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Affiliation: Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8521, Japan
SIMULTANEOUS DETERMINATION OF PROPOFOL AND DRUGS USED IN GENERAL ANESTHESIA BY DEVELOPED AND VALIDATED LIQUID CHROMATOGRAPHIC METHODS: A PRECLINICAL STUDY FOR DRUG-DRUG INTERACTION ANALYSIS

A thesis submitted to the Graduate School of Biomedical Sciences, Nagasaki University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.) in Pharmaceutical Sciences

By

MOHAMED ABOELHAMD IBRAHIM HUSSEIN

Division of Analytical Research for Pharmacoinformatics
Department of Clinical Pharmacy, Doctoral Course of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University
1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

September, 2014
Propofol (PRO), an intravenous (i.v.) hypnotic, has rapid onset and short duration of action used for both induction and maintenance of general anesthesia by a continuous infusion during surgical procedures. Total intravenous anesthesia (TIVA) is a technique of general anesthesia using a combination of i.v. administrated drugs without the traditional inhalational or balanced anesthetics. Owing to its desirable pharmacologic properties, PRO is usually used in TIVA. PRO has many properties such as antiemetic, anticonvulsant and antioxidant activities; but it has no analgesic properties. Therefore, a narcotic synthetic opioid (form fentanyl-family) is usually administered concomitantly with PRO in TIVA.

Remifentanil (REM) is an ultra-short-acting opioid appears to be an ideal analgesic (than other fentanyl-family members) used for TIVA in a combination with PRO. REM has unique properties that are different than its congeners and similar to PRO such as a rapid onset and short duration of action; as well as the independent pathway from PRO as REM’s clearance is caused by non-specific blood and tissue esterases.

The use of PRO and REM in a combination during anesthesia results in a reduction in doses requirement of both agents because they interact pharmacodynamically and act synergistically in the response of hypnosis and nociception. The TIVA with PRO and REM is a very accurate, easy, and predictable, environmentally safe and without the most common side effect after inhalational or balanced anesthesia, postoperative nausea, and vomiting technique. Unfortunately, the overdosing of PRO or REM during TIVA causes cardiovascular depression and may be death in many reported cases. Accordantly, the monitoring of PRO and REM plasma concentrations at the stable maintenance phase of anesthesia is required. Moreover, the pharmacokinetic (PK) interaction studies would clearly add to the knowledge base of specialists in the field of anesthesiology, specially the effect of REM on plasma concentration of PRO has not confirmed yet.
The published analytical methods for determination of PRO or REM in the different biological samples have been developed independently, therefore a simultaneous method for their determination is required for easily and rapid PK drug-drug interaction analysis.

On the second research point in this thesis we study the effect of drugs, used in treatment of epileptic patient, on PRO plasma concentration during anesthesia; as the epilepsy is much relevant to the anesthesia for the reasons of the interactions between the antiepileptic drugs (AEDs) and co-administrated ones, commonly postoperative seizures, and the intensive care management of status epilepticus.

Carbamazepine (CBZ) is an AED and the standard treatments for epilepsy since 1970s and still is. Unfortunately, CBZ plasma concentration is closely related to its curative effect and toxic side effects. Therefore, it is necessary to monitor its plasma concentration to improve the curative effect and decrease toxic side effects. Also, benzodiazepines such as midazolam (MDZ) are used in clinical practice mainly for the induction of anesthesia or as an anticonvulsant in patients with generalized seizures or status epilepticus. Patients with epilepsy often require anesthesia for elective and emergency surgery. For these patients, an appropriate perioperative management of administrated AEDs is vital in maintaining seizure control.

From the literatures, PRO is mainly oxidized by hepatic enzymes cytochrome P450 (CYP) 2B6 and CYP2C9. Inversely, it inhibits the activities of CYP2A1, CYP2C9, CYP2D6, and CYP3A4 enzymes. Since CYP3A4 is responsible for CBZ metabolism, co-administration of PRO and CBZ is expected to have a drug interaction. On the other hand, CBZ induces CYP1A2, CYP2C9, and CYP3A4 activities; therefore PRO that undergoes metabolism via CYP2C9 is likely to be affected. Also from the literatures, MDZ is neither inhibitor nor inducer for CYP3A4 metabolism, however it has been proposed for the hepatic CYP3A phenotyping (assessing the functional CYP3A activity).
The influence of MDZ on PK of PRO and other anesthetics has been described in details in the reported literatures. To date, there are no data that describe whether, and to what degree, CBZ affects the plasma concentration of PRO and also the published analytical methods for determination of PRO, MDZ, or CBZ in biological samples have been developed independently.

According to the mentioned above, the aim of the present thesis’s study to developed chromatographic methods used for simultaneous determination of PRO and drugs usually used in TIVA such REM and MDZ or drugs relate to patient with epilepsy and has a surgery with general anesthesia. The developed and validated methods should be sensitive and selective enough to couple with the PK drug-drug interaction analysis for the co-administrated drugs:

Chapter 1: We described a developed and validated high-performance liquid chromatography-ultraviolet detection (HPLC-UV) method for simultaneous determination of PRO and REM using fluoxetine as an internal standard (IS). A liquid-liquid extraction (LLE) procedure in a basic pH with hexane proved a successful, accurate, and precise extraction of the analytes from the pre-treated plasma samples. The linear calibration curves over the concentration ranges of 25-2500 ng/mL for both PRO and REM showed high correlation coefficients $(r) \geq 0.983$. The limit of detections (LODs) was 3 and 10 ng/mL and the limit of quantifications (LOQs) was 6 and 20 ng/mL for PRO and REM, respectively. Acceptable results for the accuracy, intra-day and inter-day precision were obtained. Unfortunately, even the developed HPLC-UV was sensitive for PRO determination in plasma, but was not sensitive enough for REM to follow its PK study after co-administration with PRO.
Chapter 2: A selective liquid chromatography-tandem mass spectrometry (LC-MS/MS), using CBZ as an IS, for simultaneous determination of PRO and REM in rat plasma was developed and validated. A LLE procedure in a basic pH with tert-butyl methyl ether was used for plasma pretreatment further analysis using electrospray ionization negative or positive (ESI⁻ or ESI⁺) modes for PRO or REM and IS, respectively.

The linear calibration curves over the concentration ranges of 0.27-10 µg/mL or 0.17-50 ng/mL for PRO and REM showed high $r$ ($\geq 0.981$). The lower LOQs were 0.27 µg/mL and 0.17 ng/mL for PRO and REM, respectively. Acceptable results for the recovery from plasma samples, accuracy, intra-day and inter-day precision and matrix effect (ME) optimization were obtained. The validated LC-MS/MS method has been successfully applied to study on the PK interactions of co-administrated PRO and REM to male Wistar rats.

Chapter 3: A sensitive and rapid HPLC-MS/MS spectrometry for simultaneous determine PRO, MDZ and CBZ in rat plasma was developed and validated. A simple and rapid solid-phase extraction procedure was used for plasma pretreatment further analysis using ESI⁻ or ESI⁺ modes for PRO or MDZ and CBZ respectively. The linear calibration curves over the concentration ranges of 1-100 (using CBZ as an IS), 1-100 (using MDZ as an IS) for PRO (two calibration curves for PRO); 2-100, and 7-1000 ng/mL for MDZ and CBZ, respectively showed a high $r$ ($\geq 0.991$). The LOQs were 0.05, 2, and 7 ng/mL for PRO, MDZ, and CBZ. Acceptable results for the recoveries from plasma samples, accuracy, intra-day and inter-day precision were obtained. The proposed method was successfully applied to study on the PK interactions of co-administrated PRO and MDZ or PRO and CBZ to rats.
Figure 1. Chemical structure of propofol (PRO, MW=178.3 g/mol) and remifentanil (REM, MW=376.5 g/mol), midazolam (MDZ, MW=325.8 g/mol), carbamazepine (CBZ, MW=236.3 g/mol) and fluoxetine (FLU, MW=345.8 g/mol).
1- Paper in Refereed Journal


2- Non-Refereed Conference Abstract (oral presentation)

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3.4. Conclusion

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<td>absorption, distribution, metabolism and elimination</td>
<td>ADME</td>
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<tr>
<td>arbitrary unit</td>
<td>au</td>
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<tr>
<td>area under the curve</td>
<td>AUC</td>
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<td>carbamazepine</td>
<td>CBZ</td>
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<td>central nervous system</td>
<td>CNS</td>
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<tr>
<td>clearance</td>
<td>Cl</td>
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<td>CLO</td>
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<tr>
<td>cytochrome P450</td>
<td>CYP</td>
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<td>electro spray ionization</td>
<td>ESI</td>
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<tr>
<td>elimination rate constant</td>
<td>$k_{el}$</td>
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<td>ethylene diamine tetraacetic acid disodium salt</td>
<td>EDAT.2Na</td>
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<td>fluoxetine</td>
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<td>food drug administration</td>
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<td>gas chromatography</td>
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<td>half-life</td>
<td>$T_{1/2}$</td>
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<td>high-performance liquid chromatography</td>
<td>HPLC</td>
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<td>HPLC-UV</td>
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<tr>
<td>ibuprofen</td>
<td>IBU</td>
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<tr>
<td>internal standard</td>
<td>IS</td>
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<td>intraperitoneal</td>
<td>i.p.</td>
</tr>
<tr>
<td>intravenous</td>
<td>i.v.</td>
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<td>limit of detection</td>
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<td>limit of quantification</td>
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<td>liquid chromatography-tandem mass spectrometry</td>
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<td>liquid-liquid extraction</td>
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<td>mass spectrometry</td>
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<td>quality control</td>
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<td>relative error</td>
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<td>relative standard division</td>
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<td>remifentanil</td>
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<td>selected reaction monitoring</td>
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<tr>
<td>signal-to-noise ratio</td>
<td>S/N</td>
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<td>solid-phase extraction</td>
<td>SPE</td>
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<td>standard division</td>
<td>SD</td>
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<tr>
<td>$t$-butyl methyl ether</td>
<td>$t$-BME</td>
</tr>
<tr>
<td>time required to reach maximum concentration</td>
<td>$T_{max}$</td>
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<tr>
<td>total intravenous anesthesia</td>
<td>TIVA</td>
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<tr>
<td>volume of distribution</td>
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<td>$\gamma$-aminobutyric acid</td>
<td>GABA</td>
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INTRODUCTION

I. GENERAL ANESTHESIA

Anesthesia refers to a condition of reduced sensibility in the body. The term comes from the Greek word aesthesia, which means “ability to sense.” The prefix a- (an-, in the presence of a vowel) is used for negation. Therefore anesthesia stands for “inability to sense” and it indicates a “condition of deprived sensibility.” Anesthesia is a reversible pharmacological state induced by the administration of anesthetic drugs. Delivery of adequate anesthesia during medical treatments ensures the patient hypnosis, analgesia and muscle relaxation 1). The types of anesthesia are broadly classified into local anesthesia, regional anesthesia (epidural and spinal) and general anesthesia. In modern clinical practice, general anesthesia is provided only when strictly necessary in order to reduce the invasiveness of the procedure. The goal in the administration of general anesthesia is to provide a stage of reversible unconsciousness with adequate analgesia and muscle relaxation for surgical procedures.

Nowadays i.v. anesthetics are largely used for induction and maintenance of general anesthesia. Fortunately, an attractive way, which may obviate the need for inhalation of a volatile liquid, is the development of the potent ultra-short-acting i.v. agents suitable for both induction and maintenance of anesthesia. In this case the anesthesia technique is called total intravenous anesthesia (TIVA), therefore, the TIVA can be defined as a technique of general anesthesia using a combination of agents given solely by the i.v. route and in the absence of all inhalational agents. The technique of TIVA has become popular and practical as it is simple, economic, environmentally safe, and associated with less postoperative nausea and vomiting than inhalational or balanced anesthesia.
Consequentially, the introduction of TIVA into the clinical procedures of anesthesia, made possible the application of a target controlled infusion (TCI) modern computer technology system through which the anesthesiologist attempts to achieve a real-time target level of drug in the plasma or effect site, according to the drug’s PK. Related to the mechanism of action, anesthetic agents have many effects on the lipids and proteins that are found in neuronal membranes. In addition, the pore-forming membrane bound proteins (ion channels) that help to establish and control the small voltage gradient that exists across the plasma membrane of all living cells. The ion channels are divided into families depending on amino acid structure and function 2):

a) Ion selective gating: as ligand-gated acetylcholine receptor, γ-aminobutyric acid receptor (GABA<sub>A</sub>) and glutamate receptors.

b) Phosphorylation gated: as adenosine triphosphate (ATP).

c) Voltage-gated: as Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup>.

There are four main classes of membrane proteins 3):

a) Enzymes, such as acetyl cholinesterase,

b) Transporters for specific molecules,

c) Neurotransmitter receptors and

d) Structural proteins.

The neurotransmitters are endogenous chemicals that transmit signals across a synapse (a structure that permits a neuron (nerve cell) to pass an electrical or chemical signal to another neural cell). The major inhibitory neurotransmitter in the vertebrate central nervous system (CNS) is γ-aminobutyric acid (GABA), whose action is produced by its selective interaction with at least two classes of receptors, namely GABA<sub>A</sub> and GABA<sub>B</sub> receptors 4, 5). Number of distinct classes of drugs such as anesthetics (PRO, benzodiazepines, benzodiazepine-like compounds and barbiturate) exert their effects by interacting with specific modulatory sites on GABA<sub>A</sub> receptors 3, 4, 6).
Propofol (Propofol®, Diprivan®)

PRO (2,6-diisopropylphenol, Figure 1) 7) is a short acting i.v. hypnotic has the advantages of rapid onset and short duration of action due to its rapid hepatic elimination. PRO is the drug of choice, widely used, for induction and maintenance of general anesthesia in humane or in the veterinary medicine 7,8). Moreover, PRO is widely used for sedation in the intensive care patients 9). PRO avoids the residual sedation, fatigue (minimal "hangover effect") and cognitive impairment of others sedative-hypnotic drugs.

PRO being extensively (98%) bounds to serum/plasma proteins and appreciably associated with or enters the blood erythrocytes as well as 10). Structurally, PRO is similar to α-tocopherol with a phenol group so it has been implicated as having antioxidant roles 11). The previous studies reported that PRO can protect the thymocytes and erythrocytes from peroxinitrite, protects the lipid against peroxidation 11,12) and macrophages from nitric oxide induced damage 13), therefore inducing or maintaining agent for patients undergoing orthopedic surgery 14). Apan et al. were suggested that PRO has antibacterial effect especially when used in combination with the ultra-short synthetic opioid remifentanil (REM) 15). After receiving a single i.v. dose of PRO, the patient loses his consciousness within 30-50 seconds and remains unconscious for about 4-6 min 16,17).

