c_{s\text{-}dct}$: 0.89, $c_{s\text{-}ddct}$: 0.89 mg / mL). Then a diluted stock standard solution was prepared by taking 200 µl of each stock standard solution, diluting with methanol in a 50 ml volumetric flask ($c_{s\text{-}ct}$: 3.7, $c_{s\text{-}dct}$: 3.6, $c_{s\text{-}ddct}$: 3.6 µg / mL). The prepared solution was labeled and stored at -20 ° C (Table 3.6).

TABLE 3.6.Preparation of Reference Standard Stock and Dilue Stock Solution								
Molecule	Salt form	Molecule weight (free form)	Molecule weight (salt form)	Potens	Factor§	Weight (mg)	Stock Standard Concentration (mg/ml)	DS Concentration (µg / mL)
S-CT	Oxalate	324.39	414.43	0.98	1.30	12	0.92	3.7
S-DCT	HCl	309.33	345.79	1.00	1.12	1	0.89	3.6
S-DDCT	HCl	296.30	332.80	1.00	1.12	1	0.89	3.6

Preparation of Calibration Standard Solutions

To prepare eight calibration standards for S-CT, S-DCT and S-DDCT in the plasma, dilute stock standard solution was spiked in different volumes to the plasma (Table 3.7). Prepared solutions are labeled and stored at -70 ° C.

TABLE 3.7.Preparation of Calibration Standard and Quality Control Samples in Plasma (ng/ml)														
Molecule	DS	Std 1	Std 2	Std3	Std 4	Std 5	Std 6	Std 7	Std8	QC1	QC2	QC3	QC4	QC5
	(µg/mL)	40 µL DS	80 µL DS	200 µL DS	400 µL DS	800 µL DS	1.2 mL DS	2 mL DS	3 mL DS	40 µL DS	120 µL DS	1.5 mL DS	2.4 mL DS	3 mL DS
		25 mL	25 mL	25 mL	25 mL	25 mL	25 mL	25 mL	25 mL	25 mL	25 mL	25 mL	25 mL	25 mL
S-CT	3.7	5.9	11.8	29.5	58.9	117.8	176.7	294.6	441.8	5.9	17.7	220.9	353.5	441.8
S-DCT	3.6	5.7	11.4	28.5	57.1	114.2	171.2	285.4	428.1	5.7	17.1	214.0	342.5	428.1
S-DDCT	3.6	5.7	11.4	28.5	57.0	114.0	170.9	284.9	427.4	5.7	17.1	213.7	341.9	427.4

Preparation of Quality Control Samples.

To prepare five different quality control samples (QCs) for S-CT, S-DCT and S-DDCT in the plasma, dilute stock standard solution was spiked in different volumes to the plasma (Table 3.7). The quality control sample 1 (QC1) is at the same level as lowest limit of quantification (LOQ) standard concentration (Standard 1). The quality control sample 2 (QC2) is three times LOQ concentration. The quality control sample 3 (QC3) was prepared at the level corresponding to the medium concentration in the standard calibration curve. The quality control sample 4 (QC4) was prepared to be 80% of the highest standard (Standard 8) concentration. The quality control sample 5 (QC5)

§ Factor= Molecule weight $_{\text{salt form}}$/ Molecule weight $_{\text{free form}}$/Potens

is at the same level as the highest standard concentration (Standard 8). Prepared solutions are labeled and stored at -70 ° C.

TABLE 3.8. Work List of Batches[**]

Sample Analysis Order	Batch 1	Batch 2	Batch 3	Acceptance criteria
1.	Methanol	Methanol	Methanol	
2.	Blank	Blank	Blank	The area of the interference from the blank samples should be less than 20% of the analyte area at the LOQ level and 5% of the internal standard area.
3.	Zero	Zero	Zero	
4.	Std1	Std1	Std1	Should be within the ± 20% limit of the nominal value
5.	Std 2	Std 2	Std 2	Should be within the ± 15% limit of the nominal value.
6.	Std 3	Std 3	Std 3	
7.	Std 4	Std 4	Std 4	
8.	Std 5	Std 5	Std 5	
9.	Std 6	Std 6	Std 6	
10.	Std 7	Std 7	Std 7	
11.	Std 8	Std 8	Std 8	
12.	Blank	Blank	Blank	
13.	QC1_1	QC1_1	QC1_1	Should be within the ± 20% limit of the nominal value
14.	QC1_2	QC1_2	QC1_2	
15.	QC1_3	QC1_3	QC1_3	
16.	QC1_4	QC1_4	QC1_4	
17.	QC1_5	QC1_5	QC1_5	
18.	QC1_6	QC1_6	QC1_6	
19.	QC2_1	QC2_1	QC2_1	
20.	QC2_2	QC2_2	QC2_2	

[**] This wok list has been repeated three times (batch 1 (day 1), batch 2 (day 2),batch 3 (day 3)) at different times.

TABLE 3.8. Work List of Batches[††] (continued)				
Sample Analysis Order	Batch 1	Batch 2	Batch 3	Acceptance criteria
21.	QC2_3	QC2_3	QC2_3	Should be within the ± 15% limit of the nominal value
22.	QC2_4	QC2_4	QC2_4	
23.	QC2_5	QC2_5	QC2_5	
24.	QC2_6	QC2_6	QC2_6	
25.	QC3_1	QC3_1	QC3_1	
26.	QC3_2	QC3_2	QC3_2	
27.	QC3_3	QC3_3	QC3_3	
28.	QC3_4	QC3_4	QC3_4	
29.	QC3_5	QC3_5	QC3_5	
30.	QC3_6	QC3_6	QC3_6	
31.	QC4_1	QC4_1	QC4_1	
32.	QC4_2	QC4_2	QC4_2	
33.	QC4_3	QC4_3	QC4_3	
34.	QC4_4	QC4_4	QC4_4	
35.	QC4_5	QC4_5	QC4_5	
36.	QC4_6	QC4_6	QC4_6	
37.	QC5_1	QC5_1	QC5_1	
38.	QC5_2	QC5_2	QC5_2	
39.	QC5_3	QC5_3	QC5_3	
40.	QC5_4	QC5_4	QC5_4	
41.	QC5_5	QC5_5	QC5_5	
42.	QC5_6	QC5_6	QC5_6	
43.	Blank	Blank	Blank	

[††] This wok list has been repeated three times (batch 1 (day 1), batch 2 (day 2),batch 3 (day 3)) at different times.

Preparation of Desipramine Internal Standard Working Solution

11 mg Desipramine hydrochloride is dissolved by methanol in a 10 ml volumetricflask. This solution labelled as IS desipramine-STOCK is stored at -20 ° C. 0.5 mL of IS desipramine-STOCK is diluted by 10 % methanol in a 1000 mL volumetricflask (c : 550 µg / mL). This solution is called IS desipramine working solution and stored at + 4 ° C.

3.3.1.3. Selectivity and Carry Over Study

Plasma samples containing LOQ concentrations and blank plasma samples prepared from at least 6 different sources were analyzed for plasma sample selectivity studies. In blank samples, the area of any interference at the retention times of S-CT, S-DCT, S-DDCT and desipramine (IS) was compared to the area of analyte at LOQ level in plasma. The area of the interference from the blank samples should be less than 20% of the analyte area at the LOQ level and 5% of the internal standard area.
For carry-over study of plasma samples, blank plasma samples were injected after the plasma samples at the highest standard concentration. The area following carry over should be less than 20% of the analyte area at the LOQ level and 5% of the internal standard area.

3.3.1.4. Calibration Curve and Quality Control Samples

In the validation method for quantitative analysis in plasma samples, S-CT was studied in the range of 5.9 - 441.80 ng / mL. Eight calibration standards (5.9, 11.8, 29.5, 58.9, 117.8, 176.7, 294.6, 441.8 ng / mL) and five quality control samples (5.9, 17.7, 220.9, 353.5, 441.8 ng / mL) were used for the validation study. The calibration range for S-DCT is 5.7 to 428.1 ng / mL. Eight calibration standards (5.7, 11.4, 28.5, 57.1, 114.2, 171.2, 285.4, 428.1 ng / ml) and five quality control samples (5.7, 17.1, 214.0, 342.5, 428.1 ng / mL) were used for the validation study. The calibration range for S-DDCT is 5.7 to 427.4 ng / mL. Eight calibration standards (5.7, 11.4, 28.5, 57.0,

114.0, 170.9, 284.9, 427.4 ng / ml) and five quality control samples (5.7, 17.1, 213.7, 341.9, 427.4 ng / mL) were used for the validation study. The concentration of escitalopram and its metabolites in plasma was calculated at eight calibration levels and compared with the most appropriate curve for calibration standards. Regression algorithm is 1/X. The amount of escitalopram and its metabolites in the quality control samples was calculated by means of the least squares method using the linear regression (the ratio of analyte area to internal standard area) by Agilent 6470 Software.

3.3.1.5. Accuracy and Precision

Accuracy is the closeness of the nominal value of the calculated value from the quality control samples as a result of the analysis and expressed as a percentage (%). The random error of the accuracy is expressed as precision. The coefficient of variation (CV) shows the value for precision.

Data obtained by analyzing QC samples and calibration standards are used to obtain the value of accuracy and precision. Accuracy is obtained by repeated analysis of the sample containing the known amount of analyte. Percent accuracy is obtained by dividing the mean of a QC concentration by the nominal concentration of that QC and multiplying by 100. Accuracy and precision are assessed in two ways: Within (intra) batch and between (inter) batch. In within (intra) batch analysis, six samples from QCs at each of five different concentration levels were prepared and analyzed. Between (inter) batch was obtained by calculating the results of three within batch analyzes that were run at different times. For the validity of accuracy and precision, the mean value of concentrations except the LOQ should be within ± 15% of the nominal value. For LOQ, this value is ± 20%

$$\text{Mean Accuracy (\%)} = (C_{mean}*100)/C_{nominal}$$

$$\text{CV (\%)} = (SD*100)/\ C_{nominal}$$

Quality control samples were analyzed 18 times at different times in the validation study for the quantitative determination of escitalopram and its metabolites in plasma. The results were evaluated according to the accuracy and precision limits given in literature.

3.3.1.6. Matrix Effect

The matrix effect in publications related to EMA 2011 and bioanalytical method studies has been reported to be determined by the CV value of the normalized internal standard (IS) matrix factor, which is calculated by analysis of different concentrations of quality control samples in matrix (plasma, urine, etc.) and absence of matrix (methanol, water, etc.), should be considered. normalize IS matrix factor should be less than 15% of the CV according to criteria.

For the matrix effect study, six samples of low (QC2) and high quality control samples (QC5) prepared in the plasma were analyzed with six samples prepared in methanol at the same concentration levels (Table 3.9). From the samples of QC2 and QC5 prepared in plasma and methanol, 500 µl was taken from each and six samples were analyzed for each sample. 100 µl of IS and 400 µl of zinc sulfate were added to these samples and vortexed for 30 seconds, and centrifuged at 15000 rpm for 8 minutes. The clear part was injected into the system by 5 µl.

TABLE 3.9. Plasma-Matrix Effect Work List

Number	Sample Type	Acceptance Criteria
1.	Methanol	
2.	Blank	
3.	Zero	
4.	Std1	should be within ± 20% of nominal value
5.	Std2	should be within ± 15% of nominal value
6.	Std3	
7.	Std4	
8.	Std6	
9.	Std7	
10.	Std8	

TABLE 3.9. Plasma-Matrix Effect Work List (continued)		
Number	Sample Type	Acceptance Criteria
11.	plasma_QC2_1	Normalized internal standard (IS) MF CV should be <15%
12.	plasma_QC2_2	
13.	plasma _QC2_3	
14.	plasma _QC2_4	
15.	plasma _QC2_5	
16.	plasma _QC2_6	
17.	plasma _QC5_1	
18.	plasma _QC5_2	
19.	plasma _QC5_3	
20.	plasma _QC5_4	
21.	plasma _QC5_5	
22.	plasma _QC5_6	
23.	methanol_QC2_1	
24.	methanol_QC2_2	
25.	methanol_QC2_3	
26.	methanol_QC2_4	
27.	methanol_QC2_5	
28.	methanol_QC2_6	
29.	methanol_QC5_1	
30.	methanol_QC5_2	
31.	methanol_QC5_3	
32.	methanol_QC5_4	
33.	methanol_QC5_5	
34.	methanol_QC5_6	

For the matrix effect calculation, the analyte matrix factor (MF) was found through first proportioning the analyte peak area in the plasma to the peak area in the methanol (absence of matrix). The internal standard (IS) matrix factor of desipramine was then calculated using the same calculation process. Finally, by dividing the MF of the analytes to the mean MF of the IS, normalized internal standard (IS) MF was found. For the validity of the study, it has been indicated in the bioanalytical method validation publications that the calculated IS normalize matrix factor should be less than 15% of the CV.

3.3.1.7. Lower Limit of Quantification (LOQ) and Sensitivity

The measured value of the lowest concentration samples prepared in plasma was compared with the nominal value to determine the accuracy and reproducible LOQ and sensitivity of the method.
For the method sensitivity, the precision and accuracy of the samples at the LOQ level of escitalopram and its metabolites were found in the acceptable range (± 20%).

3.3.1.8. Reenjection

For the re-injection study, third validation batch was re-injected into the system on the same day following the third validation batch.

3.3.1.9. Dilution

The sample was prepared in plasma at a concentration of two times the highest concentration standard (standard 8) containing escitalopram and its metabolites. 250 µL blank plasma was added to 250 µL standard sample to apply the ½ dilution procedure to the prepared sample. The resulting 500 µL diluted plasma sample was prepared according to the plasma sample preparation method and injected to the device together with freshly prepared standard and quality control samples. The comparison of results with the nominal concentration was made after the dilution correction.

3.3.1.10. Stability

In plasma samples, autosampler stability, short time room temperature and thaw-freeze stability were studied.

Auto Sampler Stability:

Six samples from both low and high concentrations of quality control samples left in the autosampler for 24 hours were analyzed with freshly prepared standards. Then, the results were evaluated at calibration standard curve.

Short Time Room Temperature Stability

Six samples from both low and high concentrations of quality control samples thawed in the room temperature and kept for 24 hours were analyzed.

Freeze and Thaw Stability

The quality control samples prepared for freeze-thaw stability were three times frozen and thawed. After the quality control samples prepared for the validation were kept at least 12 hours under appropriate storage conditions, these samples were thawed at room temperature. This process was repeated 3 times by freeze-thaw. Following freeze-thaw, the plasma sample preparation procedure was applied to the quality control samples, taking samples in the volume required for quantitative analysis. Frozen and thawed QC2 and QC5 samples were evaluated on a calibration curve prepared with freshly prepared calibration standards.

3.3.2. Validation Studies in Urine Samples

3.3.2.1. Urine Sample Preparation Method

- For urine samples taken from patients, the clear part is used by centrifuging the samples prior to analysis.
- Add 150 µL of beta glucuronidase solution to each 500 µL urine sample (patient sample, calibration and quality control samples), and hydrolyze at 70 ° C for an

hour. By adding 100 μL IS desipramine working solution and 100 μL cold acetonitrile to the hydrolyzed samples, it is vortexed for 30 sec. and centrifuged at 15000 rpm for 8 minutes. The clear part is transferred to the viels.

- To prepare a blank urine sample, add 150 μL of beta glucuronidase solution to 500 μL urine without any analytes and hydrolyze at 70 ° C for 1 hour. By adding 100 μL of deionized water and 100 μL of cold acetonitrile to hydrolyzed blank urine sample, it is vortexed for 30 seconds and centrifuged at 15000 rpm for 8 minutes. The clear part is transferred to the viels.
- To prepare a zero urine sample, add 150 μL of beta glucuronidase solution to 500 μL urine without any analytes and hydrolyze at 70 ° C for 1 hour. By adding 100 μL IS desipramine working solution and 100 μL of cold acetonitrile to hydrolyzed zero urine sample, it is vortexed for 30 seconds and centrifuged at 15000 rpm for 8 minutes. The clear part is transferred to the viels.
- The samples are placed on the device in the automatic sampler as described in the working list (Table 3.8).
- The optimum amount (5 μL) is injected into the system.

3.3.2.2. Preparation of Solutions

Preparation of Ammonium Acetate Buffer (pH = 5.0) Solvent

0.77 g of ammonium acetate is dissolved by water in a 100 mL volumetricflask. It is adjusted to pH 5 with acetic acid.

Preparation of Beta Glucuronidase Solvent (5000 Fish U)

350 mg beta glucuronidase is dissolved by 100 mM ammonium acetate buffer in a 100 mL volumetric flask.
Note: 1 g of beta glucuronidase enzyme contains 1439000 Fish U.

Preparation of Calibration Standard Solvents

To prepare eight calibration standards for S-CT, S-DCT and S-DDCT in the urine, dilute stock standard solution was spiked in different volumes to the urine (Table 3.10). Prepared solutions are labeled and stored at -70 ° C.

TABLE 3.10.Preparation of Calibration Standard and Quality Control Samples in Urine (ng/mL)

Molecule	DS	Std 1	Std 2	Std3	Std 4	Std 5	Std 6	Std 7	Std8	QC1	QC2	QC3	QC4	QC5
	(µg/mL)	15 µL DS	30 µL DS	80 µL DS	160 µL DS	320 µL DS	480 µL DS	800 µL DS	1200 µL DS	15 µL DS	45 µL DS	600 µL DS	960 µL DS	1200 µL DS
		10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL
S-CT	3.7	5.5	11.0	29.5	58.9	117.8	176.7	294.6	441.8	5.9	16.6	220.9	353.5	441.8
S-DCT	3.6	5.4	10.7	28.5	57.1	114.2	171.2	285.4	428.1	5.7	16.1	214.0	342.5	428.1
S-DDCT	3.6	5.3	10.7	28.5	57.0	114.0	170.9	284.9	427.4	5.9	16.0	213.7	341.9	427.4

Preparation of Quality Control Samples

To prepare five different quality control samples (QCs) for S-CT, S-DCT and S-DDCT in the urine, dilute stock standard solution was spiked in different volumes to the urine (Table 3.10). Prepared solutions are labeled and stored at -70 ° C.

3.3.2.3. Selectivity and Carry Over Study

Urine sample containing LOQ concentration and blank urine samples prepared from at least 6 different sources were analyzed for urine sample selectivity studies. In blank samples, the area of any interference at the retention times of S-CT, S-DCT, S-DDCT and desipramine (IS) molecules (analytes) was compared to the area of analyte at LOQ level in urine. The area of the interference from the blank samples should be less than 20% of the analyte area at the LOQ level and 5% of the internal standard area.

For carry-over study of urine samples, blank urine samples were injected after the urine sample at the highest standard concentration. The area following carry over should be less than 20% of the analyte area at the LOQ level and 5% of the internal standard area.

3.3.2.4. Calibration Curve and Quality Control Samples

In the validation method for quantitative analysis in urine samples, S-CT was studied in the range of 5.5 - 441.80 ng / mL. Eight calibration standards (5.5, 11.0, 29.5, 58.9, 117.8, 176.7, 294.6, 441.8 ng / mL) and five quality control samples (5.5, 16.6, 220.9, 353.5, 441.8 ng / mL) were used for the validation study. The calibration range for S-DCT is 5.4 to 428.1 ng / mL ng / mL. Eight calibration standards (5.4, 10.7, 28.5, 57.1, 114.2, 171.2, 285.4, 428.1 ng / mL) and five quality control samples (5.4, 16.1, 214.0, 342.5, 428.1 ng / mL) were used for the validation study. The calibration range for S-DDCT is 5.3 to 427.4 ng / mL. Eight calibration standards (5.3, 10.7, 28.5, 57.0, 114.0, 170.9, 284.9, 427.4 ng / mL) and five quality control samples (5.3, 16.0, 213.7, 341.9, 427.4 ng / mL) were used for the validation study. The concentration of S-CT and its metabolites in urine was calculated at eight calibration levels and compared with the most appropriate curve for calibration standards. Regression algorithm is 1/X. The amount of S-CT and its metabolites in the quality control samples was calculated by means of the least squares method using the linear regression (the ratio of analyte area to internal standard area) by Agilent 6470 Software.

3.3.2.5. Accuracy and Precision

Accuracy is the closeness of the nominal value of the calculated value from the quality control samples as a result of the analysis and expressed as a percentage (%). The random error of the accuracy is expressed as precision. The coefficient of variation (CV) shows the value for precision.

Data obtained by analyzing QC samples and calibration standards are used to obtain the value of accuracy and precision. Accuracy is obtained by repeated analysis of the sample containing the known amount of analyte. Percent accuracy is obtained by dividing the mean of a QC concentration by the nominal concentration of that QC and multiplying by 100. Accuracy and precision are assessed in two ways: Within (intra) batch and between (inter) batch. In within (intra) batch analysis, six samples from QCs at each of five different concentration levels were prepared and analyzed. Between

(inter) batch was obtained by calculating the results of three within batch analyzes that were run at different times. For the validity of accuracy and precision, the mean value of concentrations except the LOQ should be within ± 15% of the nominal value. For LOQ, this value is ± 20%

$$\text{Mean Accuracy (\%)} = (C_{mean}*100)/C_{nominal}$$

$$\text{CV (\%)} = (SD*100)/ C_{nominal}$$

Quality control samples were analyzed 18 times at different times in the validation study for the quantitative determination of escitalopram and its metabolites in urine. The results were evaluated according to the accuracy and precision limits given in literature.

3.3.2.6. Matrix Effect

The matrix effect in publications related to EMA 2011 and bioanalytical method studies has been reported to be determined by the CV value of the normalized internal standard (IS) matrix factor, which is calculated by analysis of different concentrations of quality control samples in matrix (plasma, urine, etc.) and absence of matrix (methanol, water, etc.), should be considered. normalize IS matrix factor should be less than 15% of the CV according to criteria.

For the matrix effect study, six samples of low (QC2) and high quality control samples (QC5) prepared in the urine were analyzed with six samples prepared in methanol at the same concentration levels (Table 3.11). From the samples QC2 and QC5 prepared in urine and methanol, 500 µl was taken from each and six samples were analyzed for each sample. 150 µL of beta glucuronidase solution is added to these samples, and allowed to hydrolyze at 70 ° C for 1 hour. 100 µL IS desipramine working solution and 100 µL cold acetonitrile are added to the hydrolyzed samples and vortexed for 30 sec. and centrifuged at 15000 rpm for 8 minutes.

TABLE 3.11.Urine- Matrix Effect Work List		
Number	Sample Type	Acceptance Criteria
1.	Methanol	
2.	Blank	
3.	Zero	
4.	Std1	Should be within ± 20% of nominal value
5.	Std2	Should be within ± 15% of nominal value
6.	Std3	
7.	Std4	
8.	Std6	
9.	Std7	
10.	Std8	
11.	Urine_QC2_1	Normalized IS matrix factor CV should be <15%
12.	Urine _QC2_2	
13.	Urine _QC2_3	
14.	Urine _QC2_4	
15.	Urine _QC2_5	
16.	Urine _QC2_6	
17.	Urine _QC5_1	
18.	Urine _QC5_2	
19.	Urine _QC5_3	
20.	Urine _QC5_4	
21.	Urine _QC5_5	
22.	Urine _QC5_6	

TABLE 3.11.Urine- Matrix Effect Work List (continued)		
Number	Sample Type	Acceptance Criteria
23.	Methanol_QC2_1	Normalized IS matrix factor CV should be <15%
24.	Methanol _QC2_2	
25.	Methanol _QC2_3	
26.	Methanol _QC2_4	
27.	Methanol _QC2_5	
28.	Methanol _QC2_6	
29.	Methanol _QC5_1	
30.	Methanol _QC5_2	
31.	Methanol _QC5_3	
32.	Methanol _QC5_4	
33.	Methanol _QC5_5	
34.	Methanol _QC5_6	

For the matrix effect calculation, the analyte matrix factor (MF) was found through first proportioning the analyte peak area in the urine to the peak area in the methanol (absence of matrix). The internal standard (IS) matrix factor of desipramine was then calculated using the same calculation process. Finally, by dividing the MF of the analytes to the mean MF of the IS, normalized internal standard (IS) MF was found. For the validity of the study, it has been indicated in the bioanalytical method validation publications that the calculated IS normalize matrix factor should be less than 15% of the CV.

3.3.2.7. Lower Limit of Quantification (LOQ) and Sensitivity

The measured value of the lowest concentration samples prepared in urine was compared with the nominal value to determine the accuracy and reproducible LOQ and sensitivity of the method.

For the method sensitivity, the precision and accuracy of the samples at the LOQ level of escitalopram and its metabolites were found in the acceptable range (± 20%).

3.3.2.8. Re-injection

For the re-injection study, third validation batch was re-injected into the system on the same day following the third validation batch.

3.3.2.9. Dilution

The sample was prepared in urine at a concentration of two times the highest concentration standard (standard 8) containing escitalopram and its metabolites. 250 µL blank urine was added to 250 µL standard sample to apply the ½ dilution procedure to the prepared sample. The resulting 500 µL diluted urine sample was prepared according to the urine sample preparation method and injected to the device together with freshly prepared standard and quality control samples. The comparison of results with the nominal concentration was made after the dilution correction.

3.3.2.10. Stability

In urine samples, autosampler stability, short time room temperature and thaw-freeze stability were studied.

Auto Sampler Stability:

Six samples from both low and high concentrations of quality control samples left in the autosampler for 24 hours were analyzed with freshly prepared standards. Then, the results were evaluated at calibration standard curve.

Short Time Room Temperature Stability

Six samples from both low and high concentrations of quality control samples thawed in the room temperature and kept for 24 hours were analyzed.

Freeze and Thaw Stability

The quality control samples prepared for freeze-thaw stability were 3 times frozen and thawed. After the quality control samples prepared for the validation were kept at least 12 hours under appropriate storage conditions, these samples were thawed at room temperature. This process was repeated 3 times by freeze-thaw. Following freeze-thaw, the urine sample preparation procedure was applied to the quality control samples, taking samples in the volume required for quantitative analysis. Frozen and thawed QC2 and QC5 samples were evaluated on a calibration curve prepared with freshly prepared calibration standards.

3.3.3. TDM Analysis of Patient Samples

Plasma. 100 µL of internal standard desipramine working solution (c: 550 µg / mL) and 400 µL 4% zinc sulfate solution were added to the 500 µL plasma sample and further vortexed for 30 sec. and centrifuged at 15000 rpm for 5 min. 5 µL from the clear part was injected into the system.

Urine. 100 µL of beta glucuronidase solution (5000 Fish U) was added to 500 µL urine sample, and it was left to hydrolyze at 65 ° C for 1 hour. 100 µL of interanal standard desipramine working solution (c: 550 µg / mL) and 100 µL of cold acetonitrile were added to the hydrolyzed samples and further vortexed for 30 sec. and centrifuged at 15000 rpm for 5 min. 5 µL was injected into the system.

3.3.4. Genotyping of Patient Samples

3.3.4.1. DNA Isolation

Isolation- Pre-processing

- The heating block was heated to 56 ° C.
- The samples were brought to room temperature.

DNA Isolation from Blood

DNA isolation from the blood was done with Invitrogen (Germany) in accordance with the manufacturer's protocol.

- Invitrogen Kit DNA isolation protocol steps

- 20 µl Proteinase K was added to the 1.5 ml eppendorf tube.
- 20 µl RNase A was added to the tube.
- 200 µl blood was transfered to tube.
- 200 µl BL buffer (binding buffer) was added to the tube and vortexed.
- It was kept in a water bath at 56 ° C for 10 min.
- 200 µl ethanol was added and vortexed.
- The supernatant was transferred to the SV column tube.
- It was centrifuged at 6000 xg for 1 min. The filtrate was discarded and the colon was placed in the recipient tube (orthicon) again.
- 500 µl wash buffer (BW 1) was added, centrifuged at 6000 xg for 1 min, filtrate was discarded, and the column was inserted back into the recipient tube (orthicon).
- 500 µl wash buffer (BW 2) was added, centrifuged at 6000 xg for 1 min. The filtrate was discarded, the spin column was placed in the eppendorf tube.
- 80 µl elution buffer was added, incubated for 1 min at room temperature, then centrifuged at 13000 xg for 1 min.
- After centrifugation, the spin column was removed and discarded. Thus, DNA was transfered into the eppendorf tube.