The rapidity in PRO onset and offset when used as a continuous i.v. infusion gave the possibility to awaken a sedated patient within minutes for evaluation and return him to sleep in seconds 18). PRO is the anesthetic of choice for ambulatory surgery in outpatients related to its rapid onset and recovery after anesthesia. Moreover the low incidence of postoperative nausea and vomiting which is a very common side-effect after inhalational or balanced anesthesia 5,19). Also PRO was shown to be a potent anticonvulsant that sometimes was effective in status epilepticus refractory to other agents 5). The antiemetic, antipruritic, antiepileptic effects of PRO are achieved at lower doses (below 0.5 µg/mL) than those required for anesthesia 20).
Furthermore, the capability of PRO to associate with the potent, rapid and ultra-short-acting synthetic opioid analgesics (such as alfentanil and its congeners) has been promoted the successful and safe using of TIVA 21. Todays (especially in the big countries), PRO usage became widespread as it has the unique pharmacological and PK properties than other the convenient used hypnotics lacked.

**Mechanism of action of PRO**

The mechanism of action of PRO involves a positive modulation of the inhibitory function of the neurotransmitter GABA through GABAA. PRO has also been reported to block Na+ and Ca2+ channels, nicotinic receptors, the N-methyl-D-aspartate receptor, and activates the rectifying outward K+ currents. In this respect and by the way, the mechanisms of PRO’s activity resemble to some extent those of carbamazepine (CBZ), and this effect may result in PRO’s action on seizure duration 22. A deeper and more discussion details for understanding the mechanism of action and structure-activity relationships of PRO was pointed in a review prepared by Trapani et al. 5.

**PK of PRO**

The PK of PRO is best described on the basis of three compartment model (Table 1) 17,18,23-25. The short effect-site equilibration half-life (T1/2) and the small central compartment are responsible for its time peak effect of only two minutes. The larger volume of distribution (Vd), combined with a clearance (Cl) that equals hepatic perfusion, are associated with a context sensitive half-time (describes the time taken for concentration in plasma of a drug to decline by 50% after the steady state of infusion) that only increases from about 20 to about 30 min with infusion durations increasing from 2 to 8 hours. Consequently, PRO is very well suited for continuous infusion administartion. As well as, its high Cl and redistribution allow for a rapid return to the consciousness even after many hours of anesthesia 26,27.
PRO is a weak organic acid that remains entirely unionized at pH 7.4. Therefore, PRO bounds to plasma proteins especially to albumin, as mentioned before. Also hemoglobin has been shown to bind PRO, however its binding properties on the erythrocytes and serum proteins remain to be precisely qualified. Although PRO is extensively bound to the plasma proteins, this does not seem to inhibit its rapid Cl and extensive tissues distribution.

Table 1. Pharmacokinetic and pharmacodynamic parameters of PRO and the four commonly opioids used with PRO.

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<th>Parameter and unit</th>
<th>PRO</th>
<th>Fentanyl</th>
<th>REM</th>
<th>Alfentanil</th>
<th>Sufentanil</th>
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<td>Vd1, L</td>
<td>4.3</td>
<td>8.9</td>
<td>5.1</td>
<td>8.9</td>
<td>14.3</td>
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<td>Vd2, L</td>
<td>24.0</td>
<td>50.3</td>
<td>9.0</td>
<td>13.8</td>
<td>63.1</td>
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<td>Vd3, L</td>
<td>238.0</td>
<td>296.0</td>
<td>6.5</td>
<td>12.1</td>
<td>262.0</td>
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<td>Cl1, L/min</td>
<td>0.7</td>
<td>0.6</td>
<td>2.5</td>
<td>0.4</td>
<td>0.9</td>
</tr>
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<td>Cl2, L/min</td>
<td>1.6</td>
<td>4.8</td>
<td>1.7</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Cl3, L/min</td>
<td>0.8</td>
<td>2.2</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>T1/2, keO, min</td>
<td>2.4</td>
<td>4.7</td>
<td>0.9</td>
<td>1.1</td>
<td>5.8</td>
</tr>
<tr>
<td>EC50, µg/L</td>
<td>340.0b</td>
<td>1.1c</td>
<td>4.7c</td>
<td>90.0c</td>
<td>0.1c</td>
</tr>
</tbody>
</table>

A Model estimation for patient 40 years, 180 cm and 80 kg; b for loss of consciousness; c optimal EC50 in the presence of PRO; Cl1= elimination clearance; Cl2= rapid distribution clearance; Cl3= slow distribution clearance; ED50= median effective concentration for loss of consciousness (PRO) or adequate analgesia (opioids); T1/2, keO= effective equilibration T1/2; Vd1= volume of central compartment; Vd2= volume of rapid equilibrating peripheral compartment; Vd3= volume of slow equilibrating peripheral compartment.

Various PKs parameter sets are available in the literatures for all of these agents, but population PK data are available only for PRO, REM and alfentanil. These population PK parameter sets may therefore be best applicable in a population that varies greatly in age, weight, and gender.

Metabolism of PRO

PRO is rapidly metabolized in the liver by conjugation to glucuronide and sulphate to produce water-soluble compounds, which are excreted by the kidneys. CYP2B6 and CYP2C9 are predominantly involved in the oxidation of PRO; other isoforms such as CYP2A6, CYP2C8, CYP2C18, CYP2C19 and CYP1A2 are also contributed at its high concentrations.
In human, PRO metabolism consists of direct conjugation of PRO to PRO-glucuronide or conjugation of the hydroxylate metabolite (2,6 diisopropyl-1-4-quinol) to three metabolites, 1-quinol glucuronide, 4-quinol glucuronide and 4-quinol sulphate as shown in Figure 2 \(^\text{32}\). About 90% of an injected dose of PRO is eliminated in urine in the form these four conjugated metabolites \(^\text{33}\).

![Figure 2. The main metabolic pathways of PRO in human \(^\text{32}\).](image)

**Side Effect of PRO**

Although PRO offers many advantages than other hypnotics, but is not free from the adverse side effects as has been reported \(^\text{34}\):
a) The inhibition of CYP2A1, CYP2C9, CYP2D6 and CYP3A4 activities.

b) Induction of a marked loss of sympathetic tone in healthy volunteers, lead to a hypotension mediated by an inhibition of the sympathetic nervous system. The impairment of the baroreflex regulatory mechanisms and loss of vascular tone in arteries, as a result of a reduced Ca\(^{2+}\) influx, may also contribute to the hypotension following induction with PRO.

c) A significant reduction in the cardiac, sympathetic baroslopes and cardiac muscle contraction as a result of reduced free systolic Ca\(^{2+}\) concentration in myocardial cells resulting in a negative inotropic state of the cardiac muscle.

d) PRO (even at low doses) depresses the ventilatory response to acute hypoxic incidents.

e) PRO infusion syndrome (especially in the children and small laboratory animal), that is acute refractory bradycardia leading to asystole, in the presence of one or more of the following: metabolic acidosis, rhabdomyolysis, hyperlipidemia, and enlarged or fatty liver and finally death.

These adverse side effects of PRO are responsible on the severe hemodynamic changes and respiratory depression.

Dosage form of PRO

PRO is highly lipophilic drug, that it was initially formulated in some surfactants, which led to a pain at the site of injection and in some cases anaphylactic reactions were reported. The recent dosage form of PRO is formulated as oil/water emulsion consist of soya bean oil, glycerol and purified egg phosphatide (Diprivan®, Zeneca UK) as 200 mg/20 mL ampules for direct i.v. infusion.
II. OPIOIDS AND ANESTHESIA

Opioid is the term used broadly to describe the compounds that work at the opioid receptors in the body. PRO-opioid together with the recent i.v. administration devices (such as TCI) improved the anesthetic depth monitoring and optimized the administrated doses. For more details, Lichtenbelt et al. were described the strategies of using of PRO-opioids in general anesthesia in different situations.

**Mechanism of Action of Opioids**

Opioids and endogenous opioids activate presynaptic receptors on GABA neurons. The varying effects of opioids may therefore be related to varying degrees of affinity for various opioid receptors. In terms of pharmacodynamics, both opioids and PRO act on γ-GABA receptors. The reason for the lower dose of PRO required for sedation in the presence of opioids may be due to this synergic effect.

**PK of Opioids**

Opioids differ in their PKs but, acting at similar receptor sites and exhibit comparable PKs. Table 1 gives an overview of representative PKs parameters of the commonly four opioids used with PRO in general anesthesia. The context sensitive half time of the four opioids gives an indication of the suitability of these agents to be given by prolonged infusion.

**Remifentanil (Ultiva®)**

REM (a hydrochloric salt of 3-[4-methoxy-carbonyl-4-[(1-oxopropyl)phenylamino]-1-pipridine]propanoic acid methylester, Figure 1), is a ultra-short-acting synthetic opioid which has a rapid onset (1 min) and offset (15 min) after an i.v. infusion regardless of the duration of its infusion.
REM remains entirely unionized at pH 7.4; it extensively (70-80%) bounds to plasma proteins especially to α₁-acid glycoprotein. The metabolism of REM in the body is completely different than other opioids used in anesthesia, as it is metabolised mainly by cholinesterases which found in the blood plasma and tissues. REM metabolite has minimal (4,600 times less potent than REM) analgesic activity and excreted by the kidneys, Figure 3.40,41).

![Chemical structure of REM](image)

Figure 3. The main metabolic pathways of REM in human.

The action of REM is not extended in patients with renal failure. Moreover, there is no risk for drug accumulation even after long-term infusion. By the way, the most attractive characteristic of REM is that, within the dose ranges studied thus far, recovery is not dependent on the infusion rate used to maintain anesthesia, the cumulative dose of REM administered, or the length of the procedure. The actual dose does not need to be adjusted for age, weight, gender, or the presence of hepatic or renal dysfunction. Therefore, REM may be described as the most "forgiving" opioid.
The anesthesia community has, in general, agreed that its properties of rapid and pleasant emergence with fewer emetic symptoms make the additional costs worthwhile. As the importance of REM in anesthesia procedures, many reviews outlined the REM’s clinical pharmacology and its applications in anesthesia. REM is very much alike other opioids pharmacodynamically; it produces the physiological changes consistent with potent μ-opioid receptor agonist activity, including analgesia and sedation with the adverse effects include, ventilatory depression, nausea, vomiting, muscular rigidity, bradycardia and pruritus. REM appears to be an ideal narcotic analgesic used for TIVA in combination with PRO, because of its independent pathway from that of PRO as well as its rapid elimination and favorable controllability. Therefore, the general anesthesia using PRO and REM is a very accurate, easy and predictable (or titratable) technique and has many advantages such as:

a) Achievement of the steady state rapidly due their rapid onset as well as a short context sensitive half-life.

b) Hemodynamic stability.

c) Rapid recovery even after long time of anesthesia.

d) Lower incidence of postoperative nausea and vomiting, as PRO has antiemetic (potentiated with REM) properties at low doses.

e) Patients like the sensation of waking up from a PRO infusion compared to patients waking up from inhaled anesthetics; those recovering from PRO feel peaceful and relaxed due to PRO stimulates areas in the limbic system that are related to pleasure (e.g. love, peace, relaxation and sexual content). More than 50% of the patients under PRO infusions have dreams; many of them report having “very nice” ones. (minimal hangover effect).

f) PRO allows encephalographic and evoked potential monitoring for ischemia and seizures, as well as PRO and REM can be used in patients in renal failure.
The Reported PK Interaction between Pro and Fentanyl-family Opioids

PRO and Fentanyl or Alfentanil PK Interactions:

It was reported that, alfentanil and fentanyl affected the PK of PRO, as its Vd₁ and Cl₁ were significantly decreased, while its Vd₃ was increased. The mechanism of actions was suggested as the phenomena that the presence of opioid with PRO, lead to potentiate its hemodynamic changes or pharmacodynamically related to the GABA receptor activates.

Inversely, alfentanil PKs were also altered in the presence of PRO (plasma concentration, 1.5 µg/mL), as a result of the inhibition of CYP3A4 which responsible for its oxidative metabolism. Therefore, a significant decreases in Cl₁, Cl₂, and Cl₃ of alfentanil were resulted.

PRO and REM PK Interactions:

A non-steady state (i.e. according to volunteers’ demands) co-administration design of PRO and REM in human was used to determine their PK interactions. Through the study, twenty healthy volunteers initially received either PRO or REM alone in a stepwise incremental and decremental fashion via TCI device. The respective second drug was infused as a fixed plasma target concentration (0-4 µg/mL for Pro and 0-4 ng/mL for Rem). As results, Rem did not alter the PK of PRO, whereas PRO causes a 41% reduction of Vd₁ and Cl₁ and a 15% reduction of the Cl₂ of REM.
III. EPILEPSY AND ANESTHESIA

Epilepsy is recurrent seizures resulting from congenital or acquired factors. The term epilepsy is derived from the Greek word *epilambanein*, meaning to attack or seize. Simple seizures involve no loss of consciousness, whereas altered levels of consciousness are seen in the complex seizures. Epilepsy is the most common serious chronic neurological disorder, with a prevalence of 0.5-1% of the population. It can be classified as generalized or partial seizures and either convulsive or non-convulsive in nature. Status epilepticus is a continuous seizure of at least 30 min duration or intermittent seizure of at least 30 min duration during which consciousness is not regained. Status epilepticus is considered a medical emergency that should be treated as quickly and aggressively as possible to prevent neuronal damage.

Antiepileptic Drugs

The history of antiepileptic drugs (AEDs) is starting with the use of bromides in the 1850s, phenobarbital, and phenytoin in the first half of the 20th century. The 1960s and 1970s saw sodium valproate (valproate) and CBZ become the standard treatments for epilepsy, and they still are. Also, PRO and a short acting benzodiazepine, midazolam (MDZ) are used in the clinical practice for the treatment of generalized seizures and status epilepticus.

Mechanism of Action of AEDs

In simple terms, a seizure can be seen as the result of imbalance between excitatory and inhibitory neuronal activity. This leads to the generation of hyper-synchronous firing of a large number of cortical neurones. Traditional AEDs exert antiseizure activity by the following mechanisms.
a) Reduce the inward voltage-gated positive currents (Na⁺ and Ca²⁺).

b) Increase inhibitory neurotransmitter activity (GABA).

c) Decrease excitatory neurotransmitter activity (glutamate and aspartate).