3.3.4.2. Real-Time Polymerase Chain Reaction (Q-PCR)

As shown, the necessary solution was prepared for the amplification of CYP2C19 * 1, * 2, * 3, * 17 Allel sites and CYP2D6*1,*2,*4,*10,*41 by the Q-PCR method. For the CYP2C19 and CYP2D6 Allelic region, the reaction mixture in a volume of 10 µl is given in the Table 3.12.

TABLE 3.12. Procedure of Real-Time Polymerase Chain Reaction (Q-PCR)	
The content of reaction	Amount (µl)
Sterile water	4,2
Master Mix	5
Assays	0,3
Mold DNA	0,5
Toplam	10

These procedures were performed in 0.5 ml ependorf tubes and the tubes were placed in a Real Time instrument and the determined pograms were applied (Table 3.13).

TABLE 3.13. CYP2C19 and CYP2D6 Allel Regions and Polymorphism Contex Sequence in Program			
Allel region	ATA	Alele	Polymorphism contex sequence
CYP2C19*2 (rs4244285)	G	G/A	TTCCCACTATCATTGATTATTTCCC[A/G]GGAACCCATAACAAATTACTTAAAA
CYP2C19*3 (rs4986893)	G	G/A	ACATCAGGATTGTAAGCACCCCCTG[A/G]ATCCAGGTAAGGCCAAGTTTTTTGC
CYP2C19*17 (rs12248560)	C	C/T	AAATTTGTGTCTTCTGTTCTCAAAG[C/T]ATCTCTGATGTAAGAGATAATGCGC
CYP2D6*2 (rs16947)	G	G/A	GAGAACAGGTCAGCCACCACTATGC[A/G]CAGGTTCTCATCATTGAAGCTGCTC
CYP2D6*4 (rs3892097)	G/C	C/T	AGACCGTTGGGGCGAAAGGGGCGTC[C/T]TGGGGGTGGGAGATGCGGGTAAGGG
CYP2D6*10 (rs1065852)	G	G/A	CCGGGCAGTGGCAGGGGGCCTGGTG[A/G]GTAGCGTGCAGCCCAGCGTTGGCGC
CYP2D6*41 (rs28371725)	C	C/T	TTCATGGGCCCCCGCCTGTACCCTT[C/T]CTCCCTCGGCCCCTGCACTGTTTCC

RT program for CYP2C19 and CYP2D6 alleles:

Hold at 95 ° C for 600 sec

at 95 ° C for 15 sec... ... ... (2-Step Amplification)

at 60 ° C for 90 sec... ... ... (2-Step Amplification)

This is done according to the order of the processes in the program. The results of CYP2C19 and CYP2D6 Allel regions were analyzed in RT-PCR device.

3.3.4.3. Polymerase Chain Reaction (PCR)

The solution required for the amplification of the 5-HTT region was prepared by the PCR method as shown in the Table 3.14.

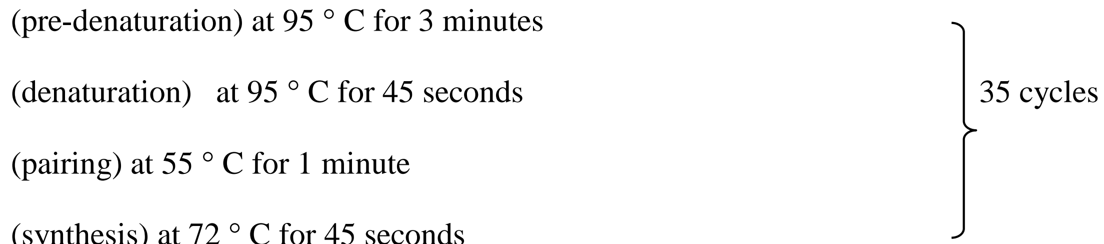
TABLE 3.14. Procedure of Polymerase Chain Reaction (PCR)

The content of reaction	Amount(µl)
Sterile water	27,2
$MgCl_2$	1
dNTP mixture	1
Buffer	5
Forward and reverse primers (to and fro)	1,5 x 1,5
Taq polymerase enzyme	0.8
Mold DNA	10
DMSO	2
Total	50

3.3.4.3.A. 5-HTT- PCR Protocol

These procedures were performed in 0.5 ml eppendorf tubes and the program was run by placing the tubes in the heat cycle device.

As the PCR loop / cycle program for the 5-HTT region :



(pre-denaturation) at 95 ° C for 3 minutes

(denaturation) at 95 ° C for 45 seconds

(pairing) at 55 ° C for 1 minute

(synthesis) at 72 ° C for 45 seconds

35 cycles

It was performed as a final extension at 72 ° C for 7 minutes.

Amplicons for the 5-HTT region, obtained after the polymerase chain reaction, were examined by 2% agarose gel electrophoresis.

3.3.4.3.B. Agarose Gel Electrophoresis

5-HTT polymorphism is studied by using the primers F5'-TCCCAGCAACTCCCTGTA-3' and R5'-GGAATACTGGTAGGGTGCAA-3'. A 2% agarose gel was prepared to identify the products amplified by PCR. For this, 0.7 g of agarose was dissolved in 35 ml of 1X TAE and boiled in a microwave oven. 2 μg / ml ethidium bromide (EtBr) was added to allow the DNA to be visualized under UV light. The appropriate comb was placed on the gel mold to form a sufficient number of wells and the gel was poured into mold until the polymer was thoroughly polymerized. 10 μl of PCR products were loaded into the wells with mixing with 2 μl loading buffer. Electrophoresis was applied at 100 V / 40 mA. Approximately 30 minutes later, PCR bands that emit radiation through the etidium bromide under UV in the examined gel were observed and compared to the standard markers. Whereas, L allele gave rise to 528 bp and S allele had 484 bp in the literatüre (47,67), in our study the method have been developed to give rise to 317 bp (L allele) and 272 bp (S allele).

4. RESULTS

4.1. Results of Analytical Method Validation in Plasma

4.1.1. Selectivity and Carry Over Study

When the chromatograms of blank samples prepared from six different plasmas were compared with the chromatograms of samples at LOQ level, it was found that the peak area of the interferences of the analytes (SCT, S-DCT, S-DDCT) at the retention time in the blank samples was less than 20% of the analyte peak area in the LOQ sample (Figure 4.1-4.3). In the same blank samples, the peak area of the interferences of the desipramine (as IS) at the retention time in the blank samples was found to be less than 5% of the desipramine peak area in the LOQ samples (Figure 4.4).

In publications on bioanalytical method validation, it is reported that the areas of the interference from the blank samples should be less than 20% of the analyte areas at the LOQ level and 5% of the internal standard area for selectivity. Considering the selectivity findings of the developed method, it is observed that it meets the criteria in related publications (Table 4.1).

For the carry over study; it was found that the area of the interferences of the analytes (SCT, S-DCT, S-DDCT) at the retention time in the injected blank sample after the highest concentration calibration standard was found to be less than 20% of the analyte peak area and less than 5% of the desipramine area in the LOQ samples. It has been observed that the analysis results meet the carryover criteria in the literature.

TABLE 4.1. S-CT, S-DCT, S-DDCT Selectivity Study in Plasma Samples									
Sample	S-CT		S-DCT		S-DDCT		Desipramine (IS)		Acceptance Criteria
	RT (min)	Area	RT (min)	Area	RT	Area	RT (min)	Area	
Blank 1	2.69	3824	2.68	-	2.63	103	3.91	-	The areas of the interference from the blank samples should be less than 20% of the analyte areas at the LOQ level and 5% of the internal standard area
Blank 2		4037		-		-		-	
Blank 3		4015		-		-		-	
Blank 4		3745		-		-		-	
Blank 5		3539		-		264		-	
Blank 6		3448		-		-		-	
Standart 1 (at LOQ concetration)		590397		65579		3496		25780469	

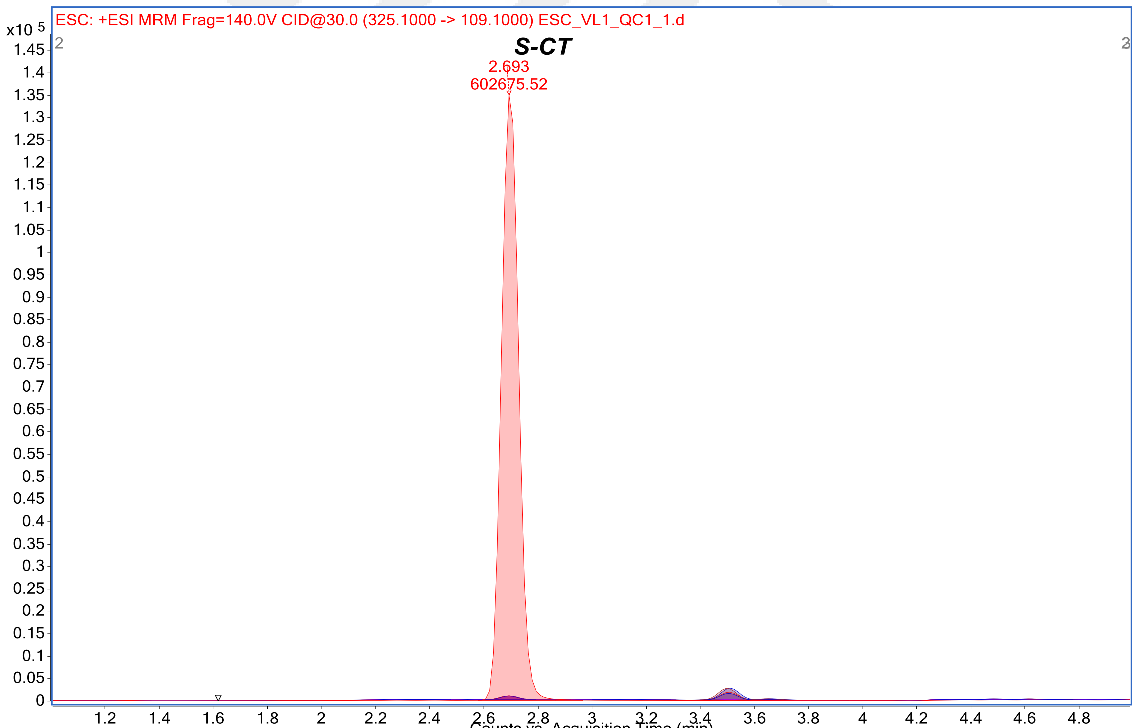


Figure 4.1. Represantation of S-CT chromatogram in plasma. Overlap of MRM chromatograms of six blank plasma samples without internal standard and without analyte with MRM chromotograms of S-CT at LOQ concentration in plasma (S-CT; m/z: 325.1>109.1; retention time (RT): 2.69 min.)

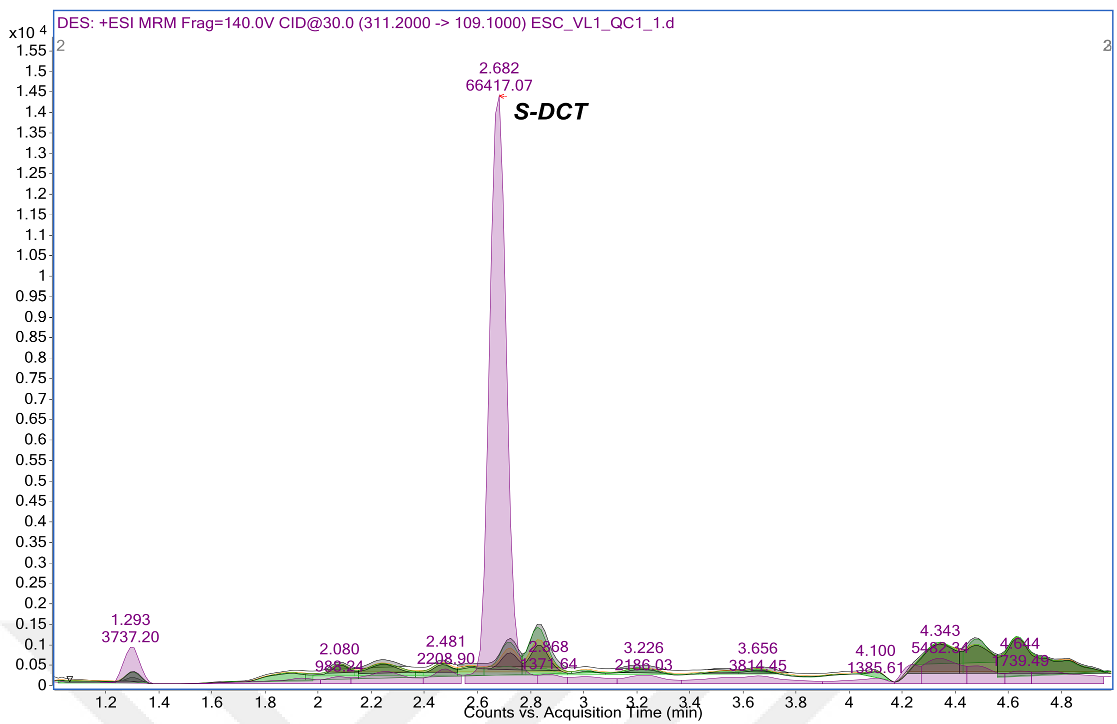


Figure 4.2 Represantation of S-DCT chromatogram in plasma. Overlap of MRM chromotograms of six blank plasma samples without internal standard and without analyte with MRM chromotograms of S-DCT at LOQ concentration in plasma (S-DCT; m/z: 311.2> 109.1; retention time (RT): 2.68 min.)

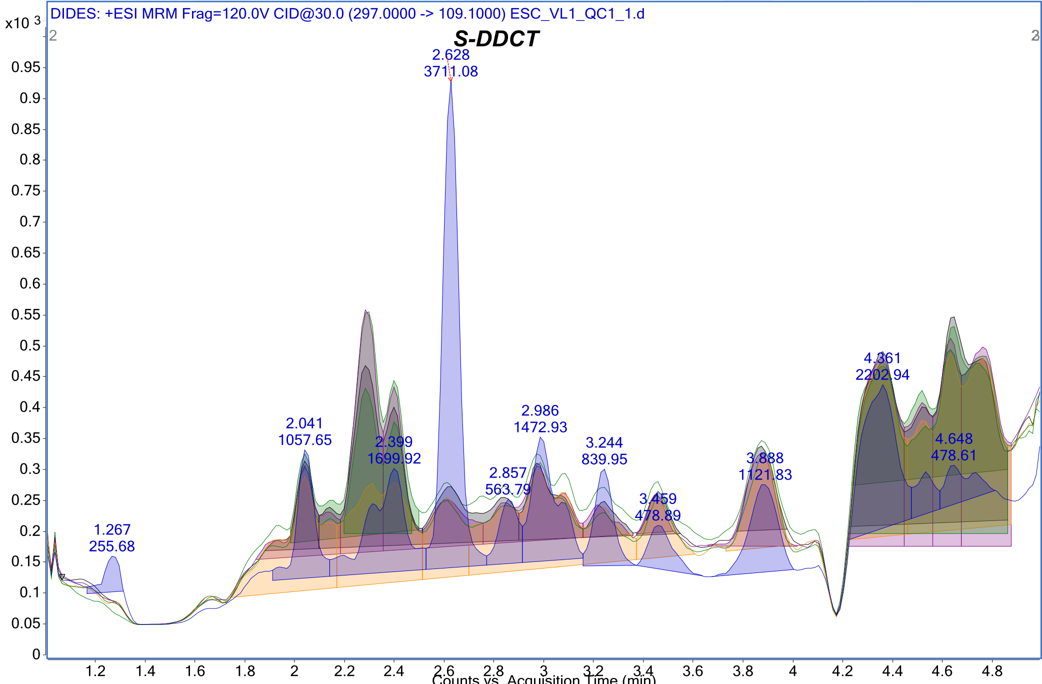


Figure 4.3. Represantation of S-DDCT chromatogram in plasma. Overlap of MRM chromotograms of six blank plasma samples without internal standard and without analyte with MRM chromatograms of S-DDCT at LOQ concentration in plasma (S-DDCT; m/z:297.0> 109.1; retention time (RT): 2.63 min.)

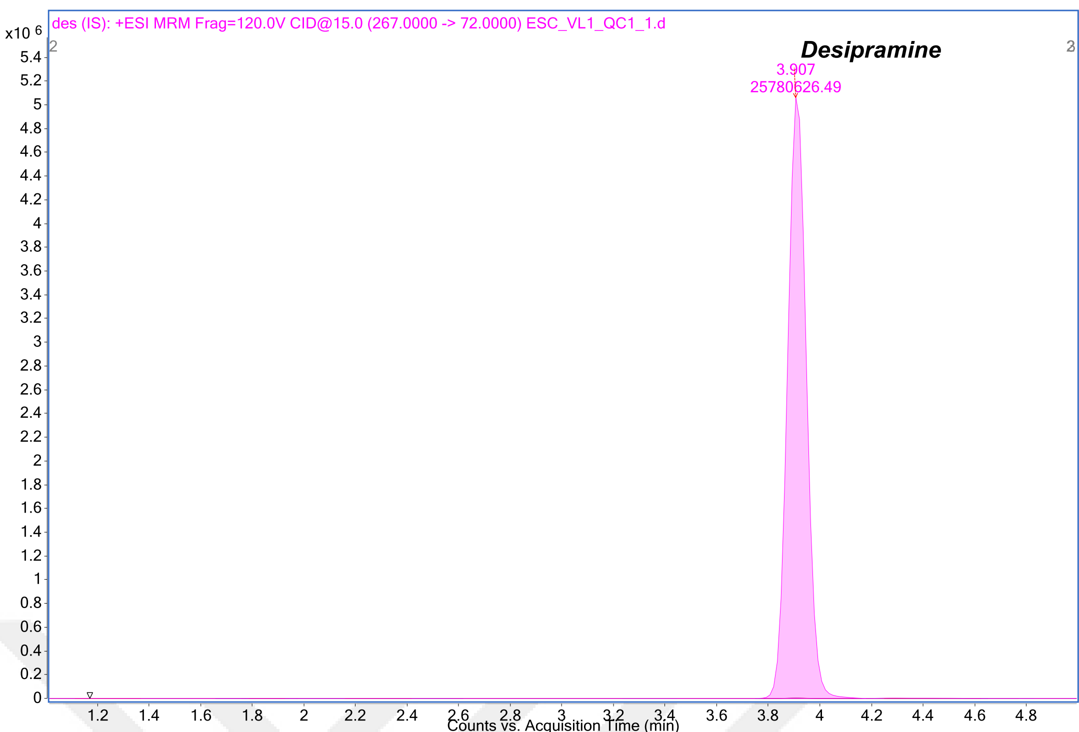


Figure 4.4 Represantation of desipramine chromatogram in plasma. Overlap of MRM chromotograms of six blank plasma samples without internal standard and without analyte with MRM chromotograms of desipramine at LOQ concentration in plasma (Desipramine (m/z: 267> 72), retention time (RT): 3.91 min.).

4.1.2. Calibration Curve and Quality Control Samples

Eight calibration standards and five quality control samples were prepared for the calibration curve. The ranges of calibration curve for the quantitative analysis of S-CT, S-DCT and S-DDCT in plasma samples are 5.9 - 441.8 ng / mL, 5.7 – 428.1 ng / mL and 5.7 – 427.4, respectively.

It has been reported in bioanalytical method validation publications that in order for calibration standards to be included in the calculation, the lowest standard concentration should be ± 20% of the lowest standard nominal value, and ± 15% of the nominal standard value of the other standard concentrations. It has also been reported that 75% of the standards for each calibration curve should met the criteria(61,64). The results met the criteria reported in bioanalytical method validation publications. Calibration curve r^2 values of escitalopram and metabolites during the validation period are given in Table 4.2.

TABLE 4.2. Calibration Curve r^2 Values of Esccitalopram and Metabolites During The Validation in Plasma		
Batch Number	Molecule	Calibration corelation coefficient (r^2)
Batch 1	S-CT	0.9916
	S-DCT	0.9929
	S-DDCT	0.9980
Batch 2	S-CT	0.9960
	S-DCT	0.9944
	S-DDCT	0.9969
Batch 3	S-CT	0.9963
	S-DCT	0.9944
	S-DDCT	0.9989

Calibration standards were analyzed as freshly prepared during validation process. Five calibration standards including one of 29.5 ng / ml for S-CT, one of 11.4 ng / ml, one 28.5 ng / ml, one of 285.4 ng / ml for S-DCT and one of 284.9 ng / ml for S-DDCT in three calibration curve during validation process were not included in the statistical calculations because the calibration standards did not meet the required criteria (± 15%) (Figure 4.5). The results included in the calculation meet the criteria for the validation study reported in the publications. Besides, it has been found that all of the quality control samples calculated by considering the calibration curve in validation study for each molecule meet the required criteria.

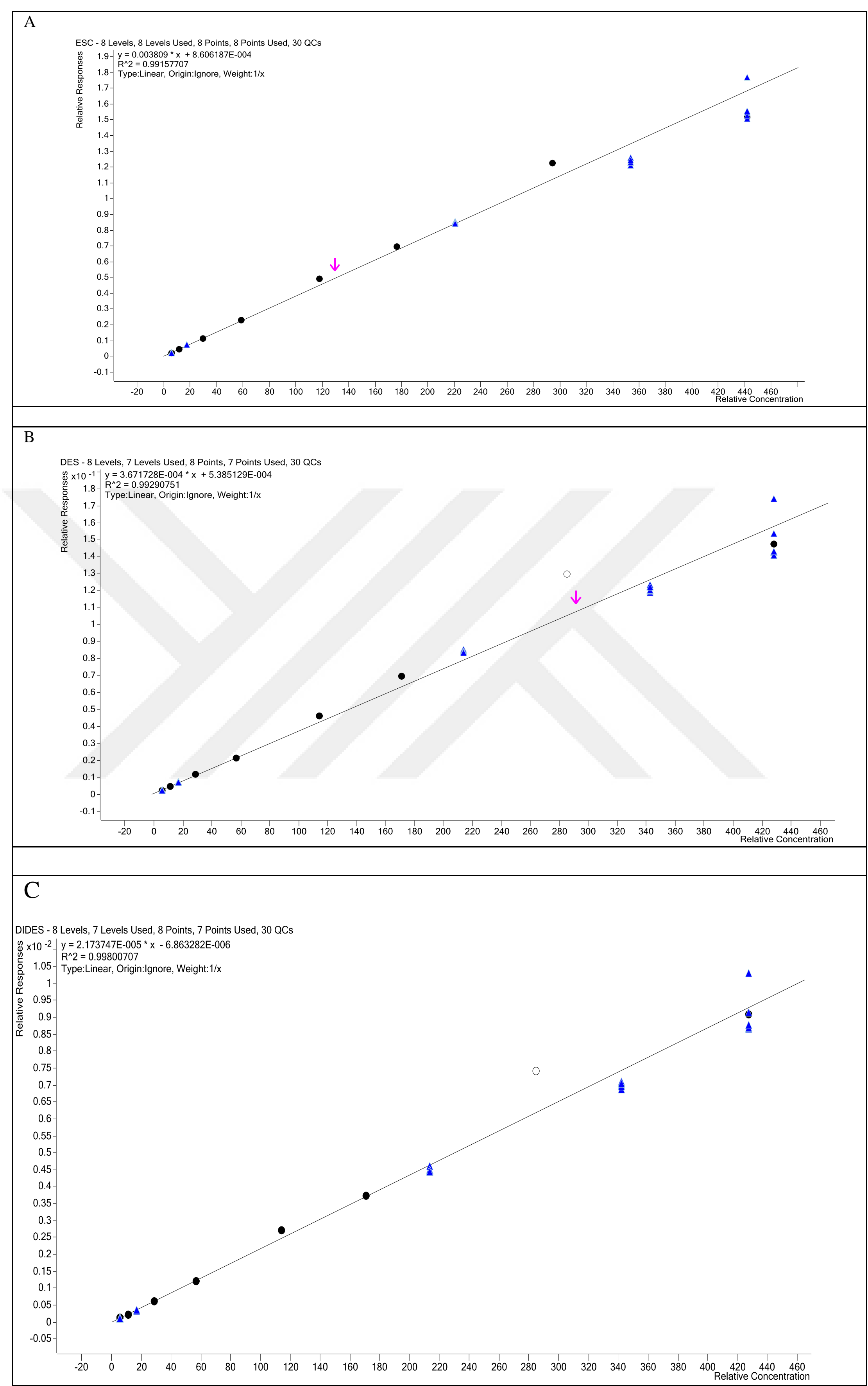


Figure 4.5 Evaluation of calibration curves of analytes in plasma (A.S-CT (r^2:0.9916), B. S-DCT (r^2: 0.9929), C. S-DDCT (r^2: 0.9980)).

4.1.3. Accuracy and Precision

The accuracy and precision of the bioanalytical method is studied by analyzing quality control samples at the lowest concentration (LOQ, QC1), at the low concentration (QC2), the medium concentration (QC3), and the high concentration levels (QC4 and QC5). Accuracy and precision are assessed in two ways: Within (intra) batch and between (inter) batch. In within (intra) batch analysis, six samples from QCs at each of five different concentration levels were prepared and 30 quality control samples in one batch in total are analyzed against the calibration standards. Between (inter) batch was obtained by calculating the results of three within batch analyzes that were run at different times.

For the validity of accuracy and precision, the mean value of concentrations except the LOQ should be within ± 15% of the nominal value. For LOQ, this value is ± 20%.

The result of accuracy and precision analysis of S-CT for within (intra) batch and between (inter) batch.

TABLE 4.3.Between (Inter) Batch Statistics of S-CT Calibration Standards Assayed During Three Days Of Validation in Plasma

Concentration	N	Obtained mean concentration (*ng*/ml)	Mean Accuray (%)	Precision (% CV)
5.9	3	5.04	85.48	1.98
11.8	3	11.87	100.72	0.99
29.5	2	30.05	101.99	3.09
58.9	3	61.61	104.59	2.61
117.8	3	126.33	107.22	1.54
176.7	3	180.55	102.16	1.14
294.6	3	313.78	106.52	2.36
441.8	3	407.96	92.33	3.69

During the validation process, one 29.5 ng / mL calibration standard was not included in the statistical calculations as it did not meet the required criteria (± 15%).The results included in the calculation meet the criteria required in the publications for the

validation study (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.3).

During the validation process, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.4).

TABLE 4.4. Between (Inter) Batch Statistics of S-CT Quality Control Samples Assayed During Three Days of Validation in Plasma

Concentration	N	Obtained mean concentration (***ng***/ml)	Mean Accuray (%)	Precision (% CV)
5.9	18	5.89	100.05	4.06
17.7	18	19.00	107.54	2.21
220.9	18	225.23	101.95	4.24
353.5	18	329.67	93.26	3.31
441.8	18	406.03	91.89	3.69

For each within (intra) batch, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.5).

TABLE 4.5.Within-Batch Statistics of S-CT Quality Control Samples Across All calibration Levels in Plasma

Batch No	Corelation Coefficient (r^2)	Mean Accuracy (%)					Precision (% CV)				
		5.9	**17.7**	**220.9**	**353.5**	**441.8**	**5.9**	**17.7**	**220.9**	**353.5**	**441.8**
Batch 1	0,9916	101.07	108.37	100.73	91.99	93.64	3.52	0.65	0.78	1.41	5.71
Batch 2	0,9960	96.74	100.78	100.16	96.40	99.89	3.05	0.92	0.69	1.91	1.81
Batch 3	0,9963	102.34	106.99	107.22	97.27	92.36	3.69	1.84	2.25	1.22	0.72

The result of accuracy and precision analysis of S-DCT for within (intra) batch and between (inter) batch.

TABLE 4.6. .Between (Inter) Batch Statistics of S-DCT Calibration Standards Assayed During Three Days of Validation in Plasma

Concentration	N	Obtained mean concentration (*ng*/ml)	Mean Accuray (%)	Precision (% CV)
5.7	3	4.67	81.71	0.83
11.4	2	11.37	99.66	0.42
28.5	2	30.49	106.84	0.52
57.1	3	59.56	104.35	3.76
114.2	3	124.03	108.65	2.81
171.2	3	177.69	103.77	5.29
285.4	2	303.26	106.26	2.48
428.1	3	397.14	92.77	5.06

During validation process, one of 11.4 ng / ml, one of 28.5 ng / ml and one of 285.4 ng / ml calibration standards were not included in the statistical calculations because they did not meet the required criteria (± 15%). The results included in the calculation meet the criteria reported in the literature for the validation study (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.6).