In terms of pharmacodynamics, valproate and PRO act on GABA receptors. The reason for the lower dose of PRO required for sedation in the presence of valproate ⁶³. In in vitro and in vivo studies, valproate were reported to inhibit CYP2C9 ²², ⁶⁴ and UDP-glucuronosyltransferase 1A9 enzyme system that is essential in the metabolism of PRO ⁶⁵. Additionally, valproate increases the rate of protein unbound free PRO in human serum, which results in an increase in sedative effect due to the free PRO ⁶⁶.

**Carbamazepine (Tegretol®)**

CBZ (5H-dibenz[b,fl]azepine-5-carboxamide, Figure 1) is a dibenzazepine, tricyclic drug used for the treatment of epilepsy, trigeminal neuralgia, and schizophrenia ⁶⁷-⁶⁹. It has been the first choice as AED for the wide range of seizure disorders in both adults and children due to its efficacy and acceptable safety profile ⁷⁰.

CBZ the main mechanism of its anticonvulsant effects through inhibit the Na⁺ channel activity ⁷¹. Unfortunately, CBZ plasma concentrations are closely related to its curative effect and toxic side effects. Therefore, it is necessary to monitor its plasma concentrations to improve the curative effect and decrease the expected toxic side effects after long term of administration ⁷²-⁷⁵.

Unlike valproate, CBZ is an inducer for several potential pathways of drug elimination, including CYPs, as well as the active transporter P-glycoprotein. To our knowledge there is no information about the possible interaction between PRO and CBZ after co-administration.
PK of CBZ

CBZ absorption following an oral administration has been shown to be slow, changeable, unpredictable, but almost complete with approximately 80-90% bioavailability. Peak plasma concentrations are usually observed between 2 to 6 hours following oral administration. CBZ is rapidly distributed into all tissues and fluids throughout the body and is reported to have Vd of approximately 1-2 L/kg.

The dose of CBZ should be adjusted to the needs of the individual to achieve adequate control of seizure activity without the toxic side effect. From the literatures, the effective total plasma concentration of CBZ is approximately 4-12 µg/mL for daily divided doses of 400 to 1200 mg. CBZ highly (70%) bounds to plasma proteins especially to albumin and α-acid glycoprotein.

Metabolism of CBZ

CBZ is mainly metabolized in the liver with approximately 5% of the drug excreted as un-changed. The hepatic metabolism of CBZ is catalyzed by CYP3A4, CYP2C8 and CYP3A5 isoforms to an active metabolite (10,11-epoxide). This active metabolite is further deactivated by the action of epoxide hydrolase to give inactive 10,11-CBZ-diol that is excreted as glucuronides conjugates by the kidneys as shown in Figure 4.

Figure 4. The main metabolic pathways of CBZ in human.
Midazolam (Dormicum®)

MDZ (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzo-diazepine, Figure 1) is a short-acting imidazobenzodiazepine with hypnotic, muscle-relaxant, anticonvulsant, and anxiolytic properties. It is used for the induction of anesthesia, sedation and the treatment of generalized seizures and status epilepticus.

**Mechanism of Action of MDZ**

MDZ binds with GABA<sub>A</sub> receptor in the CNS leads to its activation to the neurotransmitter GABA; therefore, an indirect enhancing of the inhibitory action of GABA occurs. Santhosh et al. was reported that the enhancement of the inhibitory action of GABA may enhances both the CNS and respiratory depressant effects of PRO when combined with benzodiazepines.

**PK of MDZ**

Following an oral administration, MDZ is absorbed rapidly from the gastrointestinal tract with maximum plasma concentration occurring usually within 30-90 min. but following an i.v. administration, the onset of action occurs rapidly within 1-5 min. MDZ has a Vd of 1-2.5 L/kg in normal healthy volunteers. MDZ extensively (96%) binds to plasma proteins especially to albumin.

**Metabolism of MDZ**

MDZ is metabolised mainly by the hepatic CYP3A3 isoform to an active metabolite 1'-hydroxy-MDZ, as it's the main metabolite. Other minor pharmacologically inactive metabolites such as 4-hydroxy-MDZ and 1',4-dihydroxy-MDZ are also produced. All these metabolites undergo to rapid glucuronidation and then excretion by the kidneys, as shown in Figure 5.
Compared with other benzodiazepines, MDZ has a short elimination $T_{1/2}$ of 1.5-3 h with total plasma Cl about 5.8-9 mL/min.  

From the literatures, MDZ is neither inhibitor nor inducer of CYP3A4 metabolism, however it has been proposed for the hepatic CYP3A phenotyping (assessing the functional CYP3A activity). MDZ is widely used probe in drug-drug interaction research to predict the enzyme inhibition or induction or to predict the unwanted therapeutic outcomes such as lack of therapeutic efficacy or potentially harmful response.
Since 2003, MDZ is recognized as one of the preferred in vivo probes by food and drug administration (FDA) and the pharmaceutical research and manufacturers\(^{86}\).

**PRO and MDZ PK Interactions**

The PK interactions between PRO and MDZ have been studied extensively\(^{54, 87, 88}\). Briefly, the blood concentration of PRO was significantly increased (25%) in the presence of co-administrated MDZ, related to the hemodynamic changes or pharmacodynamically both drugs act on GABA receptors. That resulted in the common phenomena that the combination between PRO and benzodiazepines may enhances both the CNS and respiratory depressant effects of PRO\(^{54, 89}\). The plasma concentration of MDZ was also increased in the presence of PRO (at plasma concentration, 1.2 µg/mL) as a possible competitive inhibition of CYP3A3, CYP3A4 and CYP3A5 isoforms responsible for MDZ metabolism\(^{87, 88}\) by PRO.

**PRO and CBZ expected PK Interactions**

As mentioned before, CBZ has a fairly narrow therapeutic range which can produce numerous of the adverse effects. CBZ is an inducer for several potential pathways of drug elimination, including CYPs such as CYP1A2, 2C9, and 3A4, as well as the active transporter P-glycoprotein. Therefore, a drug such as PRO, undergoes metabolism via these CYPs is likely to be affected after CBZ co-administration.

In addition to CBZ is primarily metabolized by CYP3A4 to an active metabolite that has about the same efficacy as the parent compound. Therefore, a drug such as PRO has an inhibition for CYP3A4 will expected to decrease the hepatic metabolism of CBZ and lead to its accumulation with the signs of toxicity\(^{90, 91}\).
IV. REPORTED ASSAY METHODS FOR PRO, REM, MDZ AND CBZ

Recently, an analytical review has been published to summarize the reported important analytical methods for determination of PRO in the different biological fluids. Since PRO binds tightly to erythrocytes (~50%) Therefore the whole blood is preferred for the analysis of PRO and its metabolites for PKs or forensic studies.

The majority of these methods were used HPLC-UV, -fluorescence, or -electrochemical detections. HPLC-UV methods (at a wavelength of 270 nm) for its determination in human plasma, blood, serum or urine; dog plasma, blood, or rat blood have been reported. LC-MS/MS methods have been used to determine PRO either by atmospheric-pressure chemical ionization without derivatization; or by negative electrospray ionization (ESI) with or without derivatization in human plasma, blood and serum, or in rat plasma and blood.

REM contains a methyl ester group in its structure, which makes it not only susceptible to enzymatic hydrolysis but also to chemical hydrolysis at physiological pH. Consequently, the addition of an acid to the collected blood after administration (citric acid (20 µL, 50% w/w)/mL of the collected blood) was needed to prevent the both types of hydrolysis.

Clinically, REM plasma concentration is 1-40 ng/mL, that is achieved after an infusion rate of 0.04-2 µg/kg/min. Because of these circumstances (REM's instability and low plasma concentrations), measurement of REM concentrations in the circulation is challenging. Most of the reported methods determined REM in whole blood which preferred to avoid time spent on plasma preparation.
Several HPLC-UV methods (at a wavelength of 210 nm) were developed in human plasma \(^{110}\) and blood \(^{98, 99, 114, 116-121}\), dog plasma and blood \(^{99, 110}\) and rat blood \(^{98, 114, 121}\). Other sensitive LC-MS/MS methods for REM analysis in plasma \(^{115, 122}\), blood \(^{123}\), and urine \(^{124}\) have been reported.

As mentioned before, monitoring the concentration of CBZ in human plasma is of great importance to clinical analysis. Hence, several publications deal with the determination of CBZ in different biological samples. HPLC-UV methods (at a wavelength of 285 nm) were reported for CBZ and its metabolite determination in human whole plasma \(^{125-134}\), blood \(^{135}\), serum \(^{136-139}\), urine \(^{127}\) and saliva \(^{128}\) or in rabbit plasma \(^{140}\). Other techniques such as liquid chromatography-mass spectrometry (LC-MS) \(^{73, 135, 141, 142}\) and LC-MS/MS \(^{73, 142, 143}\) have been reported.

Benzodiazepines are commonly administered with a potential of abuse and an environmental pollution \(^{144}\). Several non-chromatographic techniques are available for their rapid analysis in plasma, blood, or urine. These assays were depended on the available laboratory kits that are often used to provide an initial test for the enzyme immunoassay determination \(^{145}\) and linked immunosorbent assay \(^{146}\). Moreover, many analytical reviews have been published to summarize the most important reported chromatographic separations \(^{144, 147, 148}\) of MDZ and the other benzodiazepines in the different biological fluids.
CHAPTER 1

DEVELOPMENT AND VALIDATION OF AN HPLC-UV METHOD FOR SIMULTANEOUS DETERMINATION OF PROPOFOL AND REMIFENTANIL IN RAT PLASMA
1.1. BACKGROUND

As mentioned before, PRO and REM are the drugs of choice, commonly co-administered in TIVA. A little is known about PRO and REM PK interactions after co-administration. Overdosing of PRO or REM in TIVA causes cardiovascular depression as manifested by symptoms such as severe bradycardia and asystole. Clinically, REM decreases the concentration of PRO required for loss of consciousness, because they interact pharmacodynamically and act synergistically in response of hypnosis and nociception. The aim of the present study was to develop a simple and rapid HPLC-UV method for simultaneous determination of PRO and REM to investigate their PKs drug-drug interactions in rat plasma following co-administration by i.v. infusion.

1.2. MATERIAL AND METHODS

1.2.1. Reagents and Standards

PRO, sodium carbonate, sodium hydrogen carbonate, ammonium acetate, ethylacetate, hexane, ibuprofen (IBU), clonidine (CLO), methyl eugenol, methyl phenol, 4-methoxyactophenone, 2,6-di-tert-butyl-4-methyl phenol, 2,6-xylenol, t-butyl methyl ether (t-BME), diethyl ether, heptane, and acetonitrile were obtained from Wako Pure Chemicals, Ltd. (Osaka, Japan). REM hydrochloride was obtained from Janssen Pharmaceuticals, Inc. (Tokyo, Japan). Fluoxetine (FLU) was purchased from Sigma-Aldrich (Steinheim, Germany) and used as IS. All other reagents were of analytical reagent and LC grade. Water was deionized and distilled by an Aquarius GSR-500 automatic water distillation apparatus obtained from Advantec (Tokyo). Nitrogen gas of 99.999% purity was provided by Daiichisanso (Nagasaki, Japan).
1.2.2. Standard Solutions and Quality Control Samples

The stock standard solutions of PRO, REM and IS were prepared by dissolving accurately weighed individual compounds in acetonitrile to give a final concentration of 1 mg/mL (REM base was prepared by accurately weighing 1.1 mg of REM hydrochloride (equivalent to 1 mg of the free base)). Then the solutions were serially diluted with acetonitrile to obtain the working solutions at concentration range over 25-2500 ng/mL for both PRO and REM and 1 µg/mL for the IS. All the solutions were stored in a refrigerator at 4°C and brought to room temperature (25°C) before use.

The analytical standard and quality control (QC) samples were prepared by blank rat plasma (200 µL) spiked with standard working solutions (20 µL) during the study of method validation. The calibration samples were made at six calibration points of concentrations of 25, 50, 100, 500, 1000, and 2500 ng/mL for both PRO and REM while their QC samples were prepared at concentrations of 50, 500, and 2500 ng/mL.

1.2.3. HPLC-UV System and Conditions

The chromatographic separations were performed on an HPLC system, consisted of a Shimadzu LC-10ATvp LC pump (Shimadzu, Kyoto, Japan), 7125 injector with a 20-µL sample loop (Rheodyne, CA, USA), equipped with a 10 µL loop Shimadzu SPD-10A variable wavelength UV detector that set at 210 nm.

The separation was performed by using a Chromolith Performance RP-18e (100 x 4.6 mm i.d., Merck Darmstadt, Germany); with a gradient elution program with mobile phases: A (acetonitrile) and B (ammonium acetate buffer, 10 mM, pH 5.8). The gradient program was: 75% of mobile phase B for 3 min, followed by stepwise gradient from 75% to 60% until 8 min, changed to 55% until 11.8 min. the washing step by 100% mobile phase A until 15 min and then return to initial condition after 20 min.
The mobile phases were filtered through (0.45 µm nylon membrane (Omnipore™, Millipore, Tokyo), degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai)), and pumped into the column at a flow rate of 1.5 mL/min. A summary of the HPLC conditions is shown in Figure 6.

- Column: Chromolith Performance RP-18e (100 x 4.6 mm i.d.).
- Pumps: Shimadzu LC-10ATvp.
- Detector: Shimadzu SPD-10A.
- Detection wavelength, λ: 210 nm.
- Mobile phases A (acetonitrile) and B (ammonium acetate buffer (10 mM, pH 5.8)), were eluted according to gradient program.
- Flow rate: 1.5 mL/min.
- Injection volume: 20 µL.

Figure 6. HPLC conditions and the gradient elution program.

1.2.4. Plasma Collection

The plasma samples used in the present study were obtained from male Wistar rats (n=3, weight 250-320 g) purchased from Kyudo Experimental Animal (Saga, Japan). The rats were housed in the conditions of constant temperature (24°C) and provided with standard laboratory food (Oriental yeast, Tokyo) and water ad libitum. All animal procedures and care in this experiment were permitted by Nagasaki University animal care use committee (NO. 1406021153). The rats were anesthetized with ethyl carbamate (1.5 g/kg, intraperitoneal, i.p.).