TABLE 4.7. Between (Inter) Batch Statistics of S-DCT Quality Control Samples Assayed During Three Days of Validation in Plasma

Concentration	N	Obtained mean concentration (*ng*/ml)	Mean Accuray (%)	Precision (% CV)
5.7	18	5.45	95.49	4.96
17.1	18	18.46	107.85	3.70
214.0	18	221.51	103.48	5.48
342.5	18	321.79	93.96	3.80
428.1	18	392.59	91.71	5.06

During the validation process, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.7).

TABLE 4.8. Within-Batch Statistics of S-DCT Quality Control Samples Across All calibration Levels in Plasma

Batch No	Corelation Coefficient (r^2)	Mean Accuracy (%)					Precision (% CV)				
		5.7	**17.1**	**214.0**	**342.5**	**428.1**	**5.7**	**17.1**	**214.0**	**342.5**	**428.1**
Batch1	0.9929	93.45	109.31	106.44	95.91	94.75	2.89	4.19	0.90	1.43	8.18
Batch 2	0.9944	91.48	102.09	100.12	97.84	98.70	1.93	1.77	0.68	1.70	1.08
Batch 3	0.9944	101.55	105.43	107.90	96.84	91.03	1.85	3.56	1.27	1.21	0.74

For each within (intra) batch, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.8).

The result of accuracy and precision analysis of S-DDCT for within (intra) batch and between (inter) batch.

TABLE 4.9. Between (Inter) Batch Statistics of S-DDCT Calibration Standards Assayed During Three Days of Validation in Plasma

Concentration	N	Obtained mean concentration (*ng*/ml)	Mean Accuray (%)	Precision (% CV)
5.7	3	5.55	97.46	7.98
11.4	3	11.67	102.40	3.99
28.5	3	28.39	99.64	4.46
57.0	3	55.55	97.48	5.17
114.0	3	118.58	104.05	5.77
170.9	3	167.60	98.05	4.28
284.9	2	298.08	104.63	3.68
427.4	3	418.78	97.99	5.28

During validation process, one of 284.9 ng / ml calibration standard was not included in the statistical calculations because it did not meet the required criteria (± 15%).

The results included in the calculation meet the criteria reported in the literature for the validation study (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.9).

TABLE 4.10.Between (Inter) Batch Statistics of S-DDCT Quality Control Samples Assayed During Three Days of Validation in Plasma

Concentration	N	Obtained mean concentration (***ng***/ml)	Mean Accuray (%)	Precision (% CV)
5.7	18	5.69	99.76	8.30
17.1	18	16.30	95.38	5.47
213.7	18	206.07	96.44	3.75
341.9	18	319.88	93.57	3.01
427.4	18	400.88	93.80	5.28

During validation process, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.10).

TABLE 4.11. Within-Batch Statistics of S-DDCT Quality Control Samples Across All calibration Levels in Plasma

Batch No	Corelation Coefficient (r^2)	Mean Accuracy (%)					Precision (% CV)				
		5.7	**17.1**	**213.7**	**341.9**	**427.4**	**5.7**	**17.1**	**213.7**	**341.9**	**427.4**
Batch 1	0.9980	102.54	93.68	96.97	94.41	97.33	7.85	4.79	1.32	1.15	6.95
Batch 2	0.9969	103.14	105.17	99.89	101.17	99.81	9.78	5.57	0.94	1.21	0.83
Batch 3	0.9989	93.60	100.49	99.79	96.42	95.12	2.91	5.11	3.64	0.92	0.84

For each within (intra) batch, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.11).

4.1.4. Matrix Effect

For the matrix effect study, six samples of low (QC2) and high quality control samples (QC5) prepared in the plasma were analyzed with six samples prepared in methanol at the same concentration levels. For the matrix effect calculation, the analyte matrix factor (MF) was found through first proportioning the analyte peak area in the plasma to the peak area in the methanol (absence of matrix). The internal standard (IS) matrix factor of desipramine was then calculated using the same calculation process. Finally, by dividing the MF of the analytes to the mean MF of the IS, normalized internal standard (IS) MF was found (61). For the validity of the study, it has been indicated in the bioanalytical method validation publications that the calculated IS normalize matrix factor should be less than 15% of the CV.

Matrix factor calculation for internal standard (IS) in plasma

TABLE 4.12.Internal Standard (IS)- Mean Matrix Factor (IS- Mean MF) in Plasma

Sample	Plasma Area	Methanol Area	IS- MF‡‡	IS-Mean MF§§
QC2_1	17577515.19	24804225.79	0.709	0.679
QC2_2	14148472.33	24953266.77	0.567	
QC2_3	16743774.02	24859853.31	0.674	
QC2_4	17492103.66	24872584.44	0.703	
QC2_5	17645282.77	24701855.31	0.714	
QC2_6	17495080.11	24703603.82	0.708	
QC5_1	18635411.23	25457776.05	0.732	0.724
QC5_2	18624916.71	25249140.62	0.738	
QC5_3	18088997.92	25189088.93	0.718	
QC5_4	17911845.23	25023375.11	0.716	
QC5_5	17828419.62	24954022.06	0.714	
QC5_6	1789650.49	24751713.24	0.723	

‡‡ Internal Standard (IS)- Matrix Factor (IS-MF): Internal Standart Area in Plasma/ Internal Standart Area in Methanol

§§ Internal Standart (IS)- Mean Matrix Factor(IS-Mean MF): Internal Standart (IS)- Matrix Factor/ N(sample number)

Matrix effect calculation for S-CT in plasma

TABLE 4.13. S-CT- Analyte Matrix Factor (Analyte-MF) in Plasma

Sample	Plasma Area	Methanol Area	Analyte-MF***
QC2_1	1316830.08	1367807.87	0.963
QC2_2	1032538.53	1370581.06	0.753
QC2_3	1214410.23	1365545.87	0.889
QC2_4	1306575.54	1364998.58	0.957
QC2_5	1315064.89	1361907.08	0.966
QC2_6	1323666.20	1351052.28	0.980
QC5_1	27177055.52	29074677.87	0.931
QC5_2	26859651.08	28850779.77	0.931
QC5_3	26519540.65	28787217.43	0.921
QC5_4	26350402.75	28665093.96	0.919
QC5_5	26269112.43	28490666.10	0.922
QC5_6	26158088.86	28278189.40	0.925

*** Analyte Matrix Factor (Analyte-MF): Analyte Area in Plasma/ Analyte Area in Methanol

TABLE 4.14. Normalized Internal Standard (IS) Matrix Factor (Normalized IS-MF) in Plasma						
Sample	**Analyte-MF**	IS-Mean MF	Normalized IS-MF[†††]	Mean	SD	% RSD (CV)
QC2_1	0.963		1.418	1.352	0.128	9.44
QC2_2	0.753		1.109			
QC2_3	0.889		1.309			
QC2_4	0.957	0.679	1.409			
QC2_5	0.966		1.422			
QC2_6	0.980		1.443			
QC5_1	0.931		1.292	1.279	0.008	0.66
QC5_2	0.931		1.287			
QC5_3	0.921		1.273			
QC5_4	0.919		1.271			
QC5_5	0.922	0.724	1.274			
QC5_6	0.925		1.279			

[†††] Normalized internal standard (IS) Matrix Factor (Normalized IS-MF): Analyte Matrix Factor/ Internal Standart (IS)- Mean Matrix Factor

For S-CT matrix effect determination, the normalized IS matrix factor CV in the QC2 and QC5 concentrations was found to be less than 15% and was within the acceptable limits stated in the bioanalytical method validation publications (Table 4.14).

Matrix effect calculation for S-DCT in plasma

TABLE 4.15. S-DCT- Analyte Matrix Factor (Analyte-MF) in Plasma

Sample	Plasma Area	Methanol Area	Analyte-MF [‡‡‡]
QC2_1	122194.74	156143.54	0.783
QC2_2	106061.31	158147.93	0.671
QC2_3	126708.30	158377.68	0.800
QC2_4	120668.04	158728.95	0.760
QC2_5	128773.85	158376.21	0.813
QC2_6	128290.39	157906.70	0.812
QC5_1	2648935.58	3031551.22	0.874
QC5_2	2614316.89	3014742.43	0.867
QC5_3	2584519.13	3003070.48	0.861
QC5_4	2564404.29	2990346.53	0.858
QC5_5	2559077.01	2972766.81	0.861
QC5_6	2560913.10	2957814.38	0.866

[‡‡‡] Analyte Matrix Factor: Analyte Area in Plasma/ Analyte Area in Methanol

TABLE 4.16. Normalized Internal Standard (IS) Matrix Factor (Normalized IS-MF) in Plasma						
Sample	**Analyte-MF**	IS-Mean MF	Normalized IS-MF [§§§]	**Mean**	**SD**	**% RSD (CV)**
QC2_1	0.783	0.679	1.152	1.138	0.080	6.99
QC2_2	0.671		0.987			
QC2_3	0.800		1.178			
QC2_4	0.760		1.119			
QC2_5	0.813		1.197			
QC2_6	0.812		1.196			
QC5_1	0.874	0.724	1.208	1.195	0,008	0.68
QC5_2	0.867		1.199			
QC5_3	0.861		1.190			
QC5_4	0.858		1.185			
QC5_5	0.861		1.190			
QC5_6	0.866		1.197			

[§§§] Normalized internal standard (IS) Matrix Factor: Analyte Matrix Factor/ Internal Standart (IS)- Mean Matrix Factor

For the S-DCT matrix effect determination, the normalized IS matrix factor CV in the QC2 and QC5 concentrations was found to be less than 15% and was within the acceptable limits stated in the bioanalytical method validation publications (Table 4.16).

Matrix effect calculation for S-DDCT in plasma

TABLE 4.17. S-DDCT- Analyte Matrix Factor (Analyte-MF) in Plasma

Sample	Plasma Area	Methanol Area	Analyte-MF [****]
QC2_1	6489.18	9055.41	0.717
QC2_2	5047.59	9677.18	0.522
QC2_3	5642.65	9729.44	0.580
QC2_4	6611.70	9774.07	0.676
QC2_5	6057.42	9850.46	0.615
QC2_6	6573.63	9917.24	0.663
QC5_1	157496.29	209662.80	0.751
QC5_2	157403.56	210731.28	0.747
QC5_3	154870.18	209709.32	0.738
QC5_4	154990.80	210564.93	0.736
QC5_5	152216.17	209477.67	0.727
QC5_6	152622.59	208155.34	0.733

[****] Analyte Matrix Factor (Analyte-MF): Analyte Area in Plasma/ Analyte Area in Methanol

TABLE 4.18. Normalized Internal Standard (IS) Matrix Factor (Normalized IS-MF) in Plasma

Sample	Analyte-MF	IS-Mean MF	Normalized IS-MF[††††]	Mean	SD	% RSD (CV)
QC2_1	0.717	0.679	1.055	0.926	0.105	11.29
QC2_2	0.522		0.768			
QC2_3	0.580		0.854			
QC2_4	0.676		0.996			
QC2_5	0.615		0.905			
QC2_6	0.663		0.976			
QC5_1	0.751	0.724	1.038	1.021	0.012	1.22
QC5_2	0.747		1.032			
QC5_3	0.738		1.021			
QC5_4	0.736		1.017			
QC5_5	0.727		1.004			
QC5_6	0.733		1.013			

[††††] Normalized internal standard (IS) Matrix Factor (Normalized IS-MF): Analyte Matrix Factor/ Internal Standart (IS)- Mean Matrix Factor

For S-DDCT matrix effect determination, the normalized IS matrix factor CV in the QC2 and QC5 concentrations was found to be less than 15% and was within the acceptable limits stated in the bioanalytical method validation publications (Table 4.18).

4.1.5. Lower Limit of Quantification (LOQ) and Sensitivity

The accurate and reproducible LOQ that can be used for quantitative assay in plasma for escitalopram, demethylcitalopram and didemethylcitalopram is 5.9, 5.7 and 5.7 ng/mL, respectively. It has been observed that the analysis results met the LOQ and sensitivity criteria mentioned in the literature.

4.1.6. Re-injection

For the re-injection study, third validation batch was re-injected into the system on the same day following the third validation batch.

TABLE 4.19. Reinjection of S-CT Quality Control Samples for Third Validation Batch in Plasma

Concentration	N	Obtained mean concentration (ng/ml)	Mean Accuray (%)	Precision (% CV)
5.9	6	5.73	97.21	3.26
17.7	6	19.41	109.84	5.74
220.9	6	235.87	106.77	4.24
353.5	6	346.46	98.02	1.21
441.8	6	411.05	93.03	0.89

S-CT calibration standard at the concentration of 11.8 ng / ml (Std 2) was not included in the calculation because it was outside the acceptable criteria. The accuracy and precision values of the re-injected third validation batch quality control samples were found to be within the acceptable limits stated in the bioanalytical method validation publications (Table 4.19).

TABLE 4.20. Reinjection of S-DCT Quality Control Samples for Third Validation Batch in Plasma				
Concentration	N	Obtained mean concentration (ng/ml)	Mean Accuray (%)	Precision (% CV)
5.7	6	5.75	100.66	1.95
17.1	6	18.32	106.99	2.61
214.0	6	228.44	106.72	0.89
342.5	6	331.07	96.69	1.11
428.1	6	389.08	90.89	0.84

S-DCT calibration standard at the concentration of 11.4 ng / ml and 28.5 ng/ml (Std 2, Std3) was not included in the calculation because it was outside the acceptable criteria. The accuracy and precision values of the re-injected third validation batch quality control samples were found to be within the acceptable limits stated in the bioanalytical method validation publications (Table 4.20).

TABLE 4.21. Reinjection of S-DDCT Quality Control Samples for Third Validation Batch in Plasma				
Concentration	N	Obtained mean concentration (ng/ml)	Mean Accuray (%)	Precision (% CV)
5.7	6	6.07	106.57	3.87
17.1	6	17.93	104.89	2.67
213.7	6	214.79	100.52	1.30
341.9	6	337.13	98.61	1.12
427.4	6	416.84	97.54	0.97

The accuracy and precision values of the re-injected third validation batch quality control samples were found to be within the acceptable limits stated in the bioanalytical method validation publications (Table 4.21).

4.1.7. Dilution

The sample was prepared in plasma at a concentration of two times the highest concentration standard (standard 8) containing escitalopram and its metabolites. 250 µL blank plasma was added to 250 µL standard sample to apply the ½ dilution procedure to the prepared sample. The resulted 500 µL diluted plasma sample was prepared according to the plasma sample preparation method and injected to the device together with freshly prepared standard and quality control samples. The comparison of results with the nominal concentration was made after the dilution correction (Table 4.22).

TABLE 4.22. Calculation of Dilution for S-CT, S-DCT and S-DDCT in Plasma

Molecule	Nominal concentration	Obtained concentration	Calculated Concentration by dilution correction (d‡‡‡‡=2)	% RSD
S-CT	883.6	413.42	826.83	0.39
S-DCT	856.2	396.25	792.50	2.17
S-DDCT	854.8	415.00	830.00	0.15

4.1.8. Stability

Six samples from both low- and high-concentration QC samples left in autosampler for 24 hours, six samples from both low- and high-concentration QC samples left at room temperature for 24 hours and six samples from both low-concentration and high-concentration QC samples which were thawed and frozen were studied with freshly prepared calibration standards on the calibration curve. The deviations of QCs from the nominal concentration were within ± 15%.

‡‡‡‡ Correction factor (d): As the dilution method is applied in 1/2 ratio, correction is performed by extending the value with 2 in the calculations.

Autosampler Stability

TABLE 4.23. Stability of S-CT After 24 Hours on The Autosampler at Room Temperature in Plasma		
Nominal concentration (ng/ml)	**17.7**	**441.80**
Concentration (ng/ml)	19.48	405.16
	19.68	402.04
	19.90	409.28
	19.43	404.77
	19.57	404.73
	19.74	403.75
Mean	19.63	404.95
Accuracy (%)	110.92	91.66
Standart Deviation (SD)	0.18	2.40
RSD (%)	0.99	0.54

According to the results, the samples were determined to be stable for 24 hours under auto sampler conditions (Table 4.23).

TABLE 4.24. Stability of S-DCT After 24 Hours on The Autosampler at Room Temperature in Plasma		
Nominal concentration (ng/ml)	**17.1**	**428.1**
Concentration (ng/ml)	19.28	385.08
	17.72	382.74
	18.13	389.35
	18.49	386.47
	19.39	385.53
	18.70	384.91
Mean	18.62	385.68
Accuracy (%)	108.75	90.09
Standart Deviation (SD)	0.65	2.18
RSD (%)	3.80	0.51

According to the results, the samples were determined to be stable for 24 hours under auto sampler conditions (Table 4.24).

TABLE 4.25. Stability of S-DDCT After 24 Hours on The Autosampler at Room Temperature in Plasma		
Nominal concentration (ng/ml)	**17.1**	**427.4**
Concentration (ng/ml)	15.24	410.88
	17.52	405.51
	15.86	416.18
	15.55	412.24
	17.24	408.35
	15.16	407.30
Mean	16.10	410.08
Accuracy (%)	94.19	95.96
Standart Deviation (SD)	1.03	3.85
RSD (%)	6.02	0.90

According to the results, the samples were determined to be stable for 24 hours under auto sampler conditions (Table 4.25).

Short Time Room Temperature Stability

TABLE 4.26. Short Time Stability of S-CT at Room Temperature in Plasma		
Nominal concentration (ng/ml)	**17.7**	**441.8**
Concentration (ng/ml)	19.50	491.92
	19.79	401.90
	19.56	410.48
	19.54	408.98
	19.45	429.96
	19.65	408.54
Mean	19.58	425.30
Accuracy (%)	110.63	96.27
Standart Deviation (SD)	0.12	33.98
RSD (%)	0.69	7.69

Thawed and left in room temperature for 24 hours QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.26).

TABLE 4.27. Short Time Stability of S-DCT at Room Temperature in Plasma		
Nominal concentration (ng/ml)	**17.1**	**428.1**
Concentration (ng/ml)	18.21	487.70
	18.42	383.06
	19.47	389.96
	18.44	388.46
	18.74	417.46
	18.09	388.31
Mean	18.56	409.16
Accuracy (%)	108.41	95.58
Standart Deviation (SD)	0.50	40.38
RSD (%)	2.91	9.43

Thawed and left in room temperature for 24 hours QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.27).

TABLE 4.28. Short Time Stability of S-DDCT at Room Temperature in Plasma		
Nominal concentration (ng/ml)	**17.1**	**427.4**
Concentration (ng/ml)	15.18	473.36
	17.28	413.68
	16.04	418.09
	14.87	412.07
	15.60	434.92
	16.30	413.41
Mean	15.88	427.59
Accuracy (%)	92.90	100.05
Standart Deviation (SD)	0.87	23.98
RSD (%)	5.07	5.61

Thawed and left in room temperature for 24 hours QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.28).

Freeze and Thaw Stability

TABLE 4.29. Freeze and Thaw Stability of S-CT in Plasma

Nominal concentration (ng/ml)	17.7	441.8
Concentration (ng/ml)	19.45	401.79
	19.69	397.15
	20.06	403.37
	19.47	400.68
	19.66	410.04
	19.91	402.80
Mean	19.71	402.64
Accuracy (%)	111.34	91.14
Standart Deviation (SD)	0.24	4.24
RSD (%)	1.38	0.96

Frozen and thawed QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.29).

TABLE 4.30. Freeze and Thaw Stability of S-DCT in plasma

Nominal concentration (ng/ml)	17.1	428.1
Concentration (ng/ml)	18.13	386.81
	18.08	382.23
	18.70	389.09
	18.07	386.32
	19.34	394.18
	17.96	388.99
Mean	18.38	387.94
Accuracy (%)	107.36	90.62
Standart Deviation (SD)	0.54	3.95
RSD (%)	3.15	0.92

Frozen and thawed QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.30).

TABLE 4.31. Freeze and Thaw Stability of S-DDCT in Plasma		
Nominal concentration (ng/ml)	**17.1**	**427.4**
Concentration (ng/ml)	18.30	409.52
	15.05	405.43
	15.36	412.08
	17.14	407.34
	15.81	413.64
	17.17	406.38
Mean	16.47	409.06
Accuracy (%)	96.38	95.72
Standart Deviation (SD)	1.26	3.28
RSD (%)	7.39	0.77

Frozen and thawed QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.31).

4.2. Results of Analytical Method Validation in Urine

4.2.1. Selectivity and Carry Over Study

When the chromatograms of blank samples prepared from six different urines were compared with the chromatograms of samples at LOQ level, it was found that the peak area of the interferences of the analytes (SCT, S-DCT, S-DDCT) at the retention time in the blank samples was less than 20% of the analyte peak area in the LOQ sample. In the same blank samples, the peak area of the interferences of the desipramine (as IS) at the retention time in the blank samples was found to be less than 5% of the desipramine peak area in the LOQ samples (Figure 4.6).

In publications on bioanalytical method validation, it is reported that the areas of the interference from the blank samples should be less than 20% of the analyte areas at the LOQ level and 5% of the internal standard area for selectivity. Considering the

selectivity findings of the developed method, it is observed that it meets the criteria in related publications (Table 4.32).

For the carry over study; it was found that the area of the interferences of the analytes (SCT, S-DCT, S-DDCT) at the retention time in the injected blank sample after the highest concentration calibration standard was found to be less than 20% of the analyte peak area and less than 5% of the desipramine area in the LOQ samples. It has been observed that the analysis results meet the carryover criteria in the literature.

TABLE 4.32. S-CT, S-DCT, S-DDCT and Desipramine Selectivity Study in Urine Samples

Sample	S-CT		S-DCT		S-DDCT		Desipramine (IS)		Acceptance Criteria
	RT (min)	Area	RT (min)	Area	RT (min)	Area	RT (min)	Area	
Blank 1	2.71	1554	2.68	-	2.63	-	3.94	3034	The areas of the interference from the blank samples should be less than 20% of the analyte areas at the LOQ level and 5% of the internal standard area
Blank 2		1322		-		-		2399	
Blank 3		1148		-		-		2895	
Blank 4		1585		-		-		27118	
Blank 5		970		-		-		3158	
Blank 6		-		-		-		2716	
Standart 1		649520		66669		5137		34338075	

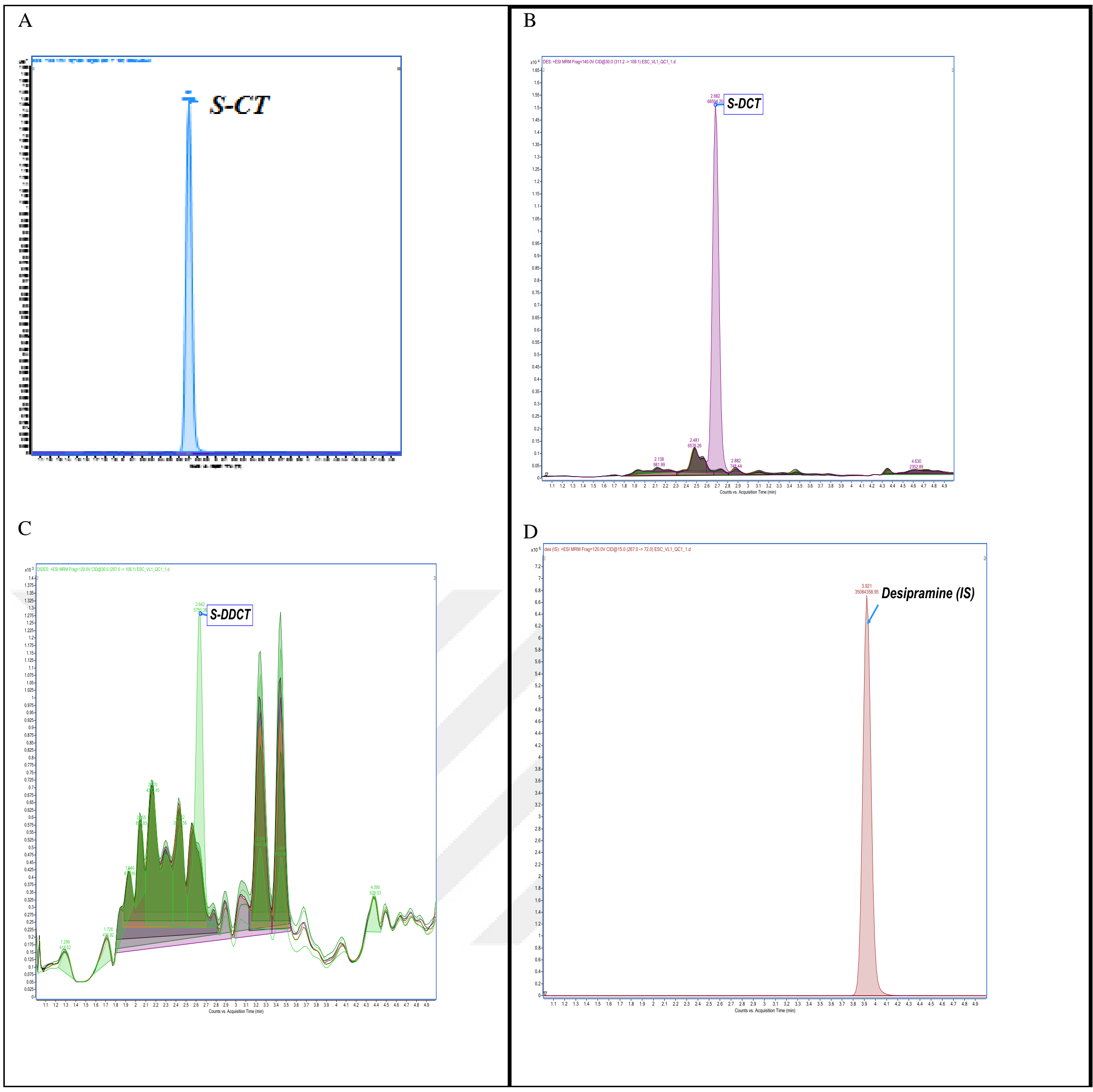


Figure 4.6. Represantation of S-CT, S-DCT, S-DDCT and desipramine chromatograms in urine. Overlap of MRM chromotograms of six blank urine samples without internal standard and without analyte with MRM chromotograms of analytes and desipramine at LOQ concentration in urine (A. S-CT (m/z: 325.1>109.1); retention time (RT): 2.71 min, B. S-DCT (m/z: 311.2> 109.1); retention time (RT): 2.68 min, C. S-DDCT (m/z:297.0> 109.1); retention time (RT): 2.63 min, Desipramine (m/z: 267> 72), retention time (RT): 3.94 min).

4.2.2. Calibration Curve and Quality Control Samples

Eight calibration standards and five quality control samples were prepared for the calibration curve. The ranges of calibration curve for the quantitative analysis of S-CT, S-DCT and S-DDCT in urine samples are 5.5 – 441.8 ng / mL, 5.4 – 428.1 ng / mL and 5.3 – 427.4, respectively.

It has been reported in bioanalytical method validation publications that in order for calibration standards to be included in the calculation, the lowest standard

concentration should be ± 20% of the lowest standard nominal value, and ± 15% of the nominal standard value of the other standard concentrations. It has also been reported that 75% of the standards for each calibration curve should meet the criteria. The results met the criteria reported in bioanalytical method validation publications. Calibration curve r^2 values of escitalopram and metabolites during the validation period are given in Table 4.33 (Figure 4.7.).

TABLE 4.33. Calibration curve r^2 values of escitalopram and metabolites during the validation in urine

Batch Number	Molecule	Calibration corelation coefficient (r^2)
	S-CT	0.9958
Batch 1	S-DCT	0.9959
	S-DDCT	0.9999
	S-CT	0.9938
Batch 2	S-DCT	0.9943
	S-DDCT	0.9996
	S-CT	0.9910
Batch 3	S-DCT	0.9926
	S-DDCT	0.9994

Calibration standards were analyzed as freshly prepared during validation process. In our study, the results included in the calculation for a total of three calibration curves meet the criteria reported in the publications for the validation study. It has also been found that all of the quality control samples calculated by considering the calibration curve in validation study for each molecule meet the required criteria.

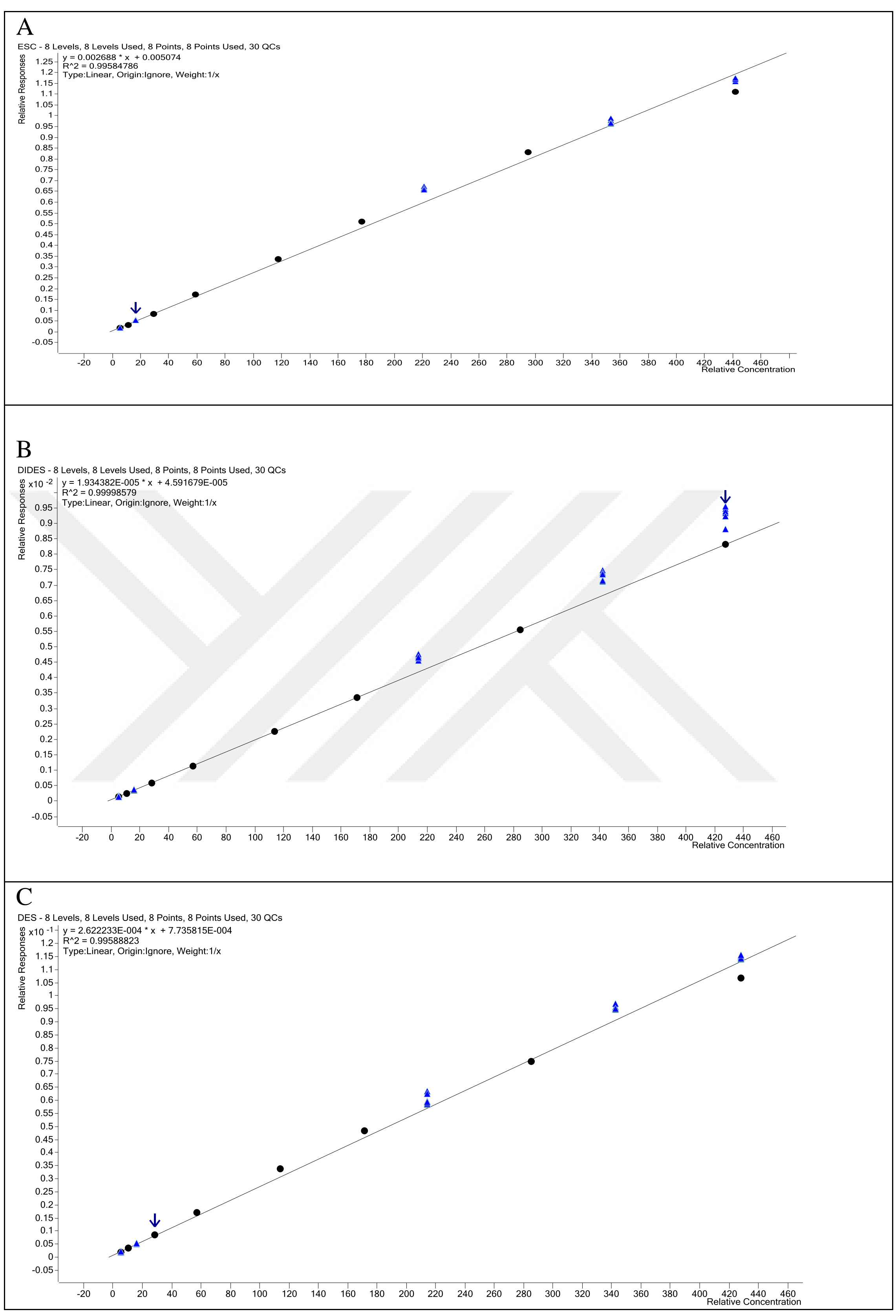


Figure 4.7 Evaluation of calibration curves of analytes in urine (A.S-CT (r^2:0.9958), B. S-DCT (r^2: 0.9959), C. S-DDCT (r^2: 0.9999))

4.2.3. Accuracy and Precision

The accuracy and precision of the bioanalytical method is studied by analyzing quality control samples at the lowest concentration (LOQ, QC1), at the low concentration (QC2), the medium concentration (QC3), and the high concentration levels (QC4 and QC5). Accuracy and precision are assessed in two ways: Within (intra) batch and between (inter) batch. In within (intra) batch analysis, six samples from QCs at each of five different concentration levels were prepared and 30 quality control samples in one batch in total are analyzed against the calibration standards. Between (inter) batch was obtained by calculating the results of three within batch analyzes that were run at different times.

For the validity of accuracy and precision, the mean value of concentrations except the LOQ should be within ± 15% of the nominal value. For LOQ, this value is ± 20%.

The result of accuracy and precision analysis of S-CT for within (intra) batch and between (inter) batch.

TABLE 4.34. Between (Inter) Batch Statistics of S-CT Calibration Standards Assayed During Three Days of Validation in Urine

Concentration	N	Obtained mean concentration (***ng***/ml)	Mean Accuray (%)	Precision (% CV)
5.5	3	4.82	87.33	5.59
11.0	3	10.38	93.90	2.79
29.5	3	29.83	101.27	2.71
58.9	3	62.73	106.48	1.94
117.8	3	124.80	105.92	2.49
176.7	3	192.41	108.87	2.72
294.6	3	306.49	104.05	2.00
441.8	3	404.34	91.51	2.73

During the validation process, all of the calibration standards included in the calculation meet the criteria reported in the literature for the validation study (the mean

value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.34)

TABLE 4.35. Between (Inter) Batch Statistics of S-CT Quality Control Samples Assayed During Three Days of Validation in Urine				
Concentration	N	Obtained mean concentration (*ng*/ml)	Mean Accuray (%)	Precision (% CV)
5.5	18	5.17	93.71	2.74
16.6	18	17.66	106.57	5.95
220.9	18	239.94	108.61	3.45
353.5	18	348.90	98.70	2.59
441.8	18	416.73	94.32	2.73

During the validation process, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.35).

TABLE 4.36. Within-Batch Statistics of S-CT Quality Control Samples Across All Calibration Levels in Urine											
Batch No	Corelation Coefficient (r^2)	Mean Accuracy (%)					Precision (% CV)				
		5.5	**16.6**	**220.9**	**353.5**	**441.8**	**5.5**	**16.6**	**220.9**	**353.5**	**441.8**
Batch 1	0.9958	95.38	111.54	111.52	101.78	98.02	2.09	2.39	1.13	0.94	0.49
Batch 2	0.9959	92.11	100.09	100.18	98.43	100.26	3.11	1.17	1.95	1.23	0.55
Batch 3	0.9999	93.65	99.48	104.99	96.31	92.27	2.27	2.15	2.98	1.29	0.34

For each within (intra) batch, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.36).

The result of accuracy and precision analysis of S-DCT for within (intra) batch and between (inter) batch.

TABLE 4.37. Between (Inter) Batch Statistics of S-DCT Calibration Standards Assayed During Three Days of Validation in Urine				
Concentration	N	Obtained mean concentration (*ng*/ml)	Mean Accuray (%)	Precision (% CV)
5.4	3	4.62	86.27	2.74
10.7	3	10.00	93.48	1.37
28.5	3	29.21	102.33	1.62
57.1	3	63.31	110.91	1.44
114.2	3	120.72	105.75	4.06
171.2	3	181.49	105.99	1.07
285.4	3	295.45	103.52	4.50
428.1	3	395.81	92.46	4.55

During the validation process, all of the calibration standards included in the calculation meet the criteria reported in the literature for the validation study (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.37)

TABLE 4.38. Between (Inter) Batch Statistics of S-DCT Quality Control Samples Assayed During Three Days of Validation in Urine				
Concentration	N	Obtained mean concentration (*ng*/ml)	Mean Accuray (%)	Precision (% CV)
5.4	18	5.13	95.91	4.43
16.1	18	17.38	108.29	4.24
214.0	18	230.65	107.76	3.31
342.5	18	342.46	99.99	4.18
428.1	18	408.17	95.34	4.55

During the validation process, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.38).

TABLE 4.39. Within-Batch Statistics of S-DCT Quality Control Samples Across All Calibration Levels in Urine											
Batch No	Corelation Coefficient (r^2)	Mean Accuracy (%)					Precision (% CV)				
		5.3	**16.1**	**214.0**	**342.5**	**428.1**	**5.3**	**16.1**	**214.0**	**342.5**	**428.1**
Batch1	0.9999	96.91	109.90	106.27	105.35	101.54	6.53	2.75	3.83	0.98	0.55
Batch2	0.9926	97.37	100.88	99.61	98.53	100.16	2.70	2.66	1.01	1.18	0.57
Batch3	0.9994	93.46	105.61	106.18	96.03	91.79	2.38	3.55	2.15	1.15	0.46

For each within (intra) batch, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.39).

The result of accuracy and precision analysis of S-DDCT for within (intra) batch and between (inter) batch.

TABLE 4.40. Between (Inter) Batch Statistics of S-DDCT Calibration Standards Assayed During Three Days of Validation in Urine				
Concentration	N	Obtained mean concentration (***ng***/ml)	Mean Accuray (%)	Precision (% CV)
5.3	3	5.15	97.20	9.97
10.7	3	10.68	99.81	4.94
28.5	3	29.12	102.18	2.55
57.0	3	57.46	100.81	1.79
114.0	3	113.20	99.30	1.22
170.9	3	172.18	100.75	0.78
284.9	3	287.28	100.83	1.13
427.4	3	423.62	99.12	9.83

During the validation process, all of the calibration standards included in the calculation met the criteria reported in the literature for the validation study (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.40)

TABLE 4.41. Between (Inter) Batch Statistics of S-DDCT Quality Control Samples Assayed During Three Days of Validation in Urine				
Concentration	N	Obtained mean concentration (*ng*/ml)	Mean Accuray (%)	Precision (% CV)
5.3	18	5.30	100.05	7.50
16.0	18	15.96	99.73	6.65
213.7	18	217.80	101.92	9.14
341.9	18	345.02	100.91	7.89
427.4	18	433.47	101.42	9.83

During the validation process, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.41).

TABLE 4.42. Within-Batch Statistics of S-DDCT Quality Control Samples Across All Calibration Levels in Urine											
Batch No	Corelation Coefficient (r^2)	Mean Accuracy (%)					Precision (% CV)				
		5.3	**16.0**	**213.7**	**341.9**	**427.4**	**5.3**	**16.0**	**213.7**	**341.9**	**427.4**
Batch1	0.9999	102.86	102.53	111.80	109.41	111.93	6.27	2.53	1.63	2.34	3.14
Batch2	0.9926	98.53	95.96	101.87	99.67	100.25	7.54	5.73	1.90	1.42	0.97
Batch3	0.9994	98.78	91.12	90.52	91.49	89.17	8.99	7.17	1.41	3.16	1.07

For each within (intra) batch, it was found out that all of the quality control samples calculated by taking the calibration curve into consideration met the required criteria (the mean value of concentrations outside the LOQ (QC1) is ± 15% of the nominal value, and this value is ± 20% for the LOQ (QC1)) (Table 4.42).

4.2.4. Matrix Effect

For the matrix effect study, six samples of low (QC2) and high quality control samples (QC5) prepared in the urine were analyzed with six samples prepared in methanol at the same concentration levels. For the matrix effect calculation, the

analyte matrix factor (MF) was found through first proportioning the analyte peak area in the plasma to the peak area in the methanol (absence of matrix).The internal standard (IS) matrix factor of desipramine was then calculated using the same calculation process. Finally, by dividing the MF of the analytes to the mean MF of the IS, normalized internal standard (IS) MF was found. For the validity of the study, it has been indicated in the bioanalytical method validation publications that the calculated IS normalize matrix factor should be less than 15% of the CV.

Matrix factor calculation for internal standart (IS) in urine

TABLE 4.43. Internal Standart (IS)- Mean Matrix Factor (IS- Mean MF) in Urine

Sample	Urine Area	Methanol Area	IS-MF[§§§§]	IS- Mean MF[*****]
QC2_1	30070397.25	37147111.38	0.809	0.802
QC2_2	30435083.83	36574890.60	0.832	
QC2_3	25225631.30	34079530.07	0.740	
QC2_4	30271521.35	36384524.93	0.832	
QC2_5	29855873.48	37041436.02	0.806	
QC2_6	29456492.95	37257117.41	0.791	
QC5_1	29197964.52	36252381.90	0.805	0.798
QC5_2	28703135.86	34409444.75	0.834	
QC5_3	28885081.82	34564239.39	0.836	
QC5_4	28521664.39	37188054.57	0.767	
QC5_5	28805285.82	37459421.62	0.769	
QC5_6	29063990.19	37460665.54	0.776	

[§§§§] Internal Standart (IS)- Matrix Factor (IS-MF): Internal Standart Area in Urine/ Internal Standart Area in Methanol

[*****] Internal Standart (IS)- Mean Matrix Factor (IS- Mean MF): Internal Standart (IS)- Matrix Factor/ N(sample number)

Matrix effect calculation for S-CT in urine

TABLE 4.44. S-CT -Analyte Matrix Factor (Analyte- MF) in Urine

Sample	Urine Area	Methanol Area	Analyte- MF
QC2_1	1559377.07	1697059.84	0.919
QC2_2	1537345.39	1662113.95	0.925
QC2_3	1339813.47	1486769.89	0.901
QC2_4	1586228.30	1659743.70	0.956
QC2_5	1562278.38	1697862.97	0.920
QC2_6	1540838.10	1707239.01	0.903
QC5_1	33429670.10	38532509.95	0.868
QC5_2	33072442.08	36545958.73	0.905
QC5_3	33261193.93	36798979.92	0.904
QC5_4	32979264.08	39304140.61	0.839
QC5_5	33293376.42	39432831.92	0.844
QC5_6	33423860.85	39367392.02	0.849

TABLE 4.45. Normalized Internal Standard (IS) Matrix Factor (Normalized IS-MF) in Urine						
Sample	Analyte-MF	IS-Mean MF	**Normalized IS-MF** [†††††]	Mean	SD	% RSD (CV)
QC2_1	0.919		1.146			
QC2_2	0.925		1.154			
QC2_3	0.901		1.124			
		0.802		**1.15**	**0.03**	**2.15**
QC2_4	0.956		1.192			
QC2_5	0.920		1.148			
QC2_6	0.903		1.126			
QC5_1	0.868		1.087			
QC5_2	0.905		1.134			
QC5_3	0.904		1.133			
		0.798		**1.09**	**0.04**	**3.42**
QC5_4	0.839		1.052			
QC5_5	0.844		1.058			
QC5_6	0.849		1.064			

[†††††] Normalized internal standard (IS) Matrix Factor (Normalized IS-MF): Analyte Matrix Factor/ Internal Standart (IS)- Mean Matrix Factor

For the escitalopram matrix factor determination, the normalized IS matrix factor CV in the QC2 and QC5 concentrations was found to be less than 15% and was within the acceptable limits stated in the bioanalytical method validation publications (Table 4.45).

Matrix effect calculation for S-DCT in urine

TABLE 4.46. S-DCT- Analyte Matrix Factor (Analyte- MF) in Urine

Sample	Urine Area	Methanol Area	Analyte- MF
QC2_1	162385.31	209250.20	0.776
QC2_2	158596.66	206523.01	0.768
QC2_3	151052.20	181622.33	0.832
QC2_4	156383.19	207813.15	0.753
QC2_5	162763.57	213119.65	0.764
QC2_6	152865.76	214509.11	0.713
QC5_1	3206554.54	3872847.57	0.828
QC5_2	3185795.93	3661210.12	0.870
QC5_3	3195005.05	3696710.25	0.864
QC5_4	3176524.66	3974430.42	0.799
QC5_5	3201637.96	3980286.53	0.804
QC5_6	3213805.84	3975033.19	0.808

TABLE 4.47. Normalized Internal Standard (IS) Matrix Factor (Normalized IS-MF) in Urine

Sample	Analyte - MF	IS- Mean MF	Normalized IS-MF‡‡‡‡‡	Mean	SD	% RSD (CV)
QC2_1	0.776	0.802	0.968	**0.96**	**0.05**	**5.03**
QC2_2	0.768		0.958			
QC2_3	0.832		1.037			
QC2_4	0.753		0.939			
QC2_5	0.764		0.953			
QC2_6	0.713		0.889			
QC5_1	0.828	0.798	1.038	**1.04**	**0.04**	**3.76**
QC5_2	0.870		1.091			
QC5_3	0.864		1.083			
QC5_4	0.799		1.002			
QC5_5	0.804		1.008			
QC5_6	0.808		1.013			

‡‡‡‡‡ Normalized internal standard (IS) Matrix Factor (Normalized IS-MF): Analyte Matrix Factor/ Internal Standart (IS)- Mean Matrix Factor

For S-DCT matrix factor determination, the normalized IS matrix factor CV in the QC2 and QC5 concentrations was found to be less than 15% and was within the acceptable limits stated in the bioanalytical method validation publications (Table 4.47).

Matrix effect calculation for S-DDCT in urine

TABLE 4.48. S-DDCT- Analyte Matrix Factor (Analyte-MF) in Urine

Sample	Urine Area	Methanol Area	Analyte - MF
QC2_1	12349.84	12333.66	1.001
QC2_2	12401.95	14680.24	0.845
QC2_3	12121.18	15526.94	0.781
QC2_4	12632.98	15341.90	0.823
QC2_5	12532.41	15094.16	0.830
QC2_6	11738.86	14918.18	0.787
QC5_1	276329.92	359487.84	0.769
QC5_2	273987.75	394632.24	0.694
QC5_3	279996.67	389931.14	0.718
QC5_4	278049.99	345571.28	0.805
QC5_5	279746.75	342051.05	0.818
QC5_6	277361.46	340317.89	0.815

TABLE 4.49. Normalized Internal Standard (IS) Matrix Factor (Normalized IS-MF) in Urine						
Sample	Analyte-MF	IS- Mean MF	Normalized IS-MF §§§§§	Mean	SD	% RSD (CV)
QC2_1	1.001	0.802	1.249	1.05	0.10	9.57
QC2_2	0.845		1.054			
QC2_3	0.781		0.974			
QC2_4	0.823		1.027			
QC2_5	0.830		1.036			
QC2_6	0.787		0.981			
QC5_1	0.769	0.798	0.963	0.97	0.07	6.86
QC5_2	0.694		0.870			
QC5_3	0.718		0.900			
QC5_4	0.805		1.008			
QC5_5	0.818		1.025			
QC5_6	0.815		1.022			

§§§§§ Normalized internal standard (IS) Matrix Factor (Normalized IS-MF): Analyte Matrix Factor/ Internal Standart (IS)- Mean Matrix Factor

For S-DDCT matrix effect determination, the normalized IS matrix factor CV in the QC2 and QC5 concentrations was found to be less than 15% and was within the acceptable limits stated in the bioanalytical method validation publications (Table 4.49).

4.2.5. Lower Limit of Quantification (LOQ) and Sensitivity

The accurate and reproducible LOQ that can be used for quantitative assay in urine for escitalopram, demethylescitalopram and didemethylescitalopram is 5.5, 5.4 and 5.3 ng/mL, respectively. It has been observed that the analysis results met the LOQ and sensitivity criteria mentioned in the literature.

4.2.6. Re-injection

For the re-injection study, third validation batch was re-injected into the system on the same day following the third validation batch.

TABLE 4.50. Reinjection of S-CT Quality Control Samples For Third Validation Batch in Urine

Concentration	N	Obtained mean concentration (ng/ml)	Mean Accuray (%)	Precision (% CV)
5.5	6	5.08	92.07	4.86
16.6	6	17.09	103.15	1.29
220.9	6	233.60	105.74	1.56
353.5	6	347.61	98.34	1.23
441.8	6	426.92	96.62	0.94

The accuracy and precision values of the re-injected third validation batch quality control samples were found to be within the acceptable limits stated in the bioanalytical method validation publications (Table 4.50).

TABLE 4.51. Reinjection of S-DCT Quality Control Samples for Third Validation Batch in Urine

Concentration	N	Obtained mean concentration (ng/ml)	Mean Accuray (%)	Precision (% CV)
5.4	5	4.97	92.96	3.80
16.1	6	17.32	107.92	2.69
214.0	6	228.98	106.98	1.76
342.5	6	334.85	97.77	1.12
428.1	6	409.30	95.61	0.92

One of 5.4 ng / ml quality control samples was not included in the statistical calculations because it did not meet the required criterion (± 20%). The accuracy and precision values of the re-injected third validation batch quality control samples were found to be within the acceptable limits stated in the bioanalytical method validation publications (Table 4.51).

TABLE 4.52. Reinjection of S-DDCT Quality Control Samples for Third Validation Batch in Urine

Concentration	N	Obtained mean concentration (ng/ml)	Mean Accuray (%)	Precision (% CV)
5.3	6	5.26	99.27	7.07
16.0	6	15.00	93.76	6.20
213.7	6	208.59	97.61	2.29
341.9	6	321.70	94.09	1.32
427.4	6	407.04	95.24	0.89

The accuracy and precision values of the re-injected third validation batch quality control samples were found to be within the acceptable limits stated in the bioanalytical method validation publications (Table 4.52).

4.2.7. Dilution

The sample was prepared in urine at a concentration of two times the highest concentration standard (standard 8) containing escitalopram and its metabolites. 250 µL blank urine was added to 250 µL standard sample to apply the ½ dilution procedure to the prepared sample. The resulting 500 µL diluted urine sample was prepared according to the urine sample preparation method and injected to the device together with freshly prepared standard and quality control samples. The comparison of results with the nominal concentration was made after the dilution correction (Table 4.53).

TABLE 4.53. Calculation of Dilution for S-CT, S-DCT and S-DDCT in Urine

Molecule	Nominal concentration (ng/mL)	Obtained concentration (ng/mL)	Calculated concentration by dilution correction (d****** =2)	% RSD
S-CT	883.6	403.06	806.12	1.31
S-DCT	856.2	391.79	783.57	1.26
S-DDCT	854.8	411.76	823.52	1.38

4.2.8. Stability

Six samples from both low- and high-concentration QC samples left in autosampler for 24 hours, six samples from both low- and high-concentration QC samples left at room temperature for 24 hours and six samples from both low-concentration and high-concentration QC samples which were thawed and frozen were studied with freshly prepared calibration standards on the calibration curve. The deviations of QCs from the nominal concentration were within ± 15%.

****** Correction factor (d): As the dilution method is applied in 1/2 ratio, correction is performed by extending the value with 2 in the calculations.

Autosampler Stability

TABLE 4.54. Stability of S-CT After 24 Hours on The Autosampler at Room Temperature in Urine		
Nominal concentration (ng/ml)	**16.6**	**441.8**
Concentration (ng/ml)	18.31	412.60
	17.95	413.23
	18.50	418.55
	18.42	413.52
	18.37	411.95
	18.41	412.28
Mean	18.33	413.69
Accuracy (%)	110.59	93.63
Standart Deviation (SD)	0,.20	2.45
RSD (%)	1.18	0.56

According to the results, the samples were determined to be stable for 24 hours under auto sampler conditions (Table 4.54).

TABLE 4.55. Stability of S-DCT After 24 Hours on The Autosampler at Room Temperature in Urine		
Nominal concentration (ng/ml)	**16.1**	**428.1**
Concentration (ng/ml)	17.77	405.06
	18.21	405.58
	18.41	410.71
	17.52	404.46
	17.76	404.21
	17.73	404.27
Mean	17.90	405.72
Accuracy (%)	111.52	94.77
Standart Deviation (SD)	0.34	2.50
RSD (%)	2.10	0.59

According to the results, the samples were determined to be stable for 24 hours under auto sampler conditions (Table 4.55).

TABLE 4.56. Stability of S-DDCT After 24 Hours on The Autosampler at Room Temperature in Urine		
Nominal concentration (ng/ml)	**16.0**	**427.4**
Concentration (ng/ml)	16.76	431.01
	16.55	433.49
	14.74	437.39
	16.20	432.24
	16.99	433.26
	15.24	425.08
Mean	16.08	432.08
Accuracy (%)	100.50	101.10
Standart Deviation (SD)	0.90	4.04
RSD (%)	5.62	0.95

According to the results, the samples were determined to be stable for 24 hours under auto sampler conditions (Table 4.56).

Short Time Room Temperature Stability

TABLE 4.57. Short Time Stability of S-CT at Room Temperature in Urine		
Nominal concentration (ng/ml)	**16.6**	**441.8**
Concentration (ng/ml)	17.93	410.52
	17.56	413.67
	18.07	416.12
	18.13	413.95
	17.91	416.09
	18.06	414.55
Mean	17.94	414.15
Accuracy (%)	108.29	93.73
Standart Deviation (SD)	0.20	2.06
RSD (%)	1.23	0.47

Thawed and left in room temperature for 24 hours QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.57).

TABLE 4.58. Short Time Stability of S-DCT at Room Temperature in Urine		
Nominal concentration (ng/ml)	**16.050**	**428.1**
Concentration (ng/ml)	16.77	404.20
	18.07	405.75
	18.15	410.36
	17.04	407.20
	17.99	408.79
	17.18	408.00
Mean	17.53	407.39
Accuracy (%)	109.24	95.16
Standart Deviation (SD)	0.61	2.19
RSD (%)	3.77	0.51

Thawed and left in room temperature for 24 hours QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.58).

TABLE 4.59. Short Time Stability of S-DDCT at Room Temperature in Urine		
Nominal concentration (ng/ml)	**16.0**	**427.4**
Concentration (ng/ml)	14.48	398.92
	14.49	401.33
	14.61	409.27
	14.85	404.25
	15.13	410.01
	15.09	411.99
Mean	14.78	405.96
Accuracy (%)	92.35	94.98
Standart Deviation (SD)	0.29	5.25
RSD (%)	1.83	1.23

Thawed and left in room temperature for 24 hours QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.59).

Freeze and Thaw stability

TABLE 4.60. Freeze and Thaw Stability of S-CT in Urine		
Nominal concentration (ng/ml)	**16.6**	**441.8**
	17.17	421.82
	16.50	423.49
	17.38	420.87
Concentration (ng/ml)	17.25	418.54
	17.28	418.31
	17.47	418.67
Mean	17.18	420.28
Accuracy (%)	103.66	95.12
Standart Deviation (SD)	0.35	2.12
RSD (%)	2.08	0.48

Frozen and thawed QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.60).

TABLE 4.61 . Freeze and Thaw Stability of S-DCT in Urine		
Nominal concentration (ng/ml)	**16.1**	**428.1**
Concentration (ng/ml)	17.29	406.85
	16.87	409.70
	17.74	406.14
	17.68	404.19
	17.78	403.58
	17.91	403.45
Mean	17.54	405.65
Accuracy (%)	109.31	94.76
Standart Deviation (SD)	0.39	2.42
RSD (%)	2.44	0.57

Frozen and thawed QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.61).

TABLE 4.62. Freeze and Thaw Stability of S-DDCT in Urine		
Nominal concentration (ng/ml)	**16.0**	**427.4**
Concentration (ng/ml)	16.00	383.95
	14.22	383.62
	14.35	383.55
	16.47	383.88
	14.41	386.62
	14.06	380.53
Mean	14.92	383.69
Accuracy (%)	93.24	89.77
Standart Deviation (SD)	1.04	1.93
RSD (%)	6.50	0.45

Frozen and thawed QC2 and QC5 samples were evaluated on calibration curve with freshly prepared calibration standards. The deviations of QC from the nominal concentration are within ± 15% (Table 4.62).

4.3. Results of Sample Analysis

4.3.1. TDM Analysis

According to DSM-IV criteria, the result of drug blood level analysis and treatment evaluation of 30 patients (9 males, 21 females) in the age range of 20-58 years who were diagnosed with major depression (MD) and take escitalopram drug treatment are given in Table 4.63. Treatment response level was determined by using the Hamilton Depression Rating Scale/HDDÖ[††††††] and the side effects that occurred during drug use were determined by examining patient files.