A cannula was inserted into the femoral artery of the rat for blood withdrawal. The blood samples were collected into eppendorf tubes contains ethylene diamine tetraacetic acid disodium salt (EDTA.2Na, 1.0 mg/mL of blood). The collected blood was centrifuged (2000g) for 10 min at 4°C, and the obtained plasma were kept at -30°C prior to use.
1.2.5. Plasma Pre-treatment

To 200 µL of rat plasma was spiked with 20 µL of PRO and REM mixture and 20 µL of IS (Stock solution concentrations in acetonitrile was 11.1 µg/mL), vortex-mixed for 10 sec. One hundred-microliter of sodium carbonate buffer (100 mM, pH 11.2) was added and vortex-mixed for 30 sec, followed by addition of 700 µL of hexane for liquid-liquid extraction (LLE) procedure. Vortex-mixed for 60 sec, then the mixture was centrifuged (2000g) for 10 min at 4°C. Six hundred-microliter of the supernatant was transferred to another vial and evaporated to be dryness under a gentle stream of N₂ gas. The residue was reconstituted with 30 µL of mobile phase and then 20 µL of reconstituted volume were injected onto the column for analysis. Triplicate measurements for each sample were performed as the amount indicated as means ± SD. The summary of the pre-treatment procedure illustrated in the Figure 7.

**Plasma, 200 µL (or + standards, 20 µL)**

- Carbonate buffer (100 mM, pH 10), 100 µL
- Vortex-mix for 30 sec
- LLE, Hexane, 700 µL
- Vortex-mix for 60 sec
- Centrifuge (2000g) at 10°C for 10 min
- **Take supernatant, 600 µL**
- Dry under N at 25°C
- Reconstitute with acetonitrile, 50 µL
- **Sample solution for HPLC analysis, 20 µL**

Figure 7. Pre-treatment procedure of PRO and REM in rat plasma for HPLC-UV method.
1.2.6. Method Validation

The method was validated for selectivity, calibration curves linearity, limit of detections (LODs), limit of quantifications (LOQs), accuracy, precision and recovery after extraction from plasma.

The selectivity was investigated by preparing and analyzing three of individual rats' blank plasma samples at the LOQs to check any interference at the retention time of the analytes.

The calibration curves linearity were assessed by preparing and analyzing the blank rat plasma spiked with PRO and REM standard of known concentrations (over the range of 25-2500 ng/mL with six spiked concentration points); triplicate measurements for each concentration were performed. The LOD was estimated as the amount of PRO or REM, which caused a signal three times to peak height of the blank response (noise). The LOQ was defined as the lowest concentration on the calibration curves of the analytes measured with acceptable precision and accuracy and with at least ten times compared to the plasma blank noise peak height.

The accuracy and precision were assessed by determining the QC samples at three concentration levels (three samples for each concentration) on the five different validation days. The accuracy was expressed as a percentage of the measured concentration over the nominal (theoretical) concentration and the precision was determined as the RSD%. The criteria used to assess the suitability of precision as the RSD% did not exceed 15%; and the accuracy was within 20% of the actual value \(^{154,155}\). The recovery (extraction efficiency) of analytes from rat plasma after the extraction procedure was determined by comparing the areas of extracted analytes with those of the non-extracted pure standards that represent 100% recovery.
1.3. RESULTS AND DISCUSSION

1.3.1. Optimization of the Assay Conditions

Following to sample preparation, an instrumental analysis should be effective to isolate the analytes of interest in biological sample from interfering compounds. Chromatography is based on the separation of analytes of interest in a mixture due to the differing time taken for each analyte to travel through a stationary phase which carried through it by a mobile phase. The main component and consider as the heart of any HPLC system is the column which can be described by its dimensions and particle size. Reversed phase (RP) column is considered the column of choice for HPLC analysis, because it provides a compatibility with aqueous and organic solutions as well as with different detection systems. However, the disadvantages of RP columns such as slow mass transfer and limitations caused by column back pressure, could be overcome by introducing the monolithic columns.

The Monolithic columns are porous rod structures characterised by mesopores and macropores. These pores provide the monolithic column with high permeability, a large number of channels and a high surface area available for reactivity. The macropores dramatically reduce the column back-pressure and allow the use of faster flow rates, thereby reducing the analysis time. While monolith is a popular material for chromatographic columns fabrication, it also plays a role in SPE sample preparation. The details of the miscellaneous applications of monolith are reviewed in a series published by Svec. In the present thesis a monolith silica-based column (Chromolith® Performance RP-18e (100 x 4.6 mm i.d., Merck, Darmstadt, Germany)) was utilized for the chromatographic separations.

Recently, mixed-mode columns were introduced to provide a practical solution to the selectivity challenge with RP and monolithic columns. It combines both RP and ion-exchange retention mechanisms.
The presence of both RP and ion-exchange functionalities eliminates the need for ion-pairing agents in the mobile phase, making it compatible with mass spectrometry (MS) detections \(^{163, 164}\). Unfortunately, the combination between RP, anion, and cation ligands perhaps is the major drawback for these types of column. As the strong ionic interaction of this stationary phase with biological matrices (some of these components present in a highly concentrations) lead to increase the time of separation and additionally a washing step after the analyte separations is needed. This issue can be resolved by increasing the ionic strength of aqueous part (buffer) in the mobile phase. However, it is important to mention that the percentages of acetonitrile higher than 50\% led to precipitation of highly used salt (in buffer phase), and the low percentages of acetonitrile likely affects the elution of highly hydrophobic compounds from the column \(^{165}\).

As the difference in the structure of PRO (acidic analyte) and REM (basic analyte), therefore we speculated, the separation by using a multimodal Scherzo SM-C18 (150 x 2 mm i.d., 3 µm, Imtakt Co., Kyoto) are going on easily. An isocratic mobile phase consist of acetonitrile-ammonium acetate buffer (10 mM, pH 6) was studied using IBU as an IS.

The preliminary experiments were directed toward the effect of various variables on the chromatographic system suitability for the standard analytes separations. The parameters which assessed involved the type and percentage of the organic modifier, concentration of ammonium acetate buffer as well as its pH as shown in Figure 8A-C. Under these conditions the retention time (using Scherzo column) of PRO, REM and IS, respectively was 10, 6 and 8 min.

The plasma samples preparation were tried with protein precipitation, LLE, and hybrid of protein precipitation and LLE. SPE procedures using monolithic (MonoSpin), Varian C18 and Bond Elut Plexa C18 cartridges also were tried.
Figure 8. Study of acetonitrile content (A), ammonium acetate buffer concentration (B) and its pH (C) in mobile phase on the retention time and peak heights of the analytes. Sample: standards concentration, 50 ng/mL for PRO and REM, IS (1µg/mL). The optimal conditions were selected as the arrows referred.
For this type of column the washing step is much necessary to elute the latent plasma peaks after analytes elution as mentioned before. Unfortunately the column washing was extended for long time (≈ 60 min) between each separation. Therefore, modifications in the HPLC-UV system and conditions were occurred by adding another pump (Shimadzu LC-10ATvp LC) to deliver the washing solution. Among the different washing solutions which used were acetonitrile-acetate buffer (50 mM, pH 3.5) (70:30%, v/v); acetonitrile-isopropanol-water (50:30:20%, v/v/v) and acetonitrile- tris(hydroxymethyl)aminomethane) buffer (100 mM, pH 7.5). As the results, the trials to optimize the Scherzo column’s washing step were unsatisfactory, and affected the experimental rapidity and reproducibility. Therefore, to complete the research point, a monolith-C18 column (Chromolith (100 x 4.6 mm i.d.) was preferred instead the Scherzo SM column.

As the presence of a suitable IS with the analytes mixture is much important during HPLC analysis, to minimize the experimental errors; analytes such as IBU, methyl eugenol, methyl phenol, 4-methoxyacetophenone, CLO, 2,6-di-tert-butyl-4-methyl phenol, 2,6-xylenol and finally FLU were checked as IS. A gradient elution using FLU as an IS was needed in order to complete separation of target analytes from plasma samples.

Sample preparation before instrumental analysis should effectively isolate the analytes from the matrix interfering compounds. However, it is a bottle-neck and consume about 80% of the analysis time. The samples free from interfering matrices not only simplify analysis but also maximize the sensitivity of detection. LLE is the traditional procedure for sample preparation in bioanalysis.

The principle behind the separation is the distribution of sample components between two immiscible liquids. The main advantages of the LLE procedure are: large samples capacity, clean extracts, and an easy approach to sample concentration.
The plasma samples in the present study were treated by a LLE as described previously, and the representative chromatograms for rat plasma blank and that spiked with analytes are showed in Figure 9.

![Chromatograms](image)

Figure 9. Chromatograms of drug free rat plasma (A) and that spiked with PRO, REM, and IS (B). Sample: spiked concentration, 50 ng/mL for PRO and REM and 1 µg/mL for IS (standard solution, 11.1 µg/mL in acetonitrile).

1.3.2. Method Validation

1.3.2.1. Selectivity

The selectivity of the proposed method was tested by analyzing different rat plasma blank for any interference. As the chromatogram of plasma blank extract and that spiked with the analytes were shown in Figure 9 there are no interfering peaks at the retention time of the all analytes. The retention time for REM, IS and PRO was 2.9, 7.9 and 12.0, respectively.
1.3.2.2. Linearity, LODs and LOQs

The calibration curves were constructed in rat plasma in the concentration ranges of 25-2500 ng/mL (six calibration points, 25, 50, 100, 500, 1000, and 2500 ng/mL) for PRO and REM. The analyte on IS peak area ratios were found to have acceptable linearity with the selected concentration range as indicated by acceptable correlation coefficients ((r) ≥ 0.983). The calibration curves’ equations for PRO and REM respectively were; y = 0.002x - 0.060 and y = 0.001x - 0.030 (where, y = peak area ratio and x = the amount of the analyte, ng/mL). The LODs and the LOQs were defined as the concentration giving peak height at a signal-to-noise (S/N) ratio of 3 and 10 were 3 and 10; 6 and 20 ng/mL, for PRO and REM respectively. The recoveries of analytes from rat plasma after the extraction were 88.3±7.6 and 68.7±5.5 as the average of low, middle, and high concentration levels for PRO and REM respectively as shown in Table 2.

1.3.2.3. Accuracy and Precision

The accuracy of the proposed method was estimated by comparing the measured (calculated) concentrations of PRO or REM in the plasma with the nominal (theoretical) concentration values of the standard in the mobile phase. The calculated accuracy for PRO and REM was found between 80.1±4.1% and 105.3±6.8% indicating an acceptable accuracy for the proposed method.

The results also showed that intra- and inter-day precisions (variations) at three different concentrations levels of PRO and REM were within the acceptable validated range as the RSD% was found less than 15% for intra- and inter-day precisions, Table 3.
Table 2. Linearity range, statistical data, and recoveries for determination of PRO and REM in spiked rat plasma.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Conc., ng/mL</th>
<th>$r^1$</th>
<th>Regression equation$^2$</th>
<th>Recovery$^3$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO</td>
<td>25-2500</td>
<td>0.997</td>
<td>$y = 0.002x -0.060$</td>
<td>88.3±7.6</td>
</tr>
<tr>
<td>REM</td>
<td>25-2500</td>
<td>0.983</td>
<td>$y = 0.001x -0.030$</td>
<td>68.7±5.5</td>
</tr>
</tbody>
</table>

$^1$: Correlation coefficient  
$^2$: Peak area ratio and x: Sample conc., ng/mL  
$^3$: Recovery vs. standard (average of low, middle, and high concentration, n=3)

Table 3. Accuracy, intra- and inter-day precision for the proposed method.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked Conc., ng/mL</th>
<th>Accuracy$^1$ % (mean±SD$^2$, n=5)</th>
<th>Precision, RSD$^3$ % (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>PRO</td>
<td>50</td>
<td>80.1±4.1</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>97.3±4.9</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>97.5±5.1</td>
<td>5.2</td>
</tr>
<tr>
<td>REM</td>
<td>50</td>
<td>105±6.8</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>94.1±4.2</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>96.3±4.3</td>
<td>4.5</td>
</tr>
</tbody>
</table>

$^1$: Accuracy % = (Found concentration/nominal concentration) × 100  
$^2$: SD = Standard division  
$^3$: RSD = Relative standard division

1.4. CONCLUSION

In the present work a simple and selective, HPLC-UV using a monolithic column was developed for simultaneous determination of PRO and REM using FLU as an IS in rat plasma. Unfortunately, the obtained sensitivity could not reach the clinical concentration of REM (plasma concentration 1-40 ng/mL) when used in clinical practices with PRO. Even the present sensitivity for PRO (plasma concentration is 1-4 µg/mL) was fulfill its clinical determination in the presence of REM. Consequently, increasing the method sensitivity was needed.
CHAPTER 2

SIMULTANEOUS DETERMINATION OF PROPOFOL AND REMIFENTANIL IN RAT PLASMA BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY: A PRECLINICAL APPLICATION TO PHARMACOKINETIC DRUG-DRUG INTERACTION ANALYSIS
2.1. BACKGROUND

MS spectrometry is closest to being an ideal chromatographic detector; it combines the optimum sensitivity with the maximum identification capability. The MS is related to gas-phase separation of ionized species according to their mass-to-charge ratio (m/z) and the resulting MS spectrum is a plot of the relative intensity versus m/z. The basic components of MS are an inlet device, ion source, ion separation system (mass analyzer, which is the "heart" of the mass spectrometer) and detector. While gas chromatography (GC) can easily be coupled to MS, LC can only be coupled via sophisticated interfaces (probes) to remove the mobile phase and ionize the analyte. Two relatively robust LC-MS interfaces related to the atmospheric pressure ionization technique; electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), which became the 'golden standard' for LC-MS system. Although both interfaces perform what called 'soft-ionization' and generate ions at atmospheric pressure, each is characterised by a specific ionization mechanisms as were described by Bruins and Kerbarle. ESI can be used in positive or negative polarity mode, dependent on the structure of the analyte of interest. Mass analysis is performed using one or two MS analyzers (MS/MS), which consist predominantly of ion traps and quadrupoles, sometimes of sector field and time-of-flight instruments. The analyzers can operate either in the full scan mode or in the more sensitive selective reaction monitoring (SRM) detecting positive or/and negative ions (earlier calling multiple reaction monitoring MRM) that is the most powerful technique for quantification of small amounts of analyte in complex matrices. The LC-MS/MS coupled on-line with ESI has been frequently used in PK studies. In the present study the change from HPLC-UV to LC-MS/MS greatly achieved the challenge for determination of PRO and REM in mixture with the required sensitivity and selectivity. An LC-MS/MS spectrometry was developed and validated using CBZ as an IS. The method was successfully applied to study the PK interactions between PRO and REM after their co-administration by i.v. infusion to male Wistar rats.
2.2. MATERIAL AND METHODS

2.2.1. Reagents and Standards

CBZ as an IS was obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Intralipid® 10% emulsion was obtained from Fresenius SE Co. (Kabi, Tokyo) and all other reagents were of analytical reagent grade; as in Chapter 1.

2.2.2. Standard Solutions and QC Samples

The stock solutions of PRO and REM respectively, 100 µg/mL and 100 ng/mL, were prepared in acetonitrile, stored at 4°C in a refrigerator, and were brought to room temperature before use. The stock solutions were serially diluted in acetonitrile to obtain the working solutions at concentrations over the range of 0.27-10 µg/mL and 0.17-50 ng/mL for PRO and REM, respectively. The analytical standard and QC samples were prepared by spiking blank rat plasma (200 µL) with standard working solutions (20 µL) during study the method validation. For in vivo PKs study the stock solutions of PRO (10 mg/mL) was prepared in Intralipid® 10% emulsion and the stock solution of REM hydrochloride (10 µg/mL) was prepared in 0.9% saline.

2.2.3. LC-MS/MS System and Conditions

A Waters 2695 separation module (Waters Co., Milford, MA, USA) was used for solvent and sample delivery. The chromatographic separations were achieved by using a Chromolith Performance RP-18e (as described in Chapter 1) at 30°C of column temperature. The mobile phase was a mixture of acetonitrile-ammonium acetate buffer (10 mM, pH 3.5) (=90:10%, v/v), pumped at a flow rate of 0.5 mL/min. A negative- and positive-ESI (ESI- and ESI+) easily changeable Quattro micro™ triple quadrupole mass spectrometer (Waters Co.) was used for mass analysis and detection.
Mass spectrometric analysis set up in the SRM mode; it was performed in a negative-ion mode for PRO and a positive-ion mode for REM and IS. Nitrogen gas was used as desolvation (500 L/h) and nebulizer (50 L/h) gas. The source and desolvation temperatures were kept at 120°C and 350°C, respectively. The selected ESI-MS/MS parameters are listed in Table 4. The LC-MS/MS system was controlled by MassLynx™ V 4.0 software (Waters Co.).

Table 4. Selected ion transitions (mass-to-charge (m/z) values) and mass spectrometric parameters for analysis of PRO, REM and IS in SRM mode.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PRO</th>
<th>REM</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor ion, m/z</td>
<td>176.94 [M-H]⁻</td>
<td>377.04 [M+H]⁺</td>
<td>237.07 [M+H]⁺</td>
</tr>
<tr>
<td>Product ion, m/z</td>
<td>160.87</td>
<td>317.04</td>
<td>220.01</td>
</tr>
<tr>
<td>Ionization</td>
<td>ESI⁻</td>
<td>ESI⁺</td>
<td>ESI⁺</td>
</tr>
<tr>
<td>Detection mode</td>
<td>SRM</td>
<td>SRM</td>
<td>SRM</td>
</tr>
<tr>
<td>ESI Capillary voltage, kV</td>
<td>-3</td>
<td>+3</td>
<td>+3</td>
</tr>
<tr>
<td>Cone voltage, V</td>
<td>-30</td>
<td>+15</td>
<td>+20</td>
</tr>
<tr>
<td>Collision energy, eV</td>
<td>11</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Ion energy 1, V</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ion energy 2, V</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Source temperature, °C</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Desolvation temperature, °C</td>
<td>350</td>
<td>350</td>
<td>350</td>
</tr>
</tbody>
</table>

2.2.4. Plasma Pre-treatment

To 200 µL of rat plasma, 20 µL of IS (spiked solution: 11.1 µg/mL in acetonitrile) was added and vortex-mixed for 10 sec and followed with 100 µL of acetonitrile for deproteinization. One hundred-microliter of sodium carbonate buffer (100 mM, pH 11.2) was added and vortex-mixed for 30 sec, followed by addition of 700 µL of t-BME for LLE. Vortex-mixed for 60 sec, then the mixture was centrifuged (2000g) for 10 min at 4°C. The supernatant was transferred to another vial and evaporated to be dryness under a gentle stream of N₂ gas.
The residue was reconstituted with 50 µL of acetonitrile and then 20 µL of reconstituted volume were injected onto the column for analysis. Triplicate measurements for each sample were performed, as the amount was indicated as means ± SD.

2.2.5. Method Validation

The validation parameters, such as method selectivity, matrix effect (ME) and reproducibility, calibration curves linearity, lower LODs and the lower LOQs, accuracy, precision and the recovery after extraction were estimated according to the food drug administration (FDA) guideline for validation of the bioanalytical methods [154]. Blank rat plasma samples obtained from ten different rats were tested for any visible interference at the retention time of the analytes. The relative ME on the spectral response of PRO and REM was assessed as described by Abay et al. [179]. In order to evaluate the absolute ME, drug free blank rat plasma (ten samples) was extracted with t-BME, dried and reconstituted using acetonitrile solutions of low (0.5 µg/mL or 1 ng/mL) and high (10 µg/mL or 50 ng/mL) concentrations levels for PRO and REM, respectively and at one concentration level of the IS (1 µg/mL), A. The standard samples which prepared in the reconstituted solution (acetonitrile) at the same concentrations without matrix components (pure standards) were used, B.

The ME was calculated as the IS normalized matrix factor (IS-MF), which is the peak area ratio in the presence of matrix for each plasma sample (A) divided by the mean of the peak area ratio in the absence of matrix (B). The calibration curves were assessed by seven concentration points (0.27, 0.5, 1, 2, 4, 6, and 10 µg/mL for PRO and 0.17, 1, 2.5, 5, 10, 30 and 50 ng/mL for REM) using same spiked volume 20 µL of IS. The LODs and lower LOQs were defined as the concentration giving peak area at S/N of 3 and 5, respectively.

The accuracy and precision of the proposed method were assessed by determining the QC plasma samples spiked at three concentration levels, 0.5, 2 and 10 µg/mL of PRO and 1, 10 and 50 ng/mL of REM (n=5).
The accuracy was expressed as a percentage of the calculated concentration on the nominal (theoretical) concentration. The intra- and inter-day precision was determined as the RSD%. The recoveries of analytes after extraction from plasma samples were determined by comparing the peak areas of extracted analytes (spiked at two concentration levels, 0.5 and 10 µg/mL for PRO and 1 and 50 ng/mL for REM, and at one concentration level, 1µg/mL for IS (n=5)) with that of the non-extracted pure standards (at the same concentration levels, n = 5).

2.2.6. **Administration Design and Sampling Time Schedule**

The administration study was performed using male Wistar rats (as described in Chapter 1). After the rats were anesthetized with ethyl carbamate (1.5 g/kg, i.p.); each animal had two indwelling cannulae: a femoral vein cannula for drug administration and a femoral artery cannula for blood sampling. PRO (dose; 125 µg/kg/min) and REM (dose; 125 ng/kg/min) were administrated by the infusion route for 60 min using a microinjection pump (CMA/100; Carnegie Medicine, Stockholm, Sweden). Then the blood samples (= 0.350 mL) were collected at time intervals of 10, 15, 25, 30-40, 45, 50, 55, 60, and 65 min as a 5 min after administration stopping as shown in Figure 10. Meanwhile a volume of 0.350 mL of saline was given to the rat after each sampling for keeping body balance.

![Figure 10. Sampling schedule for rat plasma administrated with PRO and/or REM.](image-url)
Each collected blood sample was added to tubes eppendorf contained EDTA.2Na powder as an anticoagulant for plasma preparation. To avoid REM degradation, a 30 µL of 0.2 M citric acid were added immediately for all the collected blood samples (0.350 mL). The plasma samples were prepared rapidly by centrifugation (2000g) for 10 min at 4°C then stored at -30°C until analysis. For method application into the rats, two experiments were performed. The first is sole administration of PRO or REM for 60 min of infusion period. The second, the infusion period (60 min) was divided into two parts, the first part (0-30 min), PRO or REM was administrated independently (sole), and then followed by their co-administration in the next part (30-60 min). The concentrations of PRO and REM in rat plasma were calculated from the corresponding calibration curves equation. The plasma concentration-time profile of PRO (plasma concentrations, µg/mL) and REM (plasma concentrations, ng/mL) were constructed for each individual subject after sole and after co-administration. A mathematical trapezoid method was used for area under the curve (AUC) calculations. In the experiments of sole administration and sole/co-administration, the AUCs were calculated in two separated periods, from 15 to 30 min and from 45 to 60 min. The AUC in the part of sole administration (from 15 to 30 min) and AUC in the part of co-administration (from 45 to 60 min) were compared, and also in the part of sole administration from 15-30 against the part 45-60 min. The significance difference between the AUC through the two periods were confirmed using paired Student’s t-test at the statistical significance set at $P < 0.05$.  

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2.3. RESULTS AND DISCUSSION

2.3.1. Optimization of the Assay Conditions

The monolithic column in the present study was effectively used for the present LC-MS/MS method with a short time of separation, a high mechanical stability, long operative lifetimes and much lower back pressure.

For selective analysis in the present method, the composition of the mobile phase was examined. The combination of acetonitrile and 10 mM ammonium acetate buffer was useful for separation PRO, REM and the IS. Additionally, the effect of ammonium acetate buffer pH and acetonitrile content in the mobile phase on the peak area of the analytes were examined and optimized. The maximum and constant peak area of PRO was given by more than 3.5 of pH, whereas the maximum peak area of REM at pH 3.5 was observed as in Figure 11. Therefore pH 3.5 was selected in the following experiment. Next, acetonitrile content ranging from 55 to 90% in mobile phase was examined. Increasing of the peak area of both analytes with increasing of acetonitrile content in mobile phase was observed, Figure 12. A 90% of acetonitrile content was selected in the following experiment.

Preliminary studies for selection of ISs were performed. CLO, FLU, 2,6-di-tert-butyl-4-methylphenol and finally CBZ, were examined. Among them, CBZ was eluted with similar retention time of the analytes and its extraction recovery from plasma was comparable to PRO and REM.

The present method used easily negative- and positive-ionization changeable LC-MS/MS, as PRO could be determined by ESI⁻ mode, whereas REM and IS were determined by ESI⁺ mode. In a full scan spectra, the predominant molecular ions [M-H]⁻ or [M+H]⁺ with an m/z 176.94, 377.04 and 237.07 for PRO, REM and IS, respectively were formed. The most abundant ions (of the product-ions) mass spectrums were formed at m/z 160.87, 317.04, and 194.02 for PRO, REM, and IS, respectively.
However the sensitive transition of \( m/z \ 237.07 \rightarrow 194.02 \) for IS, was less selective, with regard to the endogenous plasma matrix. Therefore, the SRM transitions of \( m/z \ 176.94 \rightarrow 160.87 \) for PRO, \( m/z \ 377.04 \rightarrow 317.04 \) for REM and \( m/z \ 237.07 \rightarrow 220.01 \) for IS were selected during the study.

The corresponding full-scan ESI-MS/MS and ESI+-MS/MS spectra for the target analytes are shown in Figure 13. The capillary and cone voltages and collision energies were studied and optimized to obtain the greatest intense of the most abundant product ions for further MS/MS experiments, as in Figure 14A-C and as in Table 4.

It was found that the source temperature and the desolvation temperature did not significantly influence the MS behaviour of these analytes and remained unchanged at the recommended value of 120°C and 350°C, respectively.

Other MS conditions, including Desolvation/Cone gas flow, RF lens voltage, and Extractor voltage, were maintained at the auto-tuned (default) values, since they did not significantly affect the collision behaviour of the analytes. The analyzer parameters were kept in recommended SIM mode, and the Entrance/Exit were modulated to -1 and +1, respectively.

At the first the ion suppression for the analytes, due to the plasma matrix, was prohibited by adding a volume acetonitrile for deproteinization. Therefore the certain acetonitrile volumes (ranging from 40 to 100 µL) were examined; the maximum peak area with 100 µL acetonitrile could be cleared from ME. Next, in order to select the proper organic extractor for LLE procedure; different organic solvents such as hexane, ethylacetate, and \( t\)-BME were examined. As the results \( t\)-BME gave the acceptable recoveries of all the analytes; 86.0±7.0% and 95.0±6.0% for PRO (0.5 and 10 µg/mL); 79.0±12.0% and 86.0±9.0% for REM (1 and 50 ng/mL) and 80.0±5.0% for IS (1 µg/mL).
Figure 11. Effect of pH of 10 mM ammonium acetate buffer in the mobile phase on the peak area of PRO and REM. Sample: standard, 0.5 µg/mL for PRO and 5 ng/mL for REM.

Figure 12. Effect of acetonitrile content in the mobile phase on the peak area of REM and REM. Sample: standard, 0.5 µg/mL for PRO and 5 ng/mL for REM.
Figure 13. Mass spectral of [M-H]⁻ ion of PRO using ESI⁻ and [M+H]⁺ of REM and IS using ESI⁺.
Figure 14. Optimized conditions for capillary (A), cone voltages (B), and collision energy (C). Sample: standard, 1 µg/mL for PRO and 5 ng/mL for REM and 1 µg/mL for IS.
2.3.2. Method Validation

2.3.2.1. Selectivity

The selectivity of the proposed method for any interference in the retention time of the analytes was estimated by analyzing ten of different blank rat plasma. Figure 15 is the representative chromatograms of drug free rat plasma and that spiked with 500 ng/mL of PRO, 5 ng/mL of REM and 1 µg/mL of IS. Moreover, the typical chromatograms of plasma after 10 min of PRO and REM co-administration are illustrated in Figure 16. The retention time of IS, PRO and REM was 3.19, 3.63 and 7.01 min, respectively.

![Representative SRM chromatograms of drug free rat plasma (A) and that spiked with PRO, REM and IS (B). Sample: spiked, 500 ng/mL for PRO, 5 ng/mL for REM and 1 µg/mL for IS (20 µL of stock solution, 11.1 µg/mL in acetonitrile).](image-url)
Figure 16. Representative SRM chromatograms of plasma sample before administration of drugs (A) and after 10 min from PRO and REM co-administration (B).