[††††††] It is a 17-question test that physicians can use to measure the severity of depression in patients. It was published by Max Hamilton in 1960. The items of scale related to sleeping difficulty, waking in the night, waking early morning, somatic symptoms, genital symptoms, losing weight and insight were scored between 0-2 and other items

TABLE 4.63. The Demographic Properties and Treatment Evaluation of 30 Patients							
Sample	Gender (Female; F, Male; M)	Age (year)	Expected S-CT concentration in plasma (ng/ml)	Obtained S-CT concentration in plasma (ng/ml)	Therapeutic Range in plasma (ng/ml)	Response	Side Effect
1.	F	33	11.6 – 30.8	13.40	15 - 80	Remission	
2.	M	34		19.16		Remission	
3.	F	26		32.28		Remission	
4.	F	40		35.87		Remission	
5.	M	30		23.90		Remission	
6.	F	36		49.32		Nonresponse/Remission after raising the drug dose (30 mg)	
7.	M	53		16.83		Partial remission	Insomnia, loss of appetite
8.	F	32		0.95		Nonresponse	
9.	F	32		31.58		Remission	
10.	F	20		22.81		Partial remission	
11.	M	30		9.66		Nonresponse	
12.	F	54		15.23		Nonresponse/Remission after antypsychotic drug addition	
13.	M	28		12.26		Nonresponse	
14.	F	50		55.25		Nonresponse/Remission after antypsychotic drug addition	
15.	F	51		27.38		Remission	
16.	F	51		27.17		Remission	
17.	F	50		63.36		Remission	

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were scored between 0-4. The highest score is 53. A score between 0-7 is an indication of no depression, 8-15 for mild depression, 16-28 for moderate depression and 29 or above for severe depression(93)

TABLE 4.63. The Demographic Properties and Treatment Evaluation of 30 Patients (continued)							
Sample	Gender (Female; F, Male; M)	Age (year)	Expected S-CT concentration in plasma (ng/ml)	Obtained S-CT concentration in plasma (ng/ml)	Therapeutic Range in plasma (ng/ml)	Response	Side Effect
18.	F	46		37.28		Remission	
19.	F	40		40.94		Remission	
20.	M	32		26.39		Remission	
21.	F	24		29.71		Remission	
22.	M	46		20.95		Remission	
23.	F	46		20.91		Remission	
24.	M	40		38.21		Remission	
25.	F	37		14.89		Nonresponse/ Drug change	
26.	F	44		2.93		Nonresponse	
27.	F	48		48.74		Partial remission	
28.	F	19		7.35		Nonresponse	
29.	F	58		25.53		Remission	Over sleep
30.	M	40		57.48		Remission	

The level of S-CT and its metabolites in plasma and urine samples of 30 psychiatric patients using 20 mg escitalopram was studied by using the method validated. As a result of the analysis, the mean values of S-CT, S-DCT and S-DDCT in plasma of patients were 27.59, 85.52 and 44.30 ng/mL respectively (see Table 4.64). At the end of the analysis, MR of S-CT and metabolites were calculated (see Table 4.64). Concentration of S-CT and its metabolites in urine was found to be above the highest calibration standard concentration of the method (see Table 4.64).

TABLE 4.64. The Level of Escitalopram and Its Metabolites in Plasma and Urine Samples of 30 Psychiatric Patients Using 20 mg Escitalopram										
Patient	Molecule	Daily dose (mg/d)	Therapeutic Range in plasma (ng/mL)	Expected plasma concentration under a given drug dose (ng/mL) ‡‡‡‡‡‡ (low-high))	Time after last dose (h)	Plasma S-CT Concentration (ng/mL)	Metabolic Ratio in plasma (MR)			Urine S-CT Concentration (ng/mL)
							S-CT/ S-DCT	S-DCT/ S-DDCT	S-CT/ S-DDCT	
1	S-CT	20	15-80	11.6 – 30.8	12	13.40	0.07	1.26	0.09	> 441.80
	S-DCT					198.04				> 428.10
	S-DDCT					156.91				> 427.40
2	S-CT	20	15-80	11.6 – 30.8	12	19.16	0.23	5.43	1.22	> 441.80
	S-DCT					85.24				> 428.10
	S-DDCT					15.70				> 427.40
3	S-CT	20	15-80	11.6 – 30.8	12	32.28	0.29	2.73	0.78	> 441.80
	S-DCT					112.78				> 428.10
	S-DDCT					41.33				> 427.40
4	S-CT	20	15-80	11.6 – 30.8	12	35.87	0.27	4.18	1.12	> 441.80
	S-DCT					133.83				> 428.10
	S-DDCT					32.04				> 427.40
5	S-CT	20	15-80	11.6 – 30.8	12	23.90	0.25	1.66	0.42	> 441.80
	S-DCT					95.04				> 428.10
	S-DDCT					57.43				> 427.40

‡‡‡‡‡‡ (C/D low and C/D high) for calculation of dose-related plasma escitalopram concentrations (C/D): 0,58-1,54) (3).

TABLE 4.64. The Level of Escitalopram and Its Metabolites in Plasma and Urine Samples of 30 Psychiatric Patients Using 20 mg Escitalopram (continued)											
Patient	Molecule	Daily dose (mg/d)	Therapeutic Range in plasma (ng/mL)	Expected plasma concentration under a given drug dose (ng/mL) * (low-high))	Time after last dose (h)	Plasma S-CT Concentration (ng/mL)	Metabolic Ratio in plasma (MR)				Urine S-CT Concentration (ng/mL)
							S-CT/S-DCT	S-DCT/S-DDCT	S-CT/ DDCT	S-	
6	S-CT	20	15-80	11.6 – 30.8	12	49.32	0.47	3.88	1.82		> 441.80
	S-DCT					104.88					> 428.10
	S-DDCT					27.06					> 427.40
7	S-CT	20	15-80	11.6 – 30.8	12	16.83	0.34	1.00	0.34		247.37
	S-DCT					49.85					> 428.10
	S-DDCT					49.69					> 427.40
8	S-CT	20	15-80	11.6 – 30.8	12	0.95	0.04	0.96	0.03		139.00
	S-DCT					27.11					> 428.10
	S-DDCT					28.27					> 427.40
9	S-CT	20	15-80	11.6 – 30.8	12	31.58	0.33	3.11	1.03		> 441.80
	S-DCT					95.27					> 428.10
	S-DDCT					30.64					> 427.40
10	S-CT	20	15-80	11.6 – 30.8	12	22.81	0.19	9.08	1.69		> 441.80
	S-DCT					122.95					> 428.10
	S-DDCT					13.54					> 427.40

TABLE 4.64. The Level of Escitalopram and Its Metabolites in Plasma and Urine Samples of 30 Psychiatric Patients Using 20 mg Escitalopram (continued)

Patient	Molecule	Daily dose (mg/d)	Therapeutic Range in plasma (ng/mL)	Expected plasma concentration under a given drug dose (ng/mL) * (low-high))	Time after last dose (h)	Plasma S-CT Concentration (ng/mL)	Metabolic Ratio in plasma (MR)				Urine S-CT Concentration (ng/mL)
							S-CT/S-DCT	S-DCT/S-DDCT	S-CT/DDCT	S-	
11	S-CT	20	15-80	11.6 – 30.8	12	9.66	0.08	1.40	0.11		195.63
	S-DCT					119.74					> 441.80
	S-DDCT					85.55					> 428.10
12	S-CT	20	15-80	11.6 – 30.8	12	15.23	0.13	0.98	0.13		> 441.80
	S-DCT					117.19					> 428.10
	S-DDCT					119.15					> 427.40
13	S-CT	20	15-80	11.6 – 30.8	12	12.26	0.17	0.72	0.12		> 441.80
	S-DCT					73.34					> 428.10
	S-DDCT					102.39					> 427.40
14	S-CT	20	15-80	11.6 – 30.8	12	55.25	1.43	11.95	17.14		> 441.80
	S-DCT					38.52					> 428.10
	S-DDCT					3.22					81.27
15	S-CT	20	15-80	11.6 – 30.8	12	27.38	0.36	1.16	0.41		> 441.80
	S-DCT					77.16					> 428.10
	S-DDCT					66.32					> 427.40

TABLE 4.64. The Level of Escitalopram and Its Metabolites in Plasma and Urine Samples of 30 Psychiatric Patients Using 20 mg Escitalopram (continued)

Patient	Molecule	Daily dose (mg/d)	Therapeutic Range in plasma (ng/mL)	Expected plasma concentration under a given drug dose (ng/mL) * (low-high))	Time after last dose (h)	Plasma S-CT Concentration (ng/mL)	Metabolic Ratio in plasma (MR)			Urine S-CT Concentration (ng/mL)
							S-CT/ S-DCT	S-DCT/ S-DDCT	S-CT/ S-DDCT	
16	S-CT	20	15-80	11.6 – 30.8	12	27.17	0.35	1.20	0.42	> 441.80
	S-DCT					77.42				> 428.10
	S-DDCT					64.81				> 427.40
17	S-CT	20	15-80	11.6 – 30.8	12	63.36	1.08	3.48	3.75	> 441.80
	S-DCT					58.80				> 428.10
	S-DDCT					16.91				> 427.40
18	S-CT	20	15-80	11.6 – 30.8	12	37.28	0.37	4.66	1.74	> 441.80
	S-DCT					100.13				> 428.10
	S-DDCT					21.49				> 427.40
19	S-CT	20	15-80	11.6 – 30.8	12	40.94	0.34	2.58	0.86	> 441.80
	S-DCT					122.14				> 428.10
	S-DDCT					47.39				> 427.40
20	S-CT	20	15-80	11.6 – 30.8	12	26.39	0.33	3.99	1.31	> 441.80
	S-DCT					80.50				> 428.10
	S-DDCT					20.18				> 427.40

TABLE 4.64. The Level of Escitalopram and Its Metabolites in Plasma and Urine Samples of 30 Psychiatric Patients Using 20 mg Escitalopram (continued)										
Patient	Molecule	Daily dose (mg/d)	Therapeutic Range in plasma (ng/mL)	Expected plasma concentration under a given drug dose (ng/mL) * (low-high))	Time after last dose (h)	Plasma S-CT Concentration (ng/mL)	Metabolic Ratio in plasma (MR)			Urine S-CT Concentration (ng/mL)
							S-CT/ S-DCT	S-DCT/ S-DDCT	S-CT/ S-DDCT	
21	S-CT	20	15-80	11.6 – 30.8	12	29.71	0.30	4.61	1.39	> 441.80
	S-DCT					98.49				> 428.10
	S-DDCT					21.38				> 427.40
22	S-CT	20	15-80	11.6 – 30.8	12	20.95	0.32	1.53	0.48	> 441.80
	S-DCT					66.52				> 428.10
	S-DDCT					43.50				> 427.40
23	S-CT	20	15-80	11.6 – 30.8	12	20.91	0.32	1.53	0.49	> 441.80
	S-DCT					65.71				> 428.10
	S-DDCT					42.85				> 427.40
24	S-CT	20	15-80	11.6 – 30.8	12	38.21	0.45	2.02	0.91	> 441.80
	S-DCT					85.06				> 428.10
	S-DDCT					42.14				> 427.40
25	S-CT	20	15-80	11.6 – 30.8	12	14.89	0.12	1.54	0.18	340.60
	S-DCT					124.96				> 428.10
	S-DDCT					81.08				> 427.40

TABLE 4.64. The Level of Escitalopram and Its Metabolites in Plasma and Urine Samples of 30 Psychiatric Patients Using 20 mg Escitalopram (continued)

Patient	Molecule	Daily dose (mg/d)	Therapeutic Range in plasma (ng/mL)	Expected plasma concentration under a given drug dose (ng/mL) * (low-high))	Time after last dose (h)	Plasma S-CT Concentration (ng/mL)	Metabolic Ratio in plasma (MR)			Urine S-CT Concentration (ng/mL)
							S-CT/ S-DCT	S-DCT/ S-DDCT	S-CT/ S-DDCT	
26	S-CT	20	15-80	11.6 – 30.8	12	2.93	0.19	0.86	0.16	377.73
	S-DCT					15.81				> 428.10
	S-DDCT					18.34				> 427.40
27	S-CT	20	15-80	11.6 – 30.8	12	48.74	1.02	3.69	3.77	> 441.80
	S-DCT					47.82				> 428.10
	S-DDCT					12.94				> 427.40
28	S-CT	20	15-80	11.6 – 30.8	12	7.35	1.28	20.25	25.92	> 441.80
	S-DCT					5.74				> 428.10
	S-DDCT					0.28				29.19
29	S-CT	20	15-80	11.6 – 30.8	12	25.54	0.36	2.39	0.87	> 441.80
	S-DCT					70.17				> 428.10
	S-DDCT					29.35				> 427.40
30	S-CT	20	15-80	11.6 – 30.8	12	57.48	0.60	2.57	1.55	> 441.80
	S-DCT					95.53				> 428.10
	S-DDCT					37.16				> 427.40

TABLE 4.64. The Level of Escitalopram and Its Metabolites in Plasma and Urine Samples of 30 Psychiatric Patients Using 20 mg Escitalopram (continued)										
Patient	Molecule	Daily dose (mg/d)	Therapeutic Range in plasma (ng/mL)	Expected plasma concentration under a given drug dose (ng/mL) * (low-high))	Time after last dose (h)	Plasma S-CT Concentration (ng/mL)	Metabolic Ratio in plasma (MR)			Urine S-CT Concentration (ng/mL)
							S-CT/ S-DCT	S-DCT/ S-DDCT	S-CT/ S-DDCT	
Mean							**0.40**	**3.55**	**2.34**	
	S-CT					**27.59**				
	S-DCT					**85.52**				
	S-DDCT					**44.30**				
SD							**0.35**	**4.01**	**5.42**	
	S-CT					**16.05**				
	S-DCT					**39.31**				
	S-DDCT					**35.45**				

4.3.2. The result of patient's genotyping

It is known that G> A nucleotide changes in CYP2C19 * 2 (rs4244285) allele in exon 5 and CYP2C19 * 3 (rs 4986893) alleles in exon 4 cause nonfunctional enzyme activities and C> T nucleotide change in CYP2C19 * 17 (rs12248560) allele in exon 5 regulatory causes increased enzyme activity (50). The results of the genotyping analysis performed to determine the CYP2C19 polymorphism in chromosome 10q24 of 30 patients are given in Table 4.65. CYP2C19 genotyping analysis revealed that 14 patients were extensive metabolizer (EM; *1 /*1), five patients were intermediate (IM; four patients *1 /* 2 and one patient *1/* 3), six patients were heterozygous rapid (Het UM; *1/*17), and five patients were homozygous rapid metabolizers (Homo UM; *17/*17) (Figure 4.8-4.10).

TABLE 4.65. The Results of The CYP2C19 Genotyping Analysis of Patients

Sample	C19 *2	C19 *3	C19 *17	Genotype	Phenotype
1	GG	GG	CT	1*17	Het. UM
2	GG	GG	CT	1*17	Het. UM
3	AG	GG	CC	1*2	IM
4	AG	GG	CC	1*2	IM
5	GG	GG	TT	17*17	Homo. UM
6	GG	GG	CT	1*17	Het. UM
7	GG	GG	CC	1*1	EM
8	GG	GG	CT	1*17	Het. UM
9	GG	GG	CC	1*1	EM
10	GG	GG	CT	1*17	Het. UM
11	GG	GG	CC	1*1	EM
12	GG	GG	CC	1*1	EM
13	GG	GG	CC	1*1	EM
14	GG	GG	CC	1*1	EM
15	GG	GG	CC	1*1	EM
16	GG	GG	CC	1*1	EM

TABLE 4.65. The Results of The CYP2C19 Genotyping Analysis of Patients (continued)					
Sample	C19 *2	C19 *3	C19 *17	Genotype	Phenotype
17	GG	GG	TT	17*17	Homo. UM
18	GG	GG	TT	17*17	Homo. UM
19	GG	GG	TT	17*17	Homo. UM
20	GG	GG	TT	17*17	Homo. UM
21	GG	GG	CT	1*17	Het. UM
22	GG	GG	CC	1*1	EM
23	GG	GG	CC	1*1	EM
24	AG	GG	CC	1*2	EM
25	GG	GG	CC	1*1	EM
26	GG	AG	CC	1*3	IM
27	AG	GG	CC	1*2	IM
28	GG	GG	CC	1*1	EM
29	GG	GG	CC	1*1	EM
30	GG	GG	CC	1*1	EM

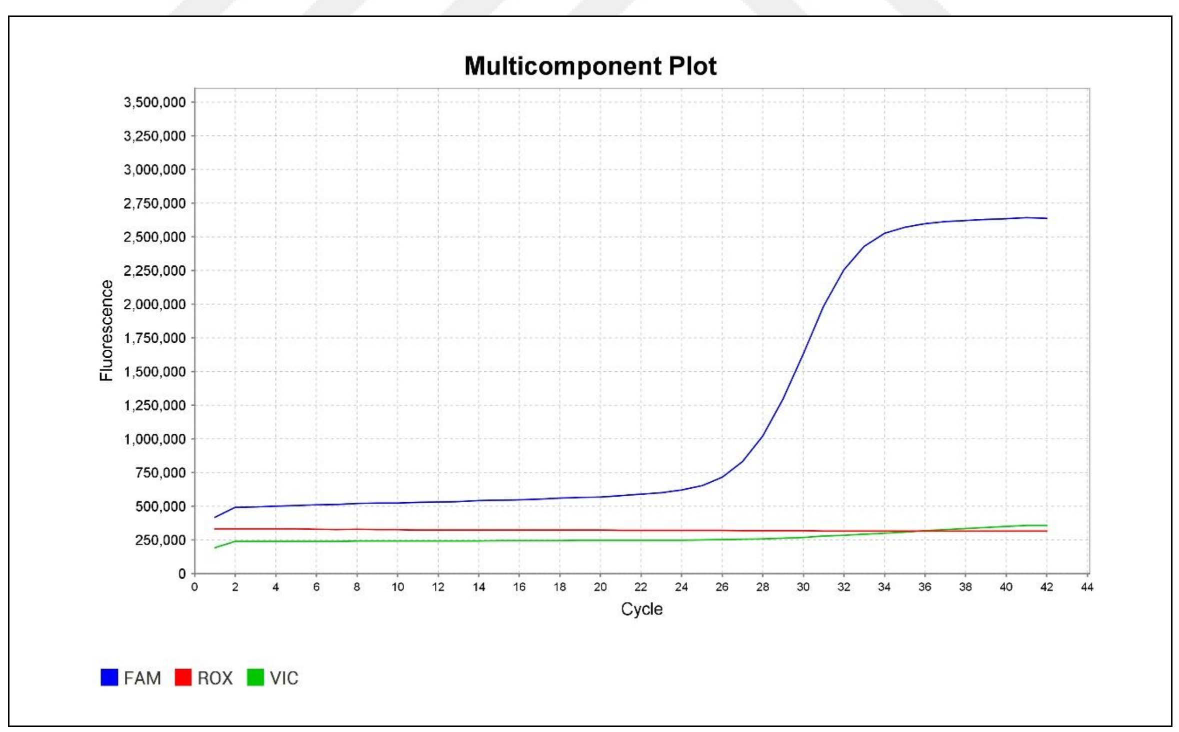

Figure 4.8 Representetive CYP2C19 wild type alleles (blue color FAM: wild type alleles; green color VIC: SNP alleles; red color ROX: baseline)

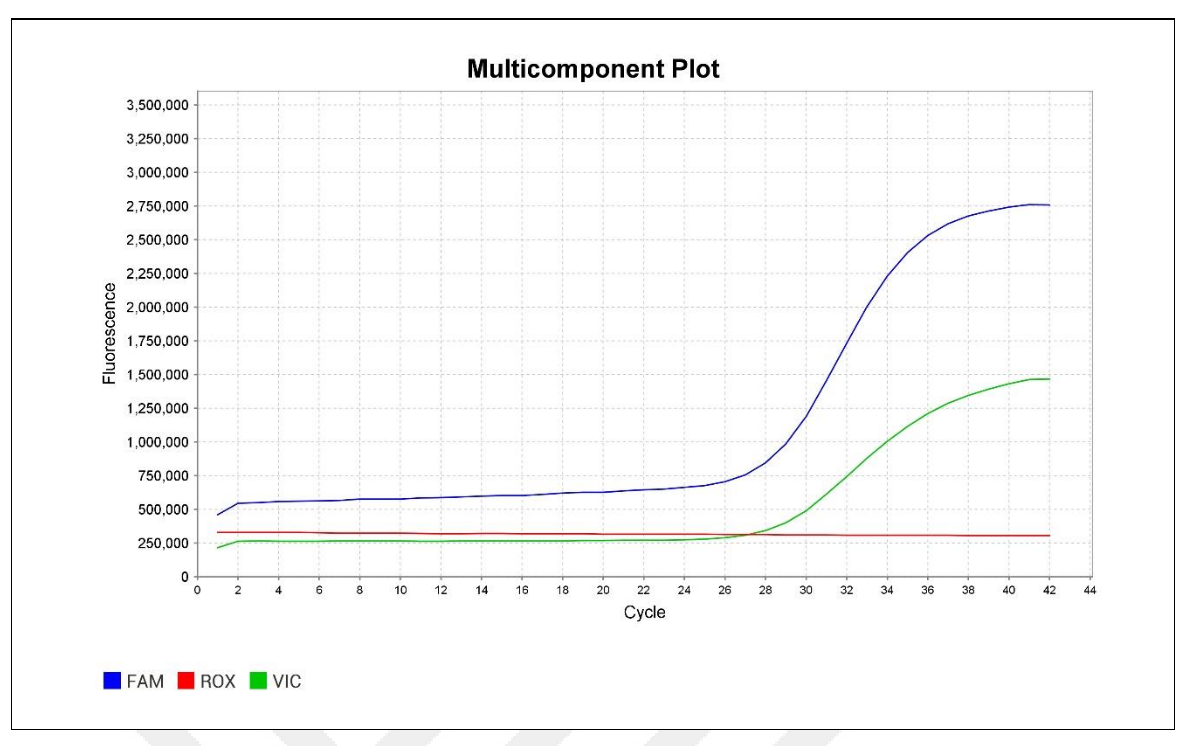

Figure 4.9 Representetive CYP2C19 heterozigot Alleles (blue color FAM: wild type alleles; green color VIC: SNP alleles; red color ROX: baseline)

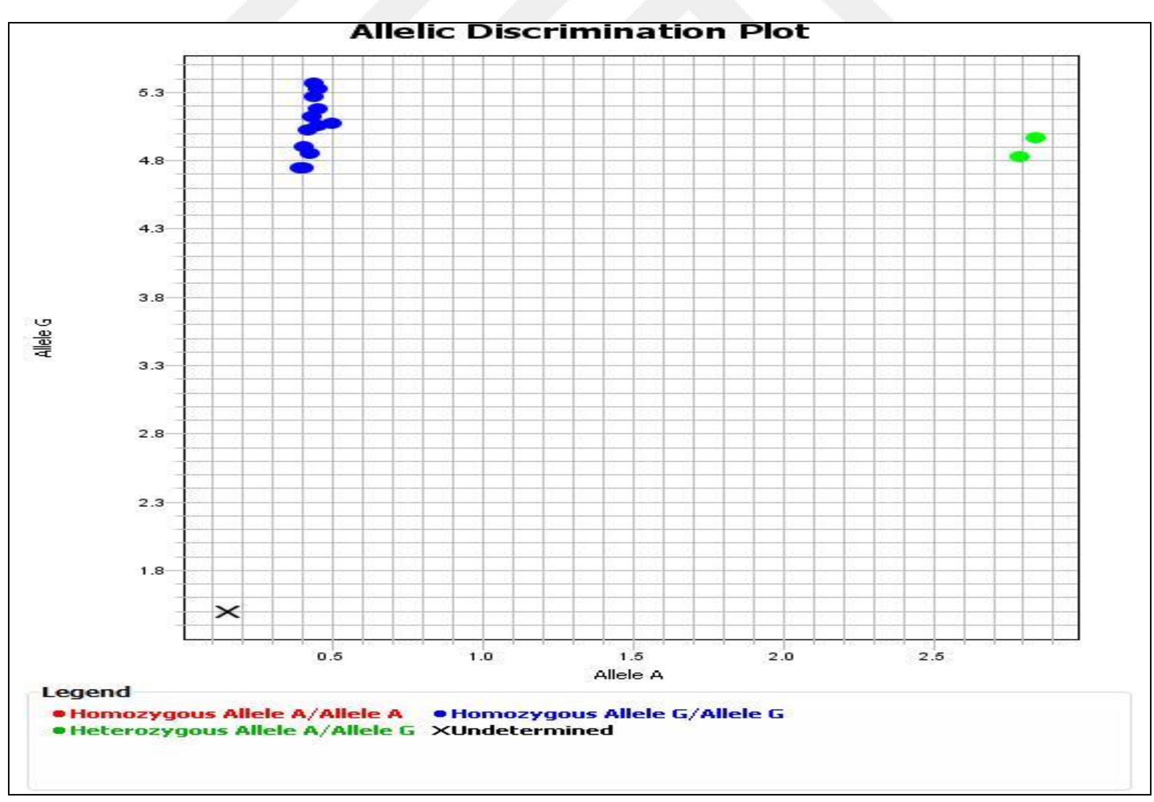

Figure 4.10 Representetive CYP2C19 Allelic discrimination Plot

When genotyping analysis results were compared to plasma escitalopram concentrations and metabolic ratios of S-CT and its metabolites, the S-CT / S-DCT metabolic ratio of five of 11 patients with Het.UM and Homo. UM genotyping was found to be below ≤ 0.25. Nine of 14 patients with EM genotyping had a metabolic ratio of S-CT / S-DCT> 0.3 (Table 4.66).

TABLE 4.66. Comparison o Escitalopram and Its Metabolites Plasma Concentration and Metabolic Ratio of 30 Patients with CYP2C19 Genotype Analysis Result

Sample	S-CT	S-DCT	S-DDCT	S-CT/S-DCT	S-DCT/S-DDCT	S-CT/S-DDCT	Genotype	Phenotype
1	13.40	198.04	156.91	0.07	1.26	0.09	1*17	Het. UM
2	19.16	85.24	15.70	0.22	5.43	1.22	1*17	Het. UM
3	32.28	112.78	41.33	0.29	2.73	0.78	1*2	IM
4	35.87	133.83	32.04	0.27	4.18	1.12	1*2	IM
5	23.90	95.04	57.43	0.25	1.66	0.42	17*17	Homo. UM
6	49.32	104.88	27.06	0.47	3.88	1.82	1*17	Het. UM
7	16.83	49.85	49.69	0.34	1.00	0.34	1*1	EM
8	0.95	27.11	28.27	0.03	0.96	0.03	1*17	Het. UM
9	31.58	95.27	30.64	0.33	3.11	1.03	1*1	EM
10	22.81	122.95	13.54	0.19	9.08	1.68	1*17	Het. UM
11	9.66	119.74	85.55	0.08	1.40	0.11	1*1	EM
12	15.23	117.19	119.15	0.13	0.98	0.13	1*1	EM
13	12.26	73.34	102.39	0.17	0.72	0.12	1*1	EM
14	55.25	38.52	3.22	1.43	11.95	17.14	1*1	EM
15	27.38	77.16	66.32	0.35	1.16	0.41	1*1	EM
16	27.17	77.42	64.81	0.35	1.19	0.42	1*1	EM
17	63.36	58.80	16.91	1.08	3.48	3.75	17*17	Homo. UM
18	37.28	100.13	21.49	0.37	4.66	1.73	17/17	Homo. UM
19	40.94	122.14	47.39	0.34	2.58	0.86	17/17	Homo. UM
20	26.39	80.50	20.18	0.33	3.99	1.31	17/17	Homo. UM
21	29.71	98.49	21.38	0.30	4.61	1.39	1*17	Het. UM
22	20.95	66.52	43.50	0.32	1.53	0.48	1*1	EM
23	20.91	65.71	42.85	0.32	1.53	0.49	1*1	EM
24	38.21	85.06	42.14	0.45	2.02	0.91	1*2	IM
25	14.89	124.96	81.08	0.12	1.54	0.18	1*1	EM
26	2.93	15.81	18.34	0.19	0.86	0.16	1*3	IM
27	48.74	47.82	12.94	1.02	3.69	3.77	1*2	IM
28	7.35	5.74	0.28	1.28	20.25	25.92	1*1	EM
29	25.54	70.17	29.35	0.36	2.39	0.87	1*1	EM
30	57.48	95.53	37.16	0.60	2.57	1.55	1*1	EM

The results of CYP2D6 genotyping analysis of 11 patients with no evaluation of CYP2C19 analysis results are given in Table 4.67.