2.3.2.2. Matrix Effect, ME

The LC-MS/MS is the instrument of choice used for quantitative bioanalysis due to its inherent sensitivity and selectivity. However, it is not free from drawbacks such as the ME phenomenon, that the co-eluting compounds may suppress or enhance the ionization of the analytes of the interest. The ME considerably affects the limit of LOD, LOQ, linearity, accuracy and precision of the developed LC-MS/MS methods. Numerous factors as endogenous phospholipids, dosing media, formulation agents and mobile phase modifiers and/or the biological samples pre-treatment and clean up procedure can causes the ME. Therefore, an assessment of the ME and a minimization of ME are critical for the reliable evaluation of newly developed LC-MS/MS methods. By the way, the current FDA guidance documents requires the study of ME as a part of the developed LC-MS/MS validation.
MF of one signifies no ME, a value of less than one suggests the suppression of ionization and a value that is greater than one signifies ionization enhancement \(^{179, 187}\). An absolute IS-MF of one is not required for a reliable analytical assay. However, the variability (RSD\%) in MFs should be less than or equal to 15\% to ensure the reproducibility of the LC-MS/MS analysis \(^{179}\).

The result of IS-MF at low concentrations of PRO and REM in plasma shows that, a slightly ME is present as indicated by values of >100\% (peak area ratio was 1.08±0.12 for PRO (0.5 µg/mL) and 1.14±0.17 for REM (1 ng/mL)) in the spiked plasma samples post-extraction. Also the ionization enhancement for PRO and REM at their high concentrations was similar and kept consistent (peak area ratio was 1.1±0.1 for PRO (10 µg/mL) and 1.2±0.1 for REM (50 ng/mL)). Moreover, the variability (RSD\%) in the present method was 11.3\% and 8.2\% for PRO (at 0.5 µg/mL and 10 µg/mL) and 15.0\% and 11.5\% for REM (at 1 ng/mL and 50 ng/mL) respectively, which indicates that the samples analysis were reproducible. Therefore, and despite a slight IS-ME was observed, the present method was considered reliable and reproducible for PRO and REM determination.

2.3.2.3. Linearity, LODs and lower LOQs

Calibration curves were constructed in rat plasma in the concentration ranges of 0.27-10 µg/mL for PRO and 0.17-50 ng/mL for REM. The analyte on IS peak area ratios were found to have a good linearity with the selected concentration range for all the analytes \((r \geq 0.976)\). The calibration curves' equations for PRO and REM respectively were; \(y = 0.02x + 0.01\) and \(y = 0.76x + 0.38\). The LODs for PRO and REM were 0.16 µg/mL and 0.10 ng/mL, whereas the lower LOQs were 0.27 µg/mL for PRO and 0.17 ng/mL for REM.

The difference in the sensitivity of the analytes was observed due to using different ion detection mode and the selected conditions were directed toward the most potent analyte (REM) which used in low plasma concentrations during the clinical practice \(^{58, 188}\).
However, the obtained sensitivity for PRO was sufficient for its determination in the plasma samples during this study; as the difference in clinically used concentrations between PRO and REM is 1:1000 times.

In a comparison with the other reported methods (used LC-MS/MS) for analysis of REM individually, the sensitivity of the present method was comparable with the reported on \(^{116}\). Moreover, the proposed method was more rapid than some of reported methods that are for determination of PRO \(^{111}\) or REM \(^{111, 122}\) in plasma.

2.3.2.4. Accuracy and Precision

The accuracy of the proposed method was estimated by comparing the measured concentrations of PRO and REM spiked in plasma with the theoretical (calculated) concentration values of the standard. The calculated accuracy (as a relative error (RE) \(\pm\) SD) for PRO or REM was found between \(-19.4\pm5.6\%\) or \(-0.0\pm5.4\%\) indicating an acceptable accuracy for the proposed method. The intra- and inter-day precisions were expressed in terms of RSD\% between the peak area values. The results showed that the intra- and inter-day variation at three different concentrations levels of PRO and REM were within the acceptable validated range as the RSD\% was found less than 15\% for intra- and inter-day precision as shown in Table 5.

### Table 5. Accuracy and precision of the proposed LC-MS/MS method.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked Conc., ng/mL</th>
<th>Accuracy % (RE(^1\pm)SD, n=5)</th>
<th>Precision, RSD% (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intra-day</td>
</tr>
<tr>
<td>PRO</td>
<td>500</td>
<td>-19.4(\pm)5.6</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>-0.0(\pm)5.4</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>-7.0(\pm)2.8</td>
<td>3.2</td>
</tr>
<tr>
<td>REM</td>
<td>10</td>
<td>-0.3(\pm)5.1</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-1.9(\pm)8.4</td>
<td>3.7</td>
</tr>
</tbody>
</table>

\(^1\)RE = Relative error (overall mean assayed concentration - added concentration)/added concentration \(\times\) 100.
2.3.2.5. Administration study of PRO and REM

The proposed method was applied to estimate drug-drug interaction after co-administration of PRO and REM to rats. The dose of PRO and REM for both sole and co-administration were 125 µg/kg/min and 125 ng/kg/min, respectively. Accordingly, these doses were included in the therapeutic range for human usage to PRO and REM co-administration \(^{188, 189}\). The plasma concentration-time profiles of both analytes for sole and sole/co-administration were constructed as shown in Figure 17 and Figure 18.

After 10 min of administration the concentration of both drugs could be confirmed to be stable. The \(AUC_{15-30\text{min}}\) and \(AUC_{45-60\text{min}}\) (sole administrations) were 7.19±0.58, 7.95±1.08 µg/mL.min for PRO; 4.42±0.11, 4.91±0.19 ng/mL.min for REM, Figure 17. The \(AUC_{15-30\text{min}}\) and \(AUC_{45-60\text{min}}\) (sole and next co-administration) were 8.40±1.22, 8.70±1.42 µg/mL.min for PRO; 5.49±0.99, 8.76±0.94 ng/mL.min for REM, Figure 18.

![Figure 17. Plasma concentration-time profiles after sole administration of PRO and REM.](image-url)
The plasma concentrations of PRO and REM seem to be constant after sole administration experiments (from 0-60 min), also PRO concentration was stable in sole as in co-administration experiments ($P = 0.44$). While a significant increasing in REM concentration after co-administration (from 30-60 min) was observed ($P = 0.01$).

As the studies before for drug-drug interactions between PRO and REM or REM-analogue (fentanyl or alfentanil) $^{58,190}$ showed that the PRO-opioid interactions. Even though ADME of REM is not the same, but the same PK alterations were obtained in the presence of co-administrated PRO. As in the two mentioned studies, the suggested mechanisms for this alteration was the hemodynamic changes which caused by PRO, we also supposed that the same cause is exist in our present study between PRO and REM. Therefore, the further studies to interpret the particular mechanism of this action are necessary.
2.4. CONCLUSION

The first and rapid LC-MS/MS method developed and validated for simultaneous determination of PRO and REM in rat plasma. The results of the validation study showed the developed method was selective, accurate, and precise to meet the requirements for determination of PRO and REM concomitantly administered. The proposed method could be successfully applied to study the PK interaction of PRO and REM in rat plasma. Moreover, the method is considered from the rare methods applied LC-MS/MS method determination for acidic (PRO) and basic (REM) analytes using an IS in rats plasma. Therefore we are convinced that our contribution is valuable as a scientific work.
CHAPTER 3
DEVELOPMENT AND VALIDATION OF LIQUID CHROMATOGRAPHY-TENDEM MASS SPECTROMETRY FOR STUDY OF THE INFLUENCE OF CO-ADMINISTRATED ANTIEPILEPTIC DRUGS ON PLASMA CONCENTRATION OF PROPOFOL DURING ANESTHESIA TO RATS
3.1. BACKGROUND

Patients with epilepsy often require anesthesia for elective and emergency surgery. Therefore, an appropriate perioperative management of AEDs is vital in maintaining seizure control in these patients. PRO and CBZ are known individually, to inhibit or induce a variety of hepatic cytochrome enzymes. Up to date, there is no data described whether and what degree CBZ affects the plasma concentration of PRO. Therefore, in the present study a rapid, sensitive, and selective LC-MS/MS method was developed and validated for simultaneous determination of PRO and CBZ. MDZ was used as an IS and as well as a valuable probe to predict the unwanted therapeutic outcomes such as lack of therapeutic efficacy or potentially harmful response during the study development. The proposed method had the opportunity to assess the PK interactions analysis between PRO and MDZ or PRO and CBZ in rat plasma.

3.2. MATERIAL AND METHODS

3.2.1. Reagents and Standards

MDZ was obtained from Wako Pure Chemicals, Ltd. (Osaka, Japan) and all other reagents were of analytical reagent grade; as in Chapter 1 and Chapter 2.

3.2.2. Standard Solutions and QC Samples

The stock solutions of PRO, MDZ, and CBZ at the concentration of 1mg/mL were prepared by dissolved an appropriate amount of each drug in acetonitrile and stored at 4°C. The working solutions were prepared by dilution of the stock solutions serially in acetonitrile to obtain the spiked plasma concentrations over the range of 0.05-100 ng/mL for PRO, 2-100 ng/mL for MDZ and 7-1000 ng/mL for CBZ.
The analytical standard and QC samples were prepared by spiking blank rat plasma with standard working solution (20 µL) during validation and each experiment run in the PK study. The QC samples were prepared at concentrations 1, 50 and 100 ng/mL for PRO; 2, 50 and 100 ng/mL for MDZ; and 7, 100 and 1000 ng/mL for CBZ. For administration study; the solutions of PRO (5 mg/mL), MDZ (2 mg/mL) and CBZ (5 mg/mL) were prepared in Intralipid® 10% emulsion.

3.2.3. LC-MS/MS System and Conditions

A Waters 2695 separation module (as described in Chapter 2) was used for solvent and sample delivery. The chromatographic separations were achieved by using a Chromolith Performance RP-18e at 30°C of column temperature. The mobile phase was a mixture of acetonitrile-ammonium acetate buffer (10 mM, pH 4) (=90:10%, v/v), pumped at a flow rate of 0.5 mL/min. An ESI− and ESI+ easily changeable Quattro micro™ triple quadrupole mass spectrometer (as described in Chapter 2) was used for mass analysis and detection. Mass spectrometric analysis was performed in a negative-ion mode for PRO and a positive-ion mode for MDZ and CBZ. Nitrogen gas was used as desolvation (500 L/h) and nebulizer (50 L/h) gas. The source and desolvation temperatures were kept at 120°C and 350°C, respectively. The selected ESI-MS/MS parameters are listed in Table 6. The LC-MS/MS system was controlled by MassLynx™ V 4.0 software (Waters Co.).
Table 6. Selected ion transitions (m/z values) and mass spectrometric parameters for analysis of PRO, MDZ, and CBZ in SRM mode.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PRO</th>
<th>MDZ</th>
<th>CBZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor ion, m/z</td>
<td>176.94 [M-H]⁻</td>
<td>326.74 [M+H]⁺</td>
<td>237.07 [M+H]⁺</td>
</tr>
<tr>
<td>Product ion, m/z</td>
<td>176.94</td>
<td>291.90</td>
<td>194.02</td>
</tr>
<tr>
<td>Ionization</td>
<td>ESI⁻</td>
<td>ESI⁺</td>
<td>ESI⁺</td>
</tr>
<tr>
<td>Detection mode</td>
<td>SRM</td>
<td>SRM</td>
<td>SRM</td>
</tr>
<tr>
<td>ESI Capillary voltage, kV</td>
<td>-3.0</td>
<td>+3.0</td>
<td>+3.0</td>
</tr>
<tr>
<td>Cone voltage, V</td>
<td>-35</td>
<td>+40</td>
<td>+25</td>
</tr>
<tr>
<td>Collision energy, eV</td>
<td>8</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Ion energy 1, V</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ion energy 2, V</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Source temperature °C</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Desolvation temperature °C</td>
<td>350</td>
<td>350</td>
<td>350</td>
</tr>
</tbody>
</table>

3.2.4. Plasma Pre-treatment

An aliquot of 200 µL of rat plasma spiked with 20 µL of MDZ or CBZ (spiked solutions concentration, 50 ng/mL of MDZ or CBZ as the ISs) was loaded to an Agilent® Bond Elut-C18 cartridge (100 mg, 1 mL), pre-conditioned with methanol then water (1 mL). The washing step was 500 µL of 10% methanol (twice) and the elution was performed with 400 µL of methanol then 20 µL of the eluent solution was inject directly to the LC-MS/MS for analysis.

3.2.5. Method Validation

The validation parameters, such as selectivity, ME and reproducibility, calibration curves linearity, LODs, LOQs, accuracy, precision, extraction recoveries and stability study were estimated according to the FDA guideline for validation of the bioanalytical methods\textsuperscript{154}. 
Blank rat plasma samples obtained from different rats were tested for any visible interference at the retention time of the analytes. In order to evaluate the absolute ME on the ionization of the analytes, blank rat plasma samples obtained from ten different rats were extracted and spiked at QC samples (low, middle, and high concentration levels of the analytes). These samples were injected together with samples containing no matrix components (pure standard in the elution solvent, 90% methanol) then the ME was calculated as IS-MF.

The two calibration curves for PRO (using 20 µL spiked volumes of ISs; CBZ or MDZ, 50 ng/mL for each) were assessed by five concentration points (1, 5, 10, 50 and 100 ng/mL); the calibration curve for MDZ (20 µL spiked volume of CBZ (IS, 50 ng/mL)) was assessed by five points (2, 8, 25, 50 and 100 ng/mL) and finally the calibration curve for CBZ (20 µL spiked volume of MDZ (IS, 50 ng/mL)) was assessed by five points (7, 25, 100, 500 and 1000 ng/mL). The LODs and LOQ were defined as the analyte's concentration that giving peak area at S/N of 3 and 10, respectively. Peak area ratios of the analytes to IS were plotted against analytes concentration. The standard curves were assessed by equations for linear regression with a weighting factor of reciprocal of concentration (1/x) in the concentration ranges as mentioned above. The analytes peaks should be identifiable, discrete, and reproducible with precision of 20% and accuracy of 80-120%. The accuracy and precision of the proposed method were assessed by determining the QC plasma samples spiked at three concentration levels (five samples for each concentration) on five different validation days. The accuracy was expressed as a percentage of the calculated concentration divided on the nominal (theoretical) concentration. The intra- and inter-day precisions were determined as the RSD%. The criteria used to assess the suitability of accuracy and precision was as follows: the accuracy was within 15% of the nominal value except at the LOQs, where it should not deviate by more than 20% and for the precision, the RSDs% did not exceed 15% except for the LOQs, 20%.
The extraction recoveries of the analytes were determined by comparing the peak areas of extracted analytes spiked at three concentration levels, for all the analytes with that of the non-extracted pure standards (at the same concentration levels (n = 5)).