TABLE 4.67. **The Results of The CYP2D6 Genotyping Analysis of Patients**						
Sample	D6 *2	D6 *4	D6 *10	D6 *41	Genotype	Phenotype
6	AG	CC	GG	CT	*2/*41	Het.EM
17	GG	CT	AG	CC	*4/*10	IM
18	GG	CT	AG	CC	*4/*10	IM
19	GG	CC	GG	CC	*1/*1	EM
20	GG	CT	AG	CC	*4/*10	IM
21	GG	CT	AG	CC	*4/*10	IM
11	AG	CC	GG	CC	*1/*2	Het.EM
12	GG	CC	GG	CC	*1/*1	EM
13	AG	CC	GG	CC	*1/*2	Het.EM
25	GG	CC	GG	CC	*1/*1	EM
26	GG	CC	AG	CC	*1/*10	Het.EM

When CYP2D6 * 2, * 4, * 10, * 41 were examined in CYP2D6 polymorphism consisting of nine exons and four introns on chromosome 22q13.1, it was found that CYP2D6 polymorphism of three of the four patients with CYP2C19 polymorphism Homo.UM were IM (* 4 / * 10) and one of them was EM (* 1 / * 1). It was found that CYP2D6 polymorphism of one of the two patients with CYP2C19 polymorphism Het. UM were IM (* 4 / * 10) and the other patient was Het.EM (* 2 / * 41). It was also found that CYP2D6 polymorphism of two of the four patients with CYP2C19 polymorphism EM were EM (* 1 / * 1) and CYP2D6 polymorphism of the other two patients were Het. EM (* 1 / * 2). CYP2D6 polymorphism of a patient with CYP2C19 polymorphism IM was Het. EM (*1 /*10) (see Table 4.68, Figure 4.11,4.12).

TABLE 4.68 Comparison of Escitalopram and Its Metabolites Plasma Concentration and Metabolic Ratios of 11 Patients with CYP2C19 and CYP2D6 Genotype Analysis Result

Sample	S-CT	S-DCT	S-DDCT	S-CT/S-DCT	S-DCT/S-DDCT	S-CT/S-DDCT	CYP2C19	CYP2D6
6	49.32	104.88	27.06	0.47	3.88	1.82	Het. UM	Het.EM
17	63.36	58.80	16.91	1.08	3.48	3.75	Homo. UM	IM
18	37.28	100.13	21.49	0.37	4.66	1.73	Homo. UM	IM
19	40.94	122.14	47.39	0.34	2.58	0.86	Homo. UM	EM
20	26.39	80.50	20.18	0.33	3.99	1.31	Homo. UM	IM
21	29.71	98.49	21.38	0.30	4.61	1.39	Het. UM	IM
11	9.66	119.74	85.55	0.08	1.40	0.11	EM	Het.EM
12	15.23	117.19	119.15	0.13	0.98	0.13	EM	EM
13	12.26	73.34	102.39	0.17	0.72	0.12	EM	Het.EM
25	14.89	124.96	81.08	0.12	1.54	0.18	EM	EM
26	2.93	15.81	18.34	0.19	0.86	0.16	IM	Het.EM

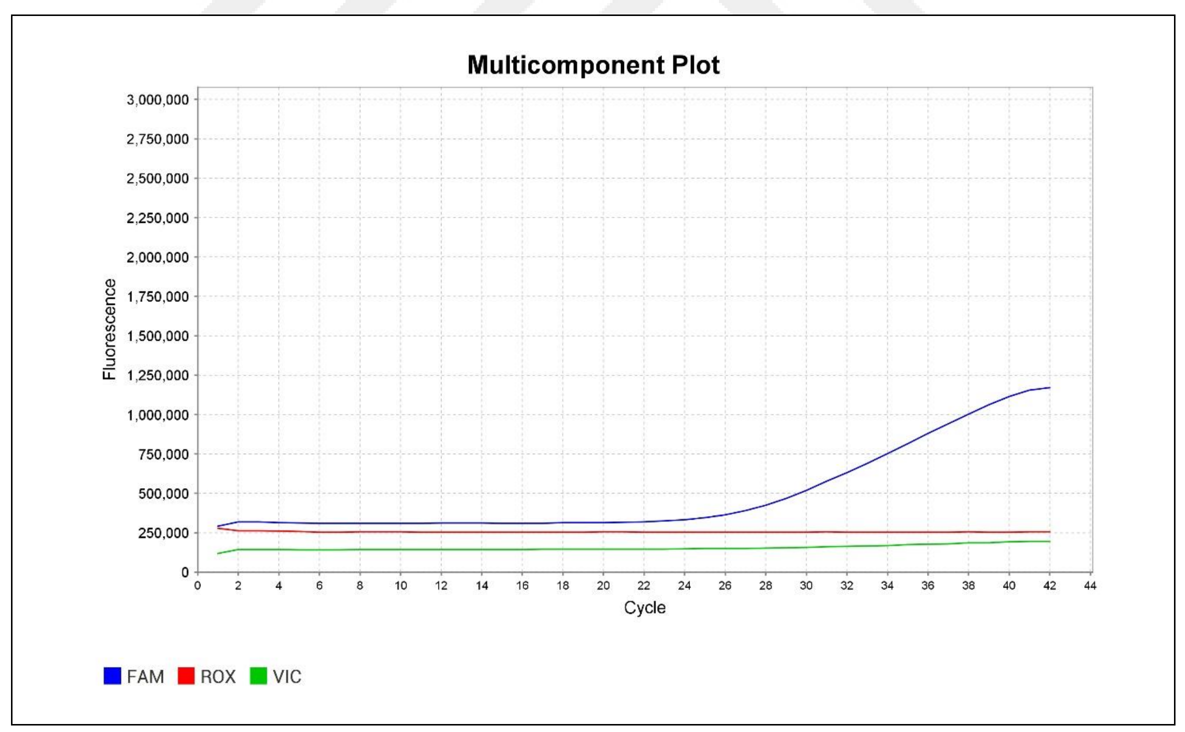

Figure 4.11 Representetive CYP2D6 wild type alleles (blue color FAM: width type alleles; green color VIC: SNP alleles; red color ROX: baseline)

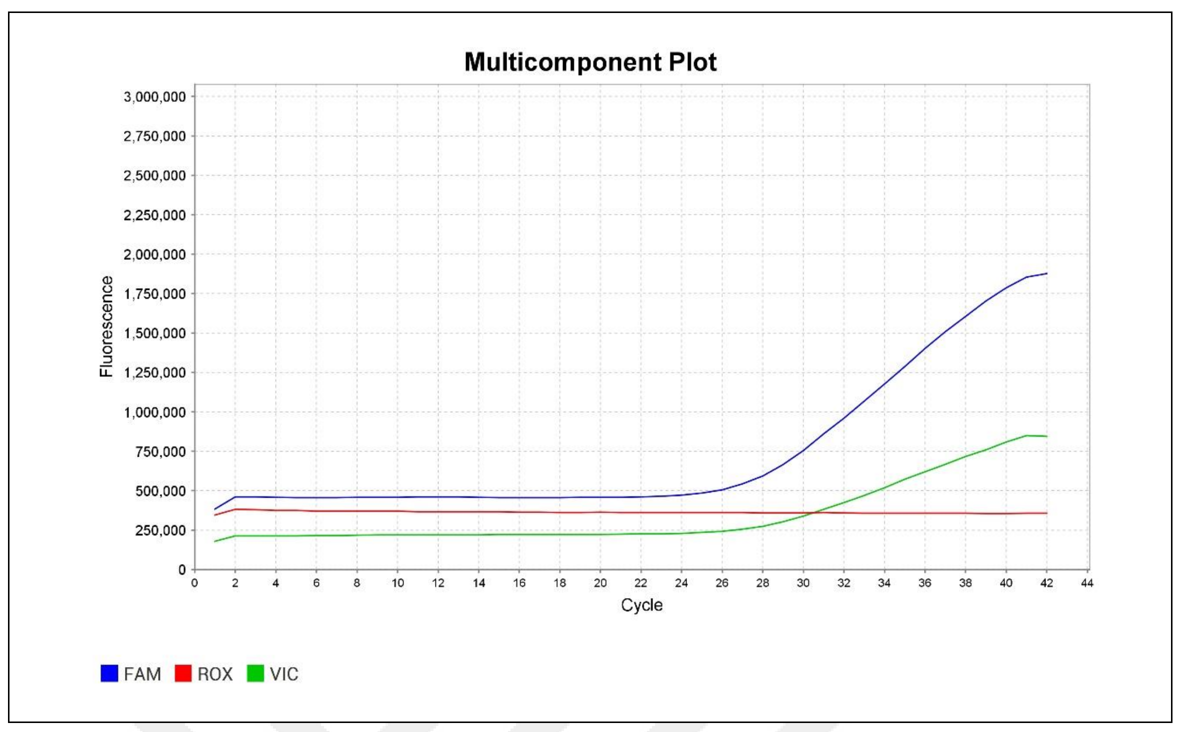

Figure 4.12 Representetive CYP2D6 heterozigot alleles (blue color FAM: width type alleles; green color VIC: SNP alleles; red color ROX: baseline)

The 5-HTT gene encodes the serotonin transporter protein consisting of 630 amino acids at a weight of 68,000 daltons and is mapped to the chromosome 17q11.1-q12 by the gene coding for SLC6A4 (Solute Carrier Family 6 Member 4).

In the transcriptional control region of the 5-HTT gene, alleles called a Long (L) form consisting of 16 repetitions of insertion of a 44 bp GC (Guanine, Cytosine) repeat sequence consisting of 20-22 bp double repeats and a Short (S) form consisting of 14 repetitions as a result of deletion ocur (Figure 4.13). According to this polymorphism, genotypes are categorized as LL, LS and SS. In our study, out of 30 patients, nine patients with LL polymorphism, eight patients with SS polymorphism and 13 patients with LS polymorphism were detected (Table 4.69).

TABLE 4.69. The Evaluation of The Relationship Between S-CT Plasma Concentration and 5-HTT Polymorphism and Drug Response

Sample	S-CT Concentration (ng/mL)	5-HTT Polymorphism	Response	Side Effect
1	13.40	LL	Remission	
2	19.16	LL	Remission	
3	32.28	LL	Remission	
4	35.87	LL	Remission	
5	23.90	SS	Remission	
6	49.32	SS	Nonresponse/Remission after raising the drug dose (30 mg)	
7	16.83	LS	Partial remission	Insomnia, loss of appetite
8	0.95	SS	Nonresponse	
9	31.58	LS	Remission	
10	22.81	LS	Partial remission	
11	9.66	LS	Nonresponse	
12	15.23	SS	Nonresponse/Remission after antypsychotic drug addition	
13	12.26	SS	Nonresponse	
14	55.25	SS	Nonresponse/Partial remission after antypsychotic drug addition	
15	27.38	LS	Remission	
16	27.17	LS	Remission	
17	63.36	LL	Remission	
18	37.28	LL	Remission	
19	40.94	LL	Remission	
20	26.39	LL	Remission	
21	29.71	LL	Remission	
22	20.95	LS	Remission	
23	20.91	LS	Remission	
24	38.21	SS	Remission	
25	14.89	LS	Nonresponse/ Drug change	
26	2.93	LS	Nonresponse	
27	48.74	LS	Partial remission	
28	7.35	LS	Nonresponse	
29	25.54	SS	Remission	Over sleep
30	57.48	LS	Remission	

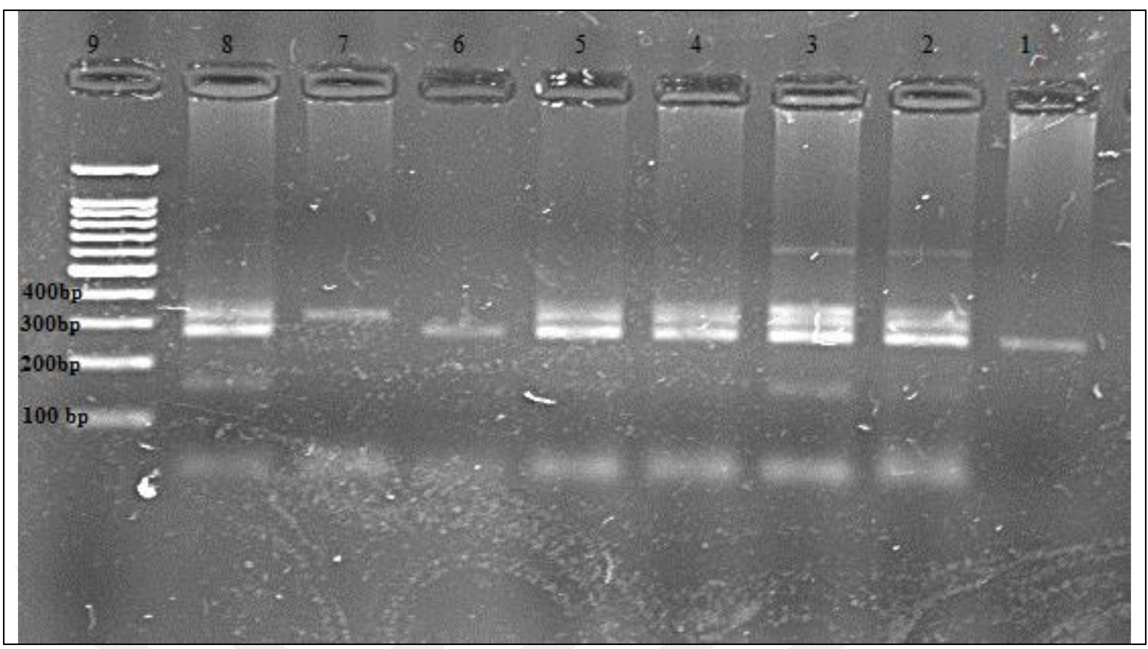

Figure 4.13 Representetive figure of Agarose gel electrophoresis and 5-HTT genotype results. Lanes; 1: SS genotype (272 bp); 2: LS genotype (317 and 272 bp); 3: LS genotype (317 and 272 bp); 4: LS genotype (317 and 272 bp); 5: LS genotype (317 and 272 bp); 6: SS genotype (272 bp); 7: LL genotype (317 bp); 8: LS genotype (317 and 272 bp); 9: DNA standart marker.

The therapeutic range of S-CT is 15-80 ng / ml. In our study, S-CT drug blood level of the nine patients with LL polymorphism was found to be in the range of 13.40 to 63.36 ng / mL (Table 4.69). When the patient files were examined, it was reported that no side effects were observed in nine patients during escitalopram administration, and the desired drug response was obtained. For 13 patients with LS polymorphism, S-CT drug blood level was found to be in the range of 2.93- 57.48 ng / ml. It has been notified in patient files that no treatment response was obtained from two patients with LS polymorphism whose S-CT drug blood level was below the therapeutic range. It has been stated that a drug change has been made for a patient with no response to drug treatment. Partial response (remission) was reported in three patients with S-CT level in the therapeutic range and response (remisison) was obtained in the remaining patients. In this group, the drug side effect (insomnia, loss of appetite) has been reported to have appeared in one person. For eight patients with SS polymorphism, S-CT drug blood level was found to be in the range of 0.95-49.32 ng / mL. Among these patients, two with S-CT drug levels below the therapeutic range were identified. When the patient files have been reviewed, it has been reported that no response was obtained from these patients. It has been reported that remission has been obtained only for one

of the patients whose S-CT level was within the therapeutic range. In other patients, partial remission or remission was reported to have been obtained by raising the drug dose or by drug addition.

4.4. Statistical Assessment

Sex

The mean level, standard deviation, and standard error of escitalopram (S-CT) plasma concentrations between male and female patient groups are summarized in Table 4.70.

TABLE 4.70. Statistical Summary of S-CT Plasma Concentration by Sex

	Sex	N	Mean	Std. Deviation	Std. Error Mean	Independent t test (Sig. (2-tailed))
Escitalopram(S-CT) concentration	Female	21	29.10	17.12	3.83	0.476
	Male	9	24.58	13.99	4.43	

Independent t test shows that there is no statistically significant effect of gender difference (Table 4.71) on escitalopram (S-CT) plasma concentration ($p > 0.05$) (Figure 4.14).

TABLE 4.71. Relationship of Sex and S-CT Plasma Concentration

Independent Samples Test	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Equal variances assumed	.881	.356	.722	28	.476	4.52350	6.26822	-8.31637	17.36337
Equal variances not assumed			.773	21,748	.448	4.52350	5.85192	-7.62079	16.66779

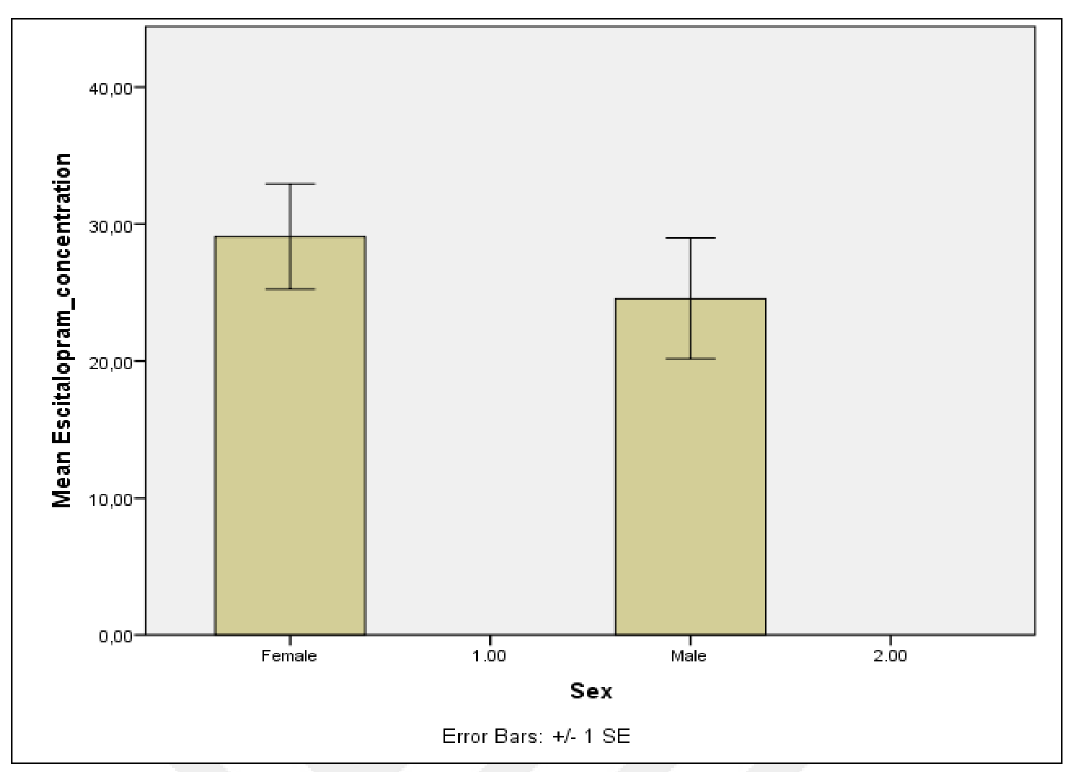

Figure 4.14 Relationship of Sex and S-CT plasma Concentration

Age

The frequency distribution of the different age groups of 30 patients is summarized in Table 4.72.

TABLE 4.72. The Frequency Distribution of The Different Age Groups of 30 Patients

Sample	Frequency	Percent	Valid Percent	Cumulative Percent
≤25	3	10.0	10.0	10.0
26-35	9	30.0	30.0	40.0
36-45	8	26.7	26.7	66.7
46-55	9	30.0	30.0	96.7
>55	1	3.3	3.3	100.0
Total	30	100.0	100.0	

When the relationship between escitalopram plasma level and age groups was examined using Kruskal Wallis test, no statistical significance was found ($p > 0.05$) in Table 4.73 (Figure 4.15.)

TABLE 4.73. Statistical Summary of The Relationship Between Escitalopram Plasma Level and Age Groups					
S-CT Concentration	≤ 25	26-35	36-45	46-55	>55
Mean	19.96	18.84	32.57	34.69	25.53[§§§§§§]
Minumum	7.35	0.95	2.93	15.23	
Maximum	29.71	32.28	57.48	63.36	
Median	22.81	19.16	37.04	27.38	
p (Krustall Wallis Asymp. Sig)			0.245		
p (One way ANOVA)			0.206		

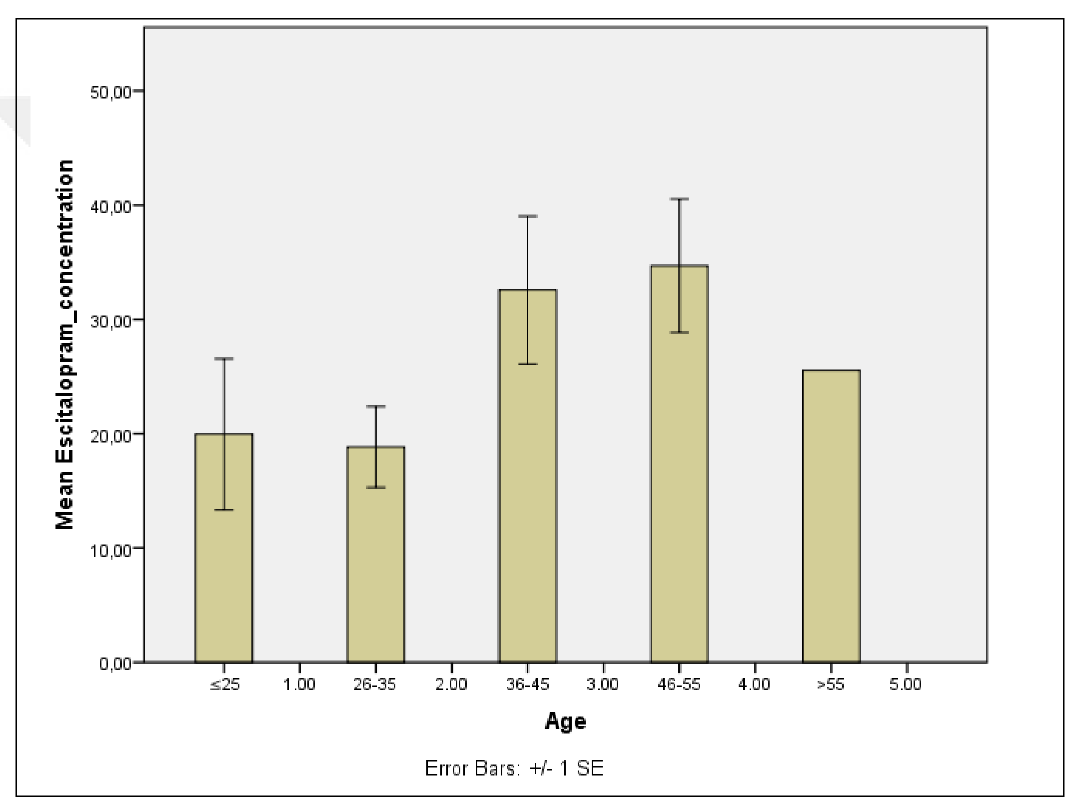

Figure 4.15 Relationship of Age and S-CT plasma Concentration

[§§§§§§] Escitalopram (S-CT) concentration is constant when Age = >55. It has been omitted.

Evaluation of Plasma S-CT Concentration and S-CT / S-DCT Metabolic Ratio by CYP2C19 Genotype Distribution

The frequency distribution of the wild type (* 1 / * 1), heterozygote (* 1 / * 2, * 1 / * 10, * 1 / * 17), * 17 / * 17 for CYP2C19 polymorphism in thirty patients was summarized in Table 4.74 (Figure 4.16).

TABLE 4.74. **The Frequency Distribution of The Cyp2c19 Polymorphism in 30 Patients**

CYP2C19	Frequency	Percent	Valid Percent	Cumulative Percent
*1/*1	14	46.7	46.7	46.7
*1/*2	4	13.3	13.3	60.0
*1/*3	1	3.3	3.3	63.3
*1/*17	6	20.0	20.0	83.3
*17/*17	5	16.7	16.7	100.0
Total	30	100.0	100.0	

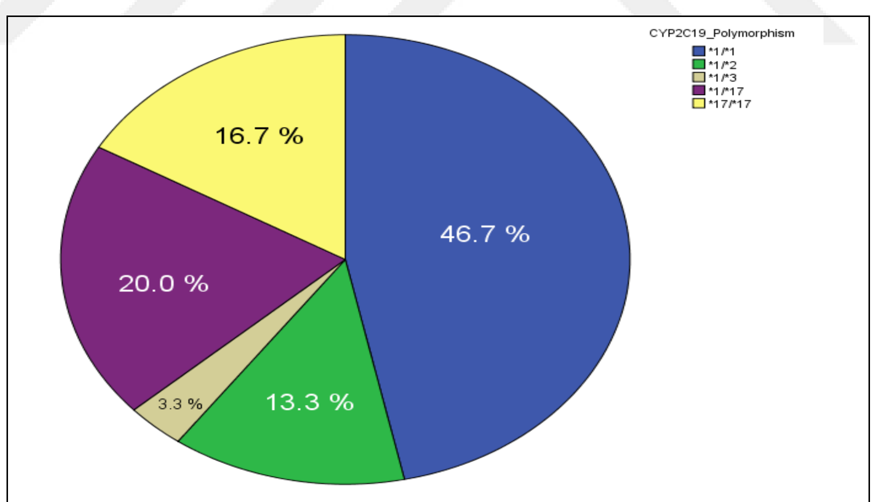

Figure 4.16 The frequency distribution of the CYP2C19polymorphism in 30 patients

The mean, median, minimum and maximum plasma S-CT concentrations of patients with CYP2C19 * 1 / * 1, (* 1 / * 2 ; * 1 /*3) and *1/*17 alleles are summarized

in Table 4.75. In addition, the Kruskall Wallis test was used to compare escitalopram (S-CT) plasma concentrations in patients with CYP2C19 * 1 / * 1, (* 1 / * 2; * 1 / * 3) and * 1 / * 17 alleles. There was no statistically significant difference between groups (p> 0.05) (Figure 4.17).

TABLE 4.75. Summary of S-CT Plasma Concentration of Patients with CYP2C19 Polymorphism

S-CT	EM (*1/*1)	IM (*1/*2; *1/*3)	Het.UM (*1/*17)
Mean	24.57	24.47	7.18
Minumum	12.26	2.93	0.95
Maximum	57.48	38.21	13.40
Median	20.95	32.28	7.18
p (Krustall Wallis Asymp Sign.)		0.150	

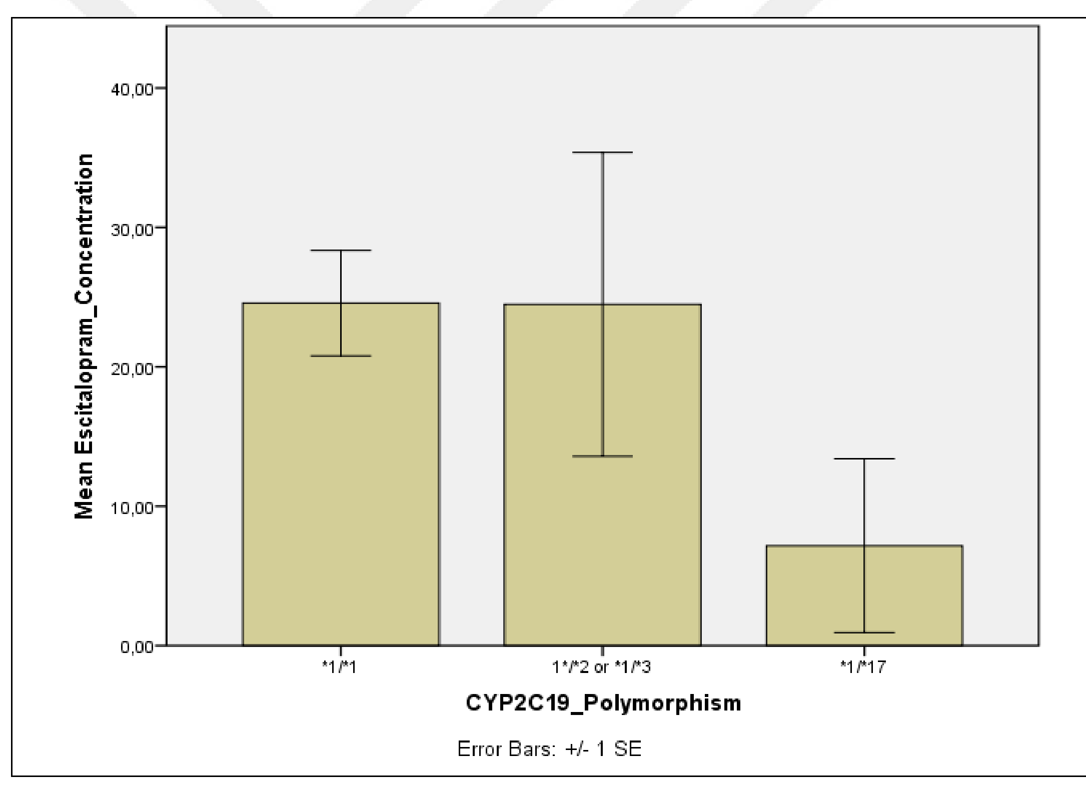

Figure 4.17 Relationship of CYP2C19 polymorphisme and S-CT plasma Concentration of patients.