The stability of analytes was assessed by determine QC samples at low, middle and high concentration levels with five measurements for each concentration, exposed to different time and temperature conditions. The stability study involved the stability after three freeze-thaw cycles; stability at room temperature (25°C) for 4 h and finally, the stability of the extracted sample for 12 h. The concentrations of the analytes for each time point in the experiments were compared to a triplicate set of freshly prepared samples in the same concentrations. In all stability experiments, analytes were assumed stable when the deviation of the mean concentrations of the test samples from the controls was within ± 20%.

3.2.6. Administration Design and Sampling Time Schedule

The administration study was performed using male Wistar rats (as described in Chapter 1 and Chapter 2). PRO in Intralipid® emulsion (0.125 µg/kg/min) was administrated to the rats by i.v. infusion route for 60 min; MDZ and CBZ in Intralipid® emulsion (2 and 5 mg/kg, i.p.) were administrated to the rats with the injection volume given as 1mL/kg of rat body weight. Following dosing administration of PRO, MDZ, and CBZ, a volume of ≈ 0.350 mL blood sample was withdrawn from femoral artery of the rat at 5, 10, 15, 20, 25, 35, 45, 55, 65, 90, 120, 180, and 240 min as shown in Figure 19. Meanwhile a volume of 0.350 mL of saline was given to the rat after each sampling for keeping body balance.

Each of the collected blood samples was added to eppendorf tubes contained EDTA.2Na powder for plasma preparation. The plasma samples were prepared directly by centrifugation (2000g) for 10 min at 4°C then stored at -30°C until analysis.
Cannula insertion in femoral
vein for i.v. infusion

MDZ or CBZ, i.p.

Stabilization
for 60 min

Cannula insertion in femoral
artery for blood samples

PRO infusion

Time after administration, min

Figure 19. Sampling schedule for rat plasma administrated with PRO (and/or) MDZ, CBZ.

3.2.7. PK Calculations

The concentrations of PRO, MDZ and CBZ in rat plasma were calculated from the corresponding calibration curves equation. The plasma concentration-time profiles of PRO, MDZ and CBZ (plasma concentrations, ng/mL) were constructed for each individual subject after sole and after co-administration. In case of PRO, a mathematical trapezoid method was used for AUC calculations. In the experiments of sole administration and co-administration, the AUCs were calculated from 10 to 240 min. Then the AUCs for sole administration and for co-administration were compared.

For MDZ and CBZ, the peak plasma concentration (C_{max}) and the time to reach the peak plasma concentration (T_{max}) were calculated form the actual plasma level data. The rate constant for plasma drug elimination (i.e. K_{el}) was calculated by the regression analysis of the monoexponential decline line of the log of plasma drug concentration versus time curve. The elimination half-life (T_{1/2}) was calculated using formula T_{1/2} = 0.693/K_{el}. The Cl was calculated as the dose/AUC for concentration versus time (AUC_{0-240}). Other parameters such as AUC_{0-240}, and the mean residence time (MRT_{0-240}) were calculated by implicating moment analysis model^{191}. All the data are presented as the mean ± standard deviation (SD, n=3). Statistical analysis was assessed by un-paired Student’s t-test with p < 0.05 being considered significant.
3.3. RESULTS AND DISCUSSION

3.3.1. Optimization of the Assay Conditions

In this study, modifications were performed to improve the previous method (Chapter 2) selectivity and sensitivity for PRO and CBZ. Moreover the modified method was extended to separate MDZ simultaneously with PRO and CBZ.

The effect of ammonium acetate buffer pH in mobile phase on the peak area of the analytes was examined. The maximum and constant peak area of PRO was given by more than 4 of pH, whereas the maximum peak area of CBZ and MDZ at pH 4 was observed as in Figure 20. Therefore pH 4 was selected in the following experiment.

![Figure 20. Effect of pH of 10 mM ammonium acetate buffer in the mobile phase on the peak area of PRO, MDZ and CBZ. Sample: standard, 2 ng/mL for PRO, and 10 ng/mL for MDZ and CBZ.](image)

A 90% content of acetonitrile content in mobile phase gave the maximum peak area for all the analytes (as the previous study in Chapter 2). In the present study MDZ was used as an IS for co-administration study between PRO and CBZ; whereas CBZ was used as an IS for co-administration study between PRO and MDZ. The method used an easily changeable negative- and positive-ionization LC-MS/MS spectrometry, as PRO could be determined by ESI- mode, whereas MDZ and CBZ were determined by ESI+ mode.
In a full scan spectra, the predominant molecular ions [M-H]⁻ or [M+H]⁺ with an m/z 176.94, 237.07 and 326.74 for PRO, CBZ and MDZ were formed. The most abundant ion of the product-ions mass spectra were formed at m/z 176.94, 194.02, and 291.90 for PRO, CBZ, and MDZ. In the present study the false m/z transition 176.94→176.94 for PRO determination in SRM was used because of the small intensity ratio of product/parent ions at the true transition 176.94→160.87. The complete separation of all analytes within 5 min was achieved without interfering peaks from plasma samples at the analytes retention time. The corresponding full-scan ESI⁻-MS/MS and ESI⁺-MS/MS spectra for PRO, MDZ and CBZ are shown in Figure 13 (Chapter 2) and in Figure 21 in this Chapter.

Figure 21. Mass spectral of [M+H]⁺ of MDZ using ESI⁺.
The capillary and cone voltages and collision energies were studied and optimized to obtain the greatest intense of the most abundant product ions for further MS/MS experiments, as in Figure 22A-C and Table 6. It was found that the source temperature and the desolvation temperature did not significantly influence the MS behaviour of these analytes and remained unchanged at the recommended value of 120°C and 350°C, respectively.

Other MS conditions, including Desolvation/Cone gas flow, RF lens voltage, and Extractor voltage were maintained at the auto-tuned (default) values, since they did not significantly affect the collision behaviour of the analytes. The analyzer parameters were kept in recommended SIM mode, and the Entrance/Exit were modulated to -1 and +1, respectively.
Figure 22. Optimized conditions for capillary (A), cone voltages (B), and collision energy (C). Sample: standard, 2 ng/mL for PRO, and 10 ng/mL for MDZ, and CBZ.
SPE is designed for rapid, selective sample preparation prior to chromatographic analysis. It is a form of digital (step-wise) chromatography designed to extract, partition, and/or adsorb one or more analytes from a liquid phase (the biological sample) onto the stationary phase (sorbent or resin). Therefore, the cartridge is designed for a single use in order to eliminate the possible carry-over problems.

In the present study, a simple, rapid, and selective clean-up procedure based on SPE for plasma extraction process was carried out. The washing and elution solvents (methanol/water mixture) were studied and optimized (Figure 23A and B) for the effective eliminate of plasma matrix components, without any further pre-treatment.

From the literatures, PRO is highly (98%) bounds with albumin and red blood cells after i.v. injection. MDZ and CBZ also are highly (96% and 70%) bound with albumin and α-acid glycoprotein. The remainder of PRO, MDZ or CBZ exists in blood as a free type. Protein-bound drugs are not detected by the present method only we detected the free type.

The recoveries of PRO, MDZ, and CBZ from rat plasma following SPE extraction were ranged from 82.9±4.8 to 107.0±7.1 and were similar at all analyte concentrations without significant concentration dependence. The reproducibility and recoveries of SPE were determined from five repetitions at three concentration levels for all the analytes, the reproducibility expressed as a RSD% as shown in Table 7.
Figure 23. Effect of methanol/water ratio (% v/v) in washing (A), or elution (B), steps on the analyte recoveries using Bond Elut-C18 cartridge. Sample: spiked concentration, 2 ng/mL for PRO and 10 ng/mL for MDZ and CBZ.

Table 7. The recoveries for PRO, MDZ and CBZ after extraction from rat plasma by SPE (n=5).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal conc., ng/mL</th>
<th>Recovery (\text{Mean} \pm \text{SD} )%</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO (CBZ IS)</td>
<td>1</td>
<td>86.7±9.9</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>107.0±7.1</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>97.8±1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>PRO (MDZ IS)</td>
<td>1</td>
<td>88.3±10.6</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>92.8±4.0</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>96.8±1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>MDZ</td>
<td>2</td>
<td>85.0±9.7</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>89.2±7.2</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>91.2±6.7</td>
<td>7.3</td>
</tr>
<tr>
<td>CBZ</td>
<td>7</td>
<td>84.4±11.0</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>86.4±7.4</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>82.9±4.8</td>
<td>5.8</td>
</tr>
</tbody>
</table>

*The recovery: was determined by comparing the area of the extracted analytes with that of the pure standards.
3.3.2. Method Validation

3.3.2.1. Selectivity

The LC-MS/MS method demonstrated high specificity because only ions derived from the analytes of interest were monitored. The selectivity of the proposed method towards endogenous plasma samples was estimated by analyzing ten of different blank and spiked rat plasma at LOQ levels. Endogenous peak at the retention time of the analytes were not observed for any plasma samples evaluated.

Representative chromatograms of blank rat plasma and that spiked with PRO (at 1 ng/mL); MDZ and CBZ at LOQ (2 and 7 ng/mL, respectively) are shown in Figure 23A and B. Moreover, the typical chromatograms of plasma after 10 min of PRO and MDZ (using CBZ, 50 ng/mL as an IS); or PRO and CBZ co-administration (using MDZ, 50 ng/mL as an IS) are illustrated in Figure 24A and B.

The retention time of CBZ, PRO, and MDZ was 3.26, 3.79, and 3.95 min, respectively. Therefore the method had a significantly shorter total running time (~5 min) as the first method for simultaneous determination of PRO, MZD, and CBZ in rat plasma.
Figure 24. Representative SRM chromatograms of drug free rat plasma (A) and that spiked with PRO, MDZ, and CBZ (B). Sample: spiked, 1 ng/mL for PRO, 2 ng/mL for MDZ, and 7 ng/mL for CBZ.
Figure 25. Representative SRM chromatograms of 10 min after co-administration of PRO and MDZ (A); PRO and CBZ (B).
3.3.2.2. ME

The IS-MF as calculated for the present work showed no significant ion suppression or enhancement at low, middle, and high concentrations of PRO, MDZ, and CBZ. The RSD% was ranged from 1.9 to 11.9% for all the analytes (Table 8), which indicates that the samples analysis were reproducible.

Table 8. Study of the absolute ME for the analytes at three concentration levels.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal conc., ng/mL</th>
<th>IS-MF</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO</td>
<td>1</td>
<td>1.0±11.9</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.1±6.3</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.1±1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>MDZ</td>
<td>2</td>
<td>0.9±8.7</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.9±5.3</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.0±4.9</td>
<td>5.1</td>
</tr>
<tr>
<td>CBZ</td>
<td>7</td>
<td>1.0±4.8</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.0±4.7</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1.1±7.3</td>
<td>6.9</td>
</tr>
</tbody>
</table>

3.3.2.3. Linearity, LODs and LOQs

Typical calibration curves for PRO, MDZ, and CBZ respectively were constructed in rat plasma in concentration ranges showed in Table 9. The analyte on IS peak area ratios were found to have a good linearity with the selected concentration ranges for all the analytes ($r \geq 0.991$). The slopes, intercepts, and $r$ values showed in Table 9. The lowest concentrations on the calibration curves for MDZ and CBZ were 2 and 7 ng/mL respectively. The analytes’s responses at these concentration levels were > 10 times of the noise baselines of the drug free rat plasma.
The accuracy and precision at these concentration levels were acceptable, with the RSD% < 20% and the RE% < 15%. Thus, the lowest concentrations on the calibration curves were acceptable as LOQ for MDZ and CBZ. The LODs were calculated to be 0.6 and 2.1 ng/mL respectively for MDZ and CBZ. For PRO the LOD was 0.02 ng/mL and LOQ was 0.05 ng/mL.

However, the obtained sensitivity for PRO, MDZ, and CBZ were sufficient for their determination in the rat plasma samples during this study with the doses that related to the clinical uses of human.

Table 9. Linearity range, slope, intercept, and the correlation coefficient (r) for the calibration curves of PRO, MDZ, and CBZ.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Conc. range, ng/mL</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO (CBZ IS, 50 ng/mL)</td>
<td>1-100</td>
<td>2.90</td>
<td>1.10</td>
<td>0.998</td>
</tr>
<tr>
<td>PRO (MDZ IS, 50 ng/mL)</td>
<td>1-100</td>
<td>1.20</td>
<td>2.20</td>
<td>0.991</td>
</tr>
<tr>
<td>MDZ (CBZ IS)</td>
<td>2-100</td>
<td>0.05</td>
<td>0.04</td>
<td>0.991</td>
</tr>
<tr>
<td>CBZ (MDZ IS)</td>
<td>7-1000</td>
<td>0.02</td>
<td>-0.06</td>
<td>0.994</td>
</tr>
</tbody>
</table>

3.3.2.4. Accuracy and Precision

The accuracy, intra- and inter-day precision data for PRO, MDZ, and CBZ are summarized in Table 10. All the values of accuracy and precision were within the recommended limits\textsuperscript{154).} The intra-day precisions ranged between 1.7% and 14.3%, and the inter-day precision was between 5.1 and 18.3; the mean errors were between -1.4±4.4 and 9.8±11.0.
Table 10. Accuracy and intra- and inter-day precision data for assays analytes in plasma.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked Conc., ng/mL</th>
<th>Accuracy % (RE±SD, n=5)</th>
<th>Precision, RSD% (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intra-day</td>
</tr>
<tr>
<td>PRO (CBZ IS)</td>
<td>5</td>
<td>8.0±6.8</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.0±9.0</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>-2.4±1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>PRO (MDZ IS)</td>
<td>5</td>
<td>7.0±7.5</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-1.6±10.1</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>-1.4±4.4</td>
<td>4.5</td>
</tr>
<tr>
<td>MDZ (CBZ IS)</td>
<td>2</td>
<td>9.8±11.0</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.6±12.0</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>2.8±5.0</td>
<td>4.9</td>
</tr>
<tr>
<td>CBZ (MDZ IS)</td>
<td>7</td>
<td>-15±8.7</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-8.6±13.0</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>5.0±9.5</td>
<td>9.1</td>
</tr>
</tbody>
</table>

3.3.2.5. Stability Study

The stability of PRO, MDZ, and CBZ in rat plasma under different storage conditions is presented in Table 11. There was no significant degradation under these conditions described in this study, since their concentrations deviated by no more than 19.7% relative to the reference nominal concentrations. No degradation products were detected under the selected MS conditions. All the analytes can be safely analyzed after stored at room temperature for 4 h, at -30°C and after three freeze-thaw cycles. Also analysis of the QC samples following SPE extraction procedure showed no significant degradation after 12 h at room temperature. These results indicated the analytes were stable under routine laboratory conditions and no specific procedures (e.g., acidification or addition organic solvents) were needed to stabilize the analytes for daily clinical drug monitoring and PK studies.
Moreover, the proposed method might be used for routine monitoring of these drugs individually or simultaneously.