The mean, median, minimum and maximum values of S-CT / S-DCT metabolic ratio (MR) of patients with CYP2C19 * 1 / * 1, (* 1 / * 2; * 1 /*3) and *1 / *17 alleles are summarized in Table 4.76.

TABLE 4.76. Statistical Summary of MR of Patients with CYP2C19 Polymorphism			
S-CT/ S-DCT	EM (*1/*1)	IM (*1/*2; *1/*3)	Het.UM (*1/*17)
Mean	0.31	0.31	0.05
Minumum	0.12	0.19	0.03
Maximum	0.60	0.45	0.07
Median	0.33	0.29	0.05
p (Krustall Wallis Asymp Sign.)		0.084	
p (One way ANOVA)		0.059	
p (TUKEY HSD)		0.052	

In addition, in order to compare the S-CT / S-DCT Metabolic Ratio (MR) in patients with CYP2C19 * 1 / * 1, (* 1 / * 2;* 1 /*3) and *1 /*17 alleles, initially Kruskall Wallis (MRIS) test, then One Way ANOVA test along with Tukey HSD were used. There was no statistically significant difference between groups in the Kruskall Wallis test (p> 0.05). As the difference was close to the significance limit in the 95% confidence interval, a second evaluation was made within the group and between groups with the One Way ANOVA test. In the One Way ANOVA test, the difference was also close to the significance limit, but a statistically significant difference was found in the MR ratios between the groups *1 /* 1 and *1 /*17 in the TUKEY HSD comparison in Table 4.77 (p <0.05) (Figure 4.18)

TABLE 4.77. Multiple Comparisons of MR of Patiens with CYP2C19 Polymorphism

Dependent Variable: MR

Tukey HSD

CYP2C19_ Polymorphism	CYP2C19_ Polymorphism	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
*1/*1	1*/*2 or *1/*3	-.00182	.08368	1.000	-.2228	.2191
	*1/*17	.25818	.09876	.052	-.0026	.5189
1*/*2 or *1/*3	*1/*1	.00182	.08368	1.000	-.2191	.2228
	*1/*17	.26000	.11728	.105	-.0497	.5697
*1/*17	*1/*1	-.25818	.09876	.052	-.5189	.0026
	1*/*2 or *1/*3	-.26000	.11728	.105	-.5697	.0497

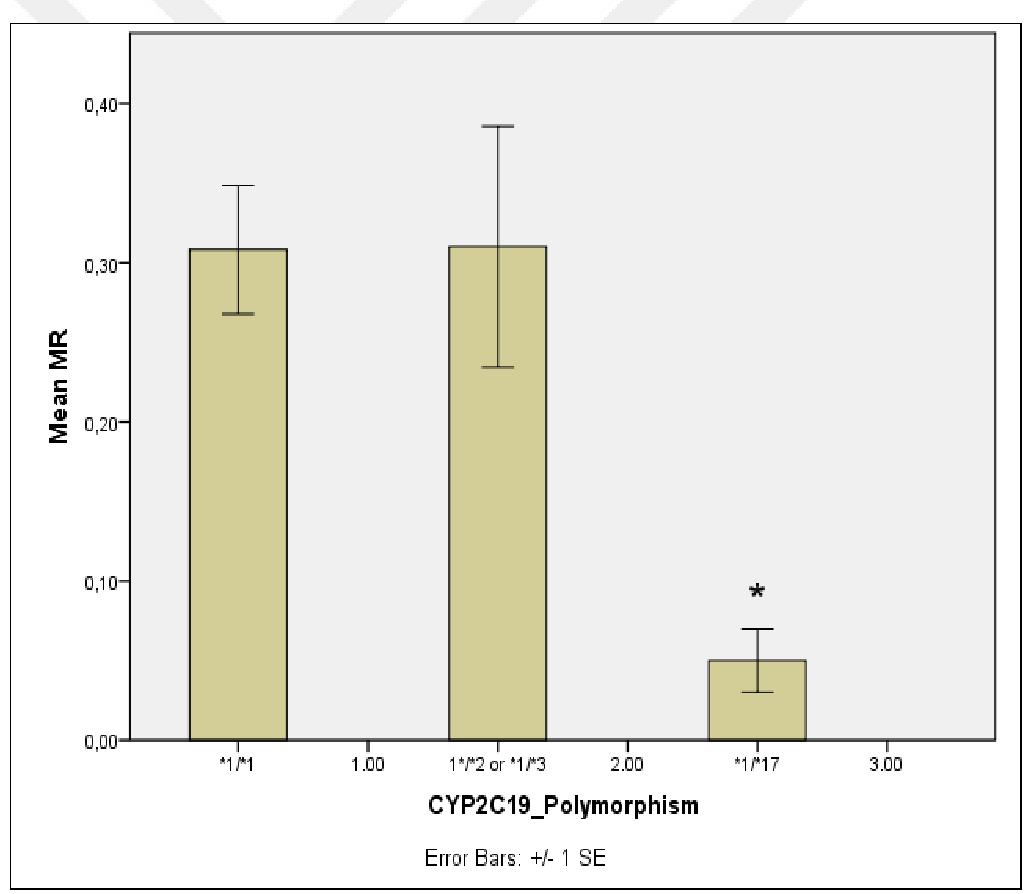

Figure 4.18 Relationship of CYP2C19 polymorphisme and MR of patients. '*' among groups (*1 /* 1 and *1 /*17) represent significant differences ($p < 0.05$) by Tukey HSD test.

Since the difference between S-CT and MR values between CYP2C19 EM (* 1 / * 1) and CYP2C19 IM (* 1 / * 2 or * 1 / * 3) groups was not statistically significant, these groups

were treated together EM-IM group and the statistical evaluation of this group with CYP2C19 * 1 / * 17 carriers was summarized in Table 4.78. The comparison between these groups was made using the Mann Whitney U test. While the difference in escitalopram concentrations between groups was statistically close to the significance limit at 95% confidence interval (p: 0.057) (Figure 4.19). The difference in MR between groups was statistically significant at 95% confidence interval (p <0.05) (Figure 4.20).

TABLE 4.78. Group Statistics S-CT Concentration and MR of Patients with CYP2C19 Polymorphism

	CYP2C19_Polymorphism	N	Mean	Std. Deviation	Std. Error Mean	Mann Whitney U Test (p)
Escitalopram (S-CT) Concentration	*1/*1 or *1/*2 or *1/*3	14	24.5450	13.24457	3.53976	0.057
	*1/*17	2	7.1750	8.80348	6.22500	
S-CT/S-DCT Metabolic Ratio (MR)	*1/*1 or *1/*2 or *1/*3	14	.3086	.12823	.03427	0.026
	*1/*17	2	.0500	.02828	.02000	

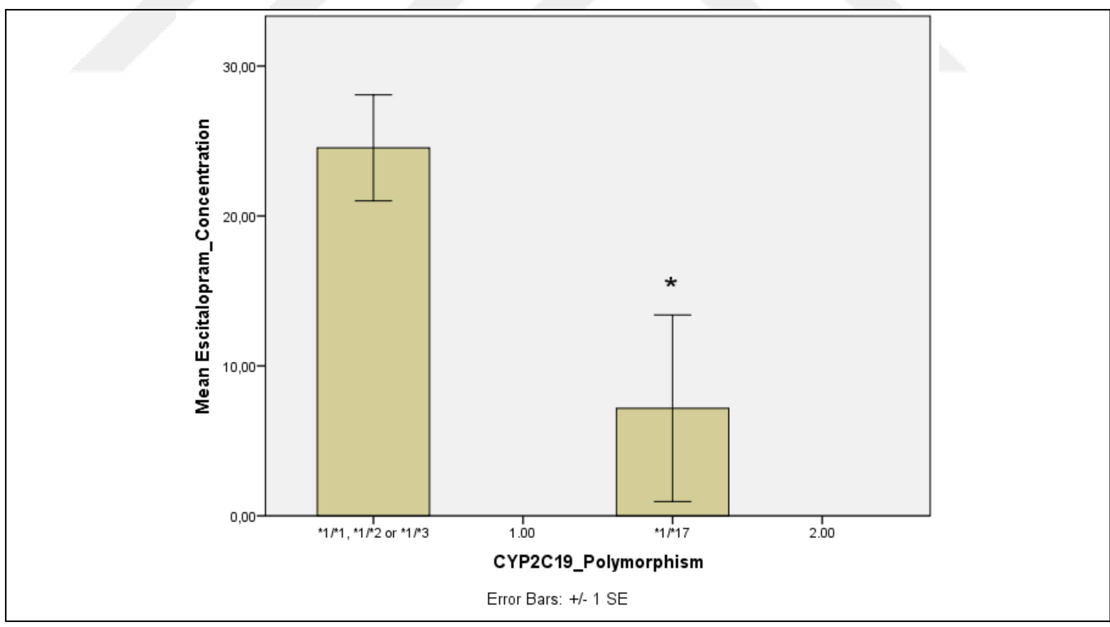

Figure 4.19 Representetive the difference in escitalopram (S-CT) concentration between groups. '*' among groups represent significant differences (p < 0.05) by Mann Whitney U test. The difference in escitalopram concentrations between groups was statistically close to the significance limit at 95% confidence interval (p: 0.057)

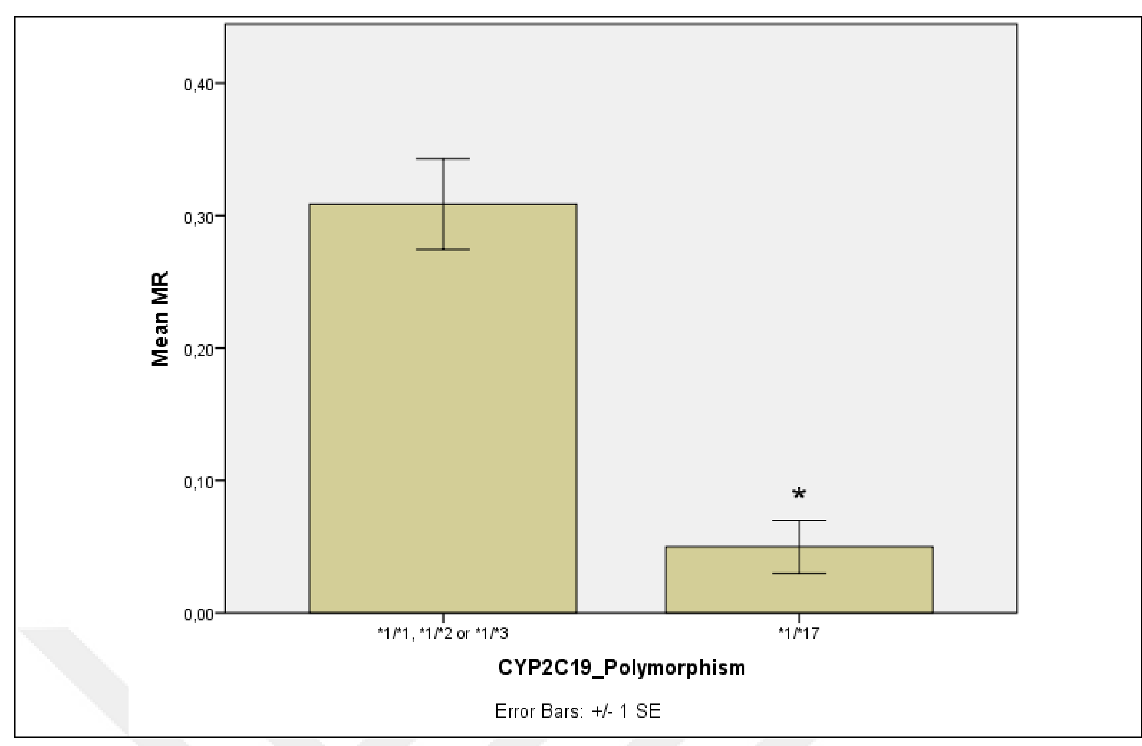

Figure 4.20 Representetive the difference in MR between groups. '*' among groups represent significant differences ($p < 0.05$) by Mann Whitney U test.

Evaluation of Metabolic Ratio of Plasma S-DCT / S-DDCT According to CYP2D6 Genotype Distribution

Frequency distributions of the wild type (* 1 / * 1), heterozygote * 1 / * 2, * 1 / * 10, * 4 / * 10 and * 2 / * 41 groups for CYP2D6 polymorphism of eleven patients were summarized in Table 4.79 (Figure 4.21)

TABLE 4.79. The Frequency Distribution of The CYP2D6 Polymorphism in 11 Patients

CYP2D6	Frequency	Percent	Valid Percent	Cumulative Percent
*1/*1	3	27.3	27.3	27.3
*1/*2	2	18.2	18.2	45.5
*1/*10	1	9.1	9.1	54.5
*4/*10	4	36.4	36.4	90.9
*2/*41	1	9.1	9.1	100.0
Total	11	100.0	100.0	

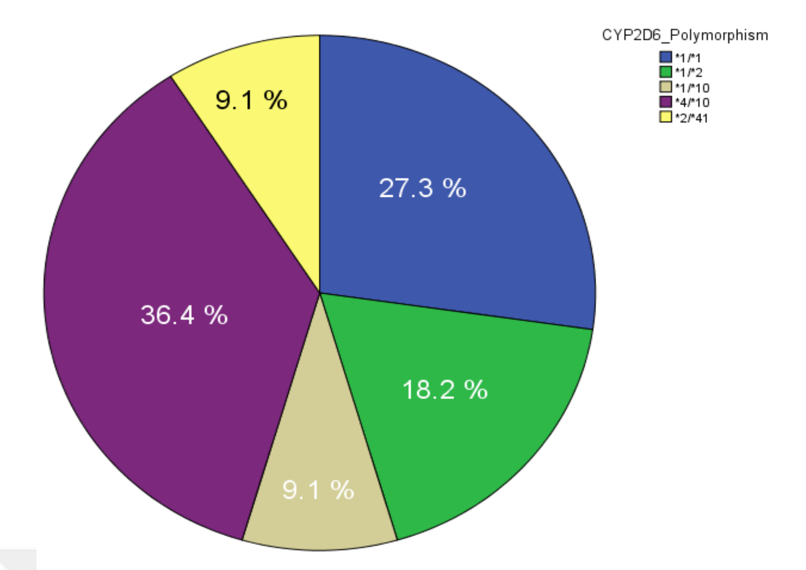

Figure 4.21 Distribution of the CYP2D6 polymorphism in 11 patients.

The mean, median, minimum and maximum values of S-DCT / S-DDCT metabolic ratio (MR) of patients with CYP2D6 * 1 / * 1, (* 1 / * 2 , * 1 /*10, *2/*41) and *4 / *10 alleles are summarized in Table 4.80.

TABLE 4.80. Statistical Summary of MR of Patients with CYP2D6 Polymorphism

S-DCT/ S-DDCT	EM (*1/*1)	Het. EM (*1/*2;*1/*10;*2/*41)	IM (*4/*10)
Mean	1.70	1.71	4.18
Minumum	0.98	0.72	3.48
Maximum	2.58	3.88	4.66
Median	1.54	1.13	4.30
p (Krustall Wallis Asymp. Sig)		0.046	
p (One way ANOVA)		0.017	
TUKEY HSD	0.035	0.025	

In addition, in order to compare the S-DCT / S-DDCT metabolic ratio (MR) of patients with CYP2D6 * 1 / * 1, (* 1 / * 2 , * 1 /*10, *2/*41) and *4 / *10 alleles, initially Kruskall Wallis test, then One Way ANOVA test along with Tukey HSD were used. In the Kruskall Wallis test, a statistically significant difference was found between the groups in the 95% confidence interval ($p < 0.05$). A second evaluation was made

within and between the groups using One-Way ANOVA test. A statistically significant difference was also found in the One Way ANOVA test ($p < 0.05$). A statistical significance was found between MR ratio of *4 / *10 group and MR ratio of other groups ($p < 0.05$) in TUKEY HSD comparison in Table 4.81 (Figure 4.22.)

TABLE 4.81. Multiple Comparisons of MR of Patiens with CYP2D6 Polymorphism

Dependent Variable: MR

Tukey HSD

CYP2D6_Polymorphism	CYP2D6_Polymorphism	Mean Difference	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
*1/*1	*1/*2, *1/*10 and *2/*41	-.01500	.79955	1.000	-2.2997	2.2697
	*4/*10	-2.48500*	.79955	.035	-4.7697	-.2003
*1/*2, *1/*10 and *2/*41	*1/*1	.01500	.79955	1.000	-2.2697	2.2997
	*4/*10	-2.47000*	.74024	.025	-4.5852	-.3548
*4/*10	*1/*1	2.48500*	.79955	.035	.2003	4.7697
	*1/*2, *1/*10 and *2/*41	2.47000*	.74024	.025	.3548	4.5852

*. The mean difference is significant at the 0.05 level.

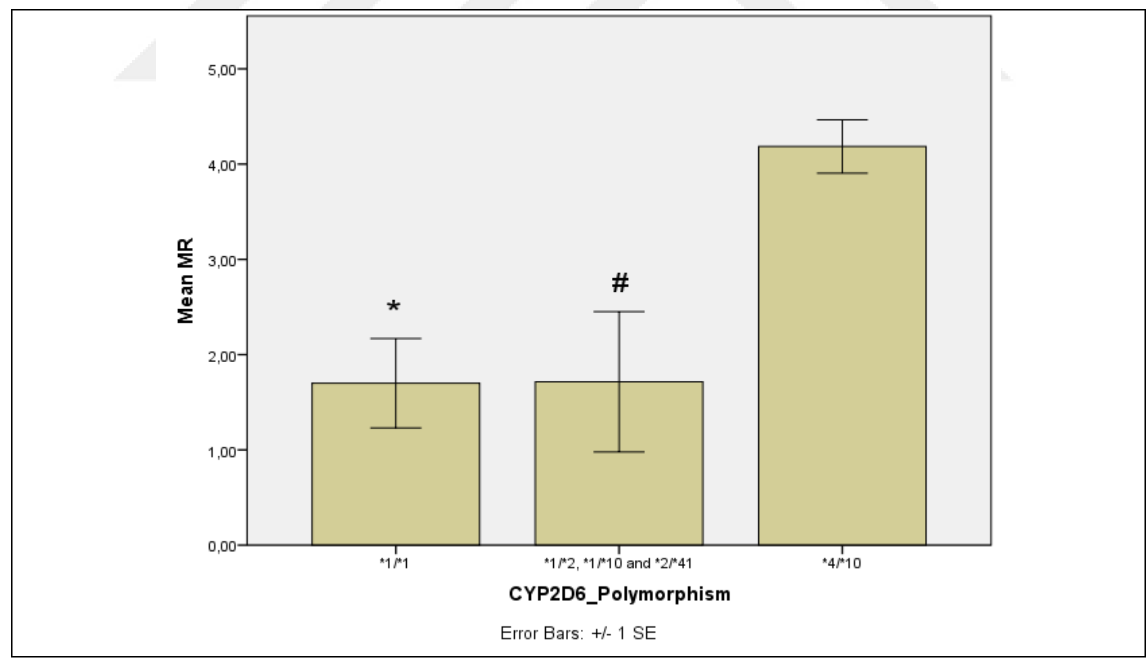

Figure 4.22 Representetive the difference in MR between groups. '*' and '#' among groups represent significant differences ($p < 0.05$) by Tukey HSD test. A statistical significance was found between MR ratio of *4 / *10 group and MR ratio of other groups.

Evaluation of Association between 5-HTT Polymorphism and Response to Treatment

The frequency distribution of the LL, LS and SS groups for the 5-HTT polymorphism of 30 patients is summarized in Table 4.82 (Figure 4.23.)**.**

TABLE 4.82. The Frequency Distribution of 5-Htt Polymorhism in 30 Patients

5-HTT	Frequency	Percent	Valid Percent	Cumulative Percent
LL	9	30.0	30.0	30.0
LS	13	43.3	43.3	73.3
SS	8	26.7	26.7	100.0
Total	30	100.0	100.0	

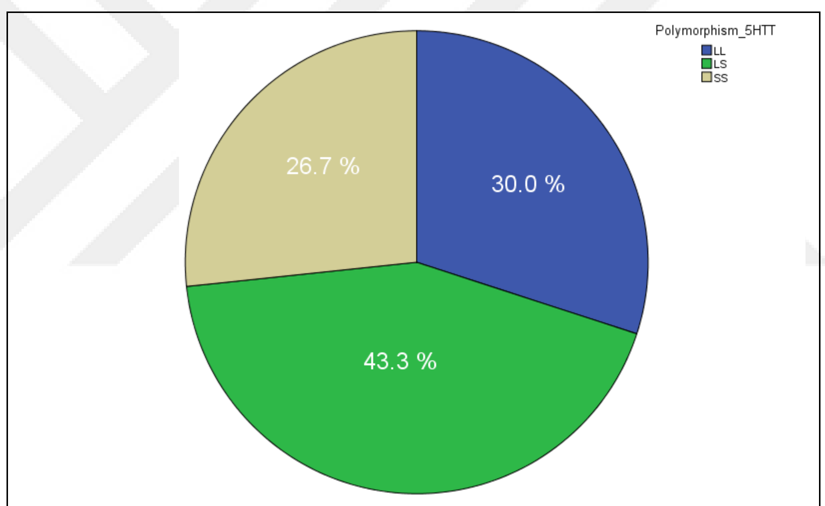

Figure 4.23 Distribution of 5-HTT polymorhism in 30 patients

Patients with LL, LS and SS polymorphisms were compared in three different groups using the Oneway Anova test to determine the effect of 5-HTT polymorphism on response during drug treatment. The difference between the groups was found to be statistically significant in Table 4.83 ($p < 0.05$).

TABLE 4.83. Evaluation of The Effect of 5-HTT Polymorphism on Response During Drug Treatment by Using ANOVA

Response

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.656	2	.828	4.813	.016
Within Groups	4.644	27	.172		
Total	6.300	29			

When the Tukey test was applied to determine the difference between the groups, the difference between the LL and SS groups was found to be statistically significant (p: 0.012 <0.05) in the 95% confidence interval in Table 4.84 (Figure 4.23)

TABLE 4.84. Multiple Comparisons of response of patiens with 5-HTT polymorphism

Dependent Variable: Response

Tukey HSD

Polymorphism_5-HTT	Polymorphism_5-HTT	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
LL	LS	.30769	.17984	.220	-.1382	.7536
	SS	.62500*	.20153	.012	.1253	1.1247
LS	LL	-.30769	.17984	.220	-.7536	.1382
	SS	.31731	.18637	.223	-.1448	.7794
SS	LL	-.62500*	.20153	.012	-1.1247	-.1253
	LS	-.31731	.18637	.223	-.7794	.1448

*. The mean difference is significant at the 0.05 level.

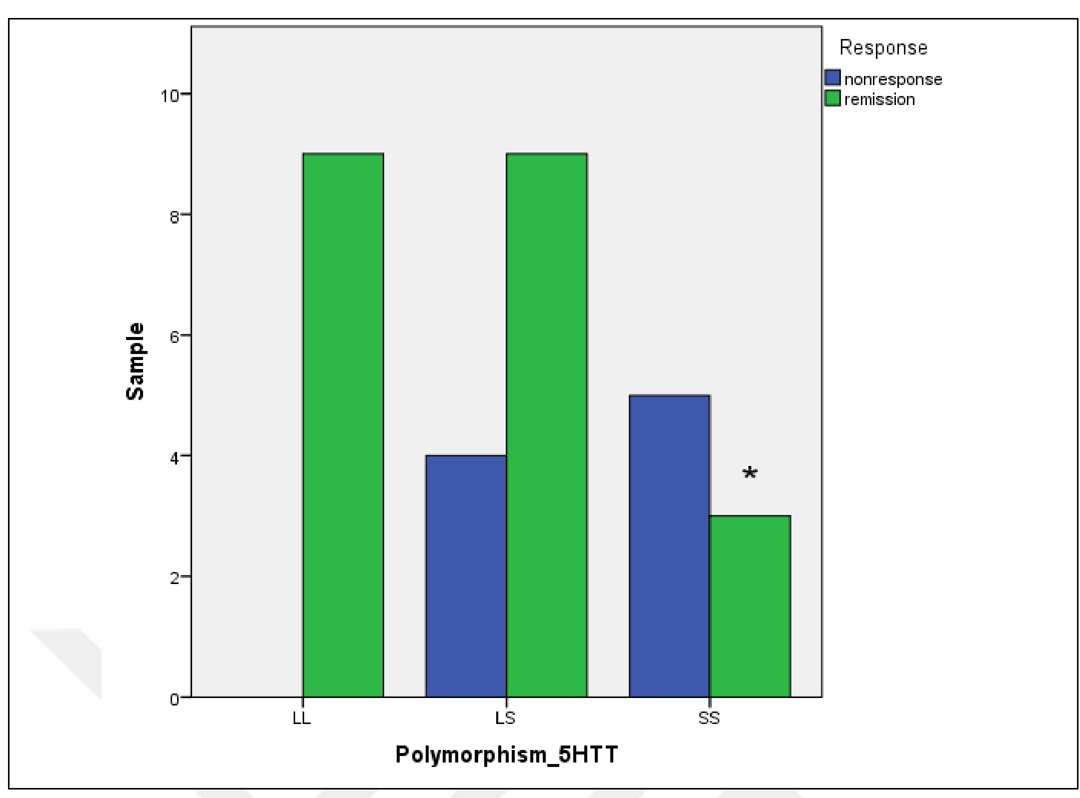

Figure 4.24 Evaluation of association between 5-HTT polymorphism and response to treatment. '*' among groups (LL and SS) represent significant differences ($p < 0.05$) by Tukey HSD test. The difference between the LL and SS groups was found to be statistically significant.

Evaluation of the association between Escitalopram (S-CT) plasma concentration and response to treatment

The mean escitalopram (S-CT) plasma concentrations of those who responded to the treatment and those who did not respond to the treatment have been summarized in the Table 4.85.

TABLE 4.85. Evaluation of The Association Between Escitalopram (S-CT) Plasma Concentration and Response to Treatment

	Response	N	Mean	Std. Deviation	Std. Error Mean	Independent T test (Sig. (2-tailed))
Escitalopram_Concentration	nonresponse	9	18.65	19.74	6.58	0.044
	remission	21	31.42	12.89	2.81	

The t-test was used to determine the effect of S-CT plasma concentration on the response to treatment (Table 4.86). Concentration value was found to be statistically significant on the response to treatment ($p < 0.05$) (Figure 4.25.)

TABLE 4.86. Statistical Summary of The Effect of S-CT Concentration of 30 patients on The Response to Treatment

Escitalopram (S-CT) Concentration	Levene's Test for Equality of Variances		t-test for Equality of Means					95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Equal variances assumed	1.936	.175	-2.114	28	.044	-12.77397	6.04343	-25.15337	-.39456
Equal variances not assumed			-1.785	11.049	.102	-12.77397	7.15514	-28.51387	2.96594

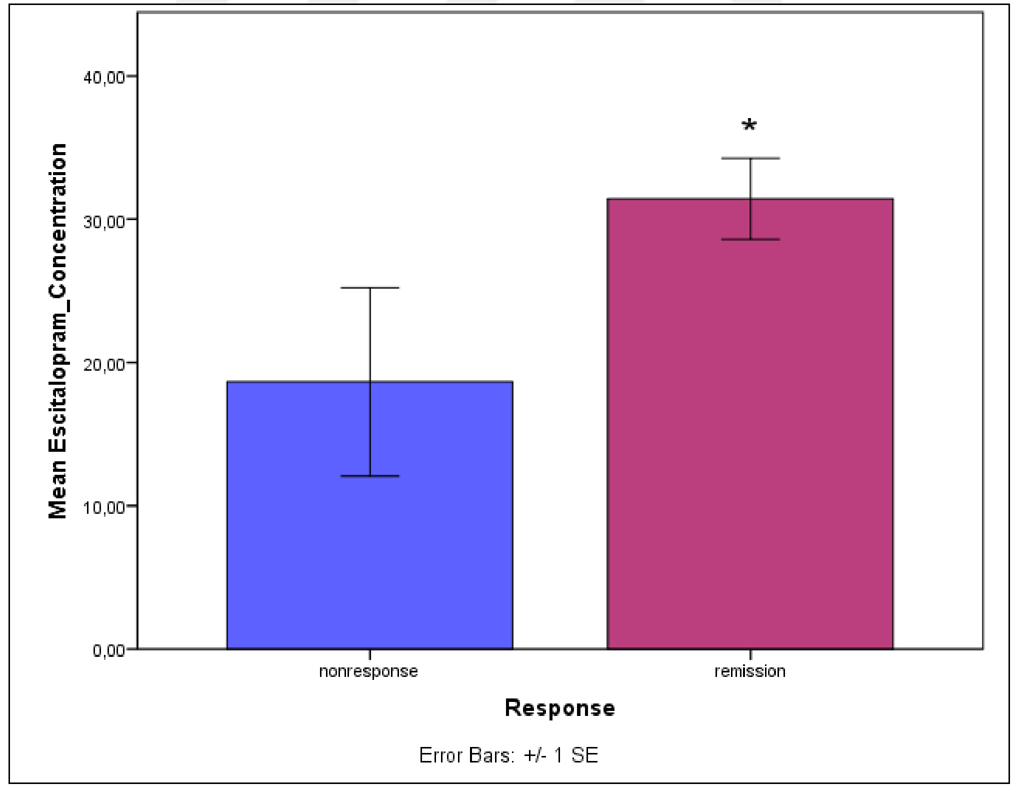

Figure 4.25 Evaluation of the association between Escitalopram (S-CT) plasma concentration and response to treatment. Concentration value was found to be statistically significant on the response to treatment. '*' represent significant differences ($p < 0.05$) by Tukey HSD test.

5. DISCUSSION and CONCLUSION

S-CT is a serotonin reuptake inhibitor frequently used in the treatment of major depression at a wide range of ages (68,69). S-CT increases serotonin levels in the presynaptic area by inhibiting serotonin transporter proteins in the brain (42,70). The therapeutic drug range is reported to be 15-80 ng / mL in the plasma (16). Although S-CT has not been associated with arrhythmia in the therapeutic drug range, it has been reported that high / toxic doses plasma S-CT levels have been associated with QT prolongation (71,72). It is known that when the individual factors such as age, sex, drug interactions affect the plasma drug levels differently (above or below expected), the physician can more easily adjust the amount of daily oral dosing, by following the drug plasma levels of patients with TDM, to provide the plasma therapeutic drug range for drug effect (73). However, for an effective TDM, it has been reported that primarily the method chosen for drug analysis should be useful and sensitive (74,75). The sensitivity of the equipment used in TDM analysis, the ease of bioanalytical method, the duration of sample preparation, the accuracy and selectivity of the method, the duration of the analysis, e.g shows the usefulness of the selected method for routine analysis (37). In our study, assay of S-CT and its metabolites (S-DCT, S-DDCT) in human plasma and urine was analyzed accurately and sensitively by the validated method. When it was looked at the quantitative analysis methods for these molecules in a study conducted in 2005, these molecules were analyzed with GC/MS (68). It has been seen that the length of sample preparation in that reference for the sample preparation procedure draws attention (76). In our study, on the other hand, it has been seen that the direct injection after protein precipitation shortened the sample preparation period.

In another study, S-CT and its metabolites were studied in the plasma with LC-MS / MS. However, urine analysis was not performed in this study (15). In yet another study, only the S-CT level was analyzed in the plasma with LC-MS/MS(16). As for our study, however, the levels of S-CT and its metabolites in plasma and urine samples have been sensitively analyzed simultaneously with a validated method.

In another study, drug analysis was performed using whole blood with LC-MS/MS. It has been seen that the duration of sample preparation in the publication is longer than the one in our study (65).

In our study, the quantitative assay of S-CT and its metabolites was achieved fast, accurately and sensitively in the plasma and urine with the validated method. Figure 4.1-4.4 shows the peak areas of the desipramine (as IS), S-CT and its metabolites in LOQ-level plasma samples compared to the interference area in six different blank samples. Also, Figure 4.1-4.4 shows the retention times of desipramine, S-CT and its metabolites in the plasma. Figure 4.5 shows the calibration curves of S-CT and its metabolites in plasma. Figure 4.6 shows the peak areas of the desipramine (as IS), S-CT and its metabolites in LOQ-level urine samples compared to the interference area in six different blank samples. Also, Figure 4.7 shows the calibration curves of S-CT and its metabolites in urine.

When the results are scrutinized, it can be seen that in the validated method, the standard area at LOQ level prepared in the urine and plasma can be easily separated from the interferences in the blank samples, and the sensitivity of the method, the accuracy and precision values could meet the required criteria (10), and thus the accuracy and precision of the method has been proven (61). Therefore, it is considered that the method validated in routine use may be useful for the analysis of plasma and

urine quantification of S-CT and its metabolites as an alternative to the methods in the literature.

In the publication of Hiemke et al. (2011), the expected drug concentration / related dose ratio (C / D (ng / mL / mg)) in the plasma for many psychotropic drugs, taking into account the daily dose of psychotropic drug used by psychiatric patients whose pharmacokinetic processes are known (total clearance, bioavailability eg.), was given in the minumum and maximum intervals (3,10). The C / D range for S-CT was reported to be 0.58-1.54 ng / mL / mg (3). When this ratio is taken into consideration, the expected S-CT concentration range for the 20 mg S-CT in the plasma can be calculated as 11.6-30.8 ng / mL. In our study, the mean plasma S-CT concentration of 30 patients using 20 mg S-CT was found to be 27.59 ng / mL. The mean S-CT plasma concentration obtained from our study was found to be within the expected S-CT plasma level range (11.6-30.8 ng / mL) as reported by Hiemke et al. (see Table 4.64). Jin et al. (2010) investigated the effect of age on S-CT exposure, indicating that S-CT plasma concentration is higher than that of younger people because they have lower clearance in elderly people (69) . In our study, however, it was found that there was no significant difference between the S-CT plasma levels in the age groups formed. The reason for the difference between the two studies is that, in contrast to the other study, the age range of the study group is closer to each other and the general age range of the study sample is narrow (range: 20-58 age) in our study, which is why that the exact distinction could not be made. Rao's (2007) study of S-CT pharmacokinetics showed that there was no significant difference on S-CT pharmacokinetics (tmax, cmax, t1 / 2) between adolescents (12-17 age) and adults (18-35 age), whereas the difference between the adults and elderly was found to be significant (9). Moreover, in the same study, it was stated that gender had no effect on S-CT level (9). In our study, the age group

formed is mainly composed of adolescents and adults. No significant difference was detected in S-CT plasma levels between these age groups ($p > 0.05$). In addition, we found that gender difference did not cause a significant difference in S-CT plasma level ($p > 0.05$). The results of the comparison between S-CT plasma level and gender and age in our study are similar to those of Rao's study (9,69).

In publications involving TDM studies, it has been reported that the plasma concentrations of drugs and metabolites can be used to calculate MR of drugs and metabolites (3,32,77). Knowledge of the MR of the drug and its metabolite has been thought to provide information about the activity of the enzyme involved in that metabolic pathway in the biotransformation of the drug (3,32). The publications have included criteria which were developed from MR of drugs such as risperidone, venlefaxin and it has also been mentioned that an assessment of the enzyme activities involved in the biotransformation of these drugs can be made. For example, the risperidone is metabolized to 9-OH risperidone (paliperidone) by the CYP2D6 enzyme. It has been reported that the risperidone / paliperidone MR in the plasma may provide insight regarding the activity of the CYP2D6 pathway (PM, EM, UM) of patients (3,32). Similarly, the venlafaxine is metabolized to the O-desmethylvenlefaxine by the CYP2D6 enzyme. Venlafaxine and O-desmethylvenlefaxine MR have also been reported to provide insight into the activity of the CYP2D6 pathway (PM, EM, UM) (32,78). In our study, with the method validated, MR of the drugs and metabolites in the plasma was calculated by analyzing S-CT and its metabolites co-occurringly (see Table 4.64). The mean S-DCT and S-DDCT concentrations of the 30 patients with mean plasma S-CT concentration of 27.59 ng/mL were 85.52 ng/mL and 44.30 ng/mL, respectively. The mean S-CT / S-DCT metabolic ratio (MR) of the escitalopram, metabolized to the S-DCT metabolite by the CYP2C19 enzyme as the major pathway,

was calculated to be 0.40 (see Table 4.64). The mean S-DCT / S-DDCT metabolic ratio (MR) of S-DCT, metabolized to the S-DDCT metabolite by the CYP2D6 enzyme was calculated to be 3.55 (see Table 4.64).

In a study published in 2007 by Reis et al., the drug blood level of S-CT and its metabolites was monitored by HPLC / FL in serum samples of 155 Swedish patients with an average age of 51 using S-CT (79). At the end of the study, the S-DCT and S-DDCT ratios in serum samples of these persons were determined as 60% and 9% of the main substance (S-CT), respectively. In our study, by using LC-MS / MS device, the mean concentration of S-CT metabolites (S-DCT and S-DDCT) in plasma samples of 30 Turkish patients with an average age of 39 using escitalopram was found higher than the mean concentration of S-CT. The reasons for the differences in analysis results between the two studies could be due to the differences in biological matrixes (serum & plasma), sampling from different races (Swedish & Turkish), individual differences (age, genetics), differences of device features used for drug blood level analysis (HPLC/FL & LC-MS/MS). When the publications are examined and laboratory studies are followed, it is known that serum and plasma are widely used as biological matrixes in drug analyzes. Given the low rate of binding of S-CT to plasma proteins, the difference in analysis between these two studies is not expected to be due to the matrix effect. It is known that the genotype characteristics of the individual, age, sex, race, environmental factors affect the results of drug analysis in the analysis of the individual drug treatment. The elimination half-time of S-CT and metabolite (S-DCT) in serum were 32.5 ± 14.2 and 54.1 ± 21.7 hours, respectively. Metabolite concentration is expected to be higher in serum because the duration of elimination of S-DCT from the serum is longer than S-CT. When we consider the individual differences in the studies, the average age of the samples of Reis etal. is 51, whereas the average age of the

samples is 39 in our study. Having a younger sample group in our study and genetic differences may have caused the difference in analysis results by affecting the rate of S-CT metabolism. Moreover, it is thought that the method differences used in the study may be influential on the differences in the analysis results. Analysis of S-CT and its metabolites in serum by Reis et al. were performed using HPLC / FL device. Although the HPLC / FL device is one of the most sensitive devices for drug analysis, the severity of the main molecule (S-CT) may be different from the intensity of the metabolites (S-DCT, S-DDCT) obtained by separation of the methyl groups (electron donor) as a result of the biotransformation of the parent molecule analyzed. Therefore, the responses obtained at the fluorescence detector may be different for each molecule. To clarify, it is known that when the donor groups are added to a molecule structure, the fluorescence intensity increases., whereas the fluorescence intensity decreases when the electron acceptors are added. In our study, plasma analysis of escitalopram and metabolites were performed using LC-MS / MS. In LC-MS / MS technique, molecules which are separated according to physicochemical properties in high pressure liquid chromatography are analyzed by mass detector. The molecules separated according to the m / z (mass / charge) ratio in the first quadrupol filter are subjected to fragmentation with a special gas of high purity called Collision Gas. The second quadrupol filter is diagnosed and quantitated through ions (daughter or production) that are formed as a result of fragmentation. Although there are many molecules with the same m / z ratio, the number of molecules with the same product (daughter) ion is very rare in nature. Thus, the LC-MS / MS technique is almost as unique as the paternity test, as well as allowing quantification of the substance at very low concentrations. There is also no need to verify the results. The fact that LC-MS / MS devices have high sensitivity, selectivity and specificity, as well as high response values for each molecule, makes it

widely used in drug analysis worldwide (80). Our study demonstrates that the method we have developed using LC-MS / MS meet the criteria specified in the relevant publications in terms of selectivity, accuracy and matrix effect values, which shows that a valid and reliable analysis of S-CT and its metabolites in plasma and urine could be studied by using the validated method. Therefore, when the results obtained from our study are evaluated, it is considered that our study has originality due to the difference of method, sampling and analysis results.

Many studies have examined the effect of polymorphic states of enzymes involved in drug metabolism on enzyme activity, drug blood levels, and response to treatment (49,81–83) Chang et al. (2014) investigated the effect of patients' CYP2C19 polymorphisms on the S-CT plasma concentration values of 847 psychiatric patients (54). It was reported in the study that compared to EM (* 1 / * 1) group, there was a 95% increase in S-CT level in those with an allele of *2 / *3 and a 30% increase in those with * 1 / * 2 and * 1 / * 3 alleles. In the same study, it was reported that compared to EM (* 1 / * 1) group,.S-CT level in plasma decreased by 14% in people with * 1 / * 17 alleles and by 36% in people with *17 /*17 alleles. When we investigated the relationship between CYP2C19 enzyme activities and S-CT plasma concentration values in 30 psychiatric patients, no statistically significant results were found ($p > 0.05$). The reason for the difference between the two studies may be that the number of samples taken for statistical evaluation in our study is not sufficient to determine the difference due to the low allele frequencies. The distribution of CYP2C19 polymorphisms of patients participating in the study is given in Table 4.74 and Figure 4.16. In CYP2C19 polymorphism distribution, 14 individuals with * 1 / * 1 allele (46.7%), four people with * 1 / *2 allele (13.3%), a person with * 1 / * 3 allele (3.3%), six people with* 1 / * 17 allele (20.0%) and five people with * 17 / * 17 allele (16.7%)

were detected. 11 of the people with the 1 * 1 allele, two with *1 / *2 allele, one with *1/*3 allele, and two with the *1/ *17 allele are included statistical calculations. Due to the fact that two of the remaining 14 individuals had autoimmune disease (FMF), the CYP2D6 enzyme activity (when the genotyping results and the S-DCT / S-DDCT ratio are considered) of a total seven with * 17 / * 17, * 1 / * 17 alleles, a patient whose plasma was taken wrong time and a total of 4 individuals with *1/ *1 allele and *1/*2 allele were different from those of CYP2D6 extensive metabolizers, these samples could not be statistically evaluated in evaluating the effect of CYP2C19 polymorphism on S-CT and its metabolites.

The mean S-CT concentration of the 11 people with *1/*1 allele included in statistical calculations is 24.57 ng / ml, 24.47 ng/ml for three patients with * 1 / * 2 and * 1 / *3 alleles and 7.17 ng / ml for two patients with *1 / *17 allele. The mean S-CT plasma concentrations of patients with CYP2C19 EM (* 1 / * 1) and IM (* 1 / * 2; * 1 / * 3) were considerably close to each other, whereas the mean S-CT plasma concentrations of patients with CYP2C19 UM (*1/*17) were found to be lower than those of EM and IM group. The difference between the mean S-CT concentrations (24.55 ng / ml) of the patients in the EM and IM groups (11 people in EM and three people in IM) and the S-CT concentrations (7.17 ng / ml) of the patients in the UM group was very close to the statistical significance limit (p: 0.057). Altar et al. (2013) reported in their study that the plasma concentration of S-CT was lower in people with fast CYP2C19 enzyme activity than in those with normal enzyme activity (81). The data in our study are consistent with the findings in this publication.

Rudberg et al. (2006) examined the MR of S-CT and its metabolite according to the CYP2C19 polymorphism distribution of 83 patients (84). 50 patients with EM * 1

/ * 1 allele and 33 patients with IM * 1/ *2 allele participated in the study. When the S-CT / S-DCT ratios of these individuals were evaluated, it was found that the S-CT / S-DCT values of the IM group were higher than those of the EM group (84). In our study, the mean MR of S-CT / S-DCT (MR; 0.31) of the 11 patients in the EM group was similar to the MR of S-CT / S-DCT (MR; 0.31) of 3 patients in the IM group and therefore a statistically significant difference could not be found (p >0.05). However, when the mean MR ofS-CT / S-DCT of these groups were compared both seperately (EM;IM) and together (EM +IM) with the mean MR ofS-CT / S-DCT (MR; 0.05) of two patients in UM group, the mean MR of S-CT / S-DCT of patients in the UM group were found to be lower than those in other groups (Table 4.76 and Table 4.78). The difference was found to be statistically significant (p <0.05) (Figure 4.20). The data obtained from our study is similar to the Rudberg et al publication. Knowing that the mean MR of patients in the EM and IM groups is different compared to the UM group, it is thought that by considering the S-CT / S-DCT value for many patients without genotyping analysis, a pre-assessment can be made regarding the CYP2C19 enzyme activity.

It is known that the enzyme CYP2D6, which plays a role in the metabolism of 20-25% of the drugs in the market, is also effective in the metabolism of S-CT(82). Over 100 genetic variants have been identified in the CYP2D6 gene on chromosome 22q13.1. However, in many publications it is noteworthy that especially CYP2D6 * 2, * 3, * 4, * 5, * 6, * 10, * 41 alleles of these variants were mentioned (82)**.** The CYP2D6 enzyme, which is involved in the conversion of S-DCT, the metabolite of S-CT to S-DDCT, is thought to be effective in the pharmacokinetics of S-CT. Therefore, in our study, CYP2D6 genotyping analysis was also performed for 11 patients with inconsistency between their mean MR of S-CT / S-DCT and CYP2C19 polymorphisms.

The distribution of CYP2D6 polymorphisms of patients participating in the study is given in Table 4.79 and Figure 4.21. In CYP2D6 polymorphism distribution, 3 people with * 1 / * 1 allele (27.3%), 2 people with * 1 / * 2 allele (18.2%), a person with * 1 / * 10 allele (9.1%), 4 people with * 4 / 10* allele (36.4%) and a person with * 2 / * 41 allele (9.1%) were detected. The mean MR of S-DCT / S-DDCT of the patients in EM (* 1 / * 1) group was 1.70, the mean MR of S-DCT / S-DDCT of those in Het. EM group (*1/*2,*1/*10,*2/*41) was 1.71 and the MR of S-DCT / S-DDCT of those in IM (*4/*10) group was found to be 4.18. While there was no statistically significant difference between MR values of patients with CYP2D6 EM and MR values of patients with CYP2D6 Het.EM ($p > 0.05$), a statistically significant difference was found between the MR values of these groups with the patients with CYP2D6 IM ($p < 0.05$) (Table 4.80, Figure 4.22). It has been reported in publications that CYP2D6 * 4 and * 10 alleles reduce enzyme activity(59). The data obtained from our study is similar to that of the related publications. Knowing that the mean MR of patients in the EM and Het. EM group is different compared to the IM group, it is thought that by considering the S-DCT / S-DDCT value for many patients without genotyping analysis, a pre-assessment can be made regarding the CYP2D6 enzyme activity.

SSRIs are known to be commonly used drugs in psychopharmacotherapy. In many studies, the pharmacodynamic mechanism of SSRIs is explained by the effect on serotonin transporter (5-HTT). 5-HTT recovers serotonin from synaptic (synaptic cleft) sites (85). S-CT located in the SSRIs group inhibits 5-HTT and prevents serotonin reuptake and increases the level of serotonin in the synaptic region (86). It is known that the 5-HTT gene has a polymorphic characteristic. In many publications, it is reported that there are variants, short (S; 484 bp) and long (L; 528 bp), commonly occurring in the promoter region of the 5-HTT gene. In our study, the variant distribution of LL, LS

and SS of 30 patients is given in Table 4.82 and Figure 4.23. It has been found that 9 patients (30%) have LL variant, 13 patients (43.3%) have LS variant, and 8 patients (26.7%) have SS variant. In a study by Samochowiec et al (2004), the effect of 5-HTT polymorphism on anxiety disorders was examined. Of the 202 healthy Caucasian in control group included in this study, 42% were reported as LL, 48% as LS and 10% as SS (87). The 5-HTT variant distribution of the patients who participated in our study is similar to the variant distribution of the control group in Samochowiec et al publication. However, the proportion of people with SS variant in the patients who participated in our study was found to be slightly higher than that in the healthy control group in the related publication. In many studies, variant variability has been reported to affect the level of 5-HTT gene expression (88). In related studies, it was noted that the 5-HTT expression decreased in those with S variant (45,89). It was reported by Mancama and Kerwin (2003) that patients in the LL, LS group had higher compliance with drug treatment than those in the SS group (88). When the response of escitalopram treatment to 30 patients with 5-HTT polymorphism distribution was examined, it was observed that the difference between the groups was statistically significant ($p <0.05$). While a statistical significance ($p < 0.05$) was detected between LL and SS groups in reponse to treatment, No statistical significance in the response to treatment was found between the LS group and the other groups ($p> 0.05$). While response to treatment was observed in the entire LL group, patients without response to the drug treatment in the SS group were observed (Table 4.84, Figure 4.24). In cases where the SS group failed to respond to the drug treatment, it has been detected from patient files that dose increase and different drug additions were performed to maintain the treatment. Many studies have reported that people with LL variants can respond better to SSRIs than those with SS

variant, and it is difficult and long to reach a remission in SS group patients (81,90,91).The data obtained from our study is similar to the data in literature.

It has been reported that in many studies studying the association between escitalopram plasma level and antidepressant effect, the antidepressant effect of escitalopram initiates by occupying at least 80% of 5-HTT (92). It has been reported that 80% of 5-HTT is loaded when escitalopram reaches 15 ng / ml in plasma. Therapeutic interval was reported to be 15-80 ng / ml in previous studies (16). In our study, the mean plasma level of 30 patients was found to be 27.59 ng / ml. Nine patients (30%) did not respond to the treatment. When 5-HTT variations of these patients were examined, it was observed that five patients were SS and four patients were from LS group. The minimum and maximum value range of S-CT drug plasma level in patients with SS group was found to be 0.95-55.25 ng / ml. Even though the S-CT drug plasma levels of these patients with the SS variant were within the therapeutic range, these concentrations were detected to be insufficient for a response to treatment. The minimum-maximum S-CT drug plasma level of the four patients in LS group was found to be 2.93-14.89 ng / ml. It has been observed that since the S-CT drug plasma levels of these patients with LL variants have not reached the lower limit of the therapeutic range (15 ng / ml), these concentrations are not sufficient for the drug response to be seen. It has been found that the mean S-CT plasma concentration of 21 patients who responded to the escitolopram treatment is 31.42 ng / ml. Nine of these patients have the LL variant, nine have the LS variant and three have the SS variant. S-CT plasma concentrations of patients with LS and SS variants in this group were found in the therapeutic range. While the S-CT plasma concentrations of eight patients in the LL variant were found to be within the therapeutic range, the S-CT plasma concentration of only one patient was 13.40 ng / ml. Although not reaching the lower limit of the

therapeutic range (15 ng / ml), it was observed that the patient received the desired response from the treatment at the detected concentration. When the association between the S-CT plasma level and response to the drug treatment was examined, it was found statistically significant ($p < 0.05$) that the S-CT drug concentration affected the response to treatment as well as the 5-HTT variation.

By using the results obtained from this study, the MR of S-CT and its metabolites were calculated. The mean of the S-CT plasma levels of the patients were found in the range of expected S-CT plasma level stated in the literature. The mean MR of individuals with mean S-CT plasma levels in this range were calculated separately for two metabolites of S-CT. It is considered that the MR results obtained from our study may provide further insight into CYP2C19 and CYP2D6 enzyme activities during drug treatment of patients using escitalopram with the expected plasma drug level mentioned in the literature.

In our study, we also analyzed S-CT and its metabolites in patient urine samples. Concentration of S-CT and its metabolites in the urine was found to be above the highest standard concentration. It has been concluded that the analysis of S-CT and its metabolites in plasma samples is faster and easier than the analysis of urine samples and also, it is possible to directly assess the patient's plasma concentration of the drug by analysis of the plasma drug concentration. Thus, the follow-up of the drug analyzes, especially in plasma samples is thought to provide the physician to be able to make easier and higher contribution in adjusting the dosage of the drug during the drug treatment of patients.

As a result; it is considered that the method developed and validated for the quantitative analysis of S-CT and its metabolites in human plasma and urine samples may contribute to the literature on account of its sensitive and easy application. Besides,

this method can be applied to other drugs in further studies. In addition, evaluation of the data obtained from our study by physicans is thought to be able to contribute to the study of patients who have S-CT treatment in the application of individual drug treatment. Also, different MR value was found from literature in our study, we think it might comes from using the different analytical method and device from literature. It is suggested that the association between MR and cytochrome P450 enzyme activities of S-CT and its metabolites in patients with different phenotypes can be assessed by performing a further study using this validated analytical method on a higher number of patients with known cytochrome p450 enzyme genotypes.

6. REFERENCES

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7. APPENDICES

7.1. Ethical Approval

Appendix A

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SAYI: B.08.6.YÖK.2.ÜS.0.05.0.06 /2017 /154 05.06.2017

Sayın Öğr. Gör. Fadime Canbolat

Üsküdar Üniversitesi Girişimsel Olmayan Araştırmalar Etik Kurulu'nun 15 Mayıs 2017 tarihinde, 05 No.lu toplantısında değerlendirmeye almış olduğu **"Genetik Polimorfizmi ve Essitalopram Monitorizasyonu Talep Edilen Hastalarda İlaç Düzeyi ile Sitokrom P450 Enzim Aktiviteleri Arasındaki İlişkinin Değerlendirilmesi"** adlı araştırma projenizin etik açıdan uygun olduğuna karar verilmiştir.

Bilgilerinizi rica ederim.

Doç. Dr. Cumhur TAŞ
Girişimsel Olmayan Araştırmalar Etik

8. CURRICULUM VITAE

Personal Information

Name	Fadime	**Surname**	Canbolat
Place of Birth	İstanbul	**Date of Birth**	05.08.1982
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Education Status

Degree	**Field**	**University**	**Year**
PhD.	Pharmaceutical Toxicology	Yeditepe University	-
Master Degree	Pharmacology&Toxicology	Selçuk University	2007
Bachelor's Degree	Chemistry	Selçuk University	2004

Career History

Position	**Place**	**Year**
Laboratory Assistant Manager	Clinical Pharmacogenetic Laboratory& Advanced Toxicology Laboratory-Üsküdar University	2010-present
Quality Manager	Advanced Toxicology Laboratory-Üsküdar University	2015-present

Scientific Studies

Articles published in national journals

S Esra, Danısman B, Özilhan S, Canbolat F, Tarhan B, Phenotype-Genotype and Therapeutic Dose Monitoring Relationship: Case Report, Turkiye Klinikleri 100 J Case Rep 2014;22(2).
Canbolat F, Kul A, Ozdemir M, Ugur Atik U, Aydin A, Ozden ST, Tarhan KN, Substance Abuse Profiles of Patients Admitted to The AMATEM (Alcohol and Drug Addiction Research, Treatment and Education Center) Unit–Turkey Turkish Journal of Pharmaceutical Sciences 2017; 14(3)

Proceedings Papers Presented in International Scientific Meetings

Relationship Between Colchine Plasma Level And Frequency Of Familial Mediterranean Fever Attacks: Poster Presentation. Annual European Congress of Rheumatology EULAR® 2015 Rome, Italy, 10 – 13 June 2015.
Relationship Between Colchine Plasma Level and Frequency of Familial Mediterranean Fever Attacks: Oral Presentation. 2015 ACR/ARHP Annual Meeting, San Francisco, CA; November 6-11,2015.

Papers presented at National Scientific Meetings

Paliperidone /Risperidone Plasma Level's Ratio Can Be Used As A Cyp2d6 PM Phenotyping Marker: Oral Presentation/ 1st Pharmacogenomics İstanbul Summit, Turkey, 27-28 Oct., 2015.

Projects

Toksik ve Bağımlılık Yapan Maddelerin Kanıta Dayalı Tayin, Tarama ve Danışma Merkezi, ISTKA. 2015