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Analyte</th>
<th>Nominal conc., ng/mL</th>
<th>Calculated conc., ng/mL</th>
<th>Mean ± SD%</th>
<th>RE(^1)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30°C freeze-thaw cycles</td>
<td>PRO (CBZ IS)</td>
<td>0.8±5.0</td>
<td>10</td>
<td>9.6±4.3</td>
<td>-3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>83.6±5.1</td>
<td>100</td>
<td>89.9±5.9</td>
<td>-14.0</td>
</tr>
<tr>
<td></td>
<td>PRO (MDZ IS)</td>
<td>1.8±6.6</td>
<td>10</td>
<td>9.0±4.1</td>
<td>-10.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>99.7±4.9</td>
<td>100</td>
<td>99.7±4.9</td>
<td>-13.5</td>
</tr>
<tr>
<td></td>
<td>MDZ</td>
<td>6.4±5.8</td>
<td>50</td>
<td>41.0±6.1</td>
<td>-18.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80.5±1.9</td>
<td>100</td>
<td>80.5±1.9</td>
<td>-19.5</td>
</tr>
<tr>
<td></td>
<td>CBZ</td>
<td>852.0±8.3</td>
<td>100</td>
<td>90.6±3.5</td>
<td>-18.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>852.0±8.3</td>
<td>90.6±3.5</td>
<td>-18.9</td>
</tr>
<tr>
<td>4 hours at room temperature</td>
<td>PRO (CBZ IS)</td>
<td>0.8±5.1</td>
<td>10</td>
<td>9.8±6.0</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86.0±5.10</td>
<td>100</td>
<td>89.9±5.9</td>
<td>-14.0</td>
</tr>
<tr>
<td></td>
<td>PRO (MDZ IS)</td>
<td>0.9±7.4</td>
<td>10</td>
<td>9.2±7.1</td>
<td>-8.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>106.0±5.3</td>
<td>100</td>
<td>106.0±5.3</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>MDZ</td>
<td>1.9±8.3</td>
<td>50</td>
<td>49.3±2.3</td>
<td>-1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>98.9±5.9</td>
<td>100</td>
<td>98.9±5.9</td>
<td>-1.1</td>
</tr>
<tr>
<td></td>
<td>CBZ</td>
<td>5.6±7.9</td>
<td>100</td>
<td>96.9±5.2</td>
<td>-18.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>835.0±2.6</td>
<td>96.9±5.2</td>
<td>-18.2</td>
</tr>
<tr>
<td>12 hours at room temperature (extracted samples)</td>
<td>PRO (CBZ IS)</td>
<td>0.9±9.7</td>
<td>10</td>
<td>10.6±2.2</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>84.0±1.2</td>
<td>100</td>
<td>84.0±1.2</td>
<td>-16.0</td>
</tr>
<tr>
<td></td>
<td>PRO (MDZ IS)</td>
<td>0.8±3.2</td>
<td>10</td>
<td>10.1±2.5</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>93.0±2.2</td>
<td>100</td>
<td>93.0±2.2</td>
<td>-7.0</td>
</tr>
<tr>
<td></td>
<td>MDZ</td>
<td>1.7±8.5</td>
<td>50</td>
<td>41.7±4.0</td>
<td>-16.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80.0±2.1</td>
<td>100</td>
<td>80.0±2.1</td>
<td>-20.0</td>
</tr>
<tr>
<td></td>
<td>CBZ</td>
<td>5.9±1.8</td>
<td>7</td>
<td>90.4±3.3</td>
<td>-11.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>823.0±1.8</td>
<td>1000</td>
<td>90.4±3.3</td>
<td>-11.6</td>
</tr>
</tbody>
</table>

\(^1\)RE = Relative error = (overall mean assayed conc. - added conc. / added conc.) x 100.
3.3.2.6. Administration Studies of PRO and MDZ or PRO and CBZ

Initially we evaluated the applicability of the proposed method to estimate drug-drug interaction after co-administration of PRO and MDZ. Teh et al.\textsuperscript{192} were examined the effect of a continuous infusion (60 min) of PRO on the human plasma concentrations of MDZ; and according to their study there were no significant increases in the concentrations of MDZ administered concurrently with PRO; this may result from the lower plasma concentrations of PRO (1.5 µg/mL) in that study. In a next studies the plasma concentration of PRO was increased to be ≈ 4 µg/mL, that resulted in the decrease of Cl of MDZ\textsuperscript{87,193}. Therefore, the PK interactions between PRO and MDZ are dose dependent.

In the present study the interactions between PRO and MDZ was confirmed and used to predict that the efficiency of therapeutic dose of PRO which responsible on the CYP3A isoforms' activity inhibition. Also a lack of any clinically significant unwanted effects on coadministration of PRO with MDZ such as the respiratory depression would have resulted from the proper selected doses of PRO and MDZ (125 µg/kg/min, \textit{i.v.} infusion and 2 mg/kg, \textit{i.p.}, respectively).

After the interaction between PRO and MDZ was successfully studied, the method was used to evaluate the PK interactions between PRO and CBZ. PRO in rat plasma was monitored after sole and co-administration with CBZ. The dose of CBZ (5 mg/kg, \textit{i.p.}) was effective dose as has been used previously for the study of PK of CBZ on rats\textsuperscript{143} or on human to evaluate the CBZ interactions with other drugs\textsuperscript{194,195}.

The plasma concentration-time profiles of PRO, MDZ, and CBZ after sole and co-administrations were constructed as shown in Figure 26A-C. PRO sole or co-administration with MDZ and/or CBZ, was determined through 60 min of infusion time and then until 240 min after the stopping of the infusion device.
Figure 26. Plasma concentration-time profiles of: PRO after sole and co-administration with MDZ or CBZ (A); sole and co-administration with PRO of MDZ or CBZ (B and C).
For PRO, after 10 min of administration, its concentration could be confirmed to be stable. The $AUC_{10-240\min}$ for sole administrations was $215.79\pm37.72$ ng/mL.min. The $AUCs_{10-240}$ after co-administration with MDZ and CBZ were $455.17\pm118.46$ and $79.45\pm39.85$ ng/mL.min, respectively. As the results revealed that the plasma concentrations of PRO were significantly increased after co-administration with MDZ ($P = 0.015$) and decreased after co-administration with CBZ ($P = 0.013$).

For both MDZ and CBZ sole and co-administrations, $T_{\text{max}, \text{min}}$ was 25 min. The mean plasma concentrations, ng/mL were evaluated for the MDZ and CBZ after sole and co-administration with PRO. The plasma concentration of both drugs was significantly ($P = 0.009$ and 0.004 for MDZ and CBZ, respectively) increased after their co-administration with PRO compared to sole administration. The $AUC_{0-240}$ was $4.90 \times 10^3\pm0.20$ after sole administration versus $6.28 \times 10^3\pm0.23$ ng/mL.min after co-administration for MDZ, and $21.54 \times 10^3\pm6.98$ after sole administration versus $41.70 \times 10^3\pm11.85$ ng/mL.min after co-administration for CBZ. The $T_{1/2, \text{min}}$ (155.93±24.50 versus 283.58±58.44 for MDZ and 69.30±0.00 versus 150.15±20.01 for CBZ); and finally the MRT$_{0-240}$, min (95.79±2.04 versus 107.39±4.48 for MDZ and 71.36±3.92 versus 94.11±7.30 for CBZ) were significantly increased as the $P$ values indicated in the Table 24 and 25.

While the $K_{\text{el}}$ was significantly decreased (at $P = 0.012$ for MDZ and 0.045 for CBZ) it was $0.005\pm0.001$ versus $0.002\pm0.001$ for MDZ; and was $0.011\pm0.004$ versus $0.005\pm0.001$ for CBZ; and also the $Cl$, mL/min for MDZ was $404.58\pm17.21$ versus $319.01\pm11.88$ and for CBZ was $246.87\pm68.18$ versus $127.31\pm39.41$ were significantly decreased in the presence of PRO compared to sole administration at $P = 0.010$ and 0.042 for MDZ and CBZ, respectively.
Table 24. PK parameters of MDZ (2 mg/kg, *i.p.*) after its sole and co-administration with PRO (125 µg/kg/mL, *i.v.* infusion for 60 min).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MDZ sole administration</th>
<th>MDZ-PRO co-administration</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$, ng/mL</td>
<td>41.11±2.49</td>
<td>44.23±1.3</td>
<td>0.150</td>
</tr>
<tr>
<td>$T_{1/2}$, min</td>
<td>155.93±24.50</td>
<td>283.58±58.44</td>
<td>0.040</td>
</tr>
<tr>
<td>AUC$_{0-240}$, ng/mL.min</td>
<td>4.90 x 10$^3$±0.20</td>
<td>6.28 x 10$^3$±0.23</td>
<td>0.009</td>
</tr>
<tr>
<td>MRT$_{0-240}$, min</td>
<td>95.79±2.04</td>
<td>107.39±4.48</td>
<td>0.030</td>
</tr>
<tr>
<td>Cl, mL/min</td>
<td>404.58±17.21</td>
<td>319.01±11.88</td>
<td>0.010</td>
</tr>
</tbody>
</table>

1 Data are expressed as mean ± SD (n=3).
2 Un-paired Student's *t*-test: *P* < 0.05, significantly different from sole administrations.

Table 25. PK parameters of CBZ (5 mg/kg, *i.p.*) after sole and co-administration with PRO (125 µg/kg/mL, *i.v.* infusion for 60 min).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CBZ sole administration</th>
<th>CBZ-PRO co-administration</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$, ng/mL</td>
<td>309.33±59.53</td>
<td>422.68±91.47</td>
<td>0.079</td>
</tr>
<tr>
<td>$T_{1/2}$, min</td>
<td>69.30±0.00</td>
<td>150.15±20.01</td>
<td>0.001</td>
</tr>
<tr>
<td>AUC$_{0-240}$, ng/mL.min</td>
<td>21.54 x 10$^3$±6.98</td>
<td>41.70 x 10$^3$±11.85</td>
<td>0.054</td>
</tr>
<tr>
<td>MRT$_{0-240}$, min</td>
<td>71.36±3.92</td>
<td>94.11±7.30</td>
<td>0.004</td>
</tr>
<tr>
<td>Cl, mL/min</td>
<td>246.87±68.18</td>
<td>127.31±39.41</td>
<td>0.042</td>
</tr>
</tbody>
</table>

The important role of the anesthetist is to minimize the risk of perioperative seizures, whilst providing adequate anesthesia to allow optimum surgical operating conditions. Moreover, the good knowledge with the PK interactions between the commonly used drugs in both anesthesia and epilepsy is essential.

This study showed the concomitant administration of CBZ with PRO significantly decreases the plasma concentration of PRO. This indicated an enhancement of PRO metabolism leading to lower plasma concentration of PRO. Such a variation would lead to sub-therapeutic in case of PRO or super-therapeutic concentration in case of CBZ. Consequence lack of sedation effect of PRO and increase the toxic effects of CBZ. There is no other study investigating the interaction between PRO and CBZ during the anesthesia in human or animal’s application. The basis for studying the interaction between PRO and CBZ was the common metabolic pathway involving the isoenzymes CYP3A4.
In rats it has been seen that the isoenzymes CYP3A4 corresponding to CYP3A4 activity in human hepatocytes 196. Hence, any drug interaction occurring due to an effect on this particular CYP isoforms. In a previous study PRO doses required for sedation and times to the recovery of the eyelash reflex and spontaneous eye opening were evaluated, the required sedative dose of PRO for patients were received oral valproate treatment for moderate or severe mental retardation was significantly lower than the normal ones. Suggesting that valproate decreases the required sedative doses of PRO, and that the normal PRO dose may be excessive for patients receiving valproate treatment and induce complications or delay recovery from anesthesia 66. Because valproate has been demonstrated to inhibit PRO metabolism in vitro 64, 65.

Inversely, in the present study the results revealed that, the co-administration of PRO and CBZ to rats, a significant \( P = 0.013 \) decrease in the plasma concentration of PRO was detected when compared with the PRO sole administration. This finding is consistent with the literature as CBZ is an inducer (its unique difference than other AEDs) for several potential pathways of drug elimination, including CYPs isoenzymes such as CYP1A2, 2C9, and 3A4, as well as the active transporter P-glycoprotein 90.

According the present study, the required dose of PRO for the sedation of epileptic patients during the anesthesia should be increased in those receiving CBZ treatment to provide the adequate sedation.

This is the first study to report the interaction between the antiepileptic CBZ with PRO. However, the importance of these findings should not be ignored. PRO is the most commonly hypnotics used in general anesthesia todays. CBZ is the most commonly used antiepileptic drugs and also indicated for trigeminal neuralgia, migraine headache and alcohol dependence, thus there is a significant percentage of population who required this drug in their life time 197.
3.4. CONCLUSION

In this study, the first and rapid LC-MS/MS for the simultaneous determination of PRO, MDZ, and CBZ in rat plasma was developed and validated. The sample pretreatment procedure was simple economical, rapid and selective for extraction of the analytes from the plasma. A detailed validation following FDA guideline indicated that the developed method had a highly sensitive, reliability, selectivity, and excellent efficiency with total running time of 5 minutes per sample, which is suitable for high-throughput PK studies.

Moreover the proposed LC-MS/MS method was successfully applied to study the PK drug-drug interactions between PRO and MDZ or between PRO and CBZ in rats. The present study showed that CBZ can alter the plasma concentration of PRO to statistically significant level and also the presence of PRO affects the PK parameters of CBZ at significant level. However, further confirmation of these findings is required in the human studies using large sample size before these results and applied in patients care.
REFERENCES


2) Ion channels.


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To the one, to whom all acknowledgments should be, to my creator the most merciful and most graceful; in the name of Almighty Allah who taught man about matters that he does not know and prayers and peace be upon our prophet Mohammed.

